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AQUEOUS SOLVATION METHOD FOR RECOMBINANT SPIDER SILK PROTEINS

by

Justin A. Jones

A dissertation submitted in partial fulfillment

of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biology

Approved:

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UTAH STATE UNIVERSITY
LOGAN, UTAH

2015

ABSTRACT

Aqueous Solvation Method for Recombinant Spider Silk Proteins

by

Justin A. Jones, Doctor of Philosophy

Utah State University, 2015

Major Professor: Randolph V. Lewis
Department: Biology

Two major hurdles face the production of recombinant spider silk protein (rSSp) based materials. First, the production of sufficient quantities of rSSp has proven difficult due to their highly repetitive nature and protein size (>250kDa). Secondly, rSSp and native silks are practically insoluble in water based solutions, necessitating the use of harsh organic solvents that can remain in the material after production. While others are working on solving production problems, this dissertation demonstrates a novel aqueous solubilization method that is rapid (<1 minute) and results in near 100% solubilization of the rSSp. From this new solubilization method films, foams, gels (hydrogels and lyogels), adhesives, coatings and fibers have been produced as well as the previously unreported sponge. All of these novel materials were derived from entirely aqueous solutions with and without minor additives to influence the final physical state of the rSSp.

159 Pages

PUBLIC ABSTRACT

Aqueous Solvation Method for Recombinant Spider Silk Proteins

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Utah State University, 2015

Major Professor: Randolph V. Lewis
Department: Biology

Spider silk is a remarkable material that has recently garnered significant international interest due to its broad applicability and natural composition. Spider silk fibers demonstrate unparalleled mechanical properties and their biocompatibility will allow them to replace products currently on the market such as fibers, threads and sutures that are made from traditional polymers. As spiders cannot be farmed, an emphasis in the Lewis lab is being placed on producing recombinant spider silk proteins (rSSP) in a variety of hosts, including alfalfa, goats, silkworms and *Escherichia. coli*. To this end, alfalfa, goats and silkworms are being generated with unique rSSP's that will improve the properties of the spun fiber as well as their recovery. A new fermentation facility is being constructed for the pilot-scale production of rSSP in *E. coli*. Novel plasmids and fermentation conditions are being developed to achieve maximum levels of production in this new facility. Recently, a new custom-engineered fiber spinning device was installed in the laboratory that allows for precise control of the process from

any point during the fibers production. With this device, advancements in fiber formation were achieved with rSSP spun utilizing both 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) dopes and a novel method of spinning rSSP's from primarily water-based solutions. Films from both HFIP and aqueous-based rSSP's were produced and a method for improving their mechanical properties was devised. Work has also begun on developing rSSP foams, hydrogels, lyogels and spray coatings.

This dissertation is dedicated to my father, Bayard Jones, who passed while I was working on this degree. He wanted nothing more than to see me finish.

Also, to my sons Andrew and Morgan Jones. Thank you for being you.
Love you guys.

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I would like to express my gratitude to all current and past team members of the aqueous group. Words cannot express how fun this ride has been.

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Finally, I would like to thank all of my friends and family for their support and encouragement. Bayard, father, your presence is felt every day and we think of you often.

Justin A. Jones

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CHAPTER 1

INTRODUCTION

In today's world of rising population and pollution levels, there is a need to replace synthetic materials with naturally derived alternatives. Alternatives that maintain or improve material performance but that are also produced using green methods and that are readily reduced in the environment to their non-polluting components. Spider silk is uniquely situated to fulfill these requirements and, due to its unique mechanical abilities, spider silk could also be utilized in entirely new material applications. Spider silk has the potential to alter the materials landscape.

Table 1: Mechanical property comparison of spider silks and common natural and synthetic material. [1,2,3,4]

Material	Strength (MPa)	Strain (%)	Toughness (KJ/kg)
Major Ampullate silk	4000	35	400
Minor Ampullate silk	1000	5	30
Flagelliform	1000	>200	400
Tubiliform silk	1000	20	100
Bombyx mori silk	600	20	60
Kevlar 49	3600	5	30
Rubber	50	850	80
Tendon	150	5	5
Bone	160	3	3

Spider silks have long been admired for their physical properties (Table 1). High energy to break, biocompatibility and biodegradation[5] are hallmark properties of the fibers. Recent research has proven that natural spider silk is as conductive as copper.[6]

Given this remarkable set of properties, extensive effort has been made to produce recombinant spider silk proteins (rSSP).

Spiders produce six different types of silk fiber and one glue. All six silk fibers and the glue are used in one or multiple phases of web development, reproduction or prey capture. Major ampullate silk is the most studied of all of the silk fibers and is used as a life-line for the spider as well as a structural component of the web.[7] Orb-weaving spiders lay down a line of major ampullate silk as they move about, much in the same way as a climber uses a belaying line. If the spider falls, they need a robust fiber to arrest their fall without causing damage to the spider. Dragline or major ampullate silk is uniquely suited to that role with 35% elasticity and a tensile strength similar to Kevlar

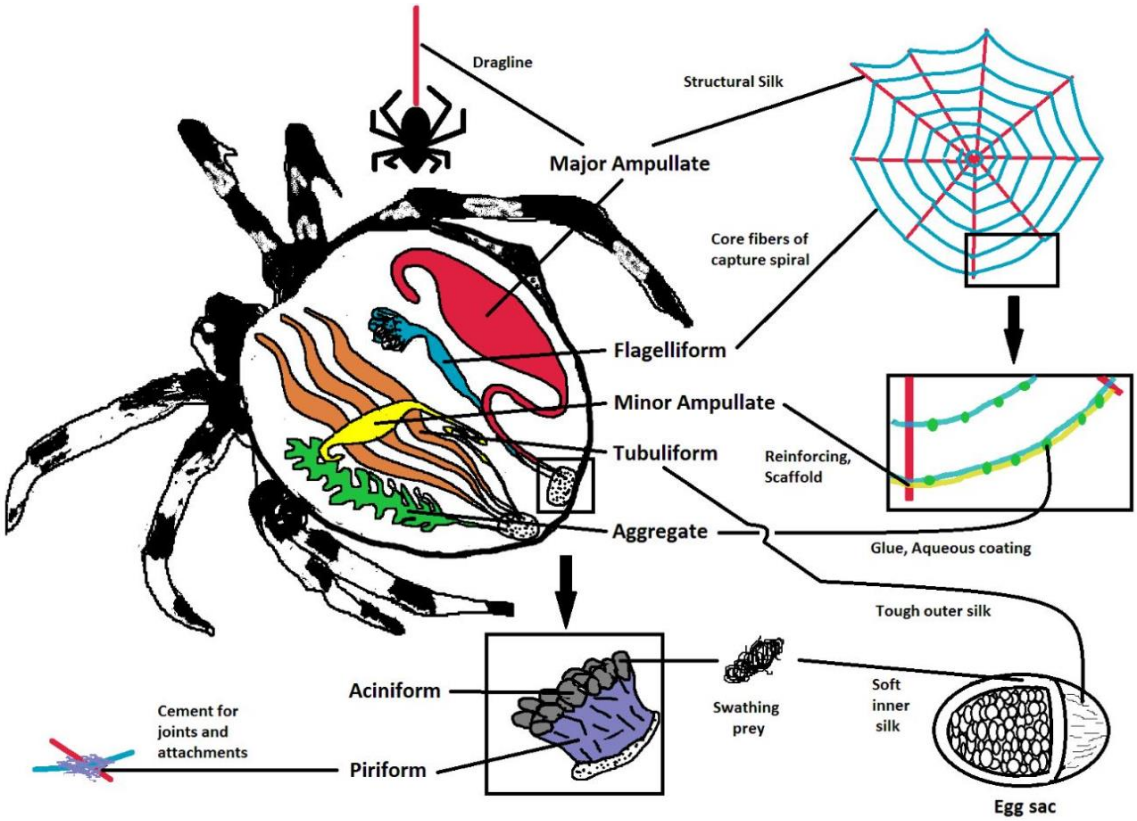

Figure 1: Diagrammatic representation of a spider, their silk protein-producing glands and resulting fiber or glue. Figure originally prepared by Dr. Michael Hinman and reproduced with his permission.

(Table 1). In its role as the structural component of the web (Figure 1), dragline needs to be robust and flexible enough to resist wind and prey damage. With its remarkable set of mechanical properties, it is well suited to that task.

Major ampullate silk is comprised of two proteins, major ampullate silk protein 1 (MaSp1)[7] and major ampullate silk protein 2 (MaSp2),[8] in approximately an 80:20 ratio. Given that there is a defined ratio of MaSp1 to MaSp2 in dragline silk, there must be a strong evolutionary reason. The mechanical properties of the fiber are that reason, and it is now understood that the two proteins contribute different structures in the fiber that, when combined, allow for the mechanical properties observed (Figure 2). MaSp2 has proline in its amino acid sequence that is used to make a β-spiral structure analogous to a slinky as well as a linker region to a □-sheet structure. MaSp1 is devoid of proline, and its structures are largely β-sheet and the stiff, rod like glycine-II helix.

Both MaSp's contain highly conserved N- and C- termini.[9,10] The C-termini has been suggested to act as a molecular switch in the conversion of the protein from a liquid crystal state within the gland to a fiber by maintaining the secreted protein in a micellar like structure.[9,11,12] The N-termini contains the secretion signal to transport the protein from the tall columnar epithelial cells into the lumen of the gland, where it can be utilized for fiber formation.[13] For the spider, the conserved N- and C-termini are essential for spinning a fiber, given their roles in solubility and secretion. However, to produce these proteins synthetically, they are not required.[14]

Minor ampullate silk fiber comprises the auxiliary spiral of the web and acts as a temporary scaffold during web construction. Minor ampullate is also comprised of two

proteins, MiSp1 and MiSp2.[15] Both are devoid of proline and mechanically they have less extensibility than the proline-containing major ampullate silk fiber. The predominant amino acid motifs are the poly-GA and poly-A β-sheet conferring sequences, as well as the GGX motif conferring the glycine-II helix (Figure 2).[16] MiSp's are also very large proteins, 315kDa and 275kDa respectively, much like the MaSp's.

Flagelliform silk fibers are involved in prey capture. With 300% elasticity and an energy-to-break similar to major ampullate silk, it is uniquely suited to this task. Flagelliform's predominant amino acid motif is GPGXX, which forms a beta-turn. When several of these motifs are strung together, they form a beta-spiral (Figure 2).[17] The beta-spiral, a similar structure found in MaSp2, acts like a slinky and the high proportion of this amino acid motif, and consequent structure, allows flagelliform to stretch to 300% of its original length and return. However, prey capture cannot be attributed to only flagelliform, as high elasticity would invoke a trampoline like effect on any prey flying into the web. The prey would hit the web, the flagelliform would absorb the impact and arrest the forward progress, and the prey would then be ejected from the web. In order to trap the insect/prey, spiders use the single non-fiber glue protein from the aggregate gland to trap the insects in their web once flagelliform has arrested the prey's progress.

Aggregate silk is one of the least studied of all the silk proteins. Choresh *et al*. demonstrated that aggregate was, in fact, two proteins coded from opposite strands of the same DNA sequence.[18] Aggregate is the only silk protein that is post-translationally modified with carbohydrates, and it shares a strong sequence similarity to chitin binding

proteins.[18] This is a highly sophisticated protein that is uniquely suited to its role as a glue protein that retains captured insects in the web.

Aciniform silk is produced to swath prey as well as to line the egg sacs of spiders. When aciniform silk was mechanically tested and compared to dragline silk, it was 50% stronger in terms of energy to break.[19] This is a remarkable feat, as the silk is largely devoid of the poly-GA and poly-A repeats that infer the strength providing crystalline regions of major and minor ampullate silk proteins.

Piriform silk fibers function to attach other fibers to surfaces. Many piriform fibers are laid down over the top of another fiber, in effect lashing the silk to virtually any surface. Piriform anchors the web to the substrate that it is built around and also anchors the spider's dragline silk. Piriform is unique from an amino acid perspective in it has exceptional motifs that contain proline.[20]

Tubuliform silk fiber is used to create the egg case that spiders lay their eggs in, and it is unusual in several ways. The first is that it is the only "seasonal" silk and is produced in the fall when spiders are ready to lay their eggs. The fiber is stiff and lacks the high glycine content seen in almost all other silk fibers.[21,22] In place of glycine, tubuliform employs serine, similar in composition to the *Bombyx mori* silk fiber.

When comparing the mechanical properties of the various fibers, what becomes immediately apparent is that major and minor dragline silks are relatively stiff and are uniquely suited to their functions in spider survival and web development. Flagelliform, aciniform and piriform are all very strong fibers that have a much higher degree of extensibility or elasticity than either major or minor ampullate. In fact, the extensibility

of aciniform is responsible for its higher energy to break than both major and minor ampullate silks. Energy to break is a measure of the area under the stress-strain curve, and increased extensibility provides for a greater area, even with the reduced tensile strength (true stress).[23] Further, the fibers can be grouped into three categories by their mechanical properties relating to their function in the spider's life cycle (survival and web development, prey capture, reproduction). Stiffer and stronger fibers are utilized in survival (major ampullate), web development (major and minor ampullate) and protecting eggs from predators (tubuliform) and cushioning them (aciniform) while highly elastic silk (flagelliform) and glue (aggregate) is used for prey capture.

A spider spins silk from water based solutions.[24] Specialized tall columnar epithelial cells produce and secrete protein into the lumen of the gland.[25] The protein solution is maintained as a viscous liquid crystal in the gland without precipitating or prematurely solidifying.[26,27,28] This is largely due to the silk orientating in a micellar-like structure in the gland.[11] The C-terminus of the major ampullate silk has a membrane-spanning-like structure of alternating hydrophobic and hydrophilic domains. The hydrophilic domains comprise the outer layer of the micelle that is in contact with water, while the hydrophobic domains are buried in the interior of the structure.[11] The liquid crystal only begins to solidify and form a fiber when a spider demands new silk fiber by pulling fiber from its spinnerets. The pulling force draws the soluble silk protein from the lumen into the neck of the gland, inducing a shear force that breaks open the micelles and exposes the hydrophobic repetitive regions to water. This induces the formation of β-sheets, as well as other structures, and transforms the liquid crystal silk

protein into a fiber.[29] As the forming fiber travels down the duct of the gland towards the spinneret, other mechanisms of fiber formation have been proposed, such as the removal of ions and a slight pH drop.[11] However, based on our experience, if a spider is carefully dissected and the major ampullate glands removed, the gland can be broken open using forceps and a fiber can be pulled directly from the luminal contents. From that observation, we begin to understand the absolutely necessary components to produce a fiber; spider silk protein solubilized in water and shear force to break open the micellar-like structures and align the protein molecules. Mimicking this process has been difficult utilizing rSSP's, primarily due to their almost complete insolubility in water, necessitating the use of 1,1,1,3,3,3,-hexafluoroisopropanol, a very harsh organic solvent.

Spiders' silk cannot be harvested for industrial purposes such as sutures or replacement tendons and ligaments. Spiders are both territorial and cannibalistic, making it impossible to farm sufficient quantities of spiders and collect silk fiber. This necessitates the production of spider silk protein in other organisms and then spinning the rSSP into fibers. However, the properties that give native spider silk fibers their remarkable mechanical properties also predispose the rSSPs solubility problems. The vast majority of rSSPs are insoluble in water due to their β-sheet content (due to glycine and alanine) and general hydrophobicity, providing a tendency to aggregate and precipitate into water insoluble forms. rSSPs are conventionally dissolved in a very harsh organic solvent HFIP to create "dopes" that can be used to create fibers, films, gels, and foams, and electrospun into fibers and mats.[30,31] HFIP has been widely used

and accepted as it is the only solvent that: 1) dissolves rSSPs at high concentrations (30% w/v) providing uniformity between various groups testing data, 2) is sufficiently volatile and miscible to be removed rapidly from the forming fiber, and 3) does not interfere with fiber formation. In addition, rSSPs are generally insoluble in aqueous solutions after purification, necessitating an organic solvent that meets the criteria outlined in 1-3.

There are significant problems with solvating rSSPs in HFIP or other organic solvents at an industrial scale. HFIP is toxic[32] to human health and to the environment and has a high likelihood of having a cytological effect due to HFIP residue in spun fibers or films.[33] The cost of purchasing HFIP ($100/g) renders it impractical as a solvent to produce synthetic fibers for medical devices on a large scale. The cost of protecting the environment and workers from exposure would drive the cost of materials produced to economically unfeasible levels. All of these negative aspects have a dramatic economic, ecological and pathological disincentive to produce rSSP based products using HFIP as a solvent. To date however, there is no working process to efficiently dissolve rSSPs in any other solvent that would be less toxic and costly.

There are some notable outliers to solvating rSSPs with HFIP. A chimeric rSSP that was soluble in Ni^{++} chromatography elution buffer (Tris-base, NaCl, Imidazole) was able to be spun into fibers.[14] The fibers produced from this chimeric sequence were short in length and impractical to produce at a large scale. Also, a series of sequences derived from aciniform silk (used for swathing and wrapping prey) that has roughly half the Gly/Ala content of major ampullate silk[26] were solvated with water and spun into fibers. In this instance, the rSSP (76kDa) was solubilized in water and fibers were spun

from that solution. However, given the reduced Gly/Ala content and thus reduced β-sheet content, the fibers were mechanically unremarkable.[24]

It is the principal goal of this dissertation to demonstrate a method that will solubilize existing water insoluble rSSPs to produce rSSP fibers and other materials using water and other green additives.

LITERATURE CITED

1. Gosline, J. M.; Guerette, P. A.; Ortlepp, C. S.; Savage, K. N. *J. Exp. Biol*. **1999**, *202*, 3295–3303.

2. Lewis, R. V. *Chem. Rev.* **2006**, *106*, 3762–3774.

3. Altman, G. H.; Diaz, F.; Jakuba, C.; Calabro, T.; Horan, R. L.; Chen, J.; Lu, H.; Richmond, J.; Kaplan, D. L. *Biomaterials* **2003**, *24*, 401–416.

4. Wohlrab, S.; Mueller, S.; Schmidt, A.; Neubauer, S.; Kessler, H.; Leal-Egana, A.; Scheibel, T. *Biomaterials* **2012**, *33*, 6650–6659.

5. Cao, Y.; Wang, B. *Int. J. Mol. Sci.* **2009**, *10*, 1514–1524.

6. Huang, X.; Liu, G.; Wang, X. *Advanced Materials* **2012**, *24*, 1482–1486

7. Xu, M.; Lewis, R. V. *P.N.A.S.* **1990**, *87*, 7120–7124.

8. Hinman, M. B.; Lewis, R. V. *J. Biol. Chem.* **1992**, *267*, 19320–19324.

9. Ittah, S.; Cohen, S.; Garty, S.; Cohn, D.; Gat, U. *Biomacromolecules* **2006**, *7*, 1790–1795.

10. Motriuk-Smith, D.; Smith, A.; Hayashi, C. Y.; Lewis, R. V. *Biomacromolecules* **2005**, *6*, 3152–3159.

11. Jin, H.-J.; Kaplan, D. L. *Nature* **2003**, *424*, 1057–1061.

12. Hagn, F.; Eisoldt, L.; Hardy, J. G.; Vendrely, C.; Coles, M.; Scheibel, T.; Kessler, H. *Nature* **2010**, *465*, 239–242.

13. Motriuk-Smith, D.; Smith, A.; Hayashi, C. Y.; Lewis, R. V. *Biomacromolecules* **2005**, *6*, 3152–3159.

14. Teulé, F.; Addison, B.; Cooper, A. R.; Ayon, J.; Henning, R. W.; Benmore, C. J.; Holland, G. P.; Yarger, J. L.; Lewis, R. V. *Biopolymers* **2012**, *97*, 418–431.

15. Colgin, M. A.; Lewis, R. V. *Protein Science* **1998**, *7*, 667–672.

16. Hayashi, C. Y.; Shipley, N. H.; Lewis, R. V. *Int. J. Biol. Macromol.* **1999**, *24*, 271–275.

17. Hayashi, C. Y.; Lewis, R. V. *Bioessays* **2001**, *23*, 750–756.

18. Choresh, O.; Bayarmagnai, B.; Lewis, R. V. *Biomacromolecules* **2009**, *10*, 2852–2856.

19. Hayashi, C. Y.; Blackledge, T. A.; Lewis, R. V. *Mol. Biol. Evol.* **2004**, *21*, 1950–1959.

20. Perry, D. J.; Bittencourt, D.; Siltberg-Liberles, J.; Rech, E. L.; Lewis, R. V. *Biomacromolecules* **2010**, *11*, 3000–3006.

21. Tian, M.; Lewis, R. V. *Biochemistry* **2005**, *44*, 8006–8012.

22. Tian, M.; Lewis, R. V. *Appl. Phys. A.* **2006**, *82*, 265–273.

23. Blackledge, T. A.; Kuntner, M.; Agnarsson, I. *Advances in Insect Physiology* **2011**, *41*, 175–262.

24. Xu, L.; Rainey, J. K.; Meng, Q.; Liu, X.-Q. *PLoS ONE* **2012**, *7*, e50227.

25. Hardy, J. G.; Romer, L. M.; Scheibel, T. R. *Polymer* **2008**, *49*, 4309–4327.

26. Vollrath, F.; Knight, D. P. *Nature* **2001**, *410*, 541–548.

27. Heim, M.; Keerl, D.; Scheibel, T. Angewandte *Chemie International Edition* **2009**, *48*, 3584–3596.

28. Dicko, C.; Kenney, J.; Knight, D.; Vollrath, F. *Biochemistry* **2004**, *43*, 14080–14087.

29. Rammensee, S.; Slotta, U.; Scheibel, T.; Bausch, A. R. *P.N.A.S.* **2008**, *105*, 6590–6595.

30. Stephens, J. S.; Fahnestock, S. R.; Farmer, R. S.; Kiick, K. L.; Chase, D. B.; Rabolt, J. F. *Biomacromolecules* **2005**, *6*, 1405–1413.

31. Bini, E.; Foo, C. W. P.; Huang, J.; Karageorgiou, V.; Kitchel, B.; Kaplan, D. L. *Biomacromolecules* **2006**, *7*, 3139–3145.

32. http://www.hexafluoroisopropanol.com/Hexafluoroisopropanol%20Material%20Safety%20Data%20Sheet.pdf

33. Hardy, J. G.; Leal-Egaña, A.; Scheibel, T. R. *Macromol. Biosci.* **2013**, *13*, 1431–1437.

CHAPTER 2

MECHANICAL AND PHYSICAL PROPERTIES OF RECOMBINANT SPIDER SILK FILMS USING ORGANIC AND AQUEOUS SOLVENTS[1]

ABSTRACT: Spider silk has exceptional mechanical and biocompatibility properties. The goal of this study was optimization of the mechanical properties of synthetic spider silk thin films made from synthetic forms of MaSp1 and MaSp2, which compose the dragline silk of *Nephila clavipes*. We increased the mechanical stress of MaSp1 and 2 films solubilized in both HFIP and water by adding glutaraldehyde and then stretching them in an alcohol based stretch bath. This resulted in stresses as high as 206 MPa and elongations up to 35%, which is 4 times higher than the as poured controls. Films were analyzed using NMR, XRD, and Raman, which showed that the secondary structure after solubilization and film formation in as-poured films is mainly a helical conformation. After the post-pour stretch in a methanol/ water bath the MaSp proteins in both the HFIP and water-based films formed aligned beta-sheets similar to those in spider silk fibers.

INTRODUCTION

Spider silk fibers have remarkable properties that could allow it to function in a variety of applications including textiles, biomedical, and manufacturing applications.[1–10] Of particular interest is dragline silk with both a high strength and elongation.[1] In recent years, producing spider silks synthetically has become a major point of emphasis because spiders cannot be farmed as they are both territorial and cannibalistic. Efforts

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1. Reprinted (adapted) with permission from (Tucker, C. L. *et al.* Mechanical and Physical Properties of Recombinant Spider Silk Films Using Organic and Aqueous Solvents. *Biomacromolecules* **15,** 3158–3170 (2014).). Copyright (2014) American Chemical Society.

to produce recombinant spider silk proteins (rSSP) have focused on the production of fibers[2–4,11,12] while comparably little effort has been expended investigating alternative forms such as films, hydrogels, lyogels, and adhesives.

Dragline silk is used as the lifeline for the spider and as structural support in the web and is one of the strongest natural fibers known to man.[1] Dragline silk is made up of two different proteins: Major ampullate silk protein 1 (MaSp1) and Major ampullate silk protein 2 (MaSp2), each with a molecular mass of around 300 kDa.[13,14] Native dragline silk is spun starting in the gland as a viscous water-based liquid crystal[15,16] in a micelle-like structure[17] in a liquid dope. Beta-sheets are induced and aligned by the friction of the duct as it decreases in diameter.[18] Beta-sheets are also formed by the removal of water from the liquid crystal[15] or micelle-like structure.[17]

Nuclear magnetic resonance (NMR),[5,19–26] Raman spectroscopy,[27,28] and X-ray diffraction (XRD),[20,29–32] show that secondary structures in spider dragline silk are mainly beta-turn, beta-sheet, and helical structures. Beta-sheets confer mechanical strength to the silk and do not allow water penetration.[26] Beta-sheets are mainly produced from the alanine-rich regions, (A_n) and $(GA)_n$ in the protein. Type IIA turns are made from the GPGXX (X is usually Y or Q) and GPGQQ repeat units, and glycine-II-helices are produced from the GGX regions.[6] These glycine-rich peptide regions allow penetration of water and increase strain, which contributes to the overall toughness of the silk.[33]

Synthetic spider silk fibers have been spun using rSSp to mimic natural spider silk properties.[2–4,11,12] It has been shown that in order to produce a strong fiber the larger the protein size the better the strength.[4] The actual spinning process is also difficult to

mimic, as current systems have a syringe and push the liquid dope out of small diameter (0.005" to 0.01" ID) PEEK tubing,[11] rather than the native pulling action. The secondary structures in the fibers need to be induced and then aligned, done by using a combination of a coagulation bath, liquid baths, and stretching.[2–4,11] The fibers then have to be woven or braided together to form a product.

Minimal research has been done on rSSp films. Recombinant spider silk film formulations have recently been found to be a promising biological material for their ability to attach and cause proliferation of fibroblast cells.[7] It was also found that the protein can be both genetically modified and chemically functionalized with cell adhesive peptides.[34] This allows for further applications in the medical industry. Silkworm and spider silk films have also been studied for their biomedical applications using fibroblasts, osteoblast-like cells, and skin cells[7–10,35,36] all showing as much attachment as traditionally used materials. The chemical stability of rSSp has also been shown to be controllable using alcohol treatments[37,38] and amino acid composition.[39,40] The mechanical properties of spider silk films have been reported, but no reports have improved on the initial properties.[41,42] Of the studies done on silkworm silk films only one was done to improve or to tailor the mechanical properties, which can make it a candidate for a biological material and scaffolds for tissue engineering.[43]

An advantage of using films over fibers is that films do not need to be woven together after processing to make functional products, which dramatically reduces the cost of production. The production of a film can be as simple as formulating a dope and pouring it. Dopes can also be modified by a change in formulation to have increased cell

attachment,[34,44] drug release,[41] and mechanical properties.[41,42] Film applications include coatings for medical devices,[45,46] skin grafts,[10,43,47] drug delivery,[41] and cellular scaffolds.[7,9,48] Improving and understanding the mechanical properties of films will provide a base for further research that tailors films to specific applications.

rSSPs are conventionally dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP)- to create "dopes" that can be used to create fibers, films, gels, and foams, as well as electrospun fibers and mats.[49–52] HFIP has been widely used and accepted as a standard solvent because it dissolves rSSPs at high concentrations (30% w/v), it is removed rapidly from the forming silk fiber, and does not interfere with fiber formation. In addition, rSSP's are generally insoluble in aqueous solutions after purification.

There are significant problems with solvating rSSPs in HFIP or other organic solvents at an industrial scale. HFIP is toxic to human health and to the environment and has a high likelihood of having a cytological effect due to residual HFIP.[52] HFIP is also not cost effective nor is it simple to work with due to the need of a controlled environment. To date however, there is no working process to efficiently dissolve rSSPs in any other solvent that would be less toxic and costly. There have been investigators that have used other solvents to produce fibers,[2,15,16] but these have diminished mechanical properties. The inability to solubilize rSSPs in aqueous solvents limits the applications of synthetic spider silk.

This study presents a novel way of processing rSSp films with solubility in HFIP and the introduction of an aqueous solvent to decrease environmental impact, cost of processing, and toxicity. Even with the change of solvent, the mechanical properties of

the films can be as high as, and in some cases, surpass those from films produced from HFIP. Post-pour processing methods were utilized to improve secondary recruitment and orientation, and thus properties.

