Production of Synthetic Spider Silk Fibers

Cameron G. Copeland

Utah State University

Follow this and additional works at: https://digitalcommons.usu.edu/etd

Part of the Engineering Commons

Recommended Citation
Copeland, Cameron G., "Production of Synthetic Spider Silk Fibers" (2016). All Graduate Theses and Dissertations. 4879.
https://digitalcommons.usu.edu/etd/4879

This Dissertation is brought to you for free and open access by the Graduate Studies at DigitalCommons@USU. It has been accepted for inclusion in All Graduate Theses and Dissertations by an authorized administrator of DigitalCommons@USU. For more information, please contact digitalcommons@usu.edu.
PRODUCTION OF SYNTHETIC SPIDER SILK FIBERS

By

Cameron G Copeland

A dissertation submitted in partial fulfillment of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biological Engineering

Approved:

________________________________                ________________________________
Dr. Randolph V. Lewis                                           Dr. Ronald Sims
Major Professor                                               Committee Member

________________________________                ________________________________
Dr. Charles Miller                                             Dr. Jon Takemoto
Committee Member                                               Committee Member

________________________________                ________________________________
Dr. David W. Britt           Dr. Mark McLellan
Committee Member           Vice President for Research &
                           Dean of the School for Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah
2016
ABSTRACT

Production of Synthetic Spider Silk Fibers

by

Cameron G Copeland, Doctor of Philosophy
Utah State University

Major Professor: Dr. Randolph V Lewis
Department: Biological Engineering

Orb-weaving spiders produce six different types of silks, each with unique mechanical properties. The mechanical properties of many of these silks, in particular the dragline silk, are of interest for various biomedical applications. Spider silk does not elicit an immune response, making it an ideal material for several applications in the medical field. However, spiders cannot be farmed for their silk as they are cannibalistic and territorial. The most reasonable alternative for producing spider silk fibers is to utilize genetic engineering to produce the proteins in a foreign host and then spin fibers from the synthetic protein. Spider silk-like proteins have been expressed in transgenic goats on a scale sufficient to spin synthetic fibers. To spin it, the protein is dissolved in a solvent to create a viscous spin dope. This spin dope is extruded into a coagulation bath where it forms a fiber. Fibers spun in this manner have poor mechanical properties and are water soluble, unlike natural spider silk. By applying a post-spin draw, the
mechanical properties of the fibers improve and they are no longer water soluble. This increase occurs because β-sheets, important secondary structures, form and begin to align parallel to the fiber axis. In previous work, post-spin draw has been applied by hand to the fibers after initial spinning. This is not a viable method for the commercial production of synthetic spider silk. The first aim of this research was to design, test, and optimize a mechanical system that can create consistent, synthetic spider silk fibers. The second aim of this research was to discover how parameters such as solvents, temperature, spinning speed, additives, and post-spin draw, among other variables, affect the properties of synthetic spider-silk proteins purified from goat milk. As part of this research, a mechanical system that can perform these treatments while the fiber is being made was designed, built and tested. This system was built with the intent to inform the creation of a process for the creation of a synthetic on an industrial level.
PUBLIC ABSTRACT
Production of Synthetic Spider Silk Fibers
Cameron G Copeland

Dragline spider silk is among the strongest known biomaterials. It is the silk used for the framework of the web and it is used to catch the spider if it falls. As such, it is stronger and much more flexible than KEVLAR©. Studies show that dragline silk is made of two proteins, Major Ampullate Spider Proteins 1 and 2 (MaSp1 and MaSp2). Due to its incredible mechanical properties, spider silk is being considered for use as a new biomaterial for drug delivery and tendon and ligament replacement/repair, as well as athletic gear, military applications, airbags, and tire cords. However, spiders can’t be farmed. Therefore, methods of mass-producing synthetic spider silk have been developed.

This study has created a process which can produce synthetic spider silk fibers with the best mechanical properties reported to date. Our process has been patented and is used to spin synthetic spider silk, silk/PHB composite fibers, silk/carbon nanotube fibers and aqueous fibers. Changing the conditions under which we create fibers, such as the solvent used to create the dope, the ratio of proteins used, the make-up of the stretch bath and the amount we stretch a fiber, can change their mechanical properties. This allows us to tailor our fibers to the application for which they are being produced.
## CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>PUBLIC ABSTRACT</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>CHAPTER ONE - Literature Review/Research Goals</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER TWO - Design of a Custom Spinning Machine for the Production of Single and Multiple Fibers</td>
<td>27</td>
</tr>
<tr>
<td>CHAPTER THREE - Development of a Process for the Spinning of Synthetic Spider Silk</td>
<td>42</td>
</tr>
<tr>
<td>CHAPTER FOUR - Tunable Fibers</td>
<td>52</td>
</tr>
<tr>
<td>CHAPTER FIVE - Apparatus &amp; Methods for Producing Fibers from Proteins</td>
<td>79</td>
</tr>
<tr>
<td>CHAPTER SIX - Conclusions</td>
<td>114</td>
</tr>
<tr>
<td>APPENDICES</td>
<td>126</td>
</tr>
<tr>
<td>VITAE</td>
<td>163</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>5</td>
</tr>
<tr>
<td>1.3</td>
<td>8</td>
</tr>
<tr>
<td>1.4</td>
<td>10</td>
</tr>
<tr>
<td>2.1</td>
<td>28</td>
</tr>
<tr>
<td>2.2</td>
<td>29</td>
</tr>
<tr>
<td>2.3</td>
<td>30</td>
</tr>
<tr>
<td>2.4</td>
<td>31</td>
</tr>
<tr>
<td>2.5</td>
<td>36</td>
</tr>
<tr>
<td>3.1</td>
<td>37</td>
</tr>
<tr>
<td>3.2</td>
<td>39</td>
</tr>
<tr>
<td>3.3</td>
<td>44</td>
</tr>
<tr>
<td>3.4</td>
<td>45</td>
</tr>
<tr>
<td>3.5</td>
<td>45</td>
</tr>
<tr>
<td>3.6</td>
<td>45</td>
</tr>
<tr>
<td>3.7</td>
<td>46</td>
</tr>
<tr>
<td>3.8</td>
<td>46</td>
</tr>
</tbody>
</table>
3.9 Mechanical testing data from synthetic fibers stretched using a dual-stretch system .................................................................46

3.10 (a) XRD pattern for as-spun synthetic spider silk. (b) XRD pattern for IPA:water stretched synthetic spider silk. (c) XRD pattern for MeOH:water treated synthetic spider silk ....................................................................................................................47

3.11 Supplementary Figure – Chart showing the maximum tensile strength of fibers produced with different ratios of IPA and water in the first bath of the double-stretch system ........................................................................................................51

4.1 Picture of the custom spinning machine used to produce synthetic spider silk fibers ........................................................................................................56

4.2 Stress vs. strain curves for comparison of acetic, formic and propionic acid spin dope solutions ........................................................................................................60

4.3 Comparison of the 2X1.5X and 1.5X2X stretched fibers ......................................................................................................................63

4.4 WAXD images for A) an IPA stretched fiber and B) a MeOH stretched fiber ........................67

Figure 1 ................................................................................................................107
Figure 2 ................................................................................................................108
Figure 3 ................................................................................................................109
Figure 4 ................................................................................................................110

6.1 Schematic of the new multi-fiber spinning head design ........................................114

6.2 Comparison of (A) hand-drawn fibers that were produced and (B) mechanically stretched fibers as part of this research .................................................................117

1D Schematic of the experimental setup .................................................................149

2D Profile of the dragline silk of N. clavipes spider under SEM imaging ..............147

3D Calibration of the temperature coefficient of resistance of the samples ...........150

4D Strong length dependence of thermal conductivities of the dragline silk of N. clavipes spider measured below ~0.001 Pa by reduced model which neglects lateral heat loss ........................................................................................................151
5D Strong length dependence of thermal diffusivities of the dragline silk of \textit{N. clavipes} spider measure below -0.001 Pa by reduced model which neglects lateral heat loss ................................................................................................................................. 151

6D Unbiased thermal conductivities of the dragline silk of \textit{N. clavipes} spider measured below –0.001 Pa by the full model ................................................................................................................................. 152