The proteins in this study are rSSp's produced in the milk of transgenic goats, derived from the *N. clavipes* major ampullate silk proteins MaSp1 and MaSp2, which combine to form the dragline fiber. The films are fabricated using a liquid dope, with primarily HFIP or water used as a solvent, cast into a mold to produce films 10-30 µm thick. The protein concentration and solvent composition are varied to increase mechanical properties. Films are post-casting processed using a combination of vapor treatments, liquid treatments, and stretching to increase stress, strain, and energy to break. To our knowledge this is the first reported rSSp film production method tailoring mechanical properties. Improving the mechanical properties of rSSP films will widen potential applications for such materials.

MATERIALS AND METHODS

MaSp1 and MaSp2 Purification

Milk from transgenic goats is first collected and frozen, then 6-8 L of milk is thawed, and defatted using a Milky cream separator (FJ60 by Clair®). The defatted milk is brought to a pH of 9 using 0.1M arginine-HCl with the milk solution at 4˚ C for 30 minutes while stirring. The solution is then clarified and concentrated using tangential flow filtration (TFF) with 750 kD and 50 kD membrane filters with the 750 KDa permeate flowing into the 50 kD with the permeate flowing back into the 750 kD[53]. The

retentate from each 750 kD column and 50 kD column are recycled through their respective columns. The rSSP's are precipitated from the 50 kD column retentate. Solid ammonium sulfate is added slowly to a concentration of 1.2M while stirring to precipitate the rSSP from the remaining milk proteins. The solution is allowed to precipitate overnight and centrifuged at 15970g for 60 min. The supernatant is removed and the pellet is washed multiple times using dH_2O followed by centrifugation at 15970g for 60 min until the conductivity of the supernatant is below 20 mS/cm. rSSP pellets are then lyophilized to remove all water and tested for purity via Western blot analysis using αM5 as a primary antibody and AP conjugated donkey anti-rabbit as a secondary antibody (Santa Cruz Biotechnology).

PDMS Mold

Water

The mold is made from a PDMS (Dow Corning) solution of 5:1 base to initiator and poured it into a 90 mm petri dish to approximately 1 mm thick. The petri dish and solution is then placed into a vacuum chamber for 20 minutes to remove all bubbles. They are then placed in an oven at 70 ºC to crosslink overnight. The solidified PDMS is removed and cut using a forceps and a razorblade to four 30 x 7 mm strips (Figure 1), with care taken to keep it clean of particulates. The mold is then thoroughly cleaned using soap and water followed by isopropanol (IPA).

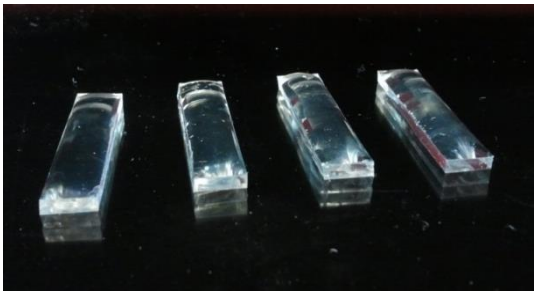

Figure 1: PDMS strips with poured spider silk dope over the top.

HFIP

The mold to form the films is made from a polydimethylsiloxane (PDMS) solution of 20:1 base to initiator and pouring it into a medium sized petri dish to 0.2 mm thick. The petri dish and solution is then put into a vacuum chamber for 20 minutes to remove all bubbles. The solution is then set overnight in an oven at 70 ºC to harden. The solidified PDMS is removed and cut using a forceps and a razorblade to four 30 x 7 mm strips keeping the PDMS clean of particulates. The PDMS strips are placed in a new petri dish side by side, avoiding touching, and a solution of 5:1 base to initiator PDMS solution is poured over the strips, with the solution at least 1 mm above the strip. The petri dish with the PDMS solution is placed into a vacuum chamber and the bubbles removed for 20 minutes, and then set overnight in an oven at 70 ºC to solidify. The PDMS is removed from petri dish and the 20:1 strips are carefully removed using forceps and a razorblade so as to not damage the 5:1 mold. The mold is then thoroughly cleaned using soap and water followed by Isopropanol (IPA).

Dope Preparation

Water

Standard water-based films are made using dopes which contain 4% MaSp1, 2% MaSp2, and 3.5% 80/20 MaSp1/MaSp2 protein dissolved in water with additive. Additives were included in the dopes to improve solubility, antibiotics and crosslinking. These additives include formic acid (FA), acetic acid, arginine and glutamic acid, Urea, ammonium hydroxide, kanamycin, glutaraldehyde (GTA), and imidazole using multiple concentrations. The dopes are microwaved, using a 700W Magic Chef household microwave, for a period of 30 seconds on full power in a sealed 3 mL Wheaton glass vial to liquefy the dope and solubilize the protein. The dope is transferred into a microcentrifuge tube and spun at 18000g for 1 min, the supernatant is transferred to another microcentrifuge tube and the centrifugation repeated to remove any particulate matter. All films are then immediately poured and spread onto four 30 x 7 mm polydimethylsiloxane (PDMS) strips with 200µL of dope on each strip.

HFIP

A standard dope contains 5% protein powder (w/v) dissolved in HFIP by overnight rotary agitation and centrifuged for 2 min at 18000g to remove any particulate matter remaining. The dope is carefully pipetted (200 µL) out of the vial and poured into a pre-made PDMS mold described above, in a chemical hood (Thermo

Scientific Hamilton Concept) with the sash opened as far as possible to slow air flow over the films and decrease drying time.

Film Formation

The dope is carefully pipetted (200 µL) out of the microcentrifuge tube and poured onto a pre-made PDMS well/strip (described above) in a Thermo Scientific Hamilton Concept chemical hood with the sash opened to provide air flow over the films and decrease drying time. After 1 day the water-based films (2 hours for HFIP-based films) are dry, and starting to peel themselves off of the strips/wells. The films are removed using forceps and the edges cut with a razor blade, producing a uniform flat film.

Post-Pour Treatments

Vapor treatment

Films were first cut using a razor blade to 3.5 x 15 mm strips and weighed to determine thickness (Equation 1). The cut films were then glued to a C-card (Supplementary Information figure S1), as described below (mechanical testing section). The films were vapor treated using different ratios of isopropanol (IPA), water, and methanol (MeOH) at room temperature. Vapor treatment consists of putting the films into a small petri dish, which is then nested into a larger petri dish with 5 mL of the treatment solution in the bottom; the lid is placed on the larger petri dish to contain

vapors. Cold treatment is simply putting the films into a closed petri dish and putting them into a refrigerator. All treatments lasted for 30 minutes.

Stretching

To stretch the films a custom made stretching device (Figure 2) was created using two, 3” x 3” x 1/4” inch (B and C) and two 3 1/8” x 3” x 1/2” (A and D) sheets of polycarbonate secured by two 1/2” dowels 3/4” from the bottom and 1/2" from the both sides and a 1/4 inch fiberglass dowel 1 3/4” from the bottom and in the center. All dowels are glued to sheets A, C, and D. A 1/8 inch all thread rod is also placed through all sheets except for the moving piece (B) which is threaded for piece B, A nut is also added flush with part D on both sides in order to make part D move. An extra nut is also placed at the extreme end at part E for ease of turning.

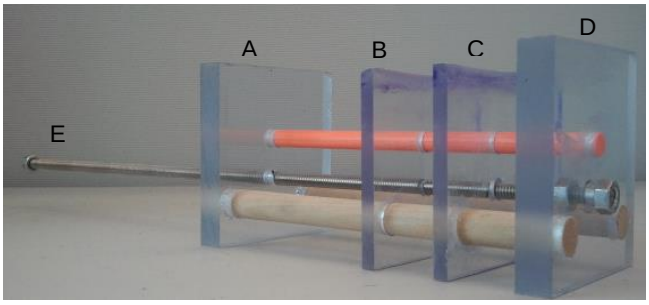

Figure 2: Diagram of the stretching apparatus used to glue as-poured films (across B and C), submerge the films in a stretch bath, and stretch the films by turning the all thread (E) clockwise.

Untreated films (dried for a 24 h) were first cut using a flat edged razor blade on a cutting board along the edges to ensure consistent thickness. The films are then cut in half lengthwise and glued to the custom made stretching apparatus described above (Figure 2). The stretching apparatus is inverted with the top of pieces B and C in a

defined mixture of alcohol and/or water with percentages measured by volume, for a period of 30 sec (2 minutes for water-based films). The apparatus is then rotated right side up and the film strips immediately stretched by turning the all thread clockwise (part E in Figure 2). With an initial film length of 8.5 mm the final length was determined by multiplying the initial length by the stretch ratio, for example a 3X stretch has a final length of 25.5 mm.

Mechanical Testing

The films, post-stretching, are cut to a specific length and width to weigh them and calculate the thickness (Equation 1) using a density for dry spider silk fiber of 1.23 g/cm.[3, 54–56] The films are then mounted on a plastic C-card (Figure S1) length wise using Loctite super glue (liquid) across an 8 mm gap.[57] After mounting, the C-card is loaded on an MTS Synergie 100 (50N load cell) by clamping the top and bottom of the film and card into the instrument with alligator clips and then cutting the side of the C-card (indicated by the dotted line in Fig. S1) so the only thing being tested is the film.[12] The film is then tested to breaking at a stretch rate of 5 mm/min, with data collection at 30 Hz to measure the film's load in order to calculate stress, strain and energy to break using MTS's TestWorks 4, 2001.

$$\text{Thickness (cm)} = \frac{\text{Weight(g)}}{1.23\left(\frac{\text{g}}{\text{cm}^3}\right)(\text{Width (cm)} * \text{Length(cm)})} \qquad (1)$$

Nuclear Magnetic Resonance (NMR)

All ^{13}C solid-state NMR data were collected on a 400 MHz Varian Wide-Bore instrument using a 1.6 mm solids triple resonance probe. Samples were packed into a 1.6 mm zirconia rotor and spun at the magic angle at 30 kHz MAS. ^{1}H - ^{13}C cross polarization conditions were calibrated using ^{13}C-enriched Glycine, and the CP condition was met by using a ramped (~15%) ^{1}H spin-lock pulse centered at 130 kHz RF field strength, and a square spin-lock pulse on the ^{13}C channel matched to the -1 spinning side bands of the Hartmann Hahn profile. All spectrum were collected using a 50 kHz spectral width, 8 ms acquisition time, 12288 scan averages, a 1 ms CP contact time, a 5 second relaxation time, and 150 kHz two-pulse-phase-modulated (TPPM) decoupling was applied on the ^{1}H channel during acquisition. 50 Hz exponential line broadening was applied to each spectra prior to Fourier transform. The ^{13}C chemical shifts are referenced externally to TMS at 0 ppm by setting the downfield resonance of adamantane to 38.56 ppm.

Raman

The films were analyzed using a home built Raman system. Films were placed bridging the space between two parallel glass slides to eliminate background and excited with a 150 mW 532 nm Coherent Sapphire SF laser focused onto the sample with a 50x magnification APO plan Mitutoyo 2.0 cm working-distance objective. The laser power was controlled using neutral density filters to make the power at the sample 28 mW which optimized the balance between signal-to-noise and sample

damage. The Raman signal was collected in back scattering geometry. The laser wavelength was discriminated from the Raman signal using an Ondax SureBlock(TM) ultra-narrow-band notch filter. An Acton Research SpectraPro 300i monochromator with a 1200 g/mm grating coupled to a PI liquid nitrogen cooled CCD detector was used to collect Raman signal for 5 acquisitions of 60 seconds each at a resolution of 1.5 cm^{-1}. Cyclohexane and acetaminophen were used as calibrants.

X-Ray Diffraction

Samples were taken to the Advanced Photon Source located at Argonne National Laboratory, Argonne IL, USA and wide-angle x-ray fiber diffraction was performed on the BioCars 14BM-C beamline using a beam energy of 12.6 keV and approximate size of 130 x 340 μm. Films were mounted and were placed at a distance of 300mm from the ADSC Quantum-315 9-panel CCD array detector. Stretched films were placed with the stretched axis parallel to the beam stop and mounted to a goniometer. The exposure time was 60 seconds for each of ten images averaged for each sample. For each sample, 5 background images were taken following each sample with the same parameters and calibrated with CeO_2. Images were then processed using Fit2D software and Matlab. The water-based MaSp2 films were contaminated while at the synchrotron source and made the x-ray diffraction data unusable.

Field Emission Scanning Electron Microscopy (FE-SEM)

The films were imaged by field emission scanning electron microscopy (FE-SEM Hitachi S-4000, Hitachi High-tech Corporation, Tokyo, Japan) to characterize their morphology. The films were mounted on an aluminum stub and coated with a gold layer 10 nm thick.

Film Functionalization

HFIP dopes were made by dissolving 50 mg of MaSp1 powder in 1 mL of HFIP and mixed overnight, 200 µL was poured into a PDMS mold (described in HFIP paper) and allowed to dry. The kanamycin containing film was made by transferring 300 µL to a new vial and adding 1 µL of kanamycin stock (15mg/mL), mixed for a minute using rotary agitation, and then 200 µL was poured into a PDMS mold.

The water-based dope was made by microwaving 15 mg MaSp1 powder in 300 µL of water for 45 seconds and pouring 200 µL onto a PDMS strip as described above. The kanamycin film was made the same way with the exception that the rSSP solution was allowed to cool at room temperature to prevent degradation of the kanamycin. One µL kanamycin (15mg/mL) was added to the dope for a final concentration of 50µg/mL. The dope was mixed for a minute using rotary agitation before pouring 200 µL onto a separate PDMS strip.

Two days after pouring the films, a lawn of *E. coli* XL1-Blue cells was established on an LB agar plate and allowed to dry for 30 min in an incubator at 37 °C. Holes (6.5

mm) were punched out of the films and a disc from each film was placed on the plate. The plates were then placed in the incubator overnight to allow cell growth.

Statistical Analysis

All statistical analyses on tensile properties were done using a one-tailed t-test assuming equal variance with a null hypothesis that the sample means are equal. A p-value of < 0.05 is considered significant.

RESULTS

Preliminary Experimentation for HFIP-based Films

To create the films, a suitable substrate was investigated to create a mold for film formation. Glass, aluminum, Teflon, and PDMS were all tested as substrates for film formation and removal. The substrate that proved to be the best was PDMS due largely to its hydrophobicity. The films could be peeled off easily after drying, which reduced mechanical damage. PDMS also provides a smooth surface free of machine marks.

The next important step was to establish the best pouring and drying method. An important factor in the pouring method was dope composition. It was found that 5% protein dopes were easy to solubilize, pour, and provided a thickness of 20 - 30 µm. To optimize the drying method, atomic force microscopy (AFM) was used to analyze surface topography. In initial work during drying, pores were created throughout the film. The pores are thought to occur due to the HFIP evaporating so quickly that it leaves holes in the films as it bubbles out. Because of this, it was thought that a slower rate of

evaporation would optimize film production. A variety of drying techniques were investigated (Table 1) in order to achieve this. Pore tomography was measured using atomic force microscopy (AFM) in tapping mode (Figure S2). The drying method that was chosen to use throughout this study is drying in a chemical hood with the sash opened as far as possible to slow the air movement. It was also assumed that because the problem of pore formation arises from HFIP evaporation, this method could be applied to all HFIP-based protein dopes.

Table 1. Comparison of pore sizes between pouring methods measure by AFM

Pouring Methods	Pore Density (pores/μm)	Pore width (nm)	Pore Depth (nm)
MaSp2 Open sash	6.8	293	4.56
MaSp2 Refrigerated	7.4	625	>80
MaSp2 Turbulent Air	0.6	6200	230
MaSp2 Vacuum Chamber	11.4	449	5.15

After optimizing the film production process, preliminary testing of un-processed films using MaSp1, MaSp2, varying ratios of MaSp1 and 2, and different dope solvent formulations including formic acid (FA) and glutaraldehyde (GTA) (Table 2) was performed. Dopes with formic acid follow the procedure of a standard dope with the exception that formic acid, 88%, is added to the dope before centrifugation and dopes with GTA have the exception that after centrifugation the dope is removed carefully from the vial and put into another vial and GTA (1 μL/mL) is added by pipette and the vial gently rotated by hand before pouring.

Table 2. Preliminary mechanical testing results with average deviations from untreated MaSp1 and MaSp2 films with different dope formulations including no additives, GTA, and 20% FA.

Protein Solution	Average Energy to Break (MJ/m^3)	Average Ultimate Stress (MPa)	Average Ultimate Strain (mm/mm)
MaSp1	2.04 ± 0.81	42.12 ± 8.52	0.068 ± 0.02
MaSp1 w/ GTA	**8.42 ± 9.67**	32.97 ± 14	**0.621 ± 0.77**
MaSp1 w/ 20% FA	2.87 ± 1.09	**50.4 ± 4.75**	0.076 ± 0.03
MaSp2	0.64 ± 0.28	29.52 ± 2.49	0.036 ± 0.01
MaSp2 w/ 20% FA	0.66 ± 0.35	44.6 ± 6.34	0.028 ± 0.01
20/80 MaSp1/MaSp2 w/ 20% FA	1.3 ± 0.74	36.56 ± 11.09	0.051 ± 0.02
50/50 MaSp1/MaSp2 w/ 20% FA	0.47 ± 0.42	34.28 ± 12.1	0.024 ± 0.01
80/20 MaSp1/MaSp2 w/ 20% FA	3.73 ± 1.88	45.21 ± 12.65	0.13 ± 0.08

All untreated films mechanical properties were mechanically tested the same day they were poured. Beta-sheet formation was measured on MaSp1 and MaSp2 films with GTA using XRD over a week after pouring, which showed little difference between the two (Figure 3A and S3). It is also evident through mechanical testing that formic acid increases stress with the highest being MaSp1 with formic acid. The addition of GTA increased strain, leading to a tripling of the energy to break for pre-processed films. MaSp1 films with formic acid were also tested after conducting a vapor treatment, which involved placing the films in a small petri dish, which was placed in a larger petri dish with the treatment liquid and the lid placed over the large petri dish. The vapor treatment time is 30 minutes and the films were tested for mechanical properties the following day (Table S1). The IPA vapor treated films produced the highest average stress 79.6, but the lowest average strain 0.03, suggesting an increase in beta-sheet content.

Preliminary Experimentation for Aqueous-based Films

With the discovery of PDMS as a suitable pouring substrate and the need for a slow drying process, the development of aqueous film formation started with changing the PDMS molds to a PDMS strip to overcome surface tension issues due to the use of water. It was then necessary to establish a dope formulation.

The stability and processing of spider silk films depend on the composition of the dope. Dope preparation began by using recombinant MaSp1, water, and formic acid (0.1, 0.5, 1, 5, 10, 15, and 20 %), acetic acid (10, 15, and 20%), arginine and glutamic acid (Arg Glu) (0.6, 12, 20, 30, 50, and 122 mM), Urea (4, 8, 160 mM), ammonium hydroxide (50, 100, and 200 mM) or Imidazole (10 and 100 mM). MaSp2 films were also made using formic acid (0.1, 2, 10, and 20%) and acetic acid (1, 5, 20%). All additives were placed into the dope prior to microwaving.

Preliminary tensile testing was done on the films as-poured (no processing). These films were screened for tensile strength, solubility, and processability. Solubility was tested by placing the films into 5 mL of DI water. Processability was determined by trying to stretch the films in different stretch baths, it was determined processable if the film stretched without breaking to a minimum of 1.5X. Films from dopes containing urea and ammonium hydroxide dissolved quickly in water (< 30 sec). Urea containing dope films also dissolved in a mixture of alcohol and water, preventing further processing of films (Table 3). The dope made with 0.1% formic acid proved to make films with a high tensile strength and process ability than the other dopes.

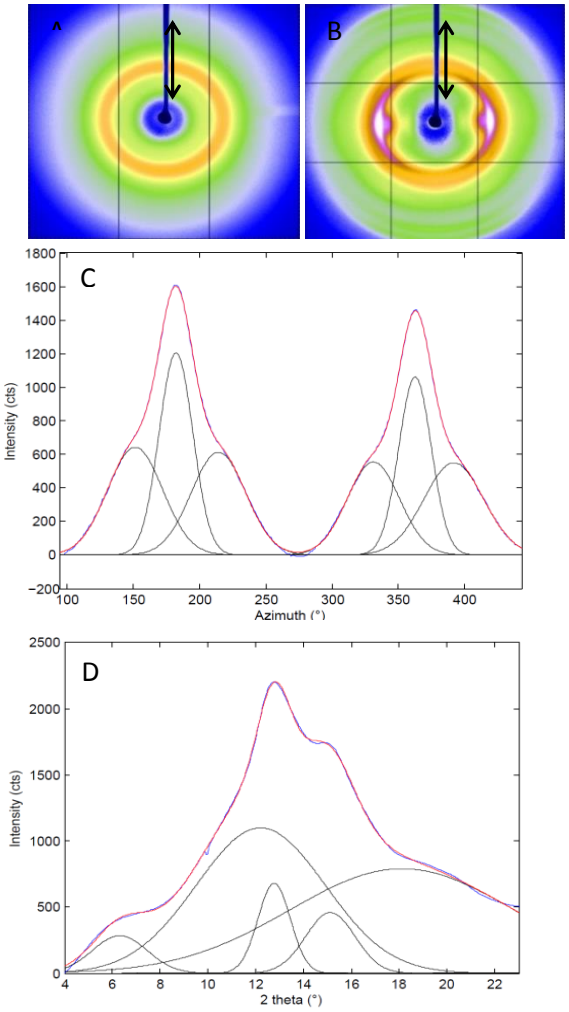

Figure 3: 2D WAXD images of MaSp1 spider silk films as-poured (A) and post-pour stretched 2.5 times its original length following an 80/20 Methanol/Water bath (B). The double arrow in (A) and (B) represents the direction of film stretch alignment which is parallel to the beamstop shadow (blue). Shown in (C) is the 1D azimuthal intensity profile of radially integrated reflections at 4.2 $Å^{-1}$ of (B) with Gaussian fits. Full 1D radial intensity azimuthally integrated profile of (D) with beamstop shadow and CCD detector lines masked and fit to 5 Gaussian components.

Tensile testing was done to understand variability between samples, structural integrity and extension of the films (Table 3). It was previously hypothesized that high extension (> 0.100) and low stress (≤ 50 MPa) led to a film that could be easily post-pour stretched as indicated by the results from the HFIP-based film. This hypothesis was

Table 3: Comparison of mechanical properties and solubility of films made from different dope formulations using MaSp1.

Additive	Concentration	Energy to Break (MJ/m^3)	Stress (MPa)	Strain (%)	Film Soluble in water
Urea	4mM	0.42 ± 0.12	50.26 ± 8.62	1.7 ± 0.3	Y
	8mM	0.43 ± 0.05	50.70 ± 3.04	1.7 ± 0.1	Y
	160mM	0.44 ± 0.14	49.97 ± 7.74	1.6 ± 0.3	Y
Arginine and Glutamic acid	0.6 mM	0.64 ± 0.22	61.82 ± 13.06	2.0 ± 0.4	N
	12 mM	0.75 ± 0.25	58.31 ± 7.94	2.3 ± 0.6	N
	20 mM	1.96 ± 3.13	50.32 ± 11.99	4.5 ± 5.3	N
	30 mM	8.71 ± 8.74	22.67 ± 2.62	43. ± 39.7	N
	50 mM	7.47 ± 6.67	15.64 ± 0.66	51.2 ± 45.3	N
	122 mM	0.07 ± 0.02	3.24 ± 0.9	3.6 ± 0.5	N
Ammonium Hydroxide	50 mM	0.41 ± 0.12	52.55 ± 6.86	1.7 ± 0.3	Y
	100 mM	0.71 ± 0.24	62.83 ± 15.49	2.5 ± 0.6	Y
	200 mM	0.68 ± 0.22	57.81 ± 11.98	2.4 ± 0.5	Y
Formic Acid	0.10%	0.61 ± 0.17	53.97 ± 4.73	2.5 ± 0.4	N
	0.50%	0.69 ± 0.19	58.15 ± 8.2	2.5 ± 0.2	N
	1%	0.84 ± 0.22	69.35 ± 7.28	2.6 ± 0.4	N
	5%	0.84 ± 0.4	65.24 ± 14.3	2.5 ± 0.7	N
	10%	0.64 ± 0.12	60.76 ± 7.52	2.4 ± 0.3	N
	15%	0.81 ± 0.04	71.36 ± 5.1	2.5 ± 0.2	N
	20%	0.87 ± 0.2	66.56 ± 7.4	2.7 ± 0.4	N
Acetic Acid	10%	2.63 ± 1.18	50.56 ± 5.63	6.9 ± 3.0	N
	15%	0.94 ± 0.22	50.35 ± 9.17	3.4 ± 1.0	N
	20%	24.28 ± 9.43	36.58 ± 2.24	82.6 ± 29.6	N

disproved as dope formulations making as-poured films with a high degree of extensibility (20% acetic acid and 30mM arginine and glutamic acid) could not be further processed. Dopes containing propionic acid (0.1 and 10%) and imidazole (10 and 100mM) were also made. Preliminary mechanical testing was not done on these films as they also broke when force was applied in the stretch bath. Films with 0.1% formic acid permitted alcohol and water treatments, as well as stretching, both of which increased mechanical properties. Due to the ease of processability, the dope formulation containing 0.1% formic acid was used for the remainder of the experiments. Additionally, 0.05% GTA was also used due to the positive results from HFIP-based films, showing that it increases both stress and strain. A similar problem was encountered when MaSp2 films were stretched using any variety of alcohol and water concentrations, breaking the films instead of actually stretching them. To solve this problem MaSp1 was mixed in with MaSp2 at different concentrations until the films were able to be processed, arriving at 80% MaSp1 and 20% MaSp2 based on weight.

Films that were made with 0.1% formic acid and 0.05% GTA were then characterized using XRD, showing that the MaSp2 films have more crystallinity than the MaSp 1 films (Figure 4 and S4). Since the pure MaSp2 films could not be post-pour stretched, it is hypothesized that the high beta-sheet content prevents the penetration of water. MaSp2 dopes also gelled faster than MaSp1 dopes after microwaving, due to the higher beta-sheet content, making it difficult to remove particulates and pour.

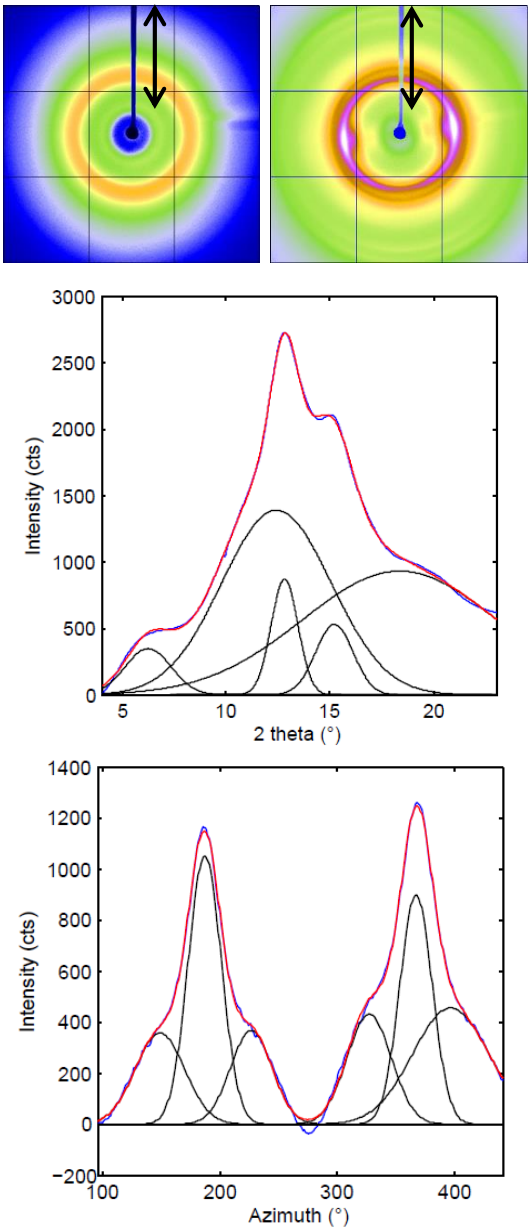

Figure 4: XRD images of as-poured spider silk films MaSp1 (A), post-pour stretched 2.5 times its original length after an 80/20 Methanol/Water bath (B), 1D radial integration profile of the whole 2D pattern of B (C), and the 1D azimuthal intensity profile of B (D). The double arrow in A and B represents the direction of film stretch alignment which is parallel to the beamstop shadow (blue).

Stretching Films

Stretching spider silk fibers has been shown to increase both stress and strain[2–4,11] by aligning secondary structure. In this study a similar technique is used to improve mechanical properties. Initially, the films were stretched by hand, but this method of stretching was both difficult and unreliable. A stretching apparatus custom made in our laboratory (Figure 2) was created to establish an easy method to create a consistent, uniform stretch. This apparatus made it possible to obtain results that were reproducible and also made it possible to stretch multiple films simultaneously. It is important to note that with HFIP-based films, formic acid impaired the post-pour stretching of the spider silk films after the stretch bath and therefore was not included in the dopes for stretched films. It is hypothesized that formic acid increases beta-sheet content preventing sufficient penetration of water or alcohols.