7D Unbiased thermal diffusivities of the dragline silk of \textit{N. clavipes} spider measured below –0.001 Pa by the full model ................................................................................................................................. 152

8D Unbiased thermal conductivity and diffusivity of the dragline silk of \textit{N. clavipes} spider obtained by linear fitting on the results determined by reduced model with respect to the square of lengths ................................................................................. 153
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Comparisons of Mechanical Properties</td>
<td>1</td>
</tr>
<tr>
<td>1.2</td>
<td>Mechanical properties of synthetic spider silk fibers when a hand post-spin draw (PSD) is applied as-spun and without post-spin treatment</td>
<td>11</td>
</tr>
<tr>
<td>2.1</td>
<td>An example of the calibration table that was used to synchronize the custom Godet with the DACA Spinline</td>
<td>32</td>
</tr>
<tr>
<td>3.1</td>
<td>Mechanical properties of synthetic spider silk fibers as-spun and with a post-spin draw (PSD) applied by hand</td>
<td>44</td>
</tr>
<tr>
<td>3.2</td>
<td>Mechanical properties of fibers processed on the single-stretch mechanical system in different stretch bath compositions</td>
<td>45</td>
</tr>
<tr>
<td>3.3</td>
<td>Mechanical testing data from synthetic fibers stretched made with the mechanical single-bath system</td>
<td>46</td>
</tr>
<tr>
<td>3.4</td>
<td>Table showing standard deviation as a percent of the average of synthetic spider silk fibers from various studies, commercial synthetic fibers, and fibers created in this study</td>
<td>47</td>
</tr>
<tr>
<td>4.1</td>
<td>Formulation of the different spin dopes created and their ability to dissolve protein, spin fibers and whether fibers produced could be subjected to a post-spin draw</td>
<td>59</td>
</tr>
<tr>
<td>4.2</td>
<td>Mechanical properties of fibers made with AA, FA, and PA dopes and stretched in either MeOH or IPA</td>
<td>61</td>
</tr>
<tr>
<td>4.3</td>
<td>Comparison of different stretch ratios in both MeOH and IPA stretch baths</td>
<td>64</td>
</tr>
<tr>
<td>4.4</td>
<td>Mechanical properties for fibers stretched 2X2X with the first bath being either 70:30 IPA:water or 80:20 MeOH:water</td>
<td>65</td>
</tr>
<tr>
<td>4.5</td>
<td>Comparison of rMaSp2 AA, FA, and PA dope fiber mechanical properties</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>Table 1</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Table 2</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>Table 3</td>
<td>97</td>
</tr>
</tbody>
</table>
6.1 The average max stress and strain of fibers processed using different stretch bath compositions .................................................................121

1A Results from hand-stretched fiber experiments ........................................128

1B Results from single mechanical stretch bath experiments .........................130

1C Results from 3 Godet mechanical double stretch-experiments ...................133

1D measured quantities for the 13 spider silk samples and determined $k$ and $\alpha$ by reduced (R) or full (F) model .................................................................151
Spider Silk Properties and Applications

Spider silks are among the strongest fibers known to mankind. Table 1.1 shows the highest recorded mechanical properties of several spider silks along with other common materials. The combination of high tensile strength and elasticity/extension make dragline silk a desirable material for many applications in several different fields$^{3-6}$.

<table>
<thead>
<tr>
<th>Material</th>
<th>Strength (MPa)</th>
<th>Strain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dragline silk</td>
<td>4000</td>
<td>35</td>
</tr>
<tr>
<td>Minor Ampullate silk</td>
<td>1000</td>
<td>5</td>
</tr>
<tr>
<td>Flagelliform</td>
<td>1000</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Tubiliform silk</td>
<td>1000</td>
<td>20</td>
</tr>
<tr>
<td><em>Bombyx mori silk</em></td>
<td>600</td>
<td>20</td>
</tr>
<tr>
<td>Kevlar 49</td>
<td>3600</td>
<td>5</td>
</tr>
<tr>
<td>Rubber</td>
<td>50</td>
<td>850</td>
</tr>
<tr>
<td>Tendon</td>
<td>150</td>
<td>5</td>
</tr>
<tr>
<td>Bone</td>
<td>160</td>
<td>3</td>
</tr>
</tbody>
</table>

$^a$Data from Gosline$^1$, Lewis$^2$, Altman$^3$

Spider silk has several other unique properties. The silk fiber has been shown in several studies to be biocompatible$^{3,7}$. Fibroblast cells, osteocytes, and mammalian cells have all been grown on natural spider silk fibers, synthetic and reconstituted spider and silkworm silk films, and in/on silk hydrogels$^{8-11}$. In many of these studies, the silk performed better than the control, generally collagen, at promoting cell growth. In regards to osteocytes, studies have shown that in addition to promoting cell proliferation, calcification was significantly increased on modified silk films$^{12}$. In addition to the cell growth studies, macrophage responses have been studied *in vitro* with no elicited immune response$^{12,13}$. 
Silk fibers and films can also be modified, at either the gene level or after the protein is produced, to contain cell binding sites, such as the amino acid sequence RGD for improved cell adhesion\textsuperscript{14}. It was shown that the modified silk structures increased cell proliferation and attachment more than the control substances, collagen and polydimethylsiloxane (PDMS), and better than the silk by itself. In vivo studies have shown that there is no significant immune response when spider silk was implanted into rats\textsuperscript{13,15}. In fact, in many of these studies, silk performed better than the materials currently in use for wound closure/repairs. Research has shown that some silks can be degraded by the body, making it a perfect candidate for tendon and ligament scaffolds, sutures, and cellular matrices\textsuperscript{16}. Spider silk protein has also been used in some noteworthy studies with pharmaceuticals for drug delivery. Researchers used silk films to store vaccines and found that they retained bioactivity longer than vaccines preserved with current storage methods even when the vaccines were stored at warmer temperatures, potentially eliminating the need for stringent storage requirements\textsuperscript{17}. Spider silk films have also been impregnated with pharmaceutically active compounds. It was found that the slow biodegradation of the silk structures provided controlled drug release\textsuperscript{10,18}.

In 2012, a paper published by Huang et. al at Iowa State claimed that natural dragline silk of \textit{N. clavipes} has a thermal conductivity comparable with that of copper\textsuperscript{19}. Thermal conductivity is a measure of how well a material transfers heat. This discovery would have opened spider silk to several other potential uses, primarily because silk is roughly one-fourth the density of copper. This would have given silk the potential to be
used in the place of copper to wick heat away from important components, reducing the weight of satellites or in other applications where weight is an issue. However, Fuente et al. studied the thermal conductivity of another orb weaver, *Araneus diadematus*, and found the thermal diffusivity (another measure of how quickly heat moves through a material) to be 400 times lower than the values reported by the group in Iowa. Both spiders are orb weavers and the differences in the genes which produce their silks are minimal. Therefore, in collaboration with the Multiscale Thermophysical Laboratory at USU, the thermal conductivity and diffusivity of natural *N. clavipes* silks and synthetic silks was investigated. As is reported in Appendix D of this dissertation, the findings were that the thermal conductivity of *N. clavipes* silk is similar to the values reported for *Araneus diadematus* silk.

**Spiders and Their Silks**

Over millions of years, orb weaving spiders have evolved the ability to make complex webs for prey capture. These webs are

![Diagram of a spider and the glands that produce each type of silk, along with descriptions on the function of each silk](image)
made of several different types of silk, each with unique mechanical properties and each produced in a different gland. In fact, orb weavers produce six types of silk and one glue, as shown in Figure 1.122.