Post-pour Processing of HFIP-based Films

The best stretching results were established by using a 2-3X stretch and testing different ratios of IPA, methanol (MeOH), and water in the bath. The results of these experiments (Table 4 and Figure 5) show that the films stretched in the 80/20 MeOH/water bath performed the best with an average energy to break more than twice that of the other films.

To examine the stretch factor on films, the 80/20 MeOH/water solution was used to determine mechanical changes in a range of stretching ratios (Figure 6). As the stretch factor increased, stress increased up to a maximum of 210 MPa, while strain

Table 4. Mechanical properties of films with average deviations after post-pour stretch using set ratios of IPA, MeOH, and water

Dope Composition + Stretch Solutions with Stretch Ratio	Average Energy to Break (MJ/m^3)	Average Ultimate Stress (MPa)	Average Ultimate Strain (mm/mm)
MaSp1 with GTA + MeOH 2 X	18.65 ± 8.95	109.61 ± 8.69	0.204 ± 0.1
MaSp1 with GTA + 50/50 IPA/water 3 X	23.14 ± 5.7	102.91 ± 12.44	0.258 ± 0.06
MaSp1 with GTA + 80/20 MeOH/water 2 X	25.8 ± 9.61	112.69 ± 15.03	0.257 ± 0.08
MaSp1 with GTA + 80/20 MeOH/water 2.75 X	**42.1 ± 9.76**	**189.39 ± 17.25**	**0.281 ± 0.05**
MaSp1 + 50/50 MeOH/IPA 2 X	23.58 ± 12.31	75.59 ± 17.66	0.334 ± 0.12
MaSp1 + 80/20 MeOH/water 2X	14.19 ± 8.57	117.4 ± 14.08	0.137 ± 0.06

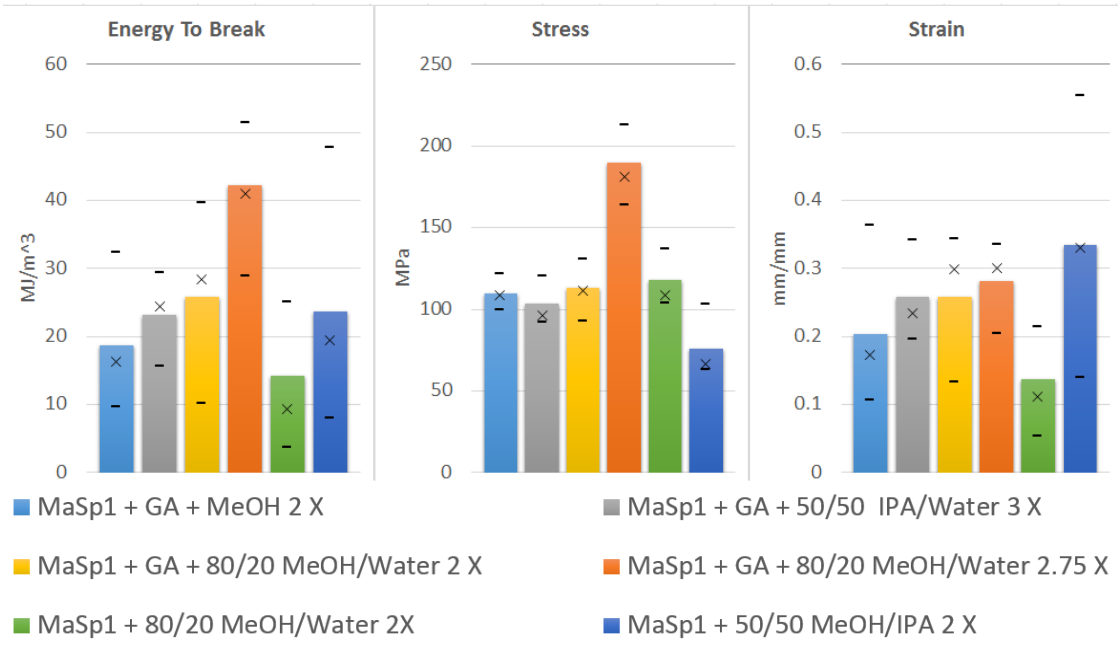

Figure 5: Bar graphs for stretched films showing average stress, strain, and energy to break with x being the median and the dashes representing minimum and maximum.

decreased by at least 25% with each incremental step. With an increased stretch factor the stress-strain graph changes, the yield strength increases, and the slope following that point increases. The films with 2.5 X stretch show a yield behavior with slight strain hardening, and the films with 2.75 and 3.25X stretch factor show strain hardening and no yielding directly after the initial jump in stress. This shows that the films can be tailored to different applications, with only a change in stretch factor.

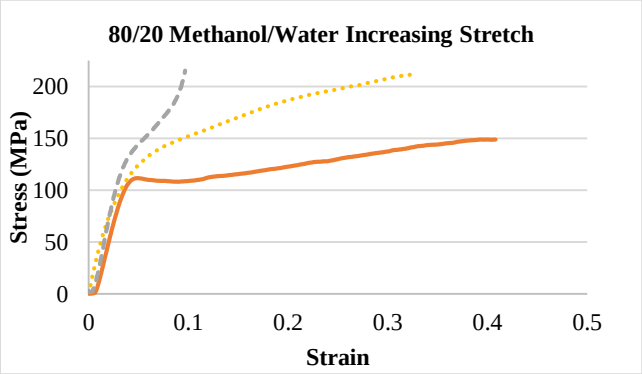

Figure 6: Select stress-strain graphs of MaSp1 samples with GTA films to illustrate the difference in stress and strain with a given stretch factor using 80/20 MeOH/Water as a stretch bath. With the following legend: 2.5X stretch (solid line), 2.75X stretch (dotted line), and 3.25X stretch (dashed line).

Previous research on mechanical properties of gelatin films has revealed that GTA can increase crosslinking of protein, which increases mechanical properties, primarily stress.[58,59] Preliminary testing showed that the spider silk films with GTA had higher strain but lower stress (Table 2). After this discovery, GTA was used in the dope for all post-pour stretched films. This produced an increase in both stress and strain and also increased consistency (Table 4). Testing showed that the GTA only helps after the films dry for a full day prior to post-pour treatment.

After establishing processing procedures, MaSp2 dopes were also made, as well as MaSp2/MaSp1 combination dopes. The resulting films were processed using 80/20 MeOH/Water and 2.5X stretch with GTA in the dope (Figure 7). There was no significant difference in stress or strain between the films that contained mixed proteins, with an average ultimate stress at 139 MPa and ultimate strain at 29.7%. The MaSp1 protein films had the highest stress (182 MPa) and the MaSp2 protein films the highest strain (33%).

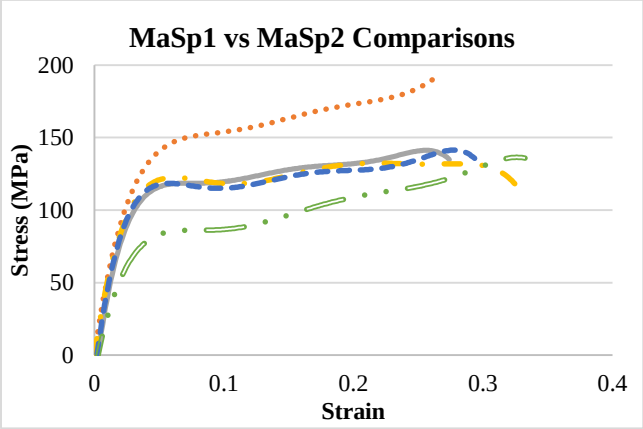

Figure 7: Stress-strain graphs comparing films composed of MaSp1, MaSp2, or a mixture of MaSp1/MaSp2; all samples received the same post-pour treatment. With the following legend: MaSp1 (dashed line), 75/25 MaSp1/MaSp2 (solid line), 50/50 MaSp1/MaSp2 (single dotted dashed line), 25/75 MaSp1/MaSp2 (dashed line) and MaSp2 (double dotted dashed line).

Post-pour Processing of Aqueous-based Films

With established procedures for post-pour stretching of HFIP-based films, the water-based films were then stretched to increase mechanical properties. The primary difference in making the change to water-based films was that they needed to soak in the stretch bath for 2 minutes instead of 30 seconds for the HFIP-based films.

Following the preliminary testing of the dope compositions, films (both MaSp1 and 80/20 MaSp1/MaSp2) with 0.1% formic acid and 0.05% GTA were stretched in a combination of water and alcohol resulting in the highest energy to break (62 MJ/m^3) for recombinant silk protein films (Table 5 and Figure 8). The results of mechanical testing also demonstrate that 80/20 (w/w) MaSp1/MaSp2 films treated in 80/20 (v/v) MeOH/water yield the highest stress with a lower stretch ratio. Using this treatment, films cannot be stretched past 2.7 X without breaking. Treating 80/20 (w/w) MaSp1/MaSp2 films in 50/50 (v/v) IPA/water increases the energy to break with a 39% strain and moderate (177 MPa) stress. With a higher stretch ratio and using the described treatment, films can be post-pour stretched up to 3.2 X their original length, although stretching past 3 X results in reduced strain.

Table 5: Mechanical properties of films with average deviations after post-pour stretch using set ratios of IPA, MeOH, and water

Material + Stretch Solutions with Stretch Ratio	Average Energy to Break (MJ/m^3)	Average Ultimate Stress (MPa)	Average Ultimate Strain (mm/mm)
MaSp1 + 50/50 IPA/Water 2.5X	30.44 ± 3.55	136.66 ± 2.06	0.253 ± 0.02
MaSp1 + 80/20 MeOH/Water 2.5X	40.6 ± 3.34	149.42 ± 7.27	0.335 ± 0.02
80/20 MaSp1/MaSp2 + 80/20 MeOH/Water 2.5X	40.58 ± 10.9	168.35 ± 20.76	0.307 ± 0.1
80/20 MaSp1/MaSp2 + 80/20 MeOH/Water 2.7X	47.06 ± 3.08	**206.81 ± 3**	0.289 ± 0.02
80/20 MaSp1/MaSp2 + 50/50 IPA/Water 3X	**52.36 ± 8.02**	183.92 ± 14.85	**0.354 ± 0.07**
80/20 MaSp1/MaSp2 + 50/50 IPA/Water 3.2X	34.58 ± 10.7	177.56 ± 3.57	0.239 ± 0.07

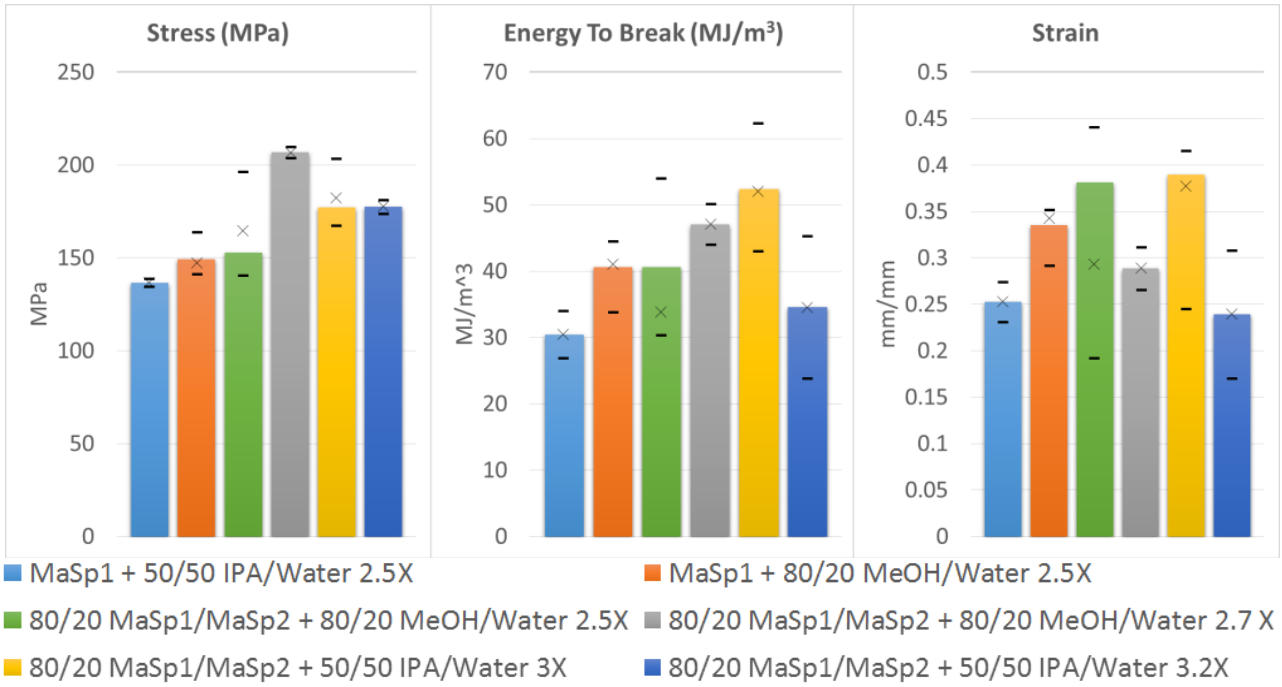

Figure 8: Histogram of the mechanical properties for stretched films showing average stress, strain, and energy to break where bar height represents the average value, and x the median with dashes representing maximum and minimum values.

The surface of the MaSp1 films were imaged using a scanning electron microscope (SEM), showing that the film after stretching remains smooth (Figure 9). It also shows that the cut edge of the film may be porous or damaged due to cutting. This is not a desirable feature, but the films need to be cut to remove the thick edges. Using these SEM images we also verified that the thickness measurements are accurate and reliable (Figure 9).

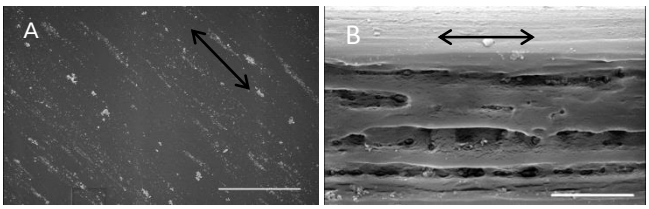

Figure 9: SEM image of the surface (A) and cut edge (B) of stretched MaSp1 films after 80/20 MeOH/water 2.5X stretch. Arrow indicates stretch direction. Scale bars: A. 30 µm, B. 12 µm.

Characterization of HFIP-based Films

MaSp1 and MaSp2 films processed using 80/20 MeOH/water stretch bath, and stretched to 2.5 X, were also characterized using XRD, the images show an increase in beta-sheet content and alignment (Figure 3B and S3B) from the films that were not stretched (Figure 3A and S3A). Wide-angle X-ray diffraction of the films yields nano-crystalline Bragg reflections and an amorphous halo. The XRD pattern shows that the crystalline structure within the stretched films is also aligned parallel to the stretch direction, with calculated Herman's orientation factors, fc, of 0.858 for MaSp1 and 0.838 for MaSp2, determined from azimuthal broadening of the equatorial reflections where f_c is calculated (equation 2) from the angle, ϕ, between the longest axis and the fiber axis.

$$f_c = \frac{3cos^2\varphi - 1}{2} \quad (2)$$

Radial integration along the equator gives the peak positions and widths of the (200) and (120) reflections which are used to calculate the a and *b* axes of the unit cell and nanocrystal dimensions. Along the meridian, the (002) reflection gives the information concerning of the *c*-axis of the unit cell. Spider silk proteins have been shown to form orthorhombic unit cells and the unit cell dimensions calculated from the peak positions of wide angle X-ray diffraction WAXD reflections are calculated from equation 3 where *d* is the peak position in d spacing and *hkl* are the Miller index notation:[60]

$$\frac{1}{d^2} = \frac{h^2}{a^2} + \frac{k^2}{b^2} + \frac{l^2}{c^2} \quad (3)$$

Radial integration along the equator (Figure 3C) and meridian were fit to Gaussian peaks and the peak positions were converted to inverse space following Bragg's Law to calculate unit cell dimensions. Average crystallite size in each dimension is calculated from the radial broadening in 2θ space using Scherrer's formula and these results are shown in Table 6.[61]

Table 6: Unit cell and crystallite dimensions calculated from WAXD.

Material	Unit cell (Å)	Crystallite (nm)
MaSp1 post-stretch film	6.90 x 9.73 x 10.50	0.80 x 3.18 x 9.99
MaSp2 post-stretch film	6.75 x 9.87 x 10.03	0.74 x 3.11 x 24.7

The crystallinity, x_c, can be estimated by radial integration of the equatorial reflections (Equation 4) which are the crystalline peaks due to Bragg diffraction relative to the full integrated peak area yielding 47.3% and 48.2% crystallinity for MaSp1 and MaSp2, respectively.[62]

$$x_c = \frac{Integrated\ equatorial\ reflections}{Full\ radial\ integration} \quad (4)$$

^{13}C solid-state NMR data collected on MaSp1 and MaSp2 films is presented in Figure 10 and the information is used to track molecular-level structural changes during the course of film production. Chemical shifts for relevant amino acids alanine, glycine, serine, proline and glutamine are indicated with dotted lines, and red arrows are used to emphasize changes to silk secondary structure during film production. For both MaSp1 and MaSp2 samples, the film progress is tracked from top to bottom; purified protein powder (10A, 10D) is solubilized in HFIP and casted as a film in PDMS wells (10B,

10E). As-poured films were then stretched 2.5X in a bath of 80/20 MeOH/water (10C, 10F). In both cases initially, the alanine-rich regions within the purified MaSp1 or MaSp2 protein powders exist primarily in a beta-sheet conformation. This is expected; the purified protein is not water soluble, presumably because of the polyalanine beta-sheet aggregates. HFIP is commonly used to solubilize large silk-like proteins because of its ability to disrupt insoluble beta-sheets and stabilize alpha-helical secondary structures.[63,64] Our NMR data indeed shows a dramatic transformation of polyalanine regions into an alpha-helical conformation for films cast from HFIP silk dopes. This is evident in the characteristic downfield and upfield shifts of Ala C_α and Ala C_β resonances, respectively, as illustrated by the outward pointing red arrows. While the majority of volatile HFIP solvent is removed via evaporation, the ^{13}C resonance near 70 ppm is attributed to residual HFIP that remains bound to the silk protein backbone. NMR data shows a transformation of polyalanine regions from helical back to beta-sheet structures when as-poured films are stretched in 80/20 MeOH/water; again this is highlighted by inward-pointing red arrows. In the case of the MaSp2 sample where serine, which is often contiguous to the polyalanine regions, is well represented, we notice a similar trend. HFIP solubilization encourages a helical structure, but a significant fraction of serine residues are driven into a beta-sheet conformation upon stretching. This structural transformation is also correlated with the loss of the HFIP resonance near 70 ppm, indicating that the helical-stabilizing organic solvent is driven away from the silk protein during the stretching procedure. NMR data therefore strongly suggests that alanine-rich repeat motifs from both MaSp1 and MaSp2 films form beta-sheet

nanocrystalline structures. This is in line with WAXD results that indicate both beta-sheet formation and axial alignment upon stretching the films in alcohol/water baths.

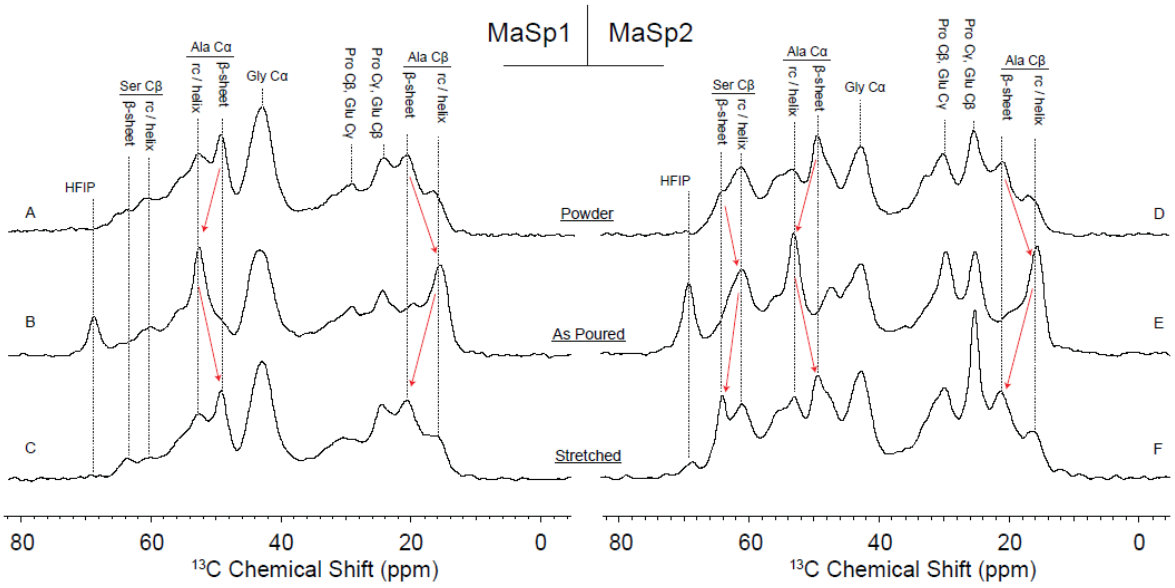

Figure 10: ^{1}H - ^{13}C CP-MAS spectra of MaSp1 films (left) and MaSp2 films (right) in various stages of production. Some resonances from dominant amino acids glycine, alanine, serine, proline and glutamine are highlighted with dotted lines, and protein secondary structure is indicated when appropriate. Red arrows are used to emphasize structural changes occurring during production. From top to bottom: Purified protein powder (A, D), as-poured films from solubilized protein in HFIP (B, E), and films stretched in 80/20 MeOH/water (C, F).

Multidimensional NMR would be necessary to extract precise chemical shifts for proline and glutamine residues, thus a complete characterization of GPGXX motifs in MaSp2 films is not possible. However, the collective chemical shifts of Pro C_γ / Glu C_β and Pro C_β / Glu C_γ at 25 and 30 ppm, respectively, are very consistent with natural dragline spider silk samples. NMR experiments on the MaSp2-rich Argiope aurantia spider dragline silk found that GPGXX motifs from MaSp2 protein exist in elastin-like type II beta-turn structures.[23] It is therefore likely that MaSp2 films share this structure. The resonance at 25 ppm from GPGXX regions also shows a narrowed line shape in

stretched MaSp2 films as compared to the protein powder and the as-poured film. This observation suggests that stretched films contain a more uniform distribution of chemical shift and therefore less heterogeneity in the distribution of molecular environments. This is consistent with XRD data that show an increase in molecular orientation upon stretching. It is concluded that the act of film stretching in alcohol/water baths not only drives out HFIP and induces beta-sheet formation of alanine-rich regions, but also improves alignment and regularity of both beta-sheet nanocrystals and elastin-like GPGXX structures.

Raman spectroscopy characterization was also done on the spider silk powder, untreated films and post-pour stretched films (Figure 11). This illustrates the secondary structure changes taking place as the MaSp1 and MaSp2 films are being processed. The powder consists primarily of beta-sheet and little helical conformation (11A, 11D). After solubilizing and pouring, the film switches to a helical conformation with little beta-sheet content (11B, 11E). After the stretch bath and subsequent stretching the film reverts back to a beta-sheet conformation bringing it full circle (11C, 11F). This increased beta-sheet content along with the alignment that occurs with stretching increases the energy to break over 20 times from the unprocessed films. Previous studies have shown β-sheet contributions at 1670 cm^{-1} and helical peaks at 1656 cm^{-1} and assigned unordered peaks near 1640 cm^{-1}. Figure 11B and 11E both appear to show an increased peak amplitude near 1656 cm^{-1} which further confirms the conversion of β-sheet secondary structure to helical and back.[27]

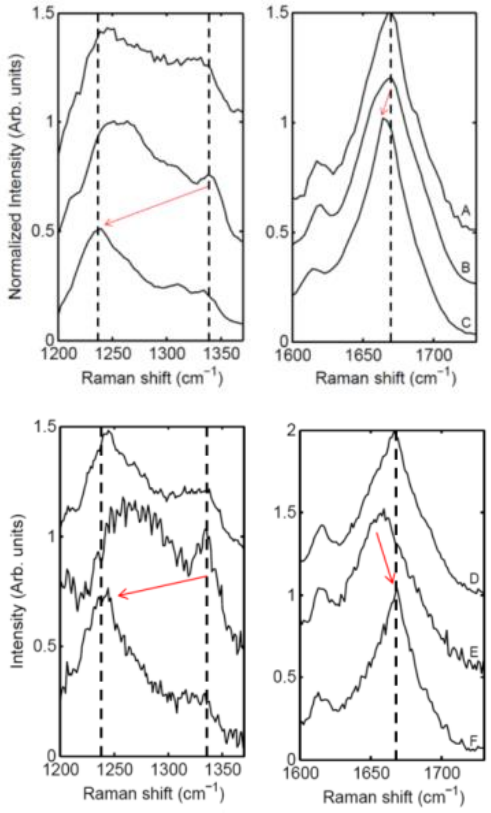

Figure 11: Raman spectra of the progression of MaSp1 films (top) and MaSp2 films (bottom) in the amide III and amide I regions. Red arrows are used to emphasize structural changes occurring during production. From top to bottom: Purified protein powder (A, D), as-poured films from solubilized protein in HFIP (B, E), and films stretched in 80/20 MeOH/water (C, F).

Characterization of Aqueous-based Films

Wide-angle X-ray diffraction of the films yields nano-crystalline Bragg reflections and an amorphous halo. The XRD pattern shows that the crystalline structure within the stretched films is also aligned parallel to the stretch direction, with a calculated Herman's orientation factor, fc, of 0.823 for MaSp1, determined from azimuthal

broadening of the equatorial reflections where f_c is calculated (as previously explained) from the angle, ϕ, between the longest axis and the fiber axis (Figure 4).

The *a* and *b* axes of the unit cell and nanocrystal dimensions were calculated as described previously. Radial integration along the equator (Figure 4C) and meridian were fit to Gaussian peaks and the peak positions were converted to inverse space following Bragg's Law to calculate unit cell dimensions. Average crystallite size in each dimension is calculated as outlined previously, results are shown in Table 7 [61]

Table 7: Unit cell and crystallite dimensions calculated from WAXD.

Material	Unit cell (Å)	Crystallite (nm)
MaSp1 post-stretch film	6.92 x 8.86 x 11.37	1.93 x 3.34 x 7.86

The crystallinity, x_c, can be estimated by radial integration of the equatorial reflections which are the crystalline peaks due to Bragg diffraction relative to the full integrated peak area as shown previously, yielding 48.8% crystallinity for MaSp1.[62]

The molecular protein structure of the films also was tracked through successive stages of film production using ^{1}H – ^{13}C CP-magic angle spinning (MAS) NMR (Figure 12). ^{13}C chemical shifts are very sensitive to protein secondary structure, and can therefore be utilized to monitor structural changes throughout film production. Chemical shifts that arise from alanine Cα and Cβ in either a beta-sheet or helical/random coil conformation are indicated with dotted lines in Figure 12. The films are essentially produced from powder to final product; initial MaSp1 protein powder

(12A) is solubilized into an aqueous-based silk dope, which is cast as an as-poured film (12B). The poured films are then submerged in a bath of 80/20 MeOH/water and stretched 2.5X (12C). The data shows that the purified MaSp1 protein powder (12A) is dominated by alanine in a beta-sheet conformation. When the silk protein is solubilized and cast into films, the data reveals that alanine originally in a beta-sheet conformation is partially converted to helical or random-coil structures. Similar to HFIP solubilization, it appears that dissolution of silk protein in an aqueous medium is correlated with a decrease in alanine adopting a beta-sheet structure (12B). However, the more stable beta-sheet structure is recovered when the as-poured films are stretched in 80/20 MeOH/water (12C). These results are consistent with trends observed for HFIP-based films with the exception that there is no HFIP peak in aqueous films. This would lead us to believe that we are essentially creating the same films using a water-based dope vs. HFIP, lowering the cost of materials, improving biocompatibility and improving the environmentally friendly aspect of this biomaterial.

Raman spectroscopy characterization was also done on the spider silk powder, untreated films and post stretch films (Figure 13). These results confirm the previous findings of NMR that the powder consists primarily of beta-sheet and little helical conformation, after solubilization and pouring, the film converts to a helical conformation with little beta-sheet content, and after stretch bath and subsequent stretching the film reverts back to beta-sheet content. These results are also similar to those found previously.

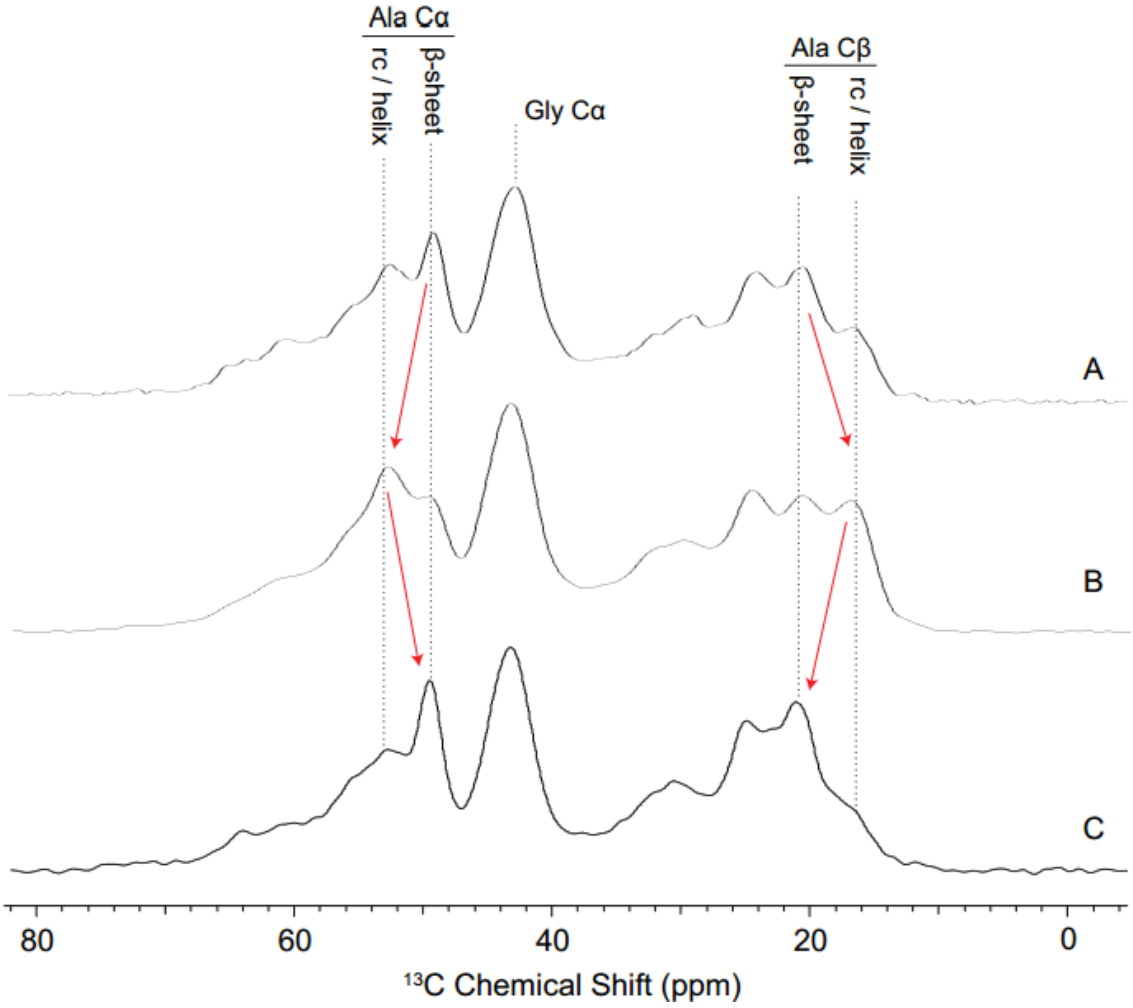

Figure 12: ^{1}H-^{13}C CP-MAS spectra of MaSp1 films in various stages of production. Resonances for alanine and glycine residues are highlighted with dotted lines, and protein secondary structure is indicated when appropriate. The data suggests that the MaSp1 starting material (A) originally contains a significant β-sheet component. The protein is then solubilized in an aqueous-based silk dope, where the β-sheet fraction is expected to have decreased during solubilization. Films poured from this dope indeed show a decrease in β-sheet content (B). β-sheet content is clearly recovered upon stretching of the as-poured films in 80/20 MeOH/H2O (C).

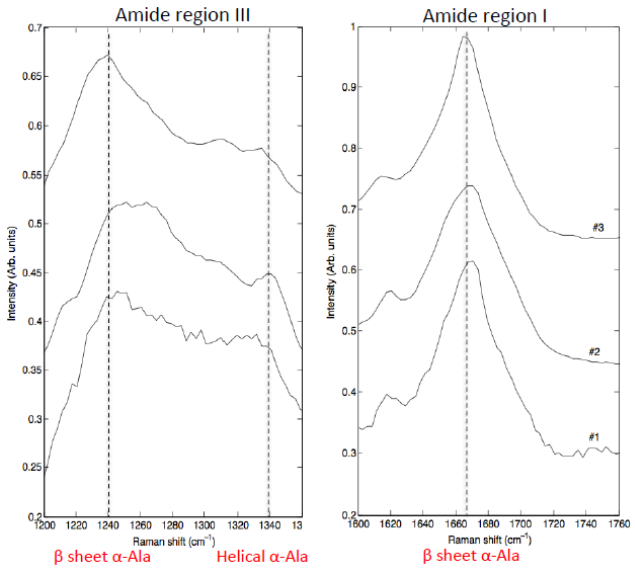

Figure 13: Raman spectra of the progression of MaSp1 films in the amide III and amide I regions. From top to bottom: Purified protein powder (1), as-poured films from solubilized protein (2), and films stretched in 80/20 MeOH/water (3).

Functionalization of Films

As proof of concept, to show the potential for these spider silk films in medical applications, two water-based films and two HFIP films were produced, the first of the two contain kanamycin in the dope and the second contain no additives. The films were placed on an agar plate that had been seeded with XL-1 Blue cells (Figure 14). Both HFIP- and water-based films containing kanamycin generated a zone of inhibition on the bacterial lawn. Water-based films without kanamycin produced no zone of inhibition; however the HFIP-based film without kanamycin produced a narrow zone of inhibition, demonstrating that there is a cytological effect (residual HFIP) (Figure 14) preventing growth of cells.[65]

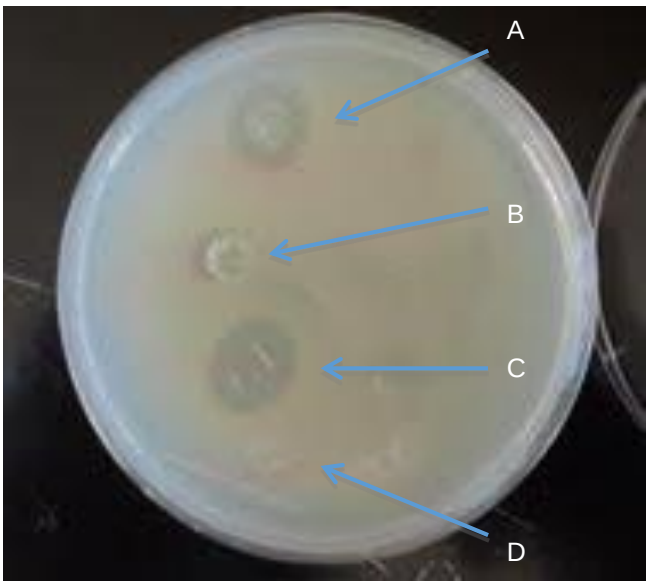

Figure 14: Zone of inhibition of films with and without kanamycin. HFIP-based film with kanamycin (A), HFIP-based film (B), water-based film with kanamycin (C), and water-based film (D).

DISCUSSION

These results show that rSSp films can be formed after dissolving them in a water or HFIP solution. The mechanical properties of as-poured films from both are similar, with the addition of formic acid increasing stress. It is clear that post-pour processing of films greatly increases the mechanical properties; these mechanical properties can be tuned to each application using a combination of dope formulation, stretch baths, and stretch ratios. The addition of GTA to the dope before pouring also increases strain in films processed in 80/20 MeOH/Water without a significant change in secondary structure suggesting that GTA may induce crosslinking between proteins. Changing the processing conditions, such as stretch baths and stretch ratios, changes the conformation of the silk protein, making the secondary structure tunable for

commercial applications. The rSSp powder is initially in a beta-sheet conformation, after dissolving in HFIP or water and pouring the protein takes a mainly random alpha-helical conformation, after post-pour stretching the protein reverts to a beta-sheet rich conformation aligned in the stretch direction which has been confirmed by a combination of WAXD, Raman, and NMR.

Table 8: Comparison of the mechanical properties of silk, collagen, and other materials[43].

Material	Form	Ultimate Stress (MPA)	Ultimate Strain (%)	References
Spider silk (dragline)	fiber	4000	35	[1]
HFIP-based Recombinant spider silk	**film**	**189**	**28**	**This study**
Water-based Recombinant spider silk	**film**	**206**	**29**	**This study**
Other Recombinant spider silk	film	54	1.8	[41]
B. mori silk fibroin	Ultrathin films	100	0.5-3.0	[43]
Collagen X-linked	film	47–72	12–16	[59]
Polylactic acid (PLA)	sheet	28–50	2–6	[43,66]
PMMA	plate	55–76	2–5	[66]

The results of these experiments also are the highest published stress and strain of any recombinant spider or silkworm silk films (Table 8), making it a strong candidate for use in a variety of products. Spider silk is a biocompatible[67] and biodegradable[41] material suitable for use in multifunctional biomaterials. The comparison of MaSp1 and

2 films also shows that with despite similar alignment and processing, the MaSp2 films do not perform as well as MaSp1 films.

The use of water instead of HFIP in the dope construct for film formation has the potential to change the processing of spider silk products due to its low cost of production and significant lowering of toxicity to the environment and people. We have been able to produce a water-based film that is similar in structure and mechanical abilities to HFIP based films, which makes the water based films even more valuable.

Thus, aqueous derived rSSP films reduce the cost of production, the toxic impact on the environment and improves biocompatibility over similar HFIP derived films. Due to the aqueous nature of the dopes, further functionalization may be more possible with aqueous films than with HFIP or other organic solvent derived rSSP materials. HFIP solvates rSSP by converting the tight beta-sheet structures to helical or random coil structures, negating the possibility of functionalizing the rSSP with protein therapeutics as they could also be denatured.

CONCLUSION

It has been shown that films produced from an aqueous dope have similar structure to those created by an HFIP dope, producing essentially the same film with a lower cost and impact on the environment. Maximum stress values of over 200 MPa were observed in processed films with a maximum energy to break over 60 MJ/m^3, and maximum strain over 40%. These values are the highest mechanical properties reported on materials used as a scaffold for cell growth (Table 8), with a stress at least double

that of all others. As well, films generated from rSSP solvated in water matched or out performed those same proteins when solvated with HFIP.

SUPPORTING INFORMATION

AFM images of MaSp2 films comparing drying methods, mechanical properties of vapor treated films, and XRD images of as-poured (water and HFIP-based) and stretched MaSp2 films (Only HFIP-based). This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

1. Lewis, R. V. *Chem. Rev.* **2006**, *106*, 3762–3774.

2. Teulé, F.; Addison, B.; Cooper, A. R.; Ayon, J.; Henning, R. W.; Benmore, C. J.; Holland, G. P.; Yarger, J. L.; Lewis, R. V. *Biopolymers* **2012**, *97*, 418–431.

3. An, B.; Jenkins, J. E.; Sampath, S.; Holland, G. P.; Hinman, M.; Yarger, J. L.; Lewis, R. *Biomacromolecules* **2012**, *13*, 3938–3948.

4. Xia, X.-X.; Qian, Z.-G.; Ki, C. S.; Park, Y. H.; Kaplan, D. L.; Lee, S. Y. *P.N.A.S.* **2010**, 107, 14059–14063.

5. Holland, G. P.; Creager, M. S.; Jenkins, J. E.; Lewis, R. V.; Yarger, J. L. *J. Am. Chem. Soc.* **2008**, *130*, 9871–9877.

6. Hayashi, C. Y.; Shipley, N. H.; Lewis, R. V. *Int. J. Biol. Macromol.* **1999**, *24*, 271–275.

7. Minoura, N.; Aiba, S.-I.; Gotoh, Y.; Tsukada, M.; Imai, Y. *J. Biomed. Mater. Res.* **1995**, *29*, 1215–1221.

8. Morgan, A. W.; Roskov, K. E.; Lin-Gibson, S.; Kaplan, D. L.; Becker, M. L.; Simon Jr., C. G. *Biomaterials* **2008**, *29*, 2556–2563.

9. Sofia, S.; McCarthy, M. B.; Gronowicz, G.; Kaplan, D. L. *J. Biomed. Mater. Res.* **2001**, *54*, 139–148.

10. Baoyong, L.; Jian, Z.; Denglong, C.; Min, L. *Burns* **2010**, *36*, 891–896.

11. Teulé, F.; Cooper, A. R.; Furin, W. A.; Bittencourt, D.; Rech, E. L.; Brooks, A.; Lewis, R. V. *Nat. Protoc.* **2009**, *4*, 341–355.

12. Albertson, A. E.; Teulé, F.; Weber, W.; Yarger, J. L.; Lewis, R. V. *J. Mech. Behav. Biomed. Mater.* **2014**, *29*, 225–234.

13. Hinman, M. B.; Lewis, R. V. *J. Biol. Chem.* **1992**, *267*, 19320–19324.

14. Xu, M.; Lewis, R. V. *Proc. Natl. Acad. Sci.* **1990**, *87*, 7120–7124.

15. Vollrath, F.; Knight, D. P. *Nature* **2001**, *410*, 541–548.

16. Xu, L.; Rainey, J. K.; Meng, Q.; Liu, X.-Q. *PLoS ONE* **2012**, *7*, e50227.

17. Jin, H.-J.; Kaplan, D. L. *Nature* **2003**, *424*, 1057–1061.

18. Chen, X.; Knight, D. P.; Vollrath, F. *Biomacromolecules* **2002**, *3*, 644–648.

19. Simmons, A.; Ray, E.; Jelinski, L. W. *Macromolecules* **1994**, *27*, 5235–5237.

20. Parkhe, A. D.; Seeley, S. K.; Gardner, K.; Thompson, L.; Lewis, R. V. *J. Mol. Recognit.* **1997**, *10*, 1–6.

21. Kümmerlen, J.; van Beek, J. D.; Vollrath, F.; Meier, B. H. *Macromolecules* **1996**, *29*, 2920–2928.

22. Jenkins, J. E.; Creager, M. S.; Lewis, R. V.; Holland, G. P.; Yarger, J. L. *Biomacromolecules* **2010**, *11*, 192–200.

23. Jenkins, J. E.; Creager, M. S.; Butler, E. B.; Lewis, R. V.; Yarger, J. L.; Holland, G. P. *Chem. Commun.* **2010**, *46*, 6714–6716.

24. Hronska, M.; van Beek, J. D.; Williamson, P. T. F.; Vollrath, F.; Meier, B. H. *Biomacromolecules* **2004**, *5*, 834–839.

25. Holland, G. P.; Jenkins, J. E.; Creager, M. S.; Lewis, R. V.; Yarger, J. L. *Biomacromolecules* **2008**, *9*, 651–657.

26. Hijirida, D. H.; Do, K. G.; Michal, C.; Wong, S.; Zax, D.; Jelinski, L. W. *Biophys. J.* **1996**, *71*, 3442–3447.

27. Lefèvre, T.; Rousseau, M.-E.; Pézolet, M. *Biophys. J.* **2007**, *92*, 2885–2895.

28. Lefèvre, T.; Paquet-Mercier, F.; Rioux-Dubé, J.-F.; Pézolet, M. *Biopolymers* **2012**, *97*, 322–336.

29. Riekel, C.; Bränden, C.; Craig, C.; Ferrero, C.; Heidelbach, F.; Müller, M. *Int. J. Biol. Macromol.* **1999**, *24*, 179–186.

30. Sampath, S.; Isdebski, T.; Jenkins, J. E.; Ayon, J. V.; Henning, R. W.; Orgel, J. P. R. O.; Antipoa, O.; Yarger, J. L. *Soft Matter* **2012**, *8*, 6713–6722.

31. Riekel, C.; Madsen, B.; Knight, D.; Vollrath, F. *Biomacromolecules* **2000**, *1*, 622–626.

32. Riekel, C.; Vollrath, F. *Int. J. Biol. Macromol.* **2001**, *29*, 203–210.

33. Hinman, M. B.; Jones, J. A.; Lewis, R. V. *Trends Biotechnol.* **2000**, *18*, 374–379.

34. Wohlrab, S.; Mueller, S.; Schmidt, A.; Neubauer, S.; Kessler, H.; Leal-Egana, A.; Scheibel, T. *Biomaterials* **2012**, *33*, 6650–6659.

35. Gomes, S.; Gallego-Llamas, J.; Leonor, I. B.; Mano, J. F.; Reis, R. L.; Kaplan, D. L. *Macromol. Biosci.* **2013**, *13*, 444–454.

36. Gomes, S.; Leonor, I. B.; Mano, J. F.; Reis, R. L.; Kaplan, D. L. *Biomaterials* **2011**, *32*, 4255–4266.

37. Spiess, K.; Wohlrab, S.; Scheibel, T. *Soft Matter* **2010**, *6*, 4168–4174.

38. Huemmerich, D.; Slotta, U.; Scheibel, T. *Appl. Phys. A* **2006**, *82*, 219–222.

39. Rabotyagova, O. S.; Cebe, P.; Kaplan, D. L. *Biomacromolecules* **2009**, *10*, 229–236.

40. Rabotyagova, O. S.; Cebe, P.; Kaplan, D. L. *Macromol. Biosci.* **2010**, *10*, 49–59.

41. Hardy, J. G.; Leal-Egaña, A.; Scheibel, T. R. *Macromol. Biosci.* **2013**, *13*, 1431–1437.

42. Krishnaji, S. T.; Bratzel, G.; Kinahan, M. E.; Kluge, J. A.; Staii, C.; Wong, J. Y.; Buehler, M. J.; Kaplan, D. L. *Adv. Funct. Mater.* **2013**, *23*, 241–253.

43. Jiang, C.; Wang, X.; Gunawidjaja, R.; Lin, Y.-H.; Gupta, M. K.; Kaplan, D. L.; Naik, R. R.; Tsukruk, V. V. *Adv. Funct. Mater.* **2007**, *17*, 2229–2237.

44. Bini, E.; Foo, C. W. P.; Huang, J.; Karageorgiou, V.; Kitchel, B.; Kaplan, D. L. *Biomacromolecules* **2006**, *7*, 3139–3145.

45. Bettinger, C. J. *Int. J. Polym.* **2010**, 59, 563–567.

46. Kim, D.-H.; Viventi, J.; Amsden, J. J.; Xiao, J.; Vigeland, L.; Kim, Y.-S.; Blanco, J. A.; Panilaitis, B.; Frechette, E. S.; Contreras, D.; Kaplan, D. L.; Omenetto, F. G.; Huang, Y.; Hwang, K.-C.; Zakin, M. R.; Litt, B.; Rogers, J. A. *Nat. Mater.* **2010**, 9, 511–517.

47. Wendt, H.; Hillmer, A.; Reimers, K.; Kuhbier, J. W.; Schäfer-Nolte, F.; Allmeling, C.; Kasper, C.; Vogt, P. M. *PLoS ONE* **2011**, *6*, e21833.

48. Vendrely, C.; Scheibel, T. *Macromol. Biosci.* **2007**, *7*, 401–409.

49. Slotta, U.; Tammer, M.; Kremer, F.; Koelsch, P.; Scheibel, T. *Supramol. Chem.* **2006**, *18*, 465–471.

50. Huemmerich, D.; Slotta, U.; Scheibel, T. *Appl. Phys. -Mater. Sci. Process.* **2006**, *82*, 219–222.

51. Krishnaji, S. T.; Huang, W.; Rabotyagova, O.; Kharlampieva, E.; Choi, I.; Tsukruk, V. V.; Naik, R.; Cebe, P.; Kaplan, D. L. *Langmuir* **2011**, *27*, 1000–1008.

52. Hardy, J. G.; Leal-Egaña, A.; Scheibel, T. R. *Macromol. Biosci.* **2013**, *13*, 1431–1437.

53. Yue, H.; N, K. C.; Carl, T. Recovery of biofilament proteins from biological fluids. WO2003057720 A2, July 17, 2003.

54. Renugopalakrishnan, V.; Lewis, R. V. *Bionanotechnology proteins to nanodevices*; Springer: Dordrecht, 2006.

55. Heim, M.; Keerl, D.; Scheibel, T. *Angew. Chem. Int. Ed.* **2009**, *48*, 3584–3596.

56. Ko, F. K.; Jovicic, J. *Biomacromolecules* **2004**, *5*, 780–785.

57. Hayashi, C. Y.; Blackledge, T. A.; Lewis, R. V. *Mol. Biol. Evol.* **2004**, *21*, 1950–1959.

58. Bigi, A.; Cojazzi, G.; Panzavolta, S.; Rubini, K.; Roveri, N. *Biomaterials* **2001**, *22*, 763–768.

59. Mallika, P.; Himabindu, A.; Shailaja, D. *J. Appl. Polym. Sci.* **2006**, *101*, 63–69.

60. Warwicker, J. O. *J. Mol. Biol.* **1960**, *2*, 350–IN1.

61. Cullity, B. In *Elements of X-ray Diffraction*; Addison-Wesley Publishing, 1959; ch. 3 p. 99.

62. Alexander, L. E. *X-ray diffraction methods in polymer science*; Wiley-Interscience (New York), 1969.

63. Zhao, C.; Yao, J.; Masuda, H.; Kishore, R.; Asakura, T. *Biopolymers* **2003**, *69*, 253–259.

64. Trabbic, K. A.; Yager, P. *Macromolecules* **1998**, *31*, 462–471.

65. Hardy, J. G.; Romer, L. M.; Scheibel, T. R. *Polymer* **2008**, *49*, 4309–4327.

66. Brandrup, J.; Immergut, E. H.; Grulke, E. A. *Polymer handbook, 4th edition*; Wiley: New York; Chichester, 2004.

67. Vollrath, F.; Barth, P.; Basedow, A.; Engström, W.; List, H. *Vivo Athens Greece* **2002**, *16*, 229–234.

CHAPTER 3

MORE THAN JUST FIBERS: AN AQUEOUS METHOD FOR THE PRODUCTION OF INNOVATIVE RECOMBINANT SPIDER SILK PROTEIN[1]

ABSTRACT: Spider silk is a striking and robust natural material that has an unrivaled combination of strength and elasticity. There are two major problems in creating materials from recombinant spider silk proteins (rSSps): expressing sufficient quantities of the large, highly repetitive proteins and solvating the naturally self-assembling proteins once produced. To address the second problem we have developed a method to rapidly dissolve rSSps in water in lieu of traditional organic solvents, and accomplish nearly 100% solvation and recovery of the protein. Our method involves generating pressure and temperature in a sealed vial by using short, repetitive bursts from a conventional microwave. The method is scalable and has been successful with all rSSps used to date. From these easily generated aqueous solutions of rSSps a wide variety of materials has been produced. Production of fibers, films, hydrogels, lyogels, sponges, and adhesives and studies of their mechanical and structural properties are reported. To our knowledge, ours is the only method that is cost effective and scalable for mass production. This solvation method allows a choice of the physical form of product to take advantage of spider silks' mechanical properties without using costly and problematic organic solvents.

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INTRODUCTION

A spider spins fibers from an aqueous solution.[1–3] The fibers, comprised nearly entirely of protein, have long been studied and admired for their combination of tenacity, elasticity and strength.[3–6] Given that spiders cannot be farmed for their silk due to their territorial and cannibalistic nature, substantial effort has been made to generate proteins synthetically. While generation of recombinant spider silk proteins (rSSps) has been successful in a variety of hosts, the vast majority of rSSps are sparingly soluble in water when produced.[7, 8] A spider's web does not dissolve in high humidity or rain, which lends some insight as to why these rSSps are so difficult to solubilize in an aqueous based solution. Highly aligned and ordered structures are present in the fibers that prevent them from dissolving and these structures are present to some degree in the rSSps when purified from their host organism.[9–12]

Spiders use complex biological and chemical mechanisms to produce their large (>250 kDa) spider silk proteins and maintain them in a liquid crystalline state within the gland.[13,14] The native N- and C-termini of the proteins have been shown to aid in the aqueous solubility of the proteins when produced by the spider.[15] The natural tendency is to include one or both of those sequences in the rSSps produced. However, inclusion of these sequences does not make the rSSps immediately water soluble. Rather, multiple steps for solubilization must be taken to first dissolve the protein in a chaotropic agent and then slowly remove it through dialysis.[8,16]

While dialysis based methods do work to generate largely aqueous soluble rSSps, it does not work in all cases (Lewis, unreported recombinant major ampullate Spidroin 1

(rMaSp1) and 2 (rMaSp2)) nor is it tenable at large-scale to produce commercially available products from rSSps. Dialysis bags and the like are functional at the laboratory scale due to their low volumes.[17–19] However, at production scales the processes for buffer removal induce major agitation in systems such as tangential flow filtration. Recombinant spider silk proteins are known to become viscous or precipitate, due to self-assembly as a result of increased shear which occurs naturally in the spider. As a result, buffer exchange is unlikely to work at large-scale as not all rSSps are soluble in guanidine or similar chaotropes and the loss of protein from multiple manipulations or precipitation during buffer exchange and centrifugation can be substantial.[20,21]

In absence of a viable large-scale method, there is a distinct need for solvating rSSps in aqueous based solutions. We report here a method for taking traditionally water insoluble rSSps and solvating them in a quick, one step process with nearly 100% solvation and recovery in water without degrading the protein (Figure S1). From these solutions fibers, films, gels, sponges, adhesives, foams, and coatings have been produced (Figure 1) and analyzed as reported below. These diverse materials have been studied for their mechanical abilities, ability to be functionalized and in appropriate cases, their protein structure.

MATERIALS AND METHODS

Protein Purification

As previously reported rMaSp1 and rMaSp2 proteins were purified from the milk of transgenic goats through tangential flow filtration, precipitation, and washing.[25]

Solubilization

A specific dope formulation and volume was placed in a glass vial (Wheaton), the mixture was sonicated at a power setting of 3 W for one minute. This suspension was then sealed and heated in a 700 W Magic Chef® microwave oven in 5-second intervals. This process was repeated until the protein was completely solubilized and the cap temperature was greater than or equal to 130 °C, which was determined using a Fluke 561 handheld infrared thermometer.

Fiber Formation

Solutions of 12% (w/v) 50/50, 80/20, and 90/10 rMaSp1/rMaSp2, respectively, with 90/10 containing 0.1% propionic acid, 10mM imidazole were solubilized. After solubilization, the solution was removed and centrifuged at 18,000 rcf for 3-minutes. The supernatant was then removed and loaded into a 3 mL glass syringe with a luer-lock (Grainger). Two needles (0.178 mm internal diameter made from PEEK tubing) are attached to the syringe through a luer-lock. The syringe and needle configuration is then loaded into a custom wet spinning extrusion device. The apparatus has controllable three godet systems that are controlled independently allowing for two separate stretches to be applied to the fiber and a fiber accumulating winder. The spinning apparatus is equipped with three baths. The first bath (bath) is filled with 100% isopropanol (IPA), the second bath (1st stretch) contains an 80/20 mixture of IPA/water and the third bath (2nd stretch) contains a 20/80 mixture of IPA/water. The fiber is formed by extruding the protein solution into the coagulation bath (IPA) at a

controlled rate. The fibers were pulled from the coagulation bath and strung through the godets, submerged in the two stretch baths, then onto the winder. Varied stretch ratios of 2.0X/2.0X, 2.5X/2.0X, 2.5X/2.5X were accumulated at unique positions along the winding drum.

Film Formation

A 3.5% (w/v) 80/20 rMaSp1/rMaSp2 protein was dissolved in water with 0.1% formic acid (Alfa Aesar) and 0.05% glutaraldehyde (Amresco) using the solubilization procedure. Films were formed by pouring the solution onto PDMS strips (Dow Corning) and allowing dehydration overnight. The films were removed and cut down to specific sizes. Films were then mounted on a custom stretching device[25] and soaked in varying alcohol and water mixtures. Following at least 3 minutes of soaking they were then stretched to 2.7X and 3X their original length, dried, and removed.

Adhesive Formation

The solubilization procedure is applied for all adhesive dopes, although dope formulations varied between materials. Formulations for samples were as follows; Wood: 20% (w/v) 50/50 rMaSp1/rMaSp2, 100mM Imidazole, 0.1% propionic acid, and 1% L-DOPA. Plastic and silicone: 12% (w/v) 50/50 rMaSp1/rMaSp2, 100mM Imidazole, 0.1% propionic acid, and 1% L-DOPA. The dope is then sprayed on the surface of the material using the airbrush and compressor mentioned in the coatings section and the same procedure. Finally, 50 µL of the adhesive solution is pipetted on substrate and the

two pieces are then gently brought together and placed on a rack. The adhesives are then dried in oven at 30 °C for 24 hours.