The silk produced by the major ampullate gland is often referred to as dragline silk. Spiders constantly lay this silk down as they move in order to catch themselves when they fall, hence the name dragline silk. Major ampullate silk also forms the framework of the web. Dragline silk has the unique combination of elasticity along with a high tensile strength, making it one of the toughest known materials. One of the unique properties of dragline silk is its ability to “supercontract” when exposed to water, meaning it loses approximately 25-40% of its length23–25. Since it is the most easily collected and the strongest silk, it is also the most studied. Major ampullate silk is composed of two different silk proteins: MaSp126 and MaSp227. Each of these proteins is very large, around 250 kDa. MaSp1 and MaSp2 proteins can be divided into three parts: the N-terminal, a massive repetitive unit, and the C-terminal. The gene sequences that make up these two proteins are highly conserved across orb-weavers21,28. The N-terminal is also a highly conserved sequence of dragline silk that contains several different possible start codons29,30. The N-terminal contains a secretion signal that allows the protein to leave the epithelial cells in the gland and travel into the lumen. The C-terminal of dragline silk is important in the storage of the spider silk protein in the gland, before the protein is formed into a fiber29,31. The repetitive portion of the dragline silk is the major contributing factor to its unique physical properties.
Tubuliform silk is produced by adult female spiders for only a short period in their life when they are ready to lay their eggs. This silk provides the outer layer of the egg sac and has very high tensile strength that protects the eggs from external forces\(^{32,33}\). The inner layer of the egg sac is made of aciniform silk. Aciniform silk is also used by orb weavers to wrap their prey. It is the weakest among the silk types in terms of tensile strength, but has an extension that is the second highest among that of orb weaver silks, at 80\%\(^{34}\).

Piriform is a specialized silk that is used for attachment or lashing\(^{35–37}\). Piriform is the least studied silk but shows great promise as a potential biomimetic adhesive. Piriform contains unique repetitive sequences whose functions and structures are currently being studied.

Minor ampullate silk is often spun along with a spider’s major ampullate silk. It provides a scaffold and helps to reinforce a spider’s web. Minor ampullate silk, unlike major silk, does not “supercontract” when exposed to water\(^{38}\). In terms of toughness, minor ampullate silk is the weakest of a spider’s silks. Genetically, minor ampullate silk contains a unique spacer sequence that has yet to be characterized\(^{39}\).

\textbf{Figure 1.2} - X-ray diffraction pattern for \textit{Nephila Clavipes} dragline silk\(^{46}\)
Other than major ampullate silk, flagelliform is the most studied silk\textsuperscript{40–42}. This highly elastic silk forms the capture spiral of the web. It combines high strength with high elongation. This elongation serves to absorb the energy of flying insects as they hit the web. Flagelliform is also the largest silk protein, with a size of approximately 320 kDa or more.

**Characterization of Silk**

Early studies by Tillinghast and Work\textsuperscript{25,43,44} showed that dragline silk was a large protein that had an unusually high percentage of glycine and alanine, more than 50% collectively. Over 90% of the sequence of dragline silk is made up of only six amino acids: glycine, alanine, glutamine, serine, proline, and arginine\textsuperscript{44}. It was not until the late 1980s and early 1990s that the genetic sequences of dragline silk proteins were determined. These studies showed that silk was a modular fiber, with distinct motifs repeated\textsuperscript{26,27}.

There are three different structural motifs in spider silk: beta-sheets, beta-spirals and glycine-II helices\textsuperscript{45}. Beta-sheets are prevalent in most silks. In dragline silk these sheets are made of poly-alanine sequences, either $A_n$ or (GA)$_n$. This structural motif is perhaps the most studied. Using X-ray diffraction, researchers have found spider silk contains an oriented, highly crystalline region\textsuperscript{46–49}. Figure 1.2 shows an X-ray diffraction pattern for natural *Nephila clavipes* major ampullate silk\textsuperscript{46}. The intense regions at the (120) and (200) reflections are used in calculating the size of crystalline structures, the percent crystallinity, and the orientation of the crystalline regions with respect to the fiber axis. Researchers have found that spider silk is roughly 28% crystalline and has an
orientation factor of 0.98, with 1.0 being perfectly oriented\textsuperscript{46}. Using NMR\textsuperscript{50–53}, Raman spectroscopy\textsuperscript{54,55}, XRD\textsuperscript{46–49}, and FTIR\textsuperscript{56}, this highly crystalline region was attributed to the beta-sheet motif. Beta-sheets are therefore mainly responsible for the high tensile strength of spider silks.

The second important structural motif found in dragline silk is the beta-spiral. Beta-spirals make up much of the non-crystalline region of spider silk fibers\textsuperscript{40,42,45}. Using molecular modeling, researchers have determined that beta-spirals form what appear to be spring-like helices that are believed to give spider silk much of its elasticity and extension. NMR data confirms the structure of proline in this conformation\textsuperscript{45}. This motif is found only as a major component in MaSp2 and flagelliform proteins. The amino acid sequence for beta-spirals is GPGXX, with XX usually being GY or QQ in MaSp 2 and GY, GS, or GA in flagelliform. This five amino acid sequence forms beta turns and several linked together form the beta-spirals. Aciniform silk is also very elastic, but has a different proline sequence\textsuperscript{34}.

The third motif that is commonly found in spider silks is the GGX motif\textsuperscript{40,45}. This motif is found in MaSp2, minor ampullate silk and flagelliform. It is the least studied in the spider silk literature. It appears from NMR\textsuperscript{50} data that the GGX motif forms a glycine-II helix, which would add to the tensile strength of silk fibers, although its precise function is still not known.
**Natural Fiber Synthesis**

Spiders form silk protein in specialized glands that feed into spinnerets that the back legs of a spider can grab and pull out as a fiber\(^{57-59}\). Figure 1.3 shows a simplified diagram of a spider’s major ampullate gland. Cells in the tail of the gland are specialized cells that produce large amounts of spider silk protein that are secreted into the lumen. These specialized cells are tall columnar cells that have a specialized golgi apparatus\(^{60}\). The protein is then stored in the lumen of the gland at ambient temperature and in an aqueous environment. When a spider pulls on the silk at the spinneret, liquid from the lumen is forced into an S-shaped duct. While moving down this duct, the fluid is transformed from a liquid solution into a solid fiber in as little as 50 milliseconds. The most popular theory on this sudden transformation is that the silk protein is stored in the lumen in a micelle-like structure\(^{61}\), allowing it to stay soluble in an aqueous solution.

When the protein is forced down the duct, shear forces act on the protein, forcing individual protein strands together, causing them to interact and form the solid spider silk fiber while water is extracted\(^{62}\). In rheological tests\(^{63-65}\), the spinning dope stored in the lumen was shown to increase dramatically in viscosity when shear forces were applied.

![Diagram of a spider silk gland](image)
Along the duct, there is a slight pH drop, from 6.6 to 6.3\textsuperscript{29,63,66}. Additionally, potassium and phosphate ions can be found in the duct, while sodium and chloride ions are removed, suggesting an ion exchange in the duct\textsuperscript{66}. It has been theorized that, along with shear forces, pH shifts and the exchange of ions are also necessary for correct fiber formation. However, synthetic silk protein and reconstituted silk fibers have both been formed into fibers successfully without a pH shift or ion exchange\textsuperscript{67–71}. Other researchers have stated that in order to correctly form recombinant spider silk fibers, the terminal ends of the protein must be included\textsuperscript{29,72}. However, researchers have formed synthetic fibers without the conserved C- and N-terminals\textsuperscript{22,67–70,73} although they may play an important role in the natural fiber spinning process.

**Synthetic Fiber Formation**

Spiders cannot be farmed for their silk because they are cannibalistic and territorial. The most reasonable alternative for producing dragline silk fibers is to utilize genetic engineering to produce the proteins in a foreign host, then spinning the fiber \textit{in vitro}. Spider silk genes have been expressed using either an exact copy of the spider’s gene sequence or by taking the genetic sequence for the silk’s structural motifs and constructing a novel spider silk-like protein. These sequences have been successfully expressed in a variety of organisms including bacteria\textsuperscript{69,70,74–78}, mammalian cells\textsuperscript{79}, Sf9 insect cells\textsuperscript{78}, \textit{Bombyx mori}\textsuperscript{80}, potato and tobacco plants\textsuperscript{81}, goats\textsuperscript{82,83}, and yeast\textsuperscript{84}. Many of these synthetic silk proteins have been produced in sufficient quantities to create fibers, films, and gels.
In order to create materials from synthetic spider silk protein, the protein must first be processed. After collection and purification, recombinant spider silk protein is a powder that is generally insoluble in water. This protein can be dissolved, generally using a chaotrophic agent such as 1,1,1,3,3,3-hexafluoro-2-proponal (HFIP) or 9M lithium bromide, to make a highly viscous spin dope. Two methods for creating aqueous spin dopes have recently been published. Heidebrecht et al. employed a method using several dialysis steps to create a spin dope. Tucker et al. used heat and pressure to solubilize recombinant spider silk protein into an aqueous solution.

Synthetic spider silk fibers have primarily been produced in two ways: wet-spinning and electrospinning. Electrospinning is a newer technology that has been used to create polymer mats that are composed of numerous nano-sized fibers. These mats are produced by applying a large positive voltage (10-25 kV) to a needle loaded with liquid polymer that is a short distance from a plate or rotating drum that is negatively charged (Figure 1.4). The electromagnetic force pulls the polymer solution to the plate or drum, forming nanofibers as it is pulled along. This method for spider silk production has the capacity to form fiber mats that could be used in for cell scaffolds and other tissue engineering applications.
Wet-spinning is the extrusion of a spin dope into a coagulation bath. For spider silk, the silk is extruded through a fine needle into an alcohol bath. The shear forces acting in the needle as the dope is extruded, coupled with the extraction of the liquid solvent by the coagulation bath, allow for the formation of fibers. In general, the initial synthetic fibers are weak and brittle. XRD of extruded fibers shows some crystalline structure in the fiber, but it is not oriented. However, using a post-spin draw on fibers can greatly increase their mechanical properties.

Table 1.2 shows the data on the effects of post-spin draws on synthetic silk fibers from other researchers. To apply a post-spin draw, the fiber is immersed in solvent, generally aqueous isopropanol or methanol, and then stretched. This process increases the mechanical properties of the fibers by increasing the degree of crystallinity in the fibers and orienting these crystals parallel to the fiber axis. In the literature and in the beginning

<table>
<thead>
<tr>
<th>Author and Citation</th>
<th>State</th>
<th>Post-Spin Method</th>
<th>Tensile Strength (MPa)</th>
<th>Strain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>An et. al\textsuperscript{67}</td>
<td>As Spun -</td>
<td>35.6</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PSD 3x, 75% IPA</td>
<td>132.5 ± 32%</td>
<td>22.8 ± 179%</td>
<td></td>
</tr>
<tr>
<td>An et. al\textsuperscript{68}</td>
<td>As Spun -</td>
<td>16.2</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PSD 3x, 75% IPA</td>
<td>28.1 ± 29%</td>
<td>26.6 ± 25%</td>
<td></td>
</tr>
<tr>
<td>Teulé et. al\textsuperscript{70}</td>
<td>As Spun -</td>
<td>28.4</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PSD 3x, 75% IPA</td>
<td>101.7 ± 10%</td>
<td>18.7 ± 74%</td>
<td></td>
</tr>
<tr>
<td>Albertson et. al\textsuperscript{89}</td>
<td>As Spun -</td>
<td>10.4</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PSD 4x, 85% IPA</td>
<td>27.1 ± 46%</td>
<td>22.0 ± 118%</td>
<td></td>
</tr>
<tr>
<td>Heidebrecht et. al\textsuperscript{85}</td>
<td>As Spun -</td>
<td>13</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PSD 6x, 75% IPA</td>
<td>370 ± 16%</td>
<td>110 ± 23%</td>
<td></td>
</tr>
<tr>
<td>Adrianos et. al\textsuperscript{90}</td>
<td>As Spun -</td>
<td>26.3</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PSD 3x, 80% IPA</td>
<td>150.6 ± 21%</td>
<td>84.5 ± 27%</td>
<td></td>
</tr>
<tr>
<td>Rothfuss &amp; Copeland, unpublished</td>
<td>As Spun -</td>
<td>49.9</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PSD 3x, 80% IPA</td>
<td>188.1 ± 22%</td>
<td>37 ± 42%</td>
<td></td>
</tr>
</tbody>
</table>
stages of the work presented in this dissertation, post-spin draws have been applied by hand to the fibers after spinning. Table 1.2 shows the results from several publications on the effect of performing a post-spin draw on as-spun fibers. Substantial variability can be seen in the mechanical properties of synthetic spider silk fibers when a stretch is performed (see Table 1.2). This variability could be the result of issues with protein quality, error in sample preparation or testing, the difficulty in performing these stretches by hand on multiple fibers, or a combination of any of these possibilities. As will be shown later, the variability seen in synthetic spider silk fibers is substantially reduced when the mechanical process developed for this dissertation is used.

In order to use synthetic spider silk for the types of commercial applications discussed previously, fibers must be spun and then a post-spin draw must be applied mechanically. It is not feasible to hand-stretch spider silk at a commercial level. Ideally, a system that can create fibers having improved and more consistent mechanical properties as compared to hand stretched fibers is desired and is the focus of this dissertation.

**Research Aims**

There were two primary aims of this research. The first aim was to create a mechanical system that can spin synthetic spider silk fibers from recombinant spider silk protein produced by transgenic goats. The fibers produced with this system needed to have mechanical properties as good as or better than hand-stretched fibers reported previously (see Table 1.2). It was desirable for the fibers to have less variability than those in published studies, equivalent to the low variability seen in commercially produced polymers. Ultimately, this process needed to be one that could be adapted to a
commercial level. The second aim of this research was to gain an understanding of how processing parameters affect the mechanical properties of silk. This would allow researchers to modify the properties of synthetic fibers in two ways: altering the genetic code and changing production parameters, such as stretch, stretch bath composition, dope additives, different silk protein ratio, etc. If possible, this same approach would be applied to various protein constructs.

The process, outlined herein, is the first of its kind to produce synthetic spider silk mechanically, without the need for tedious hand stretching techniques and a non-provisional patent has been applied for. The fibers created have consistent properties, with as good or better standard deviations than currently used industrial fibers. By changing the processing method used to create synthetic spider silk, the mechanical properties can be consistently and predictably altered to fit the needs of the end-user. This innovative approach of creating tunable spider silk fibers by changing the genetic code for the protein and the spinning process, allows for the creation of biomaterials for any number of applications.

References


10.1073/pnas.1206210109.


(24) Boutry, C.; Blackledge, T. Evolution of supercontraction in spider silk: structure-


tubuliform spidroin 1 contributes to extensibility in synthetic fibers.


(37) Blasingame, E.; Tuton-Blasingame, T.; Larkin, L.; Falick, A.; Zhao, L.; Fong, J.; Vaidyanathan, V.; Visparas, A.; Geurts, P.; Hu, X.; et al. Pyriform spidroin 1, a novel member of the silk gene family that anchors dragline silk fibers in
attachment discs of the black widow spider, Latrodectus hesperus. *J. Biol. Chem.*


(80) Teule, F.; Miao, Y.; Sohn, B.; Kim, Y.; Hull, J.; Fraser, M. J.; Lewis, R. V.; Jarvis, D. Silkworms transformed with chimeric silkworm/spider silk genes spin


CHAPTER 2

DESIGN OF A CUSTOM SPINNING MACHINE FOR THE PRODUCTION OF
SINGLE AND MULTIPLE FIBERS

This chapter details the design process of the custom spinning machine created to
spin synthetic silk as well as the various designs attempted to spin multiple fibers
simultaneously.

**Background**

Spiders produce spider silk proteins using specialized glands. These glands
produce spider silk proteins and store them in the gland’s storage area, known as the
lumen, in an aqueous state at room temperature until the silk is needed. The lumen of the
major ampullate gland, the best studied, is connected to a spinneret via an S-shaped duct.
As the protein moves through this duct, shear forces on the proteins cause them to align
and transition from a liquid to a solid\(^1,2\).

However, creating artificial spider silk fibers cannot work by the same process.
First, the spider silk protein is produced by another host organism: bacteria, goats, alfalfa,
or yeast. When purified, this protein is not in the same state as the protein stored in the
lumen of the spider’s gland, nor is it water soluble unless heat and pressure are applied.
After the protein is solubilized by heat and pressure or by using chaotrophic solvents such
as HFIP, the protein must then be extruded. This can be done with a simple extrusion
pump and has been used in several publications\(^3,4\). For the early studies presented in this
dissertation, a modified extruder originally designed for plastic or polymer extrusion was used.

The DACA Spinline

Previously, a modified DACA SpinLine system was used, as seen in Figure 2.1, to spin synthetic spider silk fibers. According to DACA Instruments, the SpinLine is a “multipurpose instrument designed to orient small quantities of polymer fibers in a precise and controlled way”\textsuperscript{5}. The extruder could be programmed to move at a desired speed, and the first Godet could be programmed to rotate at a custom speed independent of the extruder speed. This allowed for controlled extrusion and collection of fibers from a variety of spin dope viscosities. The second Godet was programmed relative to the first Godet’s speed. This allowed fibers to be stretched in between the two Godets, a common practice in polymer manufacturing\textsuperscript{6–8}.