Hydrogel Formation

Using the solubilization procedure, a solution of 6% (w/v) 50/50 rMaSp1/rMaSp1 with 2% propionic acid (Alfa Aesar) is made. After solubilization the solution was pipetted into a polypropylene tube (VWR) with a diameter of 15.5 mm upon a silicone pad (MSC Industries). The gel was then covered and allowed to cure for one hour before further processing.

Lyogel Formation

After the procedure for hydrogel formation, the hydrogels were taken and placed into a dry tube. This container was then placed into a -20 °C freezer for at least 3 hours. Once the hydrogels were sufficiently frozen they were lyophilized at -80 °C and 1 Pa, for 24 hours.

Sponge Formation

After forming a hydrogel and placing it in a water bath it was transferred to a -20 °C freezer for at least 2 hours. The tube was removed and thawed to produce a rSSp sponge.

Foam Formation

A film solution was solubilized. The liquid was then aspirated using a l mL pipette multiple times producing bubbles throughout the liquid. The sample was then left to dry.

Coating Formation

A liquid dope containing 6% (w/v) 80/20 rMaSp1/rMaSp2, 1% propionic acid (Alfa Aesar), and 100mM imidazole (Alfa Aesar) is solubilized. A layer of silk is applied from 50 cm away for 15 seconds. This is repeated as necessary using three minute dry times between layers. A G233 Master Airbrush with a 0.5 mm needle and nozzle connected to a TC-60 Master Airbrush compressor is used for the application.

Mechanical Testing of Fibers

Single fibers were removed from the winding drum and mounted on C-cards.[29] Five mounted fibers were produced for each stretch condition. The diameters of the mounted fibers were measured using a Motic BA310 microscope with 10MP Motic camera (Motic). Images were analyzed on Motic's Image plus 2.0ML software. Diameter measurements were made at three locations along the fibers axis and averaged.

Measured fibers were then mounted on an MTS Synergie 100 with custom 10-gram load cell (Transducer Techniques) by clamping the top and bottom of the C-card. The cards were cut on the edge leaving only the fiber between the load cell and moving

crosshead. Fibers were tested at 250 mm/min and 500 Hz collection rate using MTS's TestWorks 4, 2001. Data were exported to Microsoft Excel for energy to break, ultimate stress and ultimate strain determination which was done for all of the testing described below.

Mechanical Testing of Films

The films, as previously published[25], are cut to a specific length and width. The films are then mounted on a plastic C-card length wise using Loctite® super glue (liquid) across an 8 mm gap. After mounting, the C-card is loaded on an MTS Synergie 100 (50N load cell) by clamping the top and bottom of the film and card into the instrument with alligator clips and then cutting the side of the C-card so the only thing being tested is the film. The film is then tested to breaking at a stretch rate of 5 mm/min, with data collection at 30 Hz to measure the film's load in order to calculate stress, strain and energy to break using MTS's TestWorks 4, 2001.

Mechanical Testing of Adhesives

Wood samples were tested in a Tinius Olsen H50KS 50 kN using Tinius Olsen utility software in the machine utility mode testing at 10 mm/min with data collection at 8 Hz, plastic was tested on a 250 N MTS Tytron 250 at 1.3 mm/min with data collected at using Testworks4 to process information, the silicone was tested on an 50N MTS Synergie 100 at 1.3 mm/min with readings taken at 30 Hz using TestWorks4, 2001 to process information. Testing units were made by gluing samples together

(Supplementary Fig. S1) with the exception of the silicone samples, which were tested without grip support. The samples are loaded into the instruments clamping on the inside of the lips on the grip support.

Mechanical Testing of Hydrogels/Lyogels

Compressive mechanical tests were performed on the gels using an MTS Synergie 100 equipped with a 50 N load cell and 50 mm diameter aluminum compression platens using TestWorks 4 software. Prior to testing the gels, excess moisture was removed by gentle wiping and the sample was placed directly in the center of the platens. All tests were performed at room temperature with a test speed of 1.3 mm/minute until ultimate failure of the gels occurred.

RESULTS

Aqueous Solubilization and Formation

By sealing the rSSps in a vial with water and microwaving the vial for 15-60 seconds, we are able to solvate all rSSps (goat produced rMaSp1 and rMaSp2, bacterially produced native-like sequences $MaSp1_{16}$ and $Piri_4$ (piriform silk), and bacterially produced chimeric sequences FlYS, $FlYS_3$, FlAS, $FlAS_3$, and $A4S8_8$: supporting information) attempted to date.[22–24] The materials made (Figure 1) and all other data in this manuscript were produced from goat produced rMaSp1 and rMaSp2. The quick generation of heat, pressure, and potentially the known effect of microwaves spinning polar water molecules solvates up to 40% w/v rSSps solutions in a short period of time.

The method is water based, so it is environmentally friendly and non-toxic; for this reason, the use of water as the solvent also allows for easy and practical scalability.

The speed and efficiency of the method also provides a previously unknown level of versatility with respect to processing and the ability to form various materials. Fabrication and production of fibers, films, sponges, hydrogels, lyogels, foams, coatings,

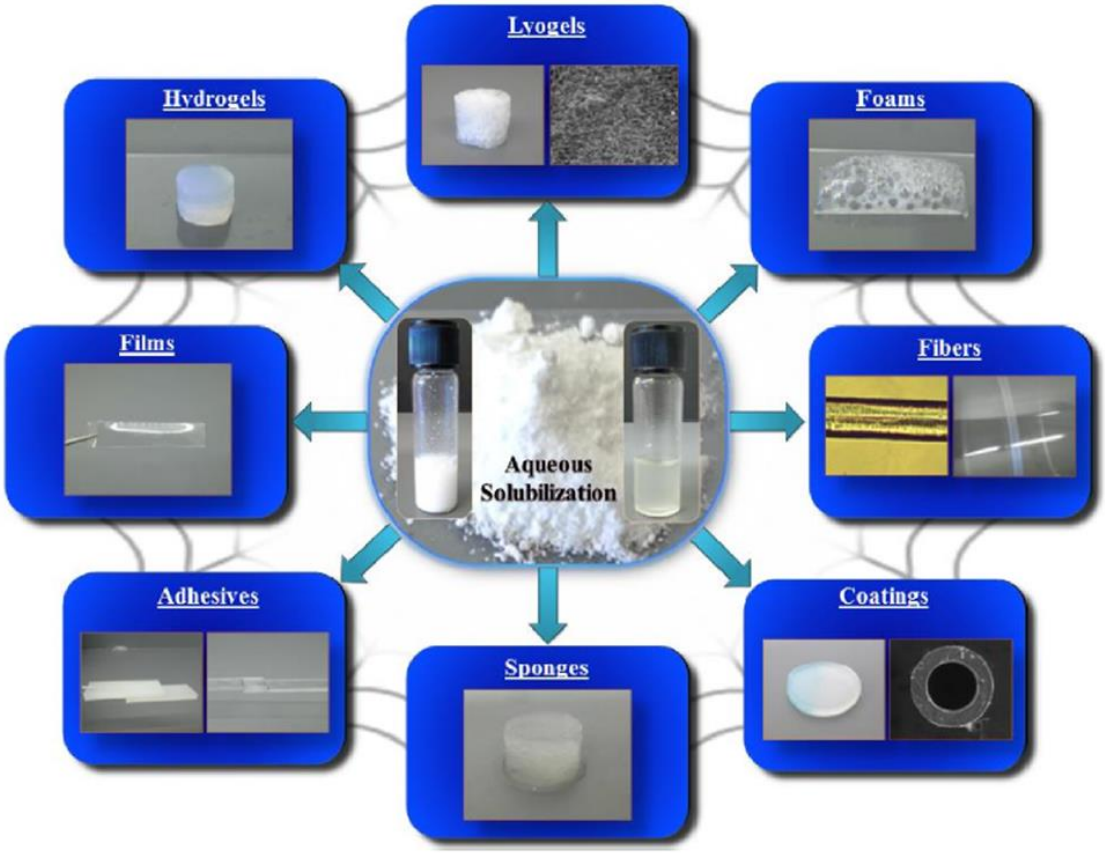

Figure 1. Aqueous method products. Schematic array of spider silk materials formed from the aqueous based solvation method. All materials are formed from lyophilized rSSps and water.

and adhesives can be achieved within a relatively short time (minutes to hours), depending on protein concentration, treatments, and desired final material. Within these final formations, it is possible to create tunable or custom features such as

variations in stress, strain or porosity by alterations to additives and post-formation treatment.[25] By using these variations, specific materials can be formed and constructed that are suitable for a variety of applications including sporting gear, industrial equipment, tissue engineering, or medical devices.[26–28]

Mechanical properties are the major focus of fibers, films, adhesives, and to some extent gels produced from this aqueous method. However, coatings, foams, and sponges are not expected to contribute substantially to a final product's mechanical properties. Rather, functionality and biocompatibility of coatings, foams, and sponges are the desired properties. The remaining categories of hydrogels, lyogels, fibers, films, and adhesives have all been tested mechanically (Figure 2). The secondary structure of fibers, films, sponges, lyogels, hydrogels, and the liquid dopes were also examined using FTIR-ATR spectroscopy (Figure S2). The structural findings indicate that the purified rSSps powder is predominantly β-sheet, the solubilized liquid rSSps dope is in a largely helical conformation that converts to a β-sheet rich material as the dope sets into its final solid form, similar to the transformation seen with 1,1,1,3,3,3-Hexafluoroisopropanol (HFIP) based dope solutions or when an alcohol/water treatment is applied to HFIP-processed materials.[25]

Aqueous-Based rSSps Fibers and Films

Fibers and films are two materials that are frequently sought for silk derived products and often these materials have the most appreciable mechanical

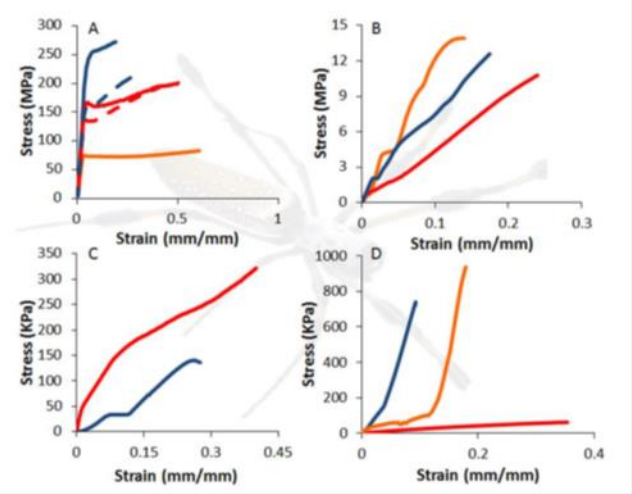

Figure 2. Mechanical properties of rSSps products. (A) A stress-strain graph of fibers (solid) and films (dashed) with maximum stress (blue), maximum strain (orange) and maximum energy to break (red). (B) Stress-strain graph of adhesive of spider silk protein on wood substrates of walnut (orange), oak (blue) and zebra wood (red). (C) A stress-strain graph of compressive properties of a lyogel (red) and a hydrogel (blue). (D) Stress-strain graph of adhesive rSSps on polycarbonate (blue), polypropylene (orange) and silicone (red).

properties.[19,25,29,30] The applications for these products range from protective materials to composites. Fibers and films can be used in the medical field to replace tendons or skin.[31,32] Published reports indicate maximum stress and strain of 508 MPa and 15% for fibers and 54 MPa and 13% for films with the silk dissolved in HFIP.[10,25]

Our method of solubilizing and spinning or casting films more closely mimics a spider's method, which does not involve organic solvents. Both films and fibers can be produced from the aqueous method (Figure 2A) Fiber mechanical properties that rival natural silks with ultimate stress values that approach 300 MPa and strains as high as 64%. Further refinement of spinning conditions and processing conditions will allow for improved mechanical ability of fibers. Films have also been produced with mechanical properties exceeding those of all other rSSps films reported with an ultimate stress as

high as 200 MPa and strain approaching 120%.[25] Strength is not the only impressive property for aqueous derived films and fibers; both the stress and strain of the materials can be manipulated to customize the mechanical properties to desired specifications. Also, fibers and films can be designed for high energy to break by varying the ratios of the rMaSp1 to rMaSp2 and stretching the fibers and films thus improving β-sheet alignment along the longitudinal axis.[25,29]

Aqueous-Based rSSps Adhesives

Adhesives are used extensively today. Spiders and other organisms use natural adhesives, glyco-proteins, and proteins, to assemble or fasten their webs or structures.[33–35] The same solutions that can be used to form fibers, films, gels, and coatings can create an adhesive as well. Using this aqueous method with rMaSp1 and rMaSp2, the solvated solutions can be used to produce rSSps glues without modification of the method or additional preparation. Due to the water based nature of this specific process the adhesives could be used within biological systems, for example, wound or surgical repair.

The solvated silk solutions were used as an adhesive on several substrates (Figure 2B,D) and tested in different loading conditions such as normal tension, compressive shear, and tensile shear (Figure S3). When these adhesive formulations are applied to substrates as a liquid, the surface is sufficiently wetted and covered by the solution allowing the adhesive to penetrate and fill cracks or pores. The rSSps adhesives are able to glue silicone materials together, with stresses exceeding 60 KPa and strain

values near 30%, mostly due to silicone's elasticity, which demonstrates the ability of the adhesives to stretch without failure (Table 1).

Table 1. Mechanical properties of different rSSp products with the mean represented followed by the standard deviation (SD) of each value.

Product	Stress (MPa)	Strain (%)	Energy to Break
Fiber	192.2 ± 51.47	28.1 ± 26.0	33.77 ±
Film	183.9 ± 14.85	35.4 ± 7.0	52.36 ± 8.02
Hydrogel	0.13 ± 0.007	31.0 ± 3.0	0.043 ±
Lyogel	0.89 ± 0.038	73.0 ± 2.6	0.251 ±
Adhesive-Wood	12.4 ± 2.52	15.4 ± 9.9	1.31 ± 1.15
Adhesive-Plastic	0.93 ± 0.23	16.4 ± 2.8	0.042 ±
Adhesive-Silicone	0.057 ± 0.005	31.0 ± 3.6	0.011 ±

Glued wood samples match or surpass the strength of conventional wood glues for most samples with stress values as high as 15 MPa and strains that approach 15%, Elmer's wood glue has maximal stress values around 10 MPa and elongations that only approach 8% before failure in our system (Figure 2B). Gorilla Glue™, a conventional adhesive with maximum stress values of 1 MPa and elongations of 3%, is substantially outperformed on all wooden substrates by the rSSps adhesives by up to an order of magnitude with stress values of 10 MPa and strains of 17% (Video S1). As a result of the increased stress and strain, these rSSps adhesives have a higher energy to break than both control adhesives tested. The maximum results for the rSSps adhesives and Elmer's wood glue previously discussed had energy to break values of 3 MJ/m3 and 0.7 MJ/m3, respectively. This increased toughness, approximately 430 % greater than Elmer's wood glue and 1300% for Gorilla Glue™, is due to the ability of the aqueous based rSSps adhesive to stretch under load (15% strain) instead of suddenly failing

without deformation of the glue (8% strain Elmer's wood glue and 3% strain for Gorilla glue™).

Aqueous-Based rSSps Gels

Hydrogels, lyogels, and sponges could not to be formed from spider silk proteins dissolved in HFIP but are readily formed with our aqueous method. The hydrogels, which are primarily composed of water (80% to 97%), are able to withstand compressive stresses up to 140 KPa and maintain their structural integrity and shape outside of a liquid suspension (Figures 2C and 3A,B). The internal organization of the hydrogels can also be varied with the use of certain treatments that induce secondary structures (reported below) within the gel (Figure S2). Other recombinant spider silk protein hydrogels have been reported in the literature with mechanical rheological stress and strain of 250 Pa and 30% respectively, but no normal compressive tests have been reported. These hydrogels were generated by a dialysis method to increase protein concentrations and remove the solubilizing agent.[16]

Lyogels are lyophilized hydrogels that possess similar traits to hydrogels but have unique mechanical properties.[36, 37] Like hydrogels, lyogels are dimensionally stable and maintain their structural stability. They can also be manipulated with post-formation treatments to alter secondary structures and macrostructures within the gel (Figure 3C). Unlike hydrogels, lyogels are able to resist mechanical loading to a greater extent with stresses approaching 300 KPa and strain near 40%, while being composed of primarily air and spider silk protein (Figure 2C). Lyogels are also able to absorb water with relative

ease, up to approximately 1600% of the original dry weight (Video S2). Although others have been able to produce both spider silk and silkworm silk derived lyogels, rarely are mechanical properties reported.[36,38,39]

A third possible material derived from a hydrogel is a rSSps sponge with distinct mechanical properties from both hydrogels and lyogels (Figure 3D-H). Sponges are

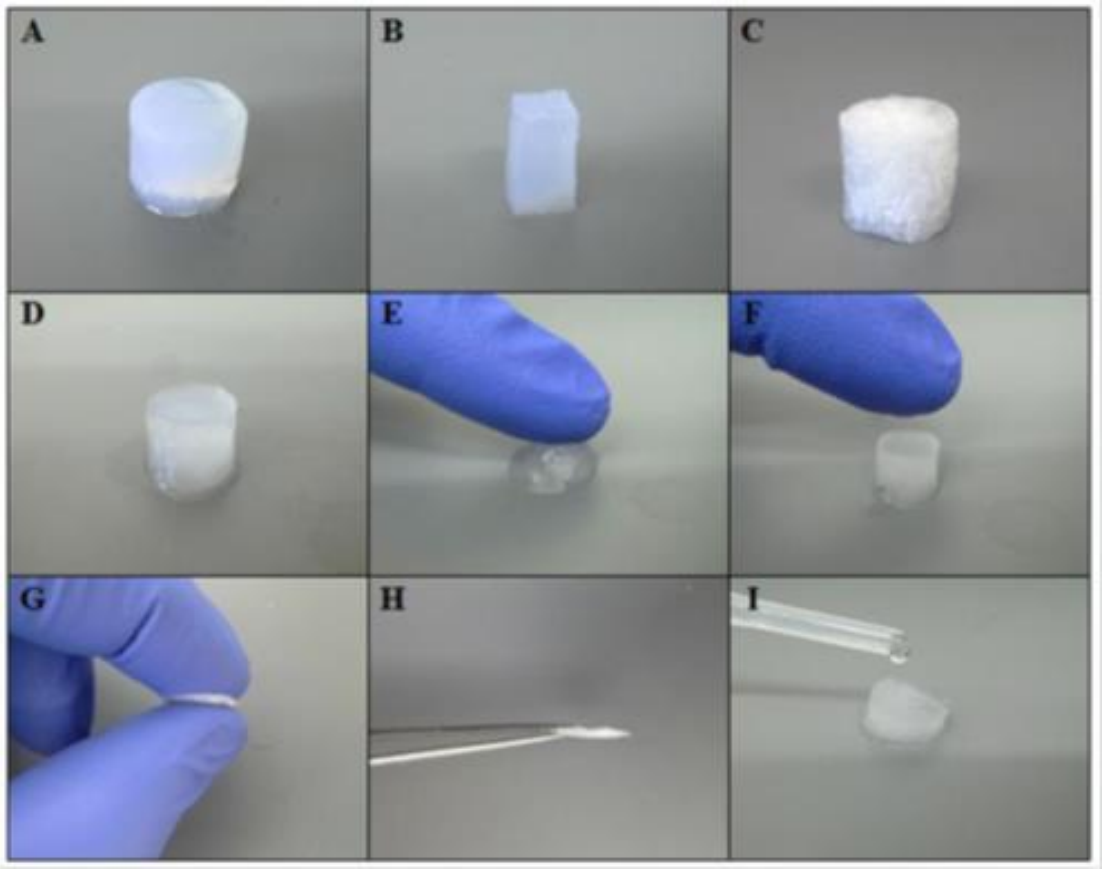

Figure 3. Spider silk protein gels and sponges. (A) rSSps hydrogel. (B) rSSps hydrogel with shape variation and stability. (C) rSSps lyogel. (D) rSSps sponge after fabrication. (E) Compressive loading of sponge and the release of water. (F) Recovery of the sponge after removal of compressive load. (G) A compressed and dehydrated sponge. (H) Compressed sponge maintaining the dehydrated shape without loading. (I) Rehydration of the compressed sponge.

formed by freezing a hydrogel in the presence of water and then allowing the hydrogel to thaw. No mechanical testing was done for sponges due to their ability to deform and

recover when rehydrated or unloaded. A silk sponge is able to go through several mechanical loadings and unloadings and recover (Figure 3) with the simple addition of water, which is absorbed into the silk matrix and returns the sponge to its original formation similar to shape memory. Compressed sponges maintain their shape when the compressive load is removed. It is only the addition of water that restores the original dimension of the sponge.

Gels and gel-derived products have proven promising in their ability to foster and support cell growth. Goat fetal fibroblast cells grown on the hydrogels show a healthy appearance and do not appear to be disturbed by the presence of the gel (Figure S4). This property is particularly important when considering this method of producing aqueous based gels for medical implant applications.

The secondary structures of the hydrogels, lyogels, and sponges, determined by FTIR-ATR spectroscopy, help explain the properties and abilities of the materials (Figure S2). When the spectra of hydrogels and lyogels are analyzed, distinct peak signatures are seen in the regions that correlate with β-sheet structures while random coils and helical regions are not as prevalent. This protein secondary structure relates to the rigidity and strength of the hydrogels and lyogels and their ability to resist or maintain their structure when compressed. However, the spectra of the sponges show that the β-sheet regions although still present, are no longer as prevalent. The increase in random coil and α-helices indicates why the sponge material is able to deform from a cylinder into a wafer through the expulsion of water, maintain the wafer shape in the

absence of compressive force, then reform to its original dimension through reabsorption of water (Video S3).

Aqueous-Based rSSps Foams and Coatings

Other materials generated from this aqueous method that are less mechanically oriented are foams and coatings. A foam is a mass of small bubbles formed on or in =

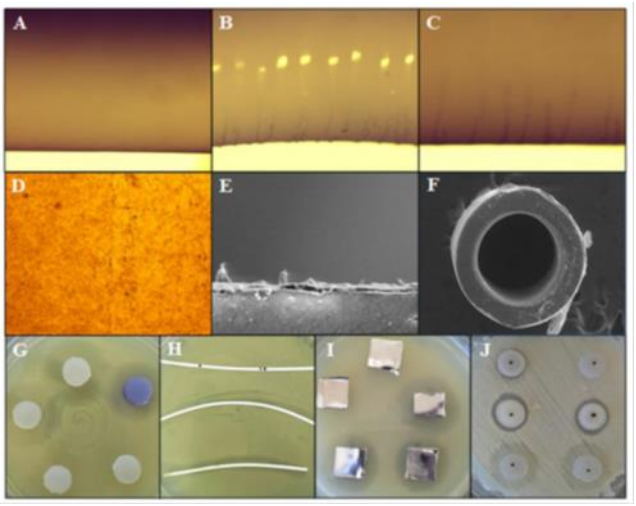

Figure 4. Various rSSps coatings. (A) An unbent silicone catheter 1.1 mm diameter coated with rSSps. (B) Silicone catheter while bent into a circle with a diameter of 2.5 cm. (C) Catheter straightened to its original form. (D) rSSps coated wafer with the coating applied to the left half. (E) SEM cross-sectional image of a rSSps coated wafer with an approximate thickness of 2 µm. (F) SEM cross-sectional image of a rSSps coated catheter with an approximate thickness of 5 µm. (G) rSSps coated silicone wafer functionalized with kanamycin that produced zones of inhibition approximately 22 mm in diameter on *E. coli*. (H) rSSps coated catheters loaded with kanamycin and zones of inhibition approximately 2.5 mm from the catheter on *E. coli*. (I) rSSps coated stainless steel functionalized with kanamycin and zones of inhibition approximately 20 mm in diameter on *E. coli*. (J) rSSps coated silicone wafers functionalized with an azole and zones of inhibition approximately 16 mm in diameter on *C. albicans*.

liquid. Foams can be used to decrease internal bleeding in trauma victims or as a cell growth matrix for research purposes (Figure 1).[31, 32] Coatings have immense potential and may be appropriate for applications such as coatings on catheters, stents, and other medical devices that would benefit from a biocompatible, functionalized coating (Figure 4).

These rSSps coatings can be applied to almost any substrate by simply administering the solubilized liquid to the surface of a substrate using an aerosolized spray, pouring, or with a submerging dip technique, depending on the application and desired surface properties. The thickness of the coating can also be varied and coatings thinner than 1 μm and as thick as 50 μm have been produced by these methods. Spider silk protein coatings are intriguing for several other reasons, the first of which is the high adherence to most surfaces, especially materials that traditionally resist coatings like medical grade silicones.[40] Another interesting feature is the ability of the rSSps coatings to resist separation upon bending or stretching and even recover from deformation from bending. This is illustrated in Figure 4: panel A is an unbent rSSps coated catheter 1.1 mm diameter; panel B is a bent rSSps coating catheter showing compression buckling lines at a 2.5 mm diameter bend; and panel C is a straightened, post-bent coated rSSps catheter showing that no cracking or breaking occurs, although minor buckling lines still remain.

A final feature is that the coatings form a biocompatible and protective layer that can be constructed to contain additives, like antibiotics, antimycotics, anti-inflammatory drugs or growth factors, which inhibit or enhance certain biological

processes. The rSSps coatings (Figure 4) contain the antibiotic kanamycin, which proved successful in preventing the growth of *Escherichia coli* for as long as two weeks as indicated by zones of inhibition around the coated silicone wafers. Common implant materials, silicone and stainless steel, have both been successfully coated and functionalized (Figure 4G-J). The dosage of an additive can be adjusted within the coating formulation to produce the desired inhibition and response (Figure 4G-J) without affecting the ability of the rSSps to coat the material. This functionalization of the coating has also been demonstrated against other organisms such as the yeast *Candida albicans*. Silicone wafers coated with rSSps containing antimycotic drugs produced a zone of inhibition when exposed to *Candida albicans*, further demonstrating the functionality of these coatings (Figure 4J).

DISCUSSION

Recombinant spider silks may offer potential solutions for a vast number of industries and applications. The various aqueous based materials presented here offer new options and possibilities for these applications. This investigation was mostly a broad approach that primarily focused on the mechanical and structural properties of the materials. Optimizations were then performed for each material to maximize these properties and determine the ranges of tunable features that were possible. It was determined through mechanical and structural analysis that most of these materials can be customized for a specific function.

The efficiency and speed of this solubilization method further increase the potential of rSSps materials. The method of solvating rSSps presented here is quick and results in nearly 100% solvation and recovery of solubilized rSSps. This method unlocks previously problematic or unattainable solutions and broadens the scope of rSSps beyond fibers into films, foams, gels, sponges, adhesives and coatings. No other single solubilization method exists from which this variety of formulations can be generated from a single rSSps and maintain the desirable characteristics of biocompatibility and mechanical abilities while being green and economically responsible. Furthermore, this aqueous method also allows for diverse functionalization of the rSSps, regardless of final forms, to allow for wound healing, implantation and tissue growth that was not available with materials based from HFIP or other organic solvent solutions. This initial inquiry into the possibilities of water-based recombinant spider silk materials demonstrated the effectiveness and initial possibilities for such materials.

CONCLUSIONS

This study has demonstrated the ability to take rSSps and solubilize them for various applications using water as a solvent. This aqueous solvation method which is safe and environmentally friendly brings recombinantly produced spider silks one step closer to commercial and large-scale uses. The use of water as the solvent mimics a spider and removes the need for costly and caustic organic solvents. Additionally, this novel process has only one major step that can be completed in a matter of seconds with a variety of solutions. Not only is this process time efficient but it has results in

nearly 100% protein solubilization, which allows for greater recovery. Finally, this process has allowed for a diverse profile of aqueous derived materials to be created. Common materials such as fibers and films are still possible from the aqueous method but more exotic materials like hydrogels, adhesives, lyogels, coatings, foams, and sponges are now possible. The reveal of this method is accompanied by characterization of these materials and their potentials.

SUPPORTING INFORMATION

Protein sequences, coomasie gel of preparation methods, comparative FTIR spectra, adhesive schematic, hydrogel cell culture figures, and related methods along with videos of adhesives, lyogels, and sponges. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

1. Gatesy, J.; Hayashi, C. Y.; Motriuk, D.; Woods, J.; Lewis, R. V. *Science* **2001**, 291, 2603–2605.

2. Vollrath, F.; Knight, D. P. *Nature* **2001**, 410, 541–548.

3. Dicko, C.; Kenney, J. M.; Knight, D.; Vollrath, F. *Biochemistry (Mosc.)* **2004**, 43, 14080–14087.

4. Hayashi, C. Y.; Shipley, N. H.; Lewis, R. V. *Int. J. Biol. Macromol.* **1999**, 24, 271–275.

5. Lewis, R. V. *Chem. Rev.* **2006**, 106, 3762–3774.

6. Cunniff, P. M.; Fossey, S. A.; Auerbach, M. A.; Song, J. W.; Kaplan, D. L.; Adams, W. W.; Eby, R. K.; Mahoney, D.; Vezie, D. L. *Polym. Adv. Technol.* **1994**, 5, 401–410.