The DACA SpinLine was purchased by Nexia Technologies, the company that, with the Lewis laboratory, created the transgenic goats. Nexia’s engineers modified the
Godet wheels, which on the original design were a single drum, to a three-drum Godet system (see Figure 2.1B). Furthermore, the Godet drums were designed with an indent – as seen in Figure 2.2 – to allow for a bath to fit underneath the Godet wheel and allow the drum to be partially submerged. The Lewis laboratory obtained this modified DACA system from Nexia in 2007, the same year the goats were acquired. This system was used to spin fibers for several publications\textsuperscript{9–13}. However, only the extrusion and winding portion of the system was used; the mechanical stretching abilities of the machine were not employed.

While useful for performing simple bench-top experiments, the programming and function of the DACA was limited: the SpinLine could not be controlled by an independent computer operating system; the handheld controller was limited in its functionality; the winding station was programmed to rotate so that only an 80mm spool could be used after the second set of Godets; the diameter could not be changed; the speed of the winder could be increased, but only by 0.001mm/min at a time. This was programmed into the winder so that as material was collected and the radius of the drum increased, the speed could be slightly increased. Controlling multiple variables on a single spin required the use of a lengthy software menu that, if not correctly navigated while changing parameters, would prematurely shut off the system.

\textbf{Figure 2.2} – Indent built into DACA Godet drums to allow the placement of a bath underneath
Initially, the goal was to use the DACA SpinLine to extrude and stretch fibers simultaneously. Experiments were performed using stretch baths, and multiple variables were tested. First, the solution the fibers were to be stretched in and the configuration of the baths were delineated. Next, multiple lengths of baths were constructed to fit under the Godets, ranging from 10” (the shortest distance possible while the two Godets were right next to each other) to 48”. It was found that optimal bath length was linked to the amount of water in the stretch bath solution; with a higher water-to-solvent ratio, shorter baths could be used. To produce fibers with the highest tensile strength, it was found that a bath length of 24” was ideal. Aligning the Godets for these experiments proved to be difficult. The Godets had to be physically lifted and moved to a new position and then a thin, metal measuring stick was used to assure that the indents of the two Godets were aligned. If there was a misalignment, then the drums of the Godet would grind into the bath, damaging them and causing the machine to shut down.

Figure 2.3 – Diagram of the custom built Godet in order to perform a double stretch with the DACA SpinLine.
Expansion of the DACA Spinline

Further research showed that a double stretch, the first occurring in an alcohol solution and the second in water, generated substantially better fiber mechanical properties. To perform a double stretch, the DACA had to be modified again. It was decided that a custom third Godet would need to be built. After analyzing the hardware and software of the DACA, it was decided that the third Godet would need to be independent of the DACA. This custom Godet would be placed before the DACA’s first Godet. The speed would be independently programmed, and then the speed of the DACA would be programmed to match. In order to spin all three drums at a speed slow enough to match the rate of extrusion, a rotisserie motor was purchased. Other available commercial motors within the allotted budget had minimum speeds significantly higher than the process required. The drums, faceplate, and gears were designed and then custom-machined by Rad Cam Inc (see Figure 2.3). The power source for the motor was a BK Precision 1785B Programmable DC Power Supply. The power supply had 16 programmable buttons for quick voltage changes. Figure 2.4 shows the system with the custom Godet now placed as the first Godet (far left in Fig. 2.4).
The DACA Spinline used m/min as its unit of speed. The speed was converted to seconds per rotation (SPR), the total time it took for the drum to rotate 360°. The relationship between m/min and SPR was a power function, $y=15.58x^{-1.012}$, with $y$ being m/min and $x$ being SPR. The SPR for the custom Godet was measured each time before using the DACA, and this data was used in correlation with the SPR for the DACA SpinLine to synchronize the new three Godet process. Table 2.1 shows an example of the calibration table that was used. Tests were performed to ensure that, after the power supply was turned on and a set voltage was supplied to the custom Godet motor, the motor would continue to spin at the same speed. The SPR were recorded when the motor first started, and then the SPR were recorded every hour for three hours. This was tested multiple times, and the consistency of the speed at a given voltage was confirmed. During these tests, it was discovered that on start-up of the machine, the SPR at a given voltage were not always the same. This meant that before each use, the custom Godet needed to be synced with the DACA Spinline, a process that generally took about 30 minutes.

<table>
<thead>
<tr>
<th>Voltage</th>
<th>DACA M/min</th>
<th>Average SPR</th>
<th>1.5X M/min</th>
<th>2X M/min</th>
<th>2.5X M/min</th>
<th>3X M/min</th>
<th>4X M/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.74</td>
<td>0.6</td>
<td>25.55</td>
<td>0.88</td>
<td>1.18</td>
<td>1.48</td>
<td>1.78</td>
<td>2.39</td>
</tr>
<tr>
<td>2.83</td>
<td>0.65</td>
<td>23.8</td>
<td>0.95</td>
<td>1.27</td>
<td>1.59</td>
<td>1.92</td>
<td>2.56</td>
</tr>
<tr>
<td>2.95</td>
<td>0.7</td>
<td>21.8</td>
<td>1.04</td>
<td>1.39</td>
<td>1.74</td>
<td>2.09</td>
<td>2.80</td>
</tr>
<tr>
<td>3.04</td>
<td>0.75</td>
<td>20.45</td>
<td>1.11</td>
<td>1.48</td>
<td>1.86</td>
<td>2.23</td>
<td>2.99</td>
</tr>
<tr>
<td>3.14</td>
<td>0.8</td>
<td>18.95</td>
<td>1.20</td>
<td>1.60</td>
<td>2.01</td>
<td>2.41</td>
<td>3.23</td>
</tr>
</tbody>
</table>
Design of a Custom Spinning Machine

While the custom Godet worked well for many experiments, the system was difficult to operate and limited in its capabilities. A new spinning machine was required. The new spinning machine needed the following specifications:

- The system required three Godets with the ability to change the speed of each one, preferably with the second and third Godets moving at a multiplier of the first.
- Each drum needed to be able to be independently positioned. Whereas the DACA Spinline had the three drums of each Godet in a set configuration, it was desirable to be able to move the Godet drums not only to increase the time a fiber spent outside of a bath and to give time and space for a heater to dry the fiber, but also to be able to insert differently sized baths into the system with ease.
- The extruder needed to be easily set into a starting position, preferably with the user being able to see when the piston was in place. The DACA SpinLine had an enclosed canister in which the Hamilton syringes sat, making it impossible to see when the piston was in place. This meant that every time the machine was used, the piston had to be removed and the starting position marked.
- The third Godet needed to employ a drum that curved to an inward point, like a V. This would force multiple fibers to come together at the end of the system to form a single yarn. The angle chosen for the V shape was 140°.
• Ideally, the machine would have a small tensiometer in the system. This could inform the user of the maximum amount of tension that could be applied to the fiber through stretching, allowing for fewer breaks while spinning.

• Pressure sensors needed to be added to the extruder in order to warn the user that there was no more spin dope left and that the needle was being put under pressure or that a blockage in the extrusion had occurred and the system needed to be shut off. Ideally, a pressure limit could be input by the user and, upon reaching that limit, the piston would stop moving.

• A sensor could be attached that would not allow the piston extruder to move past a user-determined limit.

• A different extrusion needle was needed. Using the Hamilton syringes required customizing the ferrule and cap of the syringes to fit with the PEEK tubing used for extrusion before use in the DACA. A metal syringe (to eliminate accidental breakage) that could extrude the spin dope without the need to customize the syringes before use was desired.

• The system needed a built-in microscope after the second stretch. It has been observed that the thinner the fiber is, the stronger it tends to be. An inline microscope would give the user an idea as to how the fiber might perform and enable the user to change settings during spinning to minimize fiber diameter and maximize the optical clarity of the fiber.
• The system needed to accommodate different sized spools, allow the user to input the diameter of the spool, and have the program calculate the speed at which the winder needed to rotate.