7. Chung, H.; Kim, T. Y.; Lee, S. Y. *Curr. Opin. Biotechnol.* **2012**, 23, 957–964.

8. Schacht, K.; Scheibel, T. *Biomacromolecules* **2011**, 12, 2488–2495.

9. An, B.; Jenkins, J. E.; Sampath, S.; Holland, G. P.; Hinman, M. B.; Yarger, J. L.; Lewis, R. V. *Biomacromolecules* **2012**, 13, 3938–3948.

10. Xia, X.-X.; Qian, Z.-G.; Ki, C. S.; Park, Y. H.; Kaplan, D. L.; Lee, S. Y. *Proc. Natl. Acad. Sci.* **2010**, 107, 14059-14063.

11. Holland, G. P.; Jenkins, J. E.; Creager, M. S.; Lewis, R. V.; Yarger, J. L. *Biomacromolecules* **2008**, 9, 651–657.

12. Slotta, U.; Tammer, M.; Kremer, F.; Koelsch, P.; Scheibel, T. *Supramol. Chem.* **2006**, 18, 465–471.

13. Moon, M.-J.; Townley, M. A.; Tillinghast, E. K. *Korean J. Biol. Sci.* 1998, 2, 145–152.

14. Jin, H.-J.; Kaplan, D. L. *Nature* **2003**, 424, 1057–1061.

15. Eisoldt, L.; Thamm, C.; Scheibel, T. *Biopolymers* **2012**, 97, 355–361.

16. Rammensee, S.; Huemmerich, D.; Hermanson, K. D.; Scheibel, T *Appl. Phys. A* **2006**, 82, 261–264.

17. Kim, U.-J.; Park, J.; Li, C.; Jin, H.-J.; Valluzzi, R.; Kaplan, D. L. *Biomacromolecules* **2004**, 5, 786–792.

18. Matsumoto, A.; Chen, J.; Collette, A. L.; Kim, U.-J.; Altman, G. H.; Cebe, P. Kaplan, D. L. *J. Phys. Chem. B* **2006**, 110, 21630–21638.

19. Jiang, C.; Wang, X.; Gunawidjaja, R.; Lin, Y.-H.; Gupta, M. K.; Kaplan, D.L.; Naik, R. R.; Tsukruk, V. V. *Adv. Funct. Mater.* **2007**, 17, 2229–2237.

20. Alwattari, A.; Islam, S.; Karatzas, C. N.; Rodenhiser, A. Methods and apparatus for spinning spider silk protein. WO2003060099 A2, November 6, 2006.

21. Yue, H.; Karatzas, C. N.; Turcotte, C. Recovery of biofilament proteins from biological fluids. WO2003057720 A2, July 17, 2003.

22. Hinman, M. B.; Lewis, R. V. *J. Biol. Chem.* **1992**, 267, 19320–19324.

23. Xu, M.; Lewis, R. V. *P.N.A.S.* **1990**, 87, 7120–7124.

24. Teule, F.; Furin, W. A.; Cooper, A. R.; Duncan, J. R.; Lewis, R. V. *J. Mater. Sci.* **2007**, 42, 8974–8985.

25. Tucker, C. L.; Jones, J. A.; Bringhurst, H. N.; Copeland, C. G.; Addison, J. B.; Weber, W. S.; Mou, Q.; Yarger, J. L.; Lewis, R. V. *Biomacromolecules* **2014**, 15, 3158-3170.

26. Minoura, N.; Aiba, S.-I.; Gotoh, Y.; Tsukada, M.; Imai, Y. J. *Biomed. Mater. Res.* **1995**, 29, 1215–1221.

27. Gomes, S.; Gallego-Llamas, J.; Leonor, I.B.; Mano, J.F.; Reis, R. L.; Kaplan, D. L. *Macromol. Biosci.* **2013**, 13, 444–454.

28. Gomes, S.; Leonor, I. B.; Mano, J. F.; Reis, R. L.; Kaplan, D. L. *Biomaterials* **2011**, 32, 4255–4266.

29. Albertson, A. E.; Teulé, F.; Weber, W. S.; Yarger, J. L.; Lewis, R. V. *J. Mech. Behav. Biomed. Mater.* **2014**, 29, 225–234.

30. Meinel, L.; Hofmann, S.; Karageorgiou, V.; Kirker-Head, Carl.; McCool, J.; Gronowicz G.; Zichner, L.; Langer, R.; Vunjak-Novakovic, G.; Kaplan, D. L. *Biomaterials* **2005**, 26, 147–155.

31. Widhe, M.; Bysell, H.; Nystedt, S.; Schenning, I.; Malmsten, M.; Johansson, J.; Rising, A.; Hedhammar, M. *Biomaterials* **2010**, 31, 9575–9585.

32. Spiess, K.; Lammel, A.; Scheibel, T. *Macromol. Biosci.* **2010**, 10, 998–1007.

33. Hu, X.; Vasanthavada, K.; Kohler, K.; McNary, S.; Moore, A. M. F.; Vierra, C. A. *Cell. Mol. Life Sci. CMLS* **2006**, 63, 1986–1999.

34. Opell, B. D.; Schwend, H. S. *Zool. Jena Ger.* **2009**, 112, 16–26.

35. Smith, B. L.; Schaffer, T. E.; Viani, M.; Thompson, J. B.; Frederick, N. A.; Kindt, J.; Belcher, A.; Stucky, G. D.; Morse, D. E.; Hansma, P. K. *Nature* **1999**, 399, 761–763.

36. Kundu, B.; Kundu, S. C. *Biomed. Mater.* **2013**, 8, 055003.

37. Gil, E. S.; Park, S.-H.; Tien, L. W.; Trimmer, B.; Hudson, S. M.; Kaplan, D. L. *Langmuir* **2010**, 26, 15614–15624.

38. Guziewicz, N.; Best, A.; Perez-Ramirez, B.; Kaplan, D. L. *Biomaterials* **2011**, 32, 2642–2650.

39. Li, M.; Lu, S.; Wu, Z.; Yan, H.; Mo, J.; Wang, L. *J. Appl. Polym. Sci.* **2001**, 79, 2185–2191.

40. Zeplin, P. H.; Maksimovikj, N. C.; Jordan, M. C.; Nickel, J.; Lang, G.; Leimer, A. H.; Romer, L.; Scheibel, T. *Adv. Funct. Mater.* **2014**, 24, 2658–2666.

CHAPTER 4

CONCLUSION

Recombinant spider silk protein (rSSp) has been limited in its ability to enter the materials landscape for two major reasons. The first is that it is very difficult to produce the rSSp regardless of transgenic system due to the highly repetitive nature of the silk proteins. Their underlying nucleic acid sequences cause significant problems both during transcription and translation and their protein sequences often cause aggregation issues once translated. The second reason is that traditional methods of solvating rSSp's in order to produce fibers and films require very harsh organic solvents such as HFIP. While others work to solve the expression issues of rSSp, this dissertation sets forth a new, quick and scalable method for the solvation of rSSp in water with near 100% solvation and recovery. The method is also scalable with common chemical engineering techniques.

By placing normally sparingly soluble rSSp into a sealed vial with water and then generating heat and pressure using a conventional microwave, all rSSp protein dopes attempted to date have been solvated. From those solutions fibers, films and foams have been created. As a byproduct of the aqueous method several new materials composed of rSSp have been achieved including hydrogels, lyogels, adhesives, coatings and even a never reported in the literature material, a sponge. All are unique both mechanically as well as in potential applications. Fibers have obvious benefit to the performance thread or material markets as well as in very fine sutures. The mechanical

properties demonstrated by the materials included in this thesis are remarkable in that they are comparable to fibers derived of the same protein from the conventional, but toxic HFIP solvation and spinning methods. With further development and refinement of the spinning process, and particularly with post manufacture processing, these fibers will continue to improve.

Recombinant spider silk thin films have also been accomplished through this solvation method. These films, like the fibers, were comparable if not better than films produced from identical proteins using HFIP as a solvent. Films are an intriguing product from rSSp. Biocompatible, flexible, functionalizable (ability to incorporate antibiotics, antimycotics, steroids etc) and strong, these thin films could serve a variety of uses such as cell growth matrices, wound repair substrate and degradable, flexible coatings on medical devices. Coatings are a derivation of films that are also potentially useful. Coatings can be produced by dipping the material into an aqueous rSSp solution or by spray coating. Every material attempted to date has been successfully coated including medical grade silicon and stainless steel. Again, the biocompatibility and functionalizable nature of these coatings, tunable cell adhesion[1] as well as their diverse range of coatable materials, make them an intriguing option for coating medical devices such as intravenous and urethral catheters to control infection, clotting (I.V.) and cell adhesion.

The adhesives properties of these protein based rSSps is remarkable from aqueous solutions. When compared to standard Elmer's glue and Gorilla glue, our glues perform as well as and better than both depending on the substrate, with Gorilla glue

being substantially outperformed on all substrates in our tests. (Data not shown) Interestingly, spiders produce 7 different proteins from their silk producing glands, one of which is an adhesive like protein produced from the aggregate gland.[2] This protein adhesive is highly glycosylated, similar to a mucin, and those carbohydrates provide the adhesive properties. The two proteins used in our adhesive studies are the two proteins that comprise the dragline silk, MaSp1[3] and MaSp2[4] and the spider does not use them as an adhesive in any known situation. The fact then that these two proteins alone or in combination can form an adhesive, and a mechanically robust adhesive at that, is remarkable and clearly broadens the scope of materials able to be produced from the same starting material as is used to produce the other materials presented in this thesis.

Finally, the gels and a new material, a sponge provide unique characteristics and potential applications. Hydrogels and lyogels have been reported from rSSp in the literature.[5,6] The difference between the gels produced from this aqueous method and others is dimensional stability, speed of formation and tunable properties. Reported hydrogel and lyogel formation (lyogels are lyophilized hydrogels) take many hours to form.[7]

In our method, hydrogels can be formed in minutes or the composition of the proteins and or additives altered to delay gelation. As well, by altering protein concentrations and compositions as well as post-pour treatments (EtOH, MeOH, IPA) the pore size and mechanical ability of the gel can be altered or tailored to a specific application. Sponges, a frozen and then thawed hydrogel, are an entirely new material application for rSSp and one that has to our knowledge ever been reported in the

literature. The sponge attributes are that it can be compressed to expel water and it remains in the compressed state until water is reapplied to the gel. In thinking about possible uses for such a material one quickly arrives at tissue regeneration matrices. The ability to form a sponge, remove the water, and then allow the sponge to absorb ascites fluid and stem cells to promote regeneration in whatever tissue implanted. The sponges are robust enough to be sutured in place in such applications as nerve and blood vessel regeneration.

To wrap up the project, and this dissertation, I prepared a table (Table 1) of all of the developments that have resulted from this aqueous solvation method and where the project currently stands in terms of science, publication and funding.

Table 1: Aqueous based materials current/future work with publications and funding.

Technology	Current	Future	Publication	Funding
Fibers + Films	--Improve solvation technique and understand additives --Cross-linking to improve mechanical performance	--Multi-head spinning from bacterially derived proteins	February 2015 Films published 2014	JRA with industry partners. SBIR (Phase I completed)
Gels + Sponge	--Gelation rate control --Cross-linking --Sponge formation tech --Drug release --3D printing	--Biocompatibility --Targeted drug release in-vivo --Sponge cell seeding --Directed nerve regeneration --3D printing	March-April 2015	None current and no immediate source.
Biological Stabilization	--Complete vaccine study (March 2015)	--Broaden scope of proteins --Include other industrial partners	??-IP concerns	SBIR/STTR March 2015 JRA
Coatings + Adhesives	--Characterization --Basic application techniques	--Rate control --I.V. experiments for biofouling and clotting	April-May 2015	STTR April 2015

The development of an aqueous based method of solvating normally water insoluble rSSp was a needed advancement in the spider silk community that has unlocked potential that, prior to its development, was not possible or realized. As a result, patents and scientific manuscripts have been submitted or published and funding sources unlocked to develop these new spider silk materials as well as continue to develop fibers. Continued research on fibers and films to improve spin and post-spin conditions will continue. Funding for this research has been secured through a joint research agreement with an industry partner as well as a successfully completed SBIR phase 1 grant. Phase 2 of the SBIR process is set to be submitted in March of 2015. Gels (hydrogels, lyogels and sponges): We will continue to develop the process of forming the gels and sponge through understanding the basic protein chemistry occurring that causes gelation in an attempt to control the rate of gelation, the pore sizes produced as well as the mechanical ability. A paper will be published in March or April of 2015.

Biological stabilization will continue with a vaccine stabilization study with an industry partner in March of 2015. At the conclusion of that study, a determination will be made on how to proceed, either production of the protein for stabilization or an SBIR/STTR to continue the research or both. Coatings and adhesives: The rate of adhesion will continue to be explored through additives to the aqueous solutions, such as isopropanol. Currently, the adhesion rate is too slow to be viable in most real-world applications. As well, the exploration of what additives can be included in an adhesive

or coating will continue as guided by a request for proposals for an SBIR/STTR submission regarding both intravenous and urinary catheter coatings. A preliminary scientific manuscript is in preparation for submission in April or May of 2015 with the SBIR/STTR phase 1 proposal slated for submission in April of 2015.

An aqueous solvation method for rSSp has been achieved. Through this advancement fibers, films, coatings, gels and sponges have been formed and characterized. This advancement is not the only advancement required to propel rSSp (in some form) into industrial applications. While more work is needed to solve supply problems, a method now exists to take rSSp and formulate it through a green, sustainable process.

LITERATURE CITED

1. Schacht, K.; Jüngst, T.; Schweinlin, M.; Ewald, A.; Groll, J.; Scheibel, T. Angew. *Chem. Int. Ed*. **2015**, 54, 2816-2820.
2. Choresh, O.; Bayarmagnai, B.; Lewis, R. V. *Biomacromolecules* **2009**, 10, 2852–2856. 1
3. Xu, M.; Lewis, R. V. *P.N.A.S.* **1990**, 87, 7120–7124.
4. Hinman, M. B.; Lewis, R. V. *J. Biol. Chem.* **1992**, 267, 19320–19324.
5. Gil, E. S.; Park, S.-H.; Tien, L. W.; Trimmer, B.; Hudson, S. M.; Kaplan, D. L. *Langmuir* **2010**, 26, 15614–15624.
6. Guziewicz, N.; Best, A.; Perez-Ramirez, B.; Kaplan, D. L. *Biomaterials* **2011**, 32, 2642–2650.
7. Schacht, K.; Scheibel, T. *Biomacromolecules* **2011**, 12, 2488–2495.

APPENDICES

APPENDIX A

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Title: Mechanical and Physical Properties of Recombinant Spider Silk Films Using Organic and Aqueous Solvents

Author: Chauncey L. Tucker, Justin A. Jones, Heidi N. Bringhurst, et al

Publication: Biomacromolecules

Publisher: American Chemical Society

Date: Aug 1, 2014

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APPENDIX B

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Title: More Than Just Fibers: An Aqueous Method for the Production of Innovative Recombinant Spider Silk Protein Materials

Author: Justin A. Jones, Thomas I. Harris, Chauncey L. Tucker, et al

Publication: Biomacromolecules

Publisher: American Chemical Society

Date: Apr 1, 2015

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APPENDIX C

SYNTHETIC SPIDER SILK PROTEIN COMPOSITIONS AND METHODS[1]

1. Lewis, R. V. & Jones, J. A. SYNTHETIC SPIDER SILK PROTEIN COMPOSITIONS AND METHODS. (2015). U.S. Patent Application 2015/0047532

TECHNICAL FIELD

The present disclosure relates to synthetic spider silk protein compositions. More specifically, the present disclosure relates to aqueous solutions of recombinant spider silk proteins ("rSSP") and synthetic spider silk protein compositions made from such aqueous solutions.

SUMMARY

The present disclosure in aspects and embodiments addresses these various needs and problems by providing a method of solubilizing recombinant spider silk proteins (rSSP) in an aqueous solution and related compositions. Exemplary methods include mixing rSSP with water to form a mixture, and microwaving the resulting mixture to form a solution. Optional steps also include, sonicating the mixture, centrifuging the solution, sonicating the solution, and adding various additives to the mixture. Suitable additives may be configured to decrease gel formation of the solution.

In one aspect, a method of solubilizing one or more recombinant spider silk proteins in an aqueous solution is disclosed, which includes mixing the one or more recombinant spider silk proteins with water to form a mixture in a sealed container and heating the mixture to form a solution.

In some embodiments, heating is performed with microwave irradiation. In some embodiments, the method includes sonicating the mixture. In some embodiments, the method includes sonicating the solution. In some embodiments, the method includes centrifuging the solution.

In some embodiments, the method includes providing additives for reducing gel formation in the solution. In some embodiments, the additives are selected from the group consisting of: an acid, a base, free amino acids, surfactants, and combinations thereof. In some embodiments, the additives are selected from the group consisting of: propionic acid, formic acid, acetic acid, ammonium hydroxide, L-arginine, L-glutamic acid, β-mercaptoethanol, dithiothreitol, and combinations thereof.

In some embodiments, the one or more recombinant spider silk proteins are selected from the group consisting of: M4, M5, MaSP1, a MaSP1 analogue, MaSP2, an MaSP2 analogue, and combinations thereof.

In some embodiments, the ratio of the one or more recombinant spider silk proteins to water in the mixture is from 1:10 to 1:2.

In some embodiments, the method includes obtaining a recombinant spider silk protein fiber from the mixture. In some embodiments, the method includes stretching the fiber. In some embodiments, the method includes obtaining a recombinant spider silk protein material from the mixture. In some embodiments, the method includes stretching the material.

In another aspect, recombinant spider silk protein materials prepared using techniques disclosed herein, where the material has the form of a hydrogel, lyogel, film, coating, foam, fiber, and combinations thereof. In some embodiments, the material is a hydrogel. In some embodiments, the material is a lyogel. In some embodiments, the material is a film. In some embodiments, the material is a coating. In some embodiments, the material is a foam. In some embodiments, the material is a fiber.

In another aspect, an aqueous solution of recombinant spider silk proteins is disclosed having one or more recombinant spider silk proteins and water, wherein the amount of the one or more recombinant spider silk proteins is greater than about 2% w/v. In some embodiments, the amount of the one or more recombinant spider silk proteins is less than about 50% w/v.

In some embodiments, the solution includes one or more additives for reducing gel formation. In some embodiments, the one or more additives are selected from the group consisting of: an acid, a base, free amino acids, surfactants, and combinations thereof. In some embodiments, the one or more additives are selected from the group consisting of: propionic acid, formic acid, acetic acid, ammonium hydroxide, L-arginine, L-glutamic acid, β-mercaptoethanol, dithiothreitol, and combinations thereof.

BRIEF DESCRITPION OF THE FIGURES

Figure 1A illustrates an exemplary recombinant spider silk fiber prepared according to one embodiment.

Figure 1B illustrates an exemplary recombinant spider silk fiber prepared according to one embodiment.

Figure 2 is a schematic representation of gluing boards used to characterize one embodiment.

Figure 3 is the hysteresis testing results on hydrogels according to one embodiment.

Figure 4 is a silicon wafer Purple (darkest shading) spider silk coated silicon wafer with methyl violet; next clockwise: control with spider silk coating and without kanamycin; next clockwise: Spider silk coating with 50mg/L kanamycin; next clockwise: spider silk coating with 250mg/L kanamycin; next clockwise: spider silk coating with 500mg/L kanamycin. Bacterial lawn is *E. coli* XL-1 blue cells.

Figure 5 is depiction showing silicon urinary catheters (3 french) coated with spider silk protein (top), spider silk coating loaded with 50mg/L kanamycin (middle) and spider silk coating loaded with 500mg/L kanamycin (bottom) according to one embodiment on a lawn of bacteria.

Figure 6 is depiction showing a stainless steel plate that was dip coated with recombinant spider silk protein according to one embodiment.

DETAILED DESCRIPTION

The present disclosure covers methods, compositions, reagents, and kits for making aqueous solutions of rSSP and for synthetic spider silk protein compositions derived from such solutions.

In the following description, numerous specific details are provided for a thorough understanding of specific preferred embodiments. However, those skilled in the art will recognize that embodiments can be practiced without one or more of the specific details, or with other methods, components, materials, etc. In some cases, well-known structures, materials, or operations are not shown or described in detail in order to avoid obscuring aspects of the preferred embodiments. Furthermore, the described

features, structures, or characteristics may be combined in any suitable manner in a variety of alternative embodiments. Thus, the following more detailed description of the embodiments of the present invention, as illustrated in some aspects in the drawings, is not intended to limit the scope of the invention, but is merely representative of the various embodiments of the invention.

In this specification and the claims that follow, singular forms such as “a,” “an,” and “the” include plural forms unless the content clearly dictates otherwise. All ranges disclosed herein include, unless specifically indicated, all endpoints and intermediate values. In addition, “optional” or “optionally” refer, for example, to instances in which subsequently described circumstance may or may not occur, and include instances in which the circumstance occurs and instances in which the circumstance does not occur. The terms “one or more” and “at least one” refer, for example, to instances in which one of the subsequently described circumstances occurs, and to instances in which more than one of the subsequently described circumstances occurs.

The present disclosure covers methods, compositions, reagents, and kits for making aqueous solutions of rSSP and for synthetic spider silk protein compositions derived from such solutions.

rSSP’s are conventionally dissolved in a very harsh organic solvent, 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), to create “dopes” that can be used to create fibers, films, gels and foams. HFIP has been widely used and accepted as it is the only solvent that: 1) dissolves rSSP’s at high concentrations (30% w/v) providing uniformity between various groups testing data, 2) is sufficiently volatile and miscible to be removed rapidly from

the forming fiber, 3) leaves little to no residue behind that could interfere with fiber formation. In addition, rSSP's generally are insoluble in aqueous solutions after purification, necessitating an organic solvent that meets the criteria outlined in 1-3. However, there are significant problems with solvating rSSP's in HFIP or other organic solvents.

Dissolving rSSP in HFIP and then using pressure to extrude the dope into a coagulation bath does not allow the appropriate structures to form (notably β-sheets) to an extent that the fibers or films have to be post-spin processed multiple times to achieve protein structures that result in appreciable mechanical properties. See Lazaris et al., Spider Silk Fibers Spun from Soluble Recombinant Silk Produced in Mammalian Cells, *Science* 295, 472-476 (2002) (hereinafter "Lazaris"); and Teule et al., Modifications of spider silk sequences in an attempt to control the mechanical properties of the synthetic fibers, J. Mater Sci, 42, 8974-8985 (2007) (hereinafter "Teule").

Such fiber processing methodologies include extruding the fiber into a coagulation bath that may include pure isopropanol or a mixture of isopropanol:water. The fiber may then be stretched (1.5-6X) in a second bath generally containing a mixture of isopropanol:water. A third bath may also be employed that contains pure water or a majority of water, and a second stretch applied in that bath. Water is the recurrent theme in these baths and it is the water that converts the helical structures present due to HFIP into strength providing β-sheets.

The cost of purchase and subsequent disposal of HFIP may be restrictive or prohibitive in an industrial setting of mass production. HFIP's cost of purchase is

roughly $1,000 / 100ml's of HFIP and 100ml's of HFIP would likely be capable of solvating 20-30g's of rSSP (20-30% w/v). Water is cheap even in its purest form. Per the MSDS published on Sigma Aldrich's web-site, disposal of HFIP requires; "*Dissolve or mix the material with a combustible solvent and burn in a chemical incinerator equipped with an afterburner and scrubber,*" a process that inherently has costs associated with it. Excess water can be evaporated or recycled and reused. Worker safety when utilizing such harsh, volatile solvents is also a consideration. Per the MSDS; "*Material is extremely destructive to tissue of the mucous membranes and upper respiratory tract, eyes, and skin. Cough, Shortness of breath, Headache, Nausea*" (SIC). Water has no such requirements. Finally, the process of producing rSSP products could not be considered "green" using HFIP.

rSSP's are largely insoluble in water. There are a few notable exceptions: Teule describes a series of proteins (Y_1S_8 and A_2S_8) that were produced in bacteria and purified via Ni^{++} chromatography. Short fibers were pulled straight from the eluted, pure rSSP fraction. See Teule. Lazaris describes ADF-3 (*Araneus diadematus* MaSp1) produced in mammalian cell culture. Water soluble ADF-3 was concentrated in the presence of glycine and extruded into a coagulation bath. A final example is a series of recombinant aciniform-like synthetic proteins that were able to be spun from an aqueous solution very similar to Teule 2007 (Xu 2012). See Xu et al., Recombinant Minimalist Spider Wrapping Silk Proteins Capable of Native-Like Fiber Formation. PloS-One 7(11): e50227. Doi: 10.1371/journal.pone.0050227 (2012). However, outside of this small sub-set of

rSSP's, water solubility is elusive. The majority of these proteins were much smaller than the natural proteins and thus are unlikely to make mechanically useful fibers.

U.S. Patent Application Publication No. 2011/0230911 filed by Amsilk utilizes a top down approach: genetic manipulations and expression system manipulations to try and create water soluble silk proteins. However, such processes are costly both in time to create the manipulations/cell lines and also in that the proteins appear to be expressed in mammalian cell cultures. The culture conditions for such cell lines are not only personnel and time intensive but also the ingredients and equipment are substantially more expensive than the more traditional bacterial expression systems. In addition, such methods are limiting as there are not that many iterations of various spider silk repeats that can be expressed in this manner that will result in a water soluble protein that has appreciable mechanical properties.

To address these and other challenges, this disclosure sets forth new and novel methods for solubilizing rSSP's in aqueous solutions and then creating resulting spider silk compositions therefrom. The methods and compositions described herein in embodiments create aqueous dopes from rSSPs that are otherwise not soluble in water. The methods and compositions described herein may be applied to proteins expressed by any organism, reducing the cost of production and also possibly improving the mechanical properties of the fibers, films, gels and foams by the inclusion of water in the dope.

In embodiments, methods of preparing aqueous dopes of rSSP may include the following steps: mixing rSSP, water, and optional additives; optionally sonicating the mixture; microwaving the mixture; and optionally centrifuging the microwaved mixture.

Aqueous Dopes. rSSP and water are combined to create a doping mixture of greater than about 2% w/v (e.g. 0.02g SSpS : 1 mL H_2O). In embodiments, the w/v does not typically exceed 50%. However, any percentage of less than 50% may be used.

Suitable rSSP's include: MaSp1 (as described in US Patent Nos. 7,521,228 and 5,989,894), MaSp2 (as described in US Patent Nos. 7,521,228 and 5,989,894), MiSp1 (as described in US Patent Nos. 5,733,771 and 5,756,677), MiSp2 (as described in US Patent Nos. 5,733,771 and 5,756,677) , Flagelliform (as described in US Patent No. 5,994,099), chimeric rSSP's (as described in US Patent No. 7,723,109), Pyriform, aciniform, tubuliform, aggregate gland silk proteins, and AdF-3 and AdF-4 from *araneus diadematus*. Each of the above referenced patents is herein incorporated by reference in its entirety.

Dope Additives. Various optional additives may be optionally added to the mixture. Suitable additives include compositions that contribute to the solubility of the rSSP in the solution. Some additives break or weaken disulfide bonds, thereby increasing the solubility of rSSP's. Other additives also serve to prevent hydrogel formation after the completion of the microwave step, as set forth below. If the solution forms a hydrogel quickly and the desired end product is not a gel, then

additives capable of delaying or inhibiting such a formation may be desirable. In some embodiments, multiple additives may be added to achieve desired end products.

For example, to combat hydrogel formation, various additives may be added to the suspension of rSSP and water prior to microwaving the suspension. In some embodiments, acid, base, free amino acids, surfactants, or combinations thereof may be employed to combat hydrogel formation. For example, additions of acid (formic acid and acetic acid alone or together at 0.1% to 10% v/v), base (ammonium hydroxide at 0.1% to 10% v/v), free amino acids (L-Arginine and L-Glutamic Acid at 1 to 100mM) as well as a variety of surfactants (Triton X-100 at 0.1% v/v) may be used. The additions of these various chemicals not only aid the solubility of rSSP when microwaved but in certain combinations also delay the solution from turning into a hydrogel long enough for the solution to be spun into a fiber.

By altering and adjusting the combinations of additives to the dopes, the mechanical properties of the spun fiber are significantly impacted. For example, too much acid or base may result in fibers that are brittle with little to no extensibility; too little acid or base may result in dopes where the rSSP will not solubilize to the extent necessary for fiber spinning or turns to a hydrogel quickly.