• The user needed to be able to easily and quickly adjust the winder speed. The DACA Spinline could only increase or decrease the speed of the winder by 0.001mm/min. It was desired to be able to increase or decrease the speed as a percentage of the speed of the third Godet.

• The system needed to include heating lights or elements for which the temperature could be easily adjusted during the spin.

• The system needed to be controlled with a computer rather than a dedicated controller. This would allow for easy input and the ability to fit all the spinning controls (extruder speed, first Godet speed, second and third Godet stretch ratios, and winder stations) on one screen.

• Ideally, two touch screen monitors would be placed on the system, one near the piston extruder to allow a user to monitor and control the extrusion rate, and another at the end of the system for a second user to control the stretch ratios and the winder.

• A thermometer that could be inserted into the baths and display the temperature on the screens was desirable.

• A multi-fiber extrusion head was also to be designed that could fit into the extrusion system.
• It was desired that the system be constructed of easily obtainable parts, so that, in the case of equipment breakdown, the parts could be easily acquired and replaced.

During construction, the design was reviewed and several changes were made to the machine, including how the winder stations would work and the size of the Godet drums. When a working prototype of the software was ready, time was spent inspecting it. Changes were made to how the extruder was tied to Godet speed and how the speed of the Godets was controlled, and including sliders that could change the stretch ratio. The new system is shown in Figure 2.5. After several weeks of use, errors were found in the software and hardware. Software errors were fixed by the programmer at Constellation Laboratories. Mechanical changes were made to the system, most notably inserting different types of fuses. After these changes were made, the system ran as intended.

Figure 2.5 – Photo of the USU Custom Spinning Machine.
Multi-Fiber Spinning

After several attempts to spin multiple fibers at once, a system of tubing was used with parts originally designed for chromatography systems to spin multiple fiber bundles. This system, although limited and somewhat cumbersome to put together, worked well for the spinning of eight fibers at a time. Initially these multi-fiber bundles were difficult to work with due fusing of the fibers. Fibers would fuse internally in the bundle and, as the fiber was collected on the spool, the yarn would fuse together. The

Figure 2.6 – SEM images of 8 fiber bundles produced using chromatography plumbing for the spinning head and spun using the modified procedure to prevent fusing. A) Fiber diameters are measured using SEM software and the average diameters are comparable with what is seen when spinning one fiber at a time. B) The ribbon structure adopted by the yarn can be seen here and is most likely a result of the comb structure keeping the fibers apart during the spinning process. C) Fibers were broken with tweezers and imaged. It is apparent here that the fibers are staying in pairs even when broken. D) A close-up of the fiber break points. There seems to be some fusing between fiber pairs.
mechanical properties of these bundles were below what was expected. The hypothesis was that internal fusing causes some fibers to not stretch properly, possibly causing the decrease in mechanical properties seen. To address this fusing, a special comb was made of polylactic acid (PLA) using a 3D printer that kept the fibers in four groups of two fibers, rather than a bundle of eight. Fibers would only come together into a bundle of 8 fibers at the third Godet. The fusing of the yarn to itself on the spool made it impossible to remove the yarn without it continually breaking. This fusing occurred when the fiber bundles, still wet from the stretch baths, came into contact with one another on the spool. To prevent this, changes to spinning protocol had to be made. First, the spool was moved 62cm, the farthest the electronics would allow, further away from the last Godet to allow more time for the fibers to dry before being collected. Heat lamps were placed over the silk on its way to the spool. Also a small desk fan was placed by the spool to further help drying. Yarn produced with this modification did not fuse to itself on the spool and was easily removed. Additionally, the mechanical properties of these fibers were closer to the expected values. SEM images of the fiber bundles can be seen in Figure 2.6.

The eight fiber bundles were too small and weak to be put through the electronic knitting machines used by our collaborators at Drexel University. Given the tension the knitting machines employ, it was determined that a bundle of 24 fibers would be sufficient for their process. To accomplish this, the eight fiber bundles needed to be spooled into groups of three to get 24 fiber bundles. A custom spooling process was developed. The DACA SpinLine had the option to run just the winder, independent of the rest of the system. This allowed us to use it to do our spooling. After many designs were
considered, a custom base was made that allowed the three 8-fiber spools to be placed on bearings in such a way that all three spools spun together. Then, a small tension gate was printed, again on a 3D printer, to keep the bundle tight on the new spool. This set-up allowed us make 24 fiber bundles which Drexel University was able to make kitted swatches, seen in Figure 2.7.

To eliminate the need for spooling the eight fiber bundles, and to get closer to an industrially adaptable system, a new 24-fiber spinning head is to be developed. This new head has been designed and is being machined by the Space Dynamics Laboratory at Utah State University. The advantage of working with their machine shop is that they possess the ability to produce holes in the extruder head that are 0.010in. Additionally, their laboratory has equipment that can be used to clean the nozzles, should cleaning them become an issue. The approximate date for the construction of this design is the end of 2015.

References


(5) SpinLine Page (DACA Instruments).


CHAPTER 3

DEVELOPMENT OF A PROCESS FOR THE SPINNING OF SYNTHETIC SPIDER SILK

The following chapter was published in ACS Biomaterials Science and Engineering in June of 2015. It is presented as it was in the original publication (ACS Biomaterials Science & Engineering, DOI: 10.1021/acsbiomaterials.5b00092), so tables and figures are numbered without reference to the dissertation chapter. Reproduction of this publication was done with permission from the American Chemical Society.
Development of a Process for the Spinning of Synthetic Spider Silk

Cameron G. Copeland, Brianne E. Bell, Chad D. Christensen, and Randolph V. Lewis

Department of Biological Engineering and Synthetic Biomaterials Center, Utah State University, 650 East 1600 North, Logan, Utah 84341, United States

Supporting Information

ABSTRACT: Spider silks have unique mechanical properties but current efforts to duplicate those properties with recombinant proteins have been unsuccessful. This study was designed to develop a single process to spin fibers with excellent and consistent mechanical properties. As-spun fibers produced were brittle, but by stretching the fibers the mechanical properties were greatly improved. A water-dip or water-stretch further increased the strength and elongation of the synthetic spider silk fibers. Given the promising results of the water stretch, a mechanical double-stretch system was developed. Both a methanol-water mixture and an isopropanol/water mixture were independently used to stretch the fibers with this system. It was found that the methanol mixture produced fibers with high tensile strength while the isopropanol mixture produced fibers with high elongation.

KEYWORDS: spider silk, spinning, mechanical properties, XRD, process development

INTRODUCTION

The Golden Orb Weaver, Nephila clavipes, makes six different types of silk and one glue, each with specific functions. Major ampullate silk, known commonly as dragline silk, is among the strongest known natural materials. Dragline silk has a combination of high tensile strength, elasticity, and extension, making it a desirable material for applications such as ropes or cords, medical sutures, synthetic tendons or ligaments, and sports materials. Studies have shown that dragline silk elicits almost no immunological response and even promotes cell growth, which makes it ideal for medical biomaterials.

Because of its unique properties and potential uses, dragline silk has been highly studied. Dragline silk comprises two different proteins, major ampullate spidroin 1 (MaSp1) and major ampullate spidroin 2 (MaSp2). Each of these proteins are highly repetitive and contain specific sequences which have been highly conserved. Both proteins contain a crystalline region that is formed by a poly alanine motif. This motif forms a crystalline β-sheet structure which is responsible for the high tensile strength of dragline silk. MaSp2 contains a GPGXX motif which makes up much of the presumed amorphous region of the protein and forms a β-spiral that is believed to be responsible for the elasticity of dragline silk. MaSp1 and MaSp2 are mixed in the dragline silk of N. clavipes at a ratio of 4:1 respectively, although the ratio varies in other species.

Spiders cannot be farmed for their silk, they are cannibalistic and territorial. The most reasonable alternative for producing dragline silk fibers is to utilize genetic engineering to produce the proteins in a foreign host, then spinning the fiber in vitro. Spider silk-like proteins have been expressed in a variety of organisms including bacteria, & 99 fibroin cells, and yeast, as well as in the milk of transgenic goats.
Substantial variability in the mechanical properties of stretched synthetic spider silk has been reported in various studies. For Treulé et al., Heidebrecht et al., and An et al., the standard deviations for tensile strength were 10, 16, and 32%, respectively. The standard deviations for strain values from the same studies were even higher, at 74, 23, and 179% of the average. The variability seen in most synthetic fibers could come from a variety of sources, such as an issue in protein quality, error in sample prep and testing, or from the stretching of fibers by hand. To make synthetic spider silk fibers commercially viable and possibly reduce the variability seen in most synthetic fiber properties, a mechanical system must be employed for processing these fibers. In this study, the creation of a mechanical system for processing fibers is presented that reduces variability and improves overall mechanical properties of spun fibers. This process includes the formulation of a functional spinner and the optimization of a mechanical postspin draw.

### MATERIALS AND METHODS

#### Purification of Silk Protein from Milk

The spider silk protein is synthesized by transgenic goats. There are two types of transgenic goats in the herd at Utah State University, goats that produce a MaSp1 protein analog and goats that produce a MaSp2 protein analog. The sequence of each protein is the natural spider silk protein sequence as they have been cloned previously. Both proteins are approximately 65 kDa long and contain both the repetitive region and nonrepetitive C-terminal of the protein. The protein is produced when the goats lactate and is accumulated in their milk. Milk is collected from the transgenic goats and then frozen and stored at −20°C until purification. Fat is separated from the thawed milk by a standard cream separator and the fat is discarded. The milk is combined in a 1:1 v/v ratio with 0.1 M 1-arginine-HCL (Spectrum Chemical MFG, Corp., Gardena, CA) and the pH is adjusted to 9 with 2 M sodium hydroxide (Fisher Scientific, Fair Lawn, NJ). After mixing for 30 min, the milk is loaded onto a custom system of tangential flow filters comprised of a 750 kDa hollow fiber cartridge and 50 kDa hollow fiber cartridge (GE Healthcare, Westborough, MA). The milk is filtered in a continuous loop for 24 h until concentrated and the clarified whey is collected. After filtration, the silk protein is salted out by adding ammonium sulfate (Amresco, Solon, OH) to the whey to a final concentration of 1.2 M and allowed to mix for 24 h at 4°C. The protein is then sonicated and centrifuged with distilled water until the conductivity is observed to be below 20 μS and subsequently lyophilized to dryness. SDS-PAGE is performed to confirm purity on samples using 4–20% gels (Thermo Scientific, Rockford, IL) with the manufacturer’s recommended HEPEs-SDS running buffer. The gel is stained with BioSafe Coomassie blue (Thermo Scientific, Rockford, IL). Western blots are also performed using antibodies specific to the C-terminus of rMaSp1 or rMaSp2. Recent publications have shown this method produces protein that is 90% pure.34–36

#### Spin Dope Preparation

In a ratio of 80:20, synthetic MaSp1 and MaSp2 were placed in a 4 mL glass vial with a Teflon lid (Waters Associates, Milford MA) and then HFP (HFP, Oakwood Chemical, West Columbia, SC) and 88+% formic acid (Alfa Aesar, Ward Hill, MA) was added in a 4:1 ratio in order to make a spin dope that was 25% weight protein/volume solvent (v/v). Vials were placed on a motorized rotator (Labnet, Edison NJ) and allowed to mix for 48 h at 7 rpm. Dopes were then placed in a clinical centrifuge (VWR International, Wohngen Germany) and spun for 24 h at 4100 rpm after which any impurities are removed from the top of the spin dope and the dope is removed to a new vial.

#### Spinning Process

Using a modified DACA SpinLine (DACA Instruments, Santa Barbara, CA),37–39 the spin dope is loaded into a 1 mL Hamilton gastight syringe (Hamilton Company, Reno, NV) that had 10 cm of PEER tubing (internal diameter 0.005") (SUPELCO, Bellefonte, PA) for a needle. The DACA was set to extrude the protein at a rate of 0.7 mm per minute into a 100% isopropanol (Pharmo-Products Inc., Brookfield CT) coacervation bath.

#### Single-Bath Stretching of Fibers

The fibers were submerged in either an isopropanol/water, methanol/water, or 2 M ammonium sulfate bath between two Godets, as seen in Figure 1. The two different Godets can be programmed to spin at different speeds in order to stretch the fibers while they are immersed in the stretch-bath placed between Godets. Fibers were stretched between the two godets at various speeds, from 1.5X up to 4X. Higher stretches were attempted but fibers broke before enough material was collected for testing. Alcohol that was used in the alcohol stretch-bath were either methanol (Pharmo-Products Inc., Brookfield CT) or isopropanol. Both alcohol stretch-baths were mixed with dH2O (distilled, deionized) water, with methanol mixed at a ratio of 4:1 and the isopropanol at a ratio of 8:2. For water dipping experiments, a bath of water was placed between the winder the second set of godets with a small Teflon rod placed in the bath to keep the fibers submerged while traveling through the bath. Optimization of the system was based primarily on maximizing tensile strength.

![Figure 1. Diagram of the DACA SpinLine that has been modified for single-bath stretching of fibers.](image-url)
Hand-Stretching of Fibers. Fibers that were stretched using the single-stretch system were cut into 10 cm lengths and immersed in a 10.90 x 10^5 Pa water bath. Fibers segments were immersed for 1 min before being stretched to 15 cm by gently pulling on both ends of the fiber. Fibers were then removed from the bath and held at the 15 cm length until dry (usually 1 min).

Solubility Tests of Synthetic Silks. 10 cm lengths of silk were placed in a Petri dish with ddH2O to determine their solubility in water. Fibers were left at room temperature in the Petri dishes for one month and were observed weekly.

Multibath Stretching of Fibers. A third set of Godets was built in house that could be used in conjunction with the modified DACA SpinLine. This third set of Godets allowed for two mechanical stretches to be performed on the fibers as they were made. Figure 2 shows the modified DACA SpinLine with the third set of Godets. In the first bath, the fiber was stretched to 150% of its original length (1.5x) using the same baths as for the single-bath stretch described above. Fibers were then submerged in the second bath, ddH2O water, between the second and third set of Godets stretching the treated fiber another 2x. After both stretches, the fibers had a cumulative stretch of 3x. As with the single-stretch system, optimization was done via maximizing the tensile strength of fibers.

Fiber Testing and Analysis. Commercial samples of KEVLA® para-aramid, polyester and nylon were obtained for mechanical tests. All fibers, synthetic spider silk and commercial synthetics, were tested using a procedure documented by Stauffer et al. Each fiber was taken and attached with liquid Super Glue to an X-ray film that was cut for testing purposes. The gauge length of the fiber was 19.1 mm. The diameter of the fibers was obtained by measuring each sample nine times along the length of the sample using a Mitc light microscope and supplied measuring software (Richmond, British Columbia, Canada). Fiber samples were secured to a MTS Synergy 100 (MTS Corporation, Eden Prairie, MN) test bed equipped with a custom 10 g load cell (Transducer Techniques, Temecula, CA). Samples were pulled at a rate of 5 mm/min until breaking and data accumulated at 120 Hz. All tests and measurements were performed at room temperature and the relative humidity during testing of fibers fluctuated between 20 and 26%. The recorded data was exported to Microsoft Excel and MatLab for analysis using piece-wise integration to determine energy to break.

X-ray Diffraction. Samples were taken to the Advanced Photon Source located at Argonne National Laboratory, Argonne, IL, USA and X-ray fiber diffraction was performed on the BioCars 14-BM-C beamline. Fibers were mounted and placed at a distance of 300 mm from the detector. Stretched fibers were placed with the stretched axis normal to the beamline. For a single image, data collection times were 60 s and ten images were taken for each sample. Background images were taken right after each sample was completed with identical parameters. Images were then processed using Fiji software.

RESULTS AND DISCUSSION

Single-Stretch Bath System. Fibers were successfully spun from the spin dope created. The fibers that were collected from the coagulation bath (referred to as as-spun fibers) were white in color. Fibers had an average diameter of 60.6 ± 1.9 μm. Figure 3 shows a comparison of as-spun fibers with fibers stretched to 4X their original length. Stretched fibers were less opaque under the microscope and were significantly smaller than as-spun fibers, with 2X, 3X, and 4X stretched fibers having...
an average diameter of 43.4 ± 1.7, 36.4 ± 0.4, and 32.2 ± 0.9 μm, respectively.