Exemplary additives also include compositions capable of breaking or weakening disulfide bonds, such as β-mercaptoethanol or dithiothreitol may be added to reduce bonds and increase solubility. Suitable amounts of such additives may include from about 0.1 to about 5% (v/v). In embodiments where the rSSP does not contain cysteine, the use of such additives may be unnecessary. In some embodiments employing major

ampulate silk proteins 1 and 2 (MaSp1 and MaSp2, respectfully), disulfide bonds (cysteine) are present in the C-terminus of the non-repetitive regions of MaSp1 and MaSp2. These proteins are described in U.S. Patent Numbers 7,521,228 and 5,989,894, the entirety of which is herein incorporated by reference. In addition, the C-term is present in various goat-derived spider silk proteins M4, M5 and M55 proteins, which are described in U.S. Patent Application Publication No. 20010042255 A1, the entirety of which is incorporated by reference in its entirety. In some embodiments, formic acid and/or acetic acid may be included in as little as 0.3% (v/v) but even lower amounts (0.1% v/v) are possible. Additionally, it is possible to solubilize rSSP without using any additives.

Exemplary additives are set forth in Table 1 (below), where dope formulations prepared according to the methods described herein and their resultant fibers/films mechanical properties are listed in examples that follow.

Table 1: Additives				
1	**2**	**3**	**4**	**5**
Acid	**Base**	**Free Amino Acids**	**Disulfide Reduction**	**Other**
Acetic	Ammonium Hydroxide	Arginine	β-mercaptoethanol	Triton X-100
Formic	Sodium Hydroxide	Glutamic Acid	Dithiothreitol	Glutaraldahyde
Trifluoroacetic acid		Histidine		Calcium
Other Organic Acids		Glycine		Potassium
Propionic Acid		Imidazole		Other Surfactants
		Other Free Amino Acids		Other Ions
				L-DOPA

In some embodiments, aqueous spin dopes omit additives. In some embodiments, the aqueous spin dope includes imidazole. In some embodiments, the aqueous spin dope includes propionic acid.

To formulate an aqueous solution of rSSP, additives can be chosen from any of the 5 columns. For instance one or a combination of acids can be chosen from column 1 and combined with one or combinations of free amino acids from column 3, as well as disulfide reducing compounds from column 4 and "Other" additives as required by the particular protein. Generally, it would not be useful to include both an acid from column 1 with a base from column 2. However, a base from column 2 can be combined with additives from columns 3-4.

Sonication. In some embodiments, the mixture containing water, rSSP's, and optional additives may be sonicated. The addition of sonication to the rSSP and water suspension may greatly increase the amount of solubilized protein. Sonication may be performed with any suitable sonicator, such as a Misonix 3000 with microtip at 3.0 watts) either prior to microwaving, after microwaving and cooling, or both. Thus in some embodiments, a solution formed containing water, rSSP's, and optional additives may be sonicated.

In embodiments, sonication may be employed to improve the amount of rSSP solubilized and, thus, reduce the amount of protein required to form an aqueous spin dope. Sonication also has the added benefit of producing a more homogenous solution. Sonication also improves and/or changes mechanical properties for rSSP composition products, particularly fiber mechanical properties.

For example, initial experiments required a 12.5% w/v MaSp1 analogue (125mg MaSp1 into 1ml of aqueous) in order to spin a fiber. Sonicating after microwaving reduced the concentration of MaSP1 to 5% w/v necessary to form fibers. Lower rSSP

concentrations results in more fiber spun from a given amount of protein as well as finer fibers which has been demonstrated to increase the mechanical properties in other systems (electrospinning from HFIP based dope solutions).

Microwave Irradiation. The mixture containing water, rSSP's, and optional additives may be microwaved (or otherwise irradiated with microwave radiation) prior to or after the optional sonication step. In embodiments, any microwave may be employed. In some embodiments, the mixture should be sealed prior to microwaving so as to avoid evaporation.

The mixture may be microwaved (irradiated with microwaves) for any suitable amount of time to achieve the desired end product. The time depends on the power of the microwave and the amount of solution to be microwaved. In some embodiments, the solution may be stirred or agitated during microwaving so as to evenly expose the mixture to the microwaves. Appropriate times per unit being microwaved include, for example, from 10 to 90 seconds per 1 milliliter of mixture. In some embodiments the 1ml mixture may be set at from about 10-100% power for from about 5 to 120 seconds.

Without wishing to be bound to any particularly mechanism or theory, it is believe that the irradiation of water and one or more rSSP's increases the temperature of the solution while concomitantly increasing the pressure on the constituents in solution when irradiated or otherwise heated in a sealed vessel.

After microwaving, the solution is allowed to cool and/or is taken to other processing steps, depending on the desired product.

Centrifugation. In some embodiments, the microwaved (irradiated) mixture may be optionally centrifuged. After centrifugation, the resulting supernatant may be removed and then used for rSSP compositions and further processing.

Gel Formation. Hydrogels may be generated from aqueous rSSP solutions by allowing the solubilized rSSP to cool. Additives to the dope such as acetic or formic acid can delay the formation of the hydrogel to allow the rSSP to be transferred to a mold prior to gelation. Theoretically, the variety of shapes that can be generated is limitless. The additives to the solution will change the mechanical properties of the resulting hydrogel. Hydrogel formation has been observed in solutions with as little as 3% w/v rSSP:water and all iterations greater than that. The higher the % of rSSP, the more rapidly the solution gelates. Work in other systems, Bombyx mori silk, has proven the phenomenon that increasing the ratio of silk to water improves the mechanical characteristics of the resulting hydrogel. As well, altering the temperature, pH and including calcium ions changes the properties of the gels (Kim, UJ et al., 2004, Biomacromolecules "Structure and Properties of Silk Hydrogels" Biomacromolecules 5, 786-792).

An example of a hydrogel application is illustrated in Chao et al., "Silk Hydrogel for Cartilage Tissue Engineering." Journal of Biomedical Materials Research Part B: Applied Biomaterials, Vol 95B, Issue 1 pg 84-90, 2010.

Aerogels may be formed by freezing and then lyopholizing a solution or hydrogel of rSSP. Theoretically, the shapes for these aerogels is also limitless as their starting hydrogels could be allowed to form in a mold and then frozen and lyophilized.

Film Formation. Films may be produced by pouring a dope solution onto a substrate and allowing the water and other additives to evaporate. If it is desirable to remove the film from the substrate, PDMS or Teflon allow the removal of the films. A representative dope solution is: 50mg/ml MaSP1 analogue, 1% Formic Acid, 1% acetic acid.

Films prepared by the techniques disclosed herein can vary in their dimensions. An exemplary film size in working embodiments covers a 30 x 7 mm are when the rSSP dope is poured. The film was then cut in half and the thick edges cut for a film with an average length and width of 15 x 5.5 mm and an average thickness of 25um.

Resulting films can also be stretched in 50/50 isopropyl/water bath up to 3.5X. Resulting films can also be stretched in 80/20 MeOH/water bath up to 3X.

Films may be applied as coatings or utilized after removal from a substrate.

Foam Formation. Foam may be generated from aqueous based solvents by a variety of methods and dope conditions. One method reduced to practice is to formulate a dope solution similar/identical to that described for film generation. That solution is then placed into a vacuum chamber and a vacuum applied. The solution quickly expands and forms a foam upon curing in the chamber. Additives to the dopes such as surfactants will influence final cell size and further treatment of the foam (alcohol) are possible to also change the final properties of the foams. It is also possible that foams can be generated by chemical means, mainly peroxidase reactions, to produce CO_2 that creates bubbles in the dope and upon curing a foam remains. (*See* US 20110230911 A1 Scheibel). Again, this method is also influenced by additives such as

surfactants and post formation treatments (alcohol). A final method is an extrusion method whereby the dope solution is mechanically mixed with air, or other gas, to produce foam. This method is also subject to additives and post formation treatments to alter the final foam product.

Fiber Formation. Fibers can be spun from aqueous solutions of rSSP by extrusion into a coagulation bath (alcohol) in a similar fashion as HFIP/aqueous based solutions of rSSP as described in US Patent Application Publication No. 2005/0054830. To summarize, the solubilized rSSP can be loaded into a syringe or other suitable extrusion instrument and then pushed through a fine bore needle into a bath comprised of isopropanol or other alcohol. As the rSSP drops through the alcohol, water is removed and a fiber is formed. That fiber can then be taken up or processed further by stretching in a second or even third bath comprised of alcohol(s), alcohol(s) and water or just water. Fibers have been formed from solutions with as little as 5% w/v solutions of rSSP:water. Similar 5% w/v solutions using HFIP as the solvent will not form fibers.

In some embodiments, it is unnecessary for the solution to remain liquid to form fibers. Indeed, in some embodiments, fibers may be formed from a hydrogel. For example, when forming fibers from MaSp2 proteins, the process may be stopped, the syringe immediately removed for visualization, and a hydrogel may be observed. In contrast, forming fibers from a hydrogel with MaSp1 proteins results in deleterious effects.

It is important to note that each individual rSSP, due to its unique amino acid sequence, will have different requirements for aqueous solubility. The rSSP

concentration, microwave time and power setting, amount of acid or base, and requirements for free amino acids or surfactants will be different. There does not appear to be one set of additives that achieves aqueous solubility and that also delays hydrogel formation for all rSSP's.

As an example, a 12.5% w/v solution of a MaSp1 and MaSp2 analogue can be prepared identically in terms of additives. The MaSp1 will become soluble in water easily and stay liquid for an extended period of time. The MaSp2, on the other hand, will form a hydrogel within minutes of removal from the microwave and requires more microwave time to solubilize.

The following examples are illustrative only and are not intended to limit the disclosure in any way.

EXAMPLES

Process Example- Dope Preparation: An aqueous recombinant spider silk protein (rSSP) dope solution was prepared by weighing out the rSSP such that a 1-40% (w/v) of protein was achieved in 1 ml of water. For example, 50mg's of protein in 1 ml of water yielded a 5% w/v solution of protein to water. The suspension of rSSP and water was sealed inside a 3ml glass Wheaton vial using a PTFE lined cap. The suspension and vial were then placed in a conventional 1500 watt microwave and microwaved at 50% power for 30 seconds. This solubilized the protein powder in the water.

Although this method may work to solubilize the rSSP, the solution quickly formed a hydrogel upon cooling and was generally not available thereafter to spin fibers by extrusion. If the goal of generating the aqueous dope is to form films, foams,

hydrogels or aerogels, this method may be acceptable. Microwave time may vary depending on the volume of the dope, rSSP used, additives chosen, and whether sonication is utilized.

Process Example- Sonication: The following samples were prepared, one of which was not sonicated:

(1) Dope Not Sonicated (12.5% M4, 1% acetic acid, 1% Formic Acid, 50mM L-Arg, Microwaved 30" at 50% power, centrifuged at 6000 rpm for 3 minutes, 1.5X stretch, 40X objective);

(2) Dope Sonicated (5% M4, 1% acetic acid, 1% formic acid, 50 mM L-Arg., microwaved 35 sec. at 50% power, sonicated at power level 1.5 (3 Watts) for 1.5 min., microwaved 30 sec. at 50% power, centrifuged 1 min. at 6000 rpm, 1.5X stretch**,** 40X objective.

Fibers spun from dopes that are not sonicated (Figure 1A), when analyzed microscopically, appear to have numerous lumps and discontinuities. The sonicated 5% w/v MaSP1 fibers (Figure 1B) appear much more uniform. Sonication has the added benefit of requiring lower rSSP concentrations (5% compared to >8% without sonication) to spin fibers from. Lower concentrations are advantageous as less protein is used to spin similar lengths of fiber. Thus, fiber defects when spun from aqueous dopes may be diminished by sonication of the dope.

The following examples set forth numerous rSSP sample tests and resulting data according the formulations and processing criteria set forth below:

Example Set 1 125mg's of M5 (*Nephila clavipes* MaSP2 analogue) was measured out using a fine balance into a 3ml Wheaton glass vial with PTFE seal inside a plastic cap.

Included in the dope solution was 50uL of 1M L-arginine (L-Arginine is prepared in 18.2 MOhm water), 50uL of glacial acetic acid (5% v/v), and 900uL of 18.2 MOhm water. The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a conventional microwave (GE 1.6kW) and microwaved for 30 seconds at 50% power. After microwaving, the solution was placed into a centrifuge (VWR Clinical 2000 set at 6,000 RPM) for 2 minutes to clarify. The supernatant is removed from any remaining pellet for spinning fibers or producing other materials such as films, gels or foams.

Fiber testing results (10 samples) for 1.5X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	37.25	0.75	60.05	0.02
St. Dev.	2.95	0.25	9.11	0.003

Fiber testing results (9 samples) for 2.0X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	37.40	1.98	52.94	0.047
St. Dev.	2.27	1.43		0.03

Fiber testing results (9 samples) for 2.5X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	38.91	18.07	41.64	0.68
St. Dev.	5.15	14.64	17.17	0.54

Fiber testing results (10 samples) for 3.0X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	26.71	40.25	84.54	0.57
St. Dev.	2.12	14.27	18.04	0.18

Fiber testing results (10 samples) for 3.5X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	23.07	22.73	106.65	0.25
St. Dev.	2.64	8.76	22.91	0.09

Example Set 2. 125mg's of M5 (*Nephila clavipes* MaSP2 analogue) was measured out using a fine balance into a 3ml Wheaton glass vial with PTFE seal inside a plastic cap. Included in the dope solution was 50uL of 1M L-Arginine (L-Arginine is prepared in 18.2 MOhm water), 100uL of glacial acetic acid (10% v/v), and 850uL of 18.2 MOhm water. The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a conventional microwave and microwaved for 30 seconds at 50% power. After microwaving, the solution was placed into a centrifuge (VWR Clinical 2000 set at 6,000 RPM) for 2 minutes to clarify. The supernatant was removed from any remaining pellet for spinning fibers or producing other materials such as films, gels or foams.

Fiber testing results (9 samples) 1.5X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	34.17	4.52	72.88	0.07
St. Dev.	5.74	3.10	13.83	0.05

Fiber testing results (9 samples) 2.0X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	31.02	3.92	74.05	0.08
St. Dev.	5.03	2.56	20.69	0.06

Fiber testing results (10 samples) 2.5X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	25.95	15.34	102.62	0.19
St. Dev.	1.08	13.71	17.87	0.18

Fiber testing results (10 samples) 3.0X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	25.71	41.89	87.67	0.55
St. Dev.	2.46	26.92	18.06	0.30

Example Set 3 125mg's of M5 (*Nephila clavipes* MaSP2 analogue) was measured out using a fine balance into a 3ml Wheaton glass vial with PTFE seal inside a plastic cap. Included in the dope solution was 50uL of 1M L-Arginine (L-Arginine is prepared in 18.2 MOhm water), 150uL of glacial acetic acid (15% v/v), and 800uL of 18.2 MOhm water. The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a conventional microwave and microwaved for 30 seconds at 50% power. After microwaving, the solution was placed into a centrifuge (VWR Clinical 2000 set at 6,000 RPM) for 2 minutes to clarify. The supernatant was removed from any remaining pellet for spinning fibers or producing other materials such as films, gels or foams.

Fiber testing results (10 samples) 1.5X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	39.20	3.77	69.92	0.07
St. Dev.	10.74	3.66	15.36	0.06

Fiber testing results (10 samples) 2.0X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	46.93	20.47	53.81	0.37
St. Dev.	5.23	23.18	14.17	0.35

Fiber testing results (10 samples) 2.5X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	46.05	24.95	52.00	0.49
St. Dev.	6.42	25.25	16.49	0.43

Example Set 4. 125mg's of M5 (*Nephila clavipes* MaSP2 analogue) was measured out using a fine balance into a 3ml Wheaton glass vial with PTFE seal inside a plastic cap. Included in the dope solution was 50uL of 1M L-Arginine (L-Arginine is prepared in 18.2 MOhm water), 200uL of glacial acetic acid (20% v/v), and 750uL of 18.2 MOhm water. The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a conventional microwave and microwaved for 30 seconds at 50% power. After microwaving, the solution was placed into a centrifuge (VWR Clinical 2000 set at 6,000 RPM) for 2 minutes to clarify. The supernatant was removed from any remaining pellet for spinning fibers or producing other materials such as films, gels or foams.

Fiber testing results (10 samples) 1.5X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	46.38	0.26	33.18	0.014
St. Dev.	10.11	0.11	7.64	0.003

Fiber testing results (9 samples) 2.0X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	44.65	1.09	63.71	0.02
St. Dev.	8.29	1.39	32.07	0.009

Fiber testing results (10 samples) 3.5X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	37.44	8.44	80.85	0.13
St. Dev.	2.04	11.70	8.09	0.16

Example Set 5. 125mg's of M4 (*Nephila clavipes* MaSP1 analogue) was measured out using a fine balance into a 3ml Wheaton glass vial with PTFE seal inside a plastic cap. Included in the dope solution was 50uL of 1M L-Arginine (L-Arginine is prepared in 18.2 MOhm water), 10uL of glacial acetic acid (1% v/v), 10uL of 88% Formic Acid (1% v/v), 830uL of 18.2 MOhm water. The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a conventional microwave and microwaved for 30 seconds at 50% power. After microwaving, the solution was placed into a centrifuge for 5 minutes to clarify. The supernatant was removed from any remaining pellet for spinning fibers or producing other materials such as films, gels or foams.

Fiber testing results (10 samples) 1.5X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	43.25	2.25	31.68	0.08
St. Dev.	16.23	1.25	7.83	0.04

Fiber testing results (10 samples) 2.0X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	30.02	5.02	61.68	0.09
St. Dev.	2.71	4.79	15.99	0.07

Fiber testing results (10 samples) 2.5X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	28.44	20.93	73.15	0.30
St. Dev.	3.40	18.50	30.78	0.16

Fiber testing results (9 samples) 3.0X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	27.57	3.85	33.38	0.14
St. Dev.	3.88	3.49	21.80	0.08

Example Set 6. 125mg's of M4 (*Nephila clavipes* MaSP1 analogue) was measured out using a fine balance into a 3ml Wheaton glass vial with PTFE seal inside a plastic cap. Included in the dope solution was 50uL of 1M L-Arginine (L-Arginine is prepared in 18.2 MOhm water), 10uL of glacial acetic acid (1% v/v), 30uL of 88% Formic Acid (3% v/v), and 810uL of 18.2 MOhm water. The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a conventional microwave and microwaved for 30 seconds. The solution and vial were allowed to cool and then, the solution was sonicated using a microtip on a Misonix sonicator for 1 minute at a power setting of 1.5. The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a conventional microwave and microwaved for 30 seconds at 50% power. After microwaving, the solution was placed into a centrifuge for 5 minutes to clarify. The supernatant was removed from any remaining pellet for spinning fibers or producing other materials such as films, gels or foams.

Fiber testing results (8 samples) 1.5X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	37.05	0.44	58.36	0.01
St. Dev.	3.32	0.14	13.03	0.002

Fiber testing results (9 samples) 2.0X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	49.03	0.51	33.61	0.02
St. Dev.	2.45	0.16	3.24	0.006

Fiber testing results (10 samples) 2.5X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	39.26	2.43	65.19	0.05
St. Dev.	10.08	1.96	35.24	0.04

Fiber testing results (10 samples) 3.0X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	26.42	1.12	98.28	0.02
St. Dev.	2.27	0.19	13.89	0.002

Example Set 7. 50mg's (5% w/v) of M4 (*Nephila clavipes* MaSP1 analogue) was measured out using a fine balance into a 3ml Wheaton glass vial with PTFE seal inside a plastic cap. Included in the dope solution was 50uL of 1M L-Arginine (L-Arginine is prepared in 18.2 MOhm water), 10uL of glacial acetic acid (1% v/v), and 940uL of 18.2 MOhm water. The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a conventional microwave and microwaved for 30 seconds at 50% power. After microwaving and cooling for 5 minutes, the solution was sonicated for 1 minute at 3.0 watts. After microwaving, the solution was placed into a centrifuge for 2 minutes to clarify. The supernatant was removed from any remaining pellet for spinning fibers or producing other materials such as films, gels or foams.

Fiber testing results 1.5X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	26.35	0.74	59.87	0.02
St. Dev.	0.35	0.39	8.30	0.007

Fiber testing results 3.0X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	17.30	16.31	112.10	0.16
St. Dev.	1.15	12.92	16.81	0.12

Example Set 8. 80mg's (8% w/v) of M4 (*Nephila clavipes* MaSP1 analogue) in addition to 20mg's (2% w/v) of M5(*Nephila clavipes* MaSP2 analogue) was measured out using a fine balance into a 3ml Wheaton glass vial with PTFE seal inside a plastic cap. Included in the dope solution was 50uL of 1M L-Arginine (L-Arginine is prepared in 18.2 MOhm water), 50uL of glacial acetic acid (5% v/v), and 940uL of 18.2 MOhm water. The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a conventional microwave and microwaved for 35 seconds at 50% power. After microwaving, the solution was placed into a centrifuge for 3 minutes to clarify. The supernatant was removed from any remaining pellet for spinning fibers or producing other materials such as films, gels or foams

Fiber testing results 2.0X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	31.62	1.70	68.16	0.04
St. Dev.	5.59	0.42	14.59	0.003

Fiber testing results 2.5X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	29.05	10.63	80.98	0.16
St. Dev.	1.07	3.84	10.78	0.04

Fiber testing results 3.0X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	25.48	23.31	84.91	0.31
St. Dev.	1.85	17.46	6.96	0.23

Fiber testing results 3.5X post spin stretch in an 80:20 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	27.60	14.92	79.61	0.21
St. Dev.	3.88	10.83	37.40	0.13

Example Set 9. 62.5mg's of M4 (*Nephila clavipes* MaSP1 analogue) and 62.5 mg's of M5 (*Nephila clavipes* MaSP2 analogue) was measured out using a fine balance into a 3ml Wheaton glass vial with PTFE seal inside a plastic cap. Included in the dope solution was18.2 MOhm water. The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a conventional microwave and microwaved for 5 seconds repetitively with mixing between bursts of microwave 5 total times. The solution and vial were allowed to cool and then, the solution was sonicated using a microtip on a Misonix sonicator for 1 minute at a power setting of 1.5. The PTFE sealed cap was placed on the 3ml vial tightly. After microwaving, the solution was placed into a

centrifuge for 5 minutes to clarify. The supernatant was removed from any remaining pellet for spinning fibers or producing other materials such as films, gels or foams.

Fiber testing results for a dual stretch; 2X then 1.5X post spin stretch in an 80:20 isopropanol:water and then 20:80 isopropanol:water bath.

	Diameter (µm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	54.94	16.15	43.14	0.44
St. Dev.	2.55	7.88	4.87	

Fiber testing results for a dual stretch; 1.5X then 2.0X post spin stretch in an 80:20 isopropanol:water and then 20:80 isopropanol:water bath.

	Diameter (µm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	45.41	8.12	54.24	0.17
St. Dev.	7.43	9.28	16.69	0.18

112.5mg's of M4 (*Nephila clavipes* MaSP1 analogue) and 12.5 mg's of M5 (*Nephila clavipes* MaSP2 analogue) was measured out using a fine balance into a 3ml Wheaton glass vial with PTFE seal inside a plastic cap. Included in the dope solution was18.2 MOhm water, 0.1% v/v propionic acid, and 10mM imidazole. The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a

conventional microwave and microwaved for 5 seconds repetitively with mixing between bursts of microwave 5 total times. The solution and vial were allowed to cool and then, the solution was sonicated using a microtip on a Misonix sonicator for 1 minute at a power setting of 1.5. The PTFE sealed cap was placed on the 3ml vial tightly. After microwaving, the solution was placed into a centrifuge for 5 minutes to clarify. The supernatant was removed from any remaining pellet for spinning fibers or producing other materials such as films, gels or foams.

Fiber testing results for a dual stretch; 2.0X then 2.5X post spin stretch in an 80:20 isopropanol:water and then 20:80 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	37.29	33.77	134.50	0.28
St. Dev.	2.64	33.55	38.78	0.26

Fiber testing results for a dual stretch; 2.5X then 2.0X post spin stretch in an 80:20 isopropanol:water and then 20:80 isopropanol:water bath.

	Diameter (μm)	Energy to Break (MJ/m^3)	Max Stress (MPa)	Max Strain (mm/mm)
Average	36.98	28.52	192.22	0.18
St. Dev.	3.84	11.97	51.74	0.04

Adhesives. 50mg's of M4 (*Nephila clavipes* MaSP1 analogue) was measured out using a fine balance into a 3ml Wheaton glass vial with PTFE seal inside a plastic cap. Included in the dope solution was 1000µL of 18.2 MOhm water. The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a conventional microwave and microwaved for 55 seconds. 80µl of solubilized M4 was removed from the vial and pipette onto an acrylic plastic plate and assembled with a second plate as in Figure 2. Half of the tested assemblies were scored plastic while the second half was unscored, smooth plastic. Gluing boards were heated in an oven to dry for 24 hours at 30°C. Samples were then tested with a mechanical testing frame (MTS) as shown in Figure 2 with Max Stress and Max Strain observations as reported in Table 2.

Table 2.

	Max Stress (Mpa)	**Max Strain (mm/mm)**
Scored 0	0.240553633	0.061867451
Scored 1	0.284113645	0.050748235
Scored 2	0.290257593	0.032738039
Scored 3	0.207217517	0.053287059
Scored 4	0.275858824	0.038258039
Scored 5	0.23692426	0.035489412
Average	0.255820912	0.045398039

	Max Stress (Mpa)	Max Strain (mm/mm)
Unscored 1	0.045467462	0.018403922
Unscored 2	0.087084871	0.039007059
Unscored 3	0.108057714	0.051941961
Unscored 4	0.115011765	0.027708235
Unscored 6	0.152559701	0.064506667
Average	0.101636302	0.040313569

25mg's of M4 (*Nephila clavipes* MaSP1 analogue) and 25mg's of M5 (*Nephila clavipes* MaSP2 analogue) was measured out using a fine balance into a 3ml Wheaton glass vial with PTFE seal inside a plastic cap. Included in the dope solution was 1000µL of 18.2 MOhm water. The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a conventional microwave and microwaved for 55 seconds. 80µl of solubilized M4 and M5 was removed to a plastic plate and assembled with a second plate as in Figure 1. Half of the tested assemblies were scored plastic while the second half was unscored, smooth plastic. Gluing boards were heated in an oven to dry for 24 hours at 30°C. Samples were then tested with a mechanical testing frame (MTS) as represented in Figure 2 with Max Stress and Max Strain observations as reported in Table 3.

Table 3.

	Max Stress (Mpa)	**Max Strain (mm/mm)**
Scored 0	0.405474817	0.059890196
Scored 1	0.355278111	0.051212549
Scored 2	0.509896194	0.055307451
Scored 3	0.35507744	0.064501176
Scored 4	0.300653595	0.038214902
Scored 5	0.406243752	0.052461176
Average	0.388770652	0.053597908
Unscored 1	0.267291844	0.049407843
Unscored 2	0.336299834	0.05244549
Unscored 3	0.287887532	0.04240549
Unscored 4	0.341145098	0.051715294
Unscored 6	0.336461433	0.038015686
Average	0.313817148	0.046797961

40mg's of M4 (*Nephila clavipes* MaSP1 analogue) and 10mg's of M5 (*Nephila clavipes* MaSP2 analogue) was measured out using a fine balance into a 3ml Wheaton glass vial with PTFE seal inside a plastic cap.

Included in the dope solution was 1000µL of 18.2 MOhm water. The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a

conventional microwave and microwaved for 55 seconds. 80µl of solubilized M4 and M5 was removed to a plastic plate and assembled with a second plate as in Figure 1. Half of the tested assemblies were scored plastic while the second half was unscored, smooth plastic. Gluing boards were heated in an oven to dry for 24 hours at 30°C. Samples were then tested with a mechanical testing frame (MTS) as represented in Figure 2 with Max Stress and Max Strain observations as reported in Table 4.

Table 4.

	Max Stress Mpa	**Max Strain (mm/mm)**
Scored 0	0.297577855	0.043788235
Scored 1	0318335334	0.048578824
Scored 2	0.480984237	0.074135686
Scored 3	0.299748226	0.053203922
Scored 4	0.360972549	0.049265882
Scored 5	0.372502884	0.041647843
Average	0.355020181	0.051770065
Unscored 1	0.123302074	0.019305882
Unscored 2	0.185370089	0.037901961
Unscored 3	0.107880133	0.025557647
Unscored 4	0.184345098	0.031705882

	Max Stress Mpa	**Max Strain (mm/mm)**
Unscored 6	0.167574578	0.031900392
Average	0.153694395	0.029274353

32.5mg's of M4 (*Nephila clavipes* MaSP1 analogue) and 17.5mg's of M5 (*Nephila clavipes* MaSP2 analogue) was measured out using a fine balance into a 3ml Wheaton glass vial with PTFE seal inside a plastic cap. Included in the dope solution was 1000µL of 18.2 MOhm water. The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a conventional microwave and microwaved for 90 seconds. 80µl of solubilized M4 and M5 was removed to a plastic plate and assembled with a second plate as in Figure 1. Half of the tested assemblies were scored plastic while the second half was unscored, smooth plastic. Gluing boards were heated in an oven to dry for 24 hours at 30°C. Samples were then tested with a mechanical testing frame (MTS) as represented in Figure 2 with Max Stress and Max Strain observations as reported in Table 5.

Table 5.

	Max Stress (Mpa)	**Max Strain (mm/mm)**
Scored 0	0.355832372	0.048123137
Scored 1	0.248919646	0.039603137
Scored 2	0.516693579	0.078197647

	Max Stress (Mpa)	Max Strain (mm/mm)
Scored 3	0.298771649	0.043566275
Scored 4	0.230065359	0.033040784
Scored 5	0.279468109	0.031095686
Average	0.321625119	0.045604444
Unscored 1	0.114691674	0.024512157
Unscored 2	0.21934737	0.040428235
Unscored 3	0.212504624	0.038014118
Unscored 6	0.232669566	0.037479216
Average	0.194803309	0.035108431

32.5mg's of M4 (*Nephila clavipes* MaSP1 analogue) and 17.5mg's of M5 (*Nephila clavipes* MaSP2 analogue) was measured out using a fine balance into a 3ml Wheaton glass vial with PTFE seal inside a plastic cap. Included in the dope solution was 1000µL of 18.2 MOhm water. The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a conventional microwave and microwaved for 90 seconds. 80µl of solubilized M4 and M5 was removed to a plastic plate and assembled with a second plate as in Figure 1. Half of the tested assemblies were scored plastic while the second half was unscored, smooth plastic. Gluing boards were heated in an oven to dry for 24 hours at 30°C. Samples were then tested with a mechanical testing

frame (MTS) as represented in Figure 2 with Max Stress and Max Strain observations as reported in Table 6.

Table 6.

	Max Stress (Mpa)	**Max Strain (mm/mm)**
Scored 0	0.339623222	0.043734118
Scored 1	0.323233293	0.040177255
Scored 2	0.533025759	0.05397098
Scored 3	0.307164111	0.03688
Scored 4	0.29905421	0.041872941
Scored 5	0.348283375	0.035167843
Average	0.358397328	0.04196719
Unscored 1	0.083830634	0.022381961
Unscored 2	0.141176471	0.034233725
Unscored 3	0.107584166	0.029144314
Unscored 4	0.184094118	0.047931765
Unscored 6	0.149996185	0.021408627
Average	0.133336315	0.031020078

40mg's of M4 (*Nephila clavipes* MaSP1 analogue) and 10mg's of M5 (*Nephila clavipes* MaSP2 analogue) was measured out using a fine balance into a 3ml Wheaton glass vial with PTFE seal inside a plastic cap. Included in the dope solution was 900µL of

18.2 MOhm water, 100µl 1M imidazole [10mM imidazole], and 1µl propionic acid (99%). The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a conventional microwave and microwaved for 57 seconds. 80µl of solubilized M4 and M5 was removed to a plastic plate and assembled with a second plate as in Figure 1. Half of the tested assemblies were scored plastic while the second half was unscored, smooth plastic. Gluing boards were heated in an oven to dry for 24 hours at 30°C. Samples were then tested with a mechanical testing frame (MTS) as represented in Figure 2 with Max Stress and Max Strain observations as reported in Table 7.

Table 7

	Max Stress (Mpa)	**Max Strain (mm/mm)**
Scored 0	0.191741638	0.028792941
Scored 1	0.201488595	0.034137255
Scored 2	0.300499808	0.057567843
Scored 3	0.198794537	0.039624314
Scored 5	0.322301061	0.048321569
Average	0.242965128	0.041688784
Unscored 1	0.097300369	0.026557647
Unscored 2	0.064799942	0.024059608
Unscored 3	0.074110248	0.02491451

	Max Stress (Mpa)	Max Strain (mm/mm)
Unscored 4	0.038901961	0.006124706
Unscored 6	0.171053635	0.032146667
Average	0.089233231	0.022760627

25mg's of M4 (*Nephila clavipes* MaSP1 analogue) and 25mg's of M5 (*Nephila clavipes* MaSP2 analogue) was measured out using a fine balance into a 3ml Wheaton glass vial with PTFE seal inside a plastic cap. Included in the dope solution was 900µL of 18.2 MOhm water, 100µl 1M imidazole [10mM imidazole], and 1µl propionic acid (99%). The PTFE sealed cap was placed on the 3ml vial tightly. The suspension was sonicated for 1 minute at 3 watts using a microtip on a misonix 3000 sonicator. The solution and vial were placed into a conventional microwave and microwaved for 57 seconds. The solution was clarified by centrifugation at 18kG for one minute. 80µl of solubilized M4 and M5 was removed to a plastic plate and assembled with a second plate as in Figure 1. Half of the tested assemblies were scored plastic while the second half was unscored, smooth plastic. Gluing boards were heated in an oven to dry for 24 hours at 30°C. Samples were then tested with a mechanical testing frame (MTS) as represented in Figure 2 with Max Stress and Max Strain observations as reported in Table 8.

Table 8.

	Max Stress (Mpa)	**Max Strain (mm/mm)**
Scored 0	0.308066128	0.032904314
Scored 1	0.310748299	0.038625882
Scored 2	0.408981161	0.066913725
Scored 4	0.291878897	0.048646275
Scored 5	0.347314578	0.046457255
Average	0.333397813	0.04670949
Unscored 1	0.082512119	0.022306667
Unscored 2	0.120281169	0.029595294
Unscored 3	0.173145098	0.034516078
Unscored 4	0.163302052	0.040027451
Unscored 6	0.184390619	0.036623529
Average	0.144726212	0.032613804

Silicon Adhesive. 180mg's of M4 (Nephila clavipes MaSP1 analogue) and 180mg's of M5 (Nephila clavipes MaSP2 analogue) was measured out using a fine balance into a 8ml Wheaton glass vial with PTFE seal inside a plastic cap. Included in the dope solution was 2640µL of 18.2 MOhm water, 30µl L-Dopa [stock concentration = 10mg/ml], 30µl propionic acid (99%), 300µl of imidazole [stock concentration = 100mM]. The PTFE sealed cap was placed on the 3ml vial tightly. The suspension was

sonicated for 1 minute at 3 watts using a microtip on a misonix 3000 sonicator. The solution and vial were placed into a conventional microwave and microwaved in 5 second burst until solubilized with mixing by swirling between bursts. The solution was clarified by centrifugation at 18kG for one minute. Solubilized protein is then transferred to an air sprayer bowl (Master Airbrush® Brand Model VC16-B22). Air pressure is applied and a fine mist is produced. The mist is then coated onto each silicon surface to be adhered 3 times with a 3 minute dry period between coats.

A second dope is prepared as described in the first paragraph under the heading "Silicon Adhesive" as the bulk adhesive. Approximately 100µl of that solution is then placed on top of one half of the coated silicon. The two pieces of silicon were then gently pressed together and placed into a drying oven preheated to 30°C. Adhesives were cured for 24 hours in the oven. Mechanical testing was performed on a MTS Synergy 100 by placing the ends of each piece of silicon in clamping grips and pulling on the ends until the bond failed.

Adhesive	**Max Stress (MPa)**	**Max Strain (mm/mm)**	**Energy to Break (MJ/m^3)**
Super Glue	0.0495	0.2099	0.0064
Elmers	0.0196	0.0612	0.0007
Spider Silk	0.0292	0.0819	0.0014

Foams. 144mg's of M4 (*Nephila clavipes* MaSP1 analogue) and 36mg's of M5 (*Nephila clavipes* MaSP2 analogue) was measured out using a fine balance into a 8ml Wheaton glass vial with PTFE seal inside a plastic cap.

Included in the dope solution was 2640µL of 18.2 MOhm water, 30µl L-Dopa [stock concentration = 10mg/ml], 30µl propionic acid (99%), and 300µl of imidazole [stock concentration = 1M]. The PTFE sealed cap was placed on the 3ml vial tightly. The suspension was sonicated for 1 minute at 3 watts using a microtip on a misonix 3000 sonicator. The solution and vial were placed into a conventional microwave and microwaved in 5 second burst until solubilized with mixing by swirling between bursts. The solution was clarified by centrifugation at 18kG for one minute. The solubilized protein was then placed on a glass slide and aspirated with a glass pipette until air bubbles were dispersed throughout the solution and allowed to dry on the bench. After a several hours of drying, a spongy foam remained on the glass slide.

Hydrogels. 60mg's of M4 (*Nephila clavipes* MaSP1 analogue) and 60mg's of M5 (*Nephila clavipes* MaSP2 analogue) was measured out using a fine balance into an 8ml Wheaton glass vial with PTFE seal inside a plastic cap.

Included in the dope solution was 1960µL of 18.2 MOhm water and 40µl propionic acid (99%). The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a conventional microwave and microwaved in 5 second burst, 6 times with mixing by swirling between. The suspension was sonicated for 1 minute at 3 watts using a microtip on a misonix 3000 sonicator. After 1 minute of cooling, the lid was removed and the solution poured into a circular plastic mold. The

mold is sealed on the bottom with sheet silicon to prevent the solution from leaking out of the mold. The mold can be of any dimension or shape. The solution remains in the mold until hydrogel formation and then removed by pushing the hydrogel from the mold.

60mg's of M4 (*Nephila clavipes* MaSP1 analogue) and 60mg's of M5 (*Nephila clavipes* MaSP2 analogue) was measured out using a fine balance into an 8ml Wheaton glass vial with PTFE seal inside a plastic cap.

Included in the dope solution was 1960µL of 18.2 MOhm water and 60µl propionic acid (99%).

The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a conventional microwave and microwaved in 5 second burst, 6 times with mixing by swirling between. The suspension was sonicated for 1 minute at 3 watts using a microtip on a misonix 3000 sonicator. After 1 minute of cooling, the lid was removed and the solution poured into a circular plastic mold. The mold is sealed on the bottom with sheet silicon to prevent the solution from leaking out of the mold. The mold can be of any dimension or shape. The solution remains in the mold until hydrogel formation and then removed by pushing the hydrogel from the mold.

Hydrogels were treated with various alcohols after formation by submerging the hydrogel in their respective solution for 60 minutes, then a water rinse for 60 minutes and their mechanical properties studied. They are reported in Table 9.

Table 9.

Post-Treatment	Max Stress (MPa)	Max Strain (mm/mm)	Elastic Modulus (MPa)
H20	0.01727	0.222752	0.078356
50/50 MeOH	0.027454	0.201667	0.175385
50/50 IPA	0.033131	0.250283	0.165239
50/50 EtOH	0.033621	0.175943	0.228684

Hysteresis testing of hydrogels demonstrates that the gels produced are elastic and able to survive repeated loadings. Hydrogels were of the same formulation as reported in the first two paragraphs under heading “hydrogels” above. Results of testing hydrogels is shown in Figure 3 as well as Table 10.

Table 10.

Specimen (No.)	Specimen Height (mm)	Peak Load for Entire Test (N)
1	10.3	2.546
2	10.7	2.5
3	10.8	1.603
4	11	2.921

Lyogels. Lyogels are prepared from hydrogels prepared as reported. Once a hydrogel is formed, that gel is placed into a lyopholization chamber and dried under

vacuum until water is removed leaving only the protein behind. The lyogels can then be post treated to alter the mechanical properties of the lyogel.

60mg's of M4 (*Nephila clavipes* MaSP1 analogue) and 60mg's of M5 (*Nephila clavipes* MaSP2 analogue) was measured out using a fine balance into an 8ml Wheaton glass vial with PTFE seal inside a plastic cap. Included in the dope solution was 1960µL of 18.2 MOhm water and 40µl propionic acid (99%). The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a conventional microwave and microwaved in 5 second burst, 6 times with mixing by swirling between. The suspension was sonicated for 1 minute at 3 watts using a microtip on a misonix 3000 sonicator. After 1 minute of cooling, the lid was removed and the solution poured into a circular plastic mold. The mold is sealed on the bottom with sheet silicon to prevent the solution from leaking out of the mold. The mold can be of any dimension or shape. The solution remains in the mold until hydrogel formation and then removed by pushing the hydrogel from the mold. Once the hydrogel was formed and removed from the mold, it was frozen and placed into a lyopholization bell. Vacuum was applied for 12 hours. Once dried, the lyogels were removed from the lyopholizer and treated with one of three alcohol solutions (50/50 water/isopropanol, 50/50 water/ethanol, 50/50 methanol) or water as a control and then mechanically tested by compressing them while measuring stress and strain as shown in Table 11.

Table 11.

Post Treatment	Max Stress (MPa)	Max Strain (mm/mm)	Elastic Modulus (MPa)
H20	0.93	0.76	1.00
50/50 IPA	0.14	0.37	0.47
50/50 EtOH	0.04	0.14	0.38
50/50 MeOH	0.09	0.28	0.33

Coatings. 90mg's of M4 (*Nephila clavipes* MaSP1 analogue) and 90mg's of M5 (*Nephila clavipes* MaSP2 analogue) was measured out using a fine balance into an 8ml Wheaton glass vial with PTFE seal inside a plastic cap. Included in the dope solution was 2930µl of 18.2 MOhm water, 10mM imidazole, 30µl propionic acid (99%), and 30µl L-Dopa (30ug L-Dopa). The suspension was sonicated for 1 minute at 3 watts using a microtip on a misonix 3000 sonicator. The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a conventional microwave and microwaved in 5 second burst, 6 times with mixing by swirling between. After 1 minute of cooling, the lid was removed and the solution removed to a either a dip bath or sprayer.

Dip coats were achieved by dipping samples repeatedly in the dope solution or dragging through a bath of spider silk protein. The samples are then dried completely on the bench. Dip coatings can be reapplied until the desired thickness is achieved.

Spray coatings were achieved by removing the soluble silk protein to a Master Airbrush Model G233 with a 0.2mm needle tip and spraying the solution onto the

substrate. Spray coatings can be reapplied until the desired thickness of coating is achieved.

Spray and dip coatings can be combined as well. It was observed that a light spray coating, after drying, then dip coating produced a visually impressive coating and it also appeared to adhere to the substrate to a greater degree.

Silicon wafers were coated with aqueous based recombinant spider silk proteins via the spraying method described in the first two paragraphs under the heading “Silicon Adhesives”. Antibiotics and other substances were included in the dope solutions to functionalize the coating. (Kanamycin at 10μg/wafer). Coatings were submerged in Congo Red dye (β-sheet specific dye) to allow visualization of the coating without the aid of a microscope.

All solutions were prepared as dip coats described in the second paragraph following the heading “Coatings.” Resulting products are shown in Figure 4.

Silicon urinary catheters (3 french) coated with spider silk protein (top), spider silk coating loaded with 50mg/L kanamycin (middle) and spider silk coating loaded with 500mg/L kanamycin (bottom) as shown in Figure 5.

Stainless steel can also be coated using dope solutions prepared as described in the second paragraph following the heading “Coatings.” Both spray and dip coating can be used to coat surgical stainless steel.

A stainless steel plate was dip coated with recombinant spider silk protein. Congo Red dye (stains β-sheets) was used to visualize the coating as shown in Figure 6.

Capsules. Capsules can be generated from the described aqueous methods by solvating the recombinant spider silk proteins in water and then allowing them to precipitate or by driving their precipitation via salt precipitation. When combined with another substance, such as a vaccine, the spider silk proteins encapsulate the vaccine.

Alternative Solvation Method : Autoclave. An alternative method of solvation was also tested successfully. Rather than using a microwave to irradiate an aqueous dope, heat and pressure inside of a sealed vial was performed using an autoclave.

60mg's of M4 (*Nephila clavipes* MaSP1 analogue) and 60mg's of M5 (*Nephila clavipes* MaSP2 analogue) was measured out using a fine balance into an 8ml Wheaton glass vial with PTFE seal inside a plastic cap.

Included in the dope solution was 1960µL of 18.2 MOhm water and 40µl propionic acid (99%). The PTFE sealed cap was placed on the 3ml vial tightly. The solution and vial were placed into a conventional microwave and microwaved in 5 second burst, 6 times with mixing by swirling between. The suspension was sonicated for 1 minute at 3 watts using a microtip on a misonix 3000 sonicator. After 1 minute of cooling, the lid was removed and the solution poured into a circular plastic mold. The mold is sealed on the bottom with sheet silicon to prevent the solution from leaking out of the mold. The mold can be of any dimension or shape. The solution remains in the mold until hydrogel formation and then removed by pushing the hydrogel from the mold.

The vial was placed it an autoclave for 75 minutes at 123°C and 20.1 PSI with the lid on, but not tightened. Immediately after removal from the autoclave and cooling,

the solution turned to a hydrogel even though not all of the protein was solvated (visual inspection of white precipitate in vial). However, the method did work to solvate the protein as indicated by the formation of a hydrogel.

The autoclave experiment demonstrates that microwave irradiation unexpectantly provides conditions for aqueous solvation of rSSP. Without wishing to be bound to any particular theory, the source of temperature and pressue from microwave irradiation may be uniquely suited for solvation of the proteins. Microwave irradiation is convenient as it develops heat and temperature quickly within the vial while an autoclave took 75 minutes to only partially solubilize available protein. Other methods of generating heat and pressure are available that generate higher pressure and temperature without the use of a microwave that could be used to solubilize the proteins.

The methods and compositions described herein may also be applied to other traditionally insoluble proteins. Exemplary proteins that may be used in these methods include naturally occurring and synthetic proteins associated with protein misfolding diseases such as prions (CWD, BSE, vBSE, Creutzfeldt-Jakob), Alzheimers, and Parkinsons.

Additionally, synthetically produced G-protein couple receptors (GPCR) are difficult targets as they to suffer aqueous solubility issues. Approximately 40% of drugs produced today are targeted at GPCR's. The methods described herein may also be applied to such GPCR's.

In addition, numerous proteins when expressed in E.coli, are recovered as inclusion bodies. Inclusion bodies are aggregates of the expressed protein that are also insoluble in aqueous solutions. In order to solubilize these proteins, generally high concentrations of urea are used to denature the protein(s). Once denatured, the proteins then have to be renatured into their correct conformation for them to have biological activity. That is not an easy, cheap or quick means by which to synthetically produce proteins. The methods and compositions described herein may also address such insolubility issues with such proteins associated with inclusion bodies.

Statements

1. A method of solubilizing one or more recombinant spider silk proteins in an aqueous solution, comprising:

mixing the one or more recombinant spider silk proteins with water to form a mixture in a sealed container;

heating the mixture to form a solution.

2. The method of claim 1, wherein the heating is performed with microwave irradiation.

3. The method of any one of claims 1-2, further comprising sonicating the mixture.

4. The method of any one of claims 1-3, further comprising sonicating the solution.

5. The method of any one of claims 1-4, further comprising centrifuging the solution.

6. The method of any one of claims 1-5, further comprising providing additives for reducing gel formation in the solution.

7. The method of claim 6, wherein the additives are selected from the group consisting of: an acid, a base, free amino acids, surfactants, and combinations thereof.

8. The method of claim 6, wherein the additives are selected from the group consisting of: propionic acid, formic acid, acetic acid, ammonium hydroxide, L-arginine, L-glutamic acid, β-mercaptoethanol, dithiothreitol, and combinations thereof.

9. The method of any one of claims 1-8, wherein the one or more recombinant spider silk proteins are selected from the group consisting of: M4, M5, MaSP1, a MaSP1 analogue, MaSP2, an MaSP2 analogue, and combinations thereof.

10. The method of any one of claims 1-9, wherein the ratio of the one or more recombinant spider silk proteins to water in the mixture is from 1:10 to 1:2.

11. The method of any one of claims 1-10, further comprising obtaining a recombinant spider silk protein fiber from the mixture.

12. The method of any one of claims 1-11, further comprising stretching the fiber.

13. A recombinant spider silk protein material prepared according to any one of claims 1-12, having the form of a hydrogel, lyogel, film, coating, foam, fiber, and combinations thereof.

14. An aqueous solution of recombinant spider silk proteins, comprising: one or more recombinant spider silk proteins and water, wherein the amount of the one or more recombinant spider silk proteins is greater than about 2% w/v.

15. The aqueous solution of claim 14, wherein the amount of the one or more recombinant spider silk proteins is less than about 50% w/v.

16. The aqueous solution of any one of claims 14 and 15, further comprising one or more additives for reducing gel formation.

17. The aqueous solution of claim 16, wherein the one or more additives are selected from the group consisting of: an acid, a base, free amino acids, surfactants, and combinations thereof.

18. The aqueous solution of claim 16, wherein the one or more additives are selected from the group consisting of: propionic acid, formic acid, acetic acid, ammonium hydroxide, L-arginine, L-glutamic acid, β-mercaptoethanol, dithiothreitol, and combinations thereof.

It will be appreciated that variations of the above-disclosed and other features and functions, or alternatives thereof, may be desirably combined into many other different systems or applications. Also, various presently unforeseen or unanticipated alternatives, modifications, variations or improvements therein may be subsequently

made by those skilled in the art, and are also intended to be encompassed by the following claims.

WHAT IS CLAIMED IS:

1. A method of solubilizing one or more recombinant spider silk proteins in an aqueous solution, comprising:

mixing the one or more recombinant spider silk proteins with water to form a mixture in a sealed container;

heating the mixture to form a solution.

2. The method of claim 1, wherein the heating is performed with microwave irradiation.

3. The method of claim 1, further comprising sonicating the mixture.

4. The method of claim 1, further comprising sonicating the solution.

5. The method of claim 1, further comprising centrifuging the solution.

6. The method of claim 1, further comprising providing additives for reducing gel formation in the solution.

7. The method of claim 6, wherein the additives are selected from the group consisting of: an acid, a base, free amino acids, surfactants, and combinations thereof.

8. The method of claim 6, wherein the additives are selected from the group consisting of: propionic acid, formic acid, acetic acid, ammonium hydroxide, L-arginine, L-glutamic acid, β-mercaptoethanol, dithiothreitol, and combinations thereof.

9. The method of claim 1, wherein the one or more recombinant spider silk proteins are selected from the group consisting of: M4, M5, MaSP1, a MaSP1 analogue, MaSP2, an MaSP2 analogue, and combinations thereof.

10. The method of claim 1, wherein the ratio of the one or more recombinant spider silk proteins to water in the mixture is from 1:10 to 1:2.

11. The method of claim 1, further comprising obtaining a recombinant spider silk protein fiber from the mixture.

12. The method of claim 11, further comprising stretching the fiber.

13. A recombinant spider silk protein material prepared according to claim 12, having the form of a hydrogel, lyogel, film, coating, foam, fiber, and combinations thereof.

14. An aqueous solution of recombinant spider silk proteins, comprising: one or more recombinant spider silk proteins and water, wherein the amount of the one or more recombinant spider silk proteins is greater than about 2% w/v.

15. The aqueous solution of claim 14, wherein the amount of the one or more recombinant spider silk proteins is less than about 50% w/v.

16. The aqueous solution of claim 14, further comprising one or more additives for reducing gel formation.

17. The aqueous solution of claim 16, wherein the one or more additives are selected from the group consisting of: an acid, a base, free amino acids, surfactants, and combinations thereof.

18. The aqueous solution of claim 16, wherein the one or more additives are selected from the group consisting of: propionic acid, formic acid, acetic acid, ammonium hydroxide, L-arginine, L-glutamic acid, β-mercaptoethanol, dithiothreitol, and combinations thereof.

19. A hydrogel made from mixing the one or more recombinant spider silk proteins with water to form a mixture in a sealed container and heating the mixture to form a solution.

20. The hydrogel of claim 19, further comprising an antibiotic material.

ABSTRACT OF THE DISCLOSURE

A method for solubilizing recombinant spider silk proteins in an aqueous solutions, where the method includes mixing recombinant spider silk proteins with water to form a mixture and heating the mixture in a closed vessel to form a solution.

VITA

JUSTIN A. JONES

Professional Experience

INSTITUTION	DEGREE	DATE(s)	FIELD OF STUDY
• University of Wyoming	B.S.	1998	Molecular Biology
• University of Wyoming	M.S.	2000	Molecular Biology
• Utah State University	Ph.D.	2015	Biology

Professional Experience

2000-2001 University of Wyoming Research Scientist, Laramie, WY
2001-2002 AspenBio, Inc. Littleton, CO Protein Production / Research Scientist
2002–2009 Manager, Macromolecular Core Equipment Facility, University of Wyoming Laramie, WY
2008-2010 Biotech Consultant, Advanced Biosolutions, Colorado and California
2009-2011 Director, Macromolecular Core Equipment Facility, Unive of Wyoming, Laramie, WY
2011- Sr. Research Scientist, Laboratory Manager, Utah State University, Dr. Lewis Lab

Honors

2008 College of Agriculture Dean's outstanding professional staff award.

Selected Peer-reviewed Publications

- Hinman, M.B., J.A. Jones, and R.V. Lewis, Synthetic spider silk: a modular fiber. Trends Biotechnol 18(9): p.374-9, 2000
- Brooks BD, Alberston AE, Jones JA, Speare JO, Lewis RV. Efficient screening of high-signal and low-background antibody pairs in the bio-bar code assay using prio protein as the target, Anal Biochem. Nov 1;382(1):60-2 Epub 2008 Jul 17, 2008
- Amanda Brooks, Shane R. Nelson, Justin A. Jones, Courtney Koenig, Michael Hinman, Shane Stricker and Randolph V. Lewis. Distinct contributions of model MaSp1 and MaSp2 Like peptides to the mechanical properties of synthetic major ampullate silk fibers as revealed in silico, Nanotechnology, Science and Applications 1:9-16, 2008
- Creager, Melinda; Jenkins, Janelle; Thagard, Leigh; Brooks, Amanda; Jones, Justin; Lewis, Randy; Holland, Gregory; Yarger, Jeffery, Solid-State NMR Comparison of Various Spiders' Dragline Silk Fiber, Biomacromolecules , 11(8):2039-2043, 2010
- Steinkraus, H.B.; Rothfuss, H.; Jones, J.A.; Dissen, E.; Shefferly, E.; and Lewis, R.V; The absence of detectable fetal microchimerism in nontransgenic goats (*Capra aegagrus hircus*) bearing transgenic offspring, Journal of Animal Science published online October 7, 2011.
- Adrianos SL, Teule F, Hinman MB, Jones JA, Weber WS, Yarger JL, and Lewis RV. Nephila clavipes Flagelliform silk-like GGX motifs contribute to extensibility and spacer motifs contribute to strength in synthetic spider silk fibers, Biomacromolecules Jun 10;14(6):1751-60, 2013
- Chauncey L Tucker, Justin A. Jones, Heidi Bringhurst, Cameron G Copeland, John Bennett Addison, Warner S Weber, Quishi Mou, Jeffry L Yarger, Randolph V Lewis; Mechanical and Physical Properties of Recombinant Spider Silk Films Using Organic and Aqueous Solvents. Biomacromolecules, 15(8):3158-3170, 2014.
- An, B., Tang-Schomer, M. D., Huang, W., He, J., Jones, J. A., Lewis, R. V., & Kaplan, D. L. (2015). Physical and biological regulation of neuron regenerative growth and network formation on recombinant dragline silks. Biomaterials, *48*, 137–146. http://doi.org/10.1016/j.biomaterials.2015.01.044

- Jones, J. A., Harris, T. I., Tucker, C. L., Berg, K. R., Christy, S. Y., Day, B. A., … Lewis, R. V. (2015) More Than Just Fibers: An Aqueous Method for the Production of Innovative Recombinant Spider Silk Protein Materials. Biomacromolecules. http://doi.org/10.1021/acs.biomac.5b00226, 2015.

Presentations:

- Waters Corporation Symposium, Protein Aggregation: Kill and Cure. Investigations of Spider Silk and Prions, 2008
- Tahoe Transgenic Animal Conference "Spider Goats; The trials and tribulations of breeding, rearing and milking goats that express a naturally self associating protein", 2009
- Craniomaxillofacial Meeting at the University of Michigan. Presentation regarding the capability of spider silk produced from transgenic goats to aid the development of bone, skin, ligament and tendon regeneration matrices, 2009
- University of Wyoming, Department of Animal Science Seminar. "CWD Biomarkers", 2009
- Rocky Mountain Biological Engineering Symposium. "The Road to Solvation is Paved With Good Applications." 2015

Funded Grants written or co-written:

- USDA-CSREES 2005-35603-16255 Development of Blood Protein Assays for Prions in Mammalian TSE's
- DOD-DURIP 2006 Purchase of AKTA Explorer and Beckman ProteomeLab PF2D systems in support of prior DOD funding.
- Wyoming Livestock Disease Partnership: Immunological Protein Response to TSE Prion Infection
- Army-DOD SBIR Phase I. 2014 Award