As-spun fibers were difficult to handle as they were brittle and weak, with an average tensile strength of 32.6 ± 7.4 MPa and an average strain of 0.011 ± 0.003 mm/mm. To process fibers, the second set of godets was set to 2X, 3X, and 4X to stretch the fiber. The stretch-bath between the godets was filled with an 80:20 mixture of IPA and water. Figure 4 shows the stress vs strain curves of fibers that were stretched in the described manner. All fibers had improved mechanical properties when compared to the as-spun fibers. As the stretch ratio was increased the tensile strength of the fibers increased, measuring 84.0 ± 9, 143.2 ± 13.5, and 174.3 ± 20.2 MPa for the 2X, 3X, and 4X fibers, respectively. The standard deviation of the tensile strength for the mechanical stretches is improved in comparison to those reported for hand-stretched fibers. The strain of the fibers decreased with increasing stretch ratio, going from 0.56 ± 0.25 mm/mm at a 2X stretch to 0.26 ± 0.11 mm/mm at a 3X stretch to 0.07 ± 0.03 mm/mm at a 4X stretch. From the data it would appear that an increase in tensile strength is connected to decreased strain. Stretching the fiber to 4 times its original length instead of 2 times its original length yielded over a 200% increase in tensile strength but decreased the strain of the 4X fiber to 15% of that of the 2X fiber. The
<table>
<thead>
<tr>
<th>Material</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>KEVLAR</td>
<td>24.89</td>
</tr>
<tr>
<td>para-aramid</td>
<td>27.17</td>
</tr>
<tr>
<td>nylon</td>
<td>8.15</td>
</tr>
<tr>
<td>polyester</td>
<td>15.99</td>
</tr>
<tr>
<td>synthetic spider silk by Teulé et al</td>
<td>110.11</td>
</tr>
<tr>
<td>synthetic spider silk by Ro et al</td>
<td>77.79</td>
</tr>
<tr>
<td>synthetic spider silk by Heidebrecht et al</td>
<td>21.81</td>
</tr>
<tr>
<td>MeOH:water 1:5 x 2x fibers</td>
<td>13.26</td>
</tr>
<tr>
<td>IPA:water 1:5 x 2x fibers</td>
<td>19.01</td>
</tr>
</tbody>
</table>

**The commercial fibers and fibers from this study used n = 10 for calculations of standard deviation and the other values are as reported.

Toughness for the samples, that is the energy required to break the sample, also decreases with decreasing elongation. When placed in water, the as-spun fibers dissolve. Two 10 cm lengths of 3x stretch were placed in a Petri dish with DI water to determine their solubility in water. After one month the fibers were intact, indicating that the fibers in the alcohol/water bath change their structure so that they are no longer soluble in water.

Natural dragline silk has the unique property of supercontracting in water, with studies reporting the silk losing between 25 and 40% of its original length. With this in mind, 10 cm sections of fibers of 3x stretched fibers were cut and then dipped in DI water for 1 min. Fibers were then removed and their length was measured. On average, the stretched fibers contracted 36% of their original length with a standard deviation of 11%. This implies that the structure of the stretched fibers is similar to natural spider silk.

Different baths were attempted in the single mechanical stretch system to explore the effects of different liquids on the fiber’s mechanical properties. Three different baths were attempted: 80:20 IPA and water, 50:50 IPA and water and 2 M ammonium sulfate. These baths were chosen based on hand-stretching experiments using a variety of bath compositions. Ethanol was also attempted in the hand-stretching experiments but did not perform well. Fibers were stretched successfully in each of the three baths, though fibers in the 50:50 IPA:water bath often broke while at a 3x stretch. As such, a 2.5x stretch was gathered and analyzed for the 50:50 bath. The 2 M ammonium sulfate bath would leave the fibers with salt residue on the outside. To remove residue, the fiber was run through a water bath after the second set of godets before being collected on the winder. Table 2 shows the mechanical properties of fibers collected from each of these stretch-bath experiments. The 50:50 bath shows a large decrease in tensile strength when compared to the 80:20 bath, but has significantly improved strain, with the average sample being pulled to 143% of its original length before breaking. This confirms what other researchers have found using NMR and XRD, that with increasing water concentration comes increased mobility of the protein in the fiber. The 2 M ammonium sulfate fibers had nearly identical tensile strength values as the 80:20 IPA:water stretched fibers at 145.45 ± 15.99 MPa as compared to 143.16 ± 13.46 MPa of the 80:20 IPA:water bath. However, the strain of these fibers was better, averaging 0.553 mm/mm as compared to the 0.258 of the 80:20 samples.

**Post Single-Stretch Treatments.** Teulé et al demonstrated that dipping hand-drawn fibers in water after being drawn and dried improved tensile strength and strain, they recorded a 2.8 fold increase in strain. It was theorized that the higher strain observed in the 2 M ammonium sulfate stretched samples was a potential result of being washed in a water bath prior to collection rather than a consequence of the stretch-bath. To determine this, we decided to do a water dip experiment using fibers that were spun using the 80:20 IPA:water stretch-bath and stretched to 3x their original length. A water bath was placed before the winder to dip the fibers before collection, just as they had been for the 2 M ammonium sulfate bath. Furthermore, it was theorized that stretching the sample a second time in water, rather than simply dipping it in water, would increase mechanical properties. To test our two-stretch theory, we collected fibers that were stretched to 3x their original length in the 80:20 IPA:water bath. These fibers were cut into 10 cm segments and then placed in a 10:90 IPA:water bath. Fibers were allowed to soak for 1 min and then fibers were pulled by hand from both sides to a length of 15 cm, meaning they were stretched to 1.5 times their original length. The results from these two experiments can be seen in Figure 5.

The 80:20 IPA:water stretched fibers dipped in water with no stretch before being collected had a tensile strength of 141.1 ± 19.5 MPa, which was statistically identical to the tensile

![Figure 8](image-url)
strength of fibers that were not dipped in water as well as to the 2 M ammonium sulfate stretched fibers (p-value <0.05). However, the strain for these fibers increased from 0.26 ± 0.11 mm/mm without the water treatment to 0.66 ± 0.10 mm/mm with the water treatment, an increase of over 250%. The increase in strain over the 2 M ammonium sulfate fibers was also found to be statistically significant. With this increase in strain, the toughness also increased 213%, to 74.64 MJ/m^2 with the water treatment from 34.63 MJ/m^2 without the water treatment.

Fibers that were stretched a second time had an average tensile strength of 210.9 ± 15.7 MPa, significantly higher than that of the fibers stretched once. The average strain of these samples was 0.50 ± 0.05, slightly higher than the strain of the single stretched fibers. Fibers were stretched to 1.5X the original length, this was after being stretched 3X. This means that the total stretch applied to these fibers was 4.5X. Compare the properties of these fibers to the properties of the fibers that were stretched only once, but to 4X their original length (see Figure 4). The 4X fibers had an average tensile strength of 174.3 MPa with an average strain of 0.07 mm/mm, whereas the increase in length that they were stretched twice to a total of 4.5X their original length had a tensile strength of 210.9 MPa and a strain of 0.30 mm/mm. This shows the benefits of stretching the fibers twice, first in an alcohol bath, and second in a water bath. These results led to the creation of our two-stretch mechanical system.

**Double-Stretch Bath System.** For the second bath in the double-stretch system, two different mixtures were used: DI water and a 90:10 water/IPA mixture. In initial tests, the 100% DI water bath produced fibers that had tensile strengths 30% higher and strain values 85% higher than the 90:10 water/IPA bath. Therefore, for all experiments a 100% DI water was used as the second stretch bath.

Researchers have used varying ratios of isopropanol and water to treat and improve synthetic fibers. To determine the optimal ratio of isopropanol and water, 4X spun fibers with isopropanol-water ratios of 90:10, 80:20, 70:30, 60:40, and 50:50 in the first bath. Maximum tensile strength was the desired property. Results can be seen in Figure S1. A ratio of 70:30 IPA : water resulted in fibers with the highest tensile strength, and all further experiments utilized this IPA:water ratio.

Additionally, other researchers have treated spider silk fibers with methanol, and seen improved properties. As such, a mixture of methanol and water at a ratio of 80:20 MeOH:water was used in the first bath of the three godet double stretch system. For both the IPA:water bath fibers and the MeOH:water bath fibers the same stretch ratios were collected. In the first bath the fibers were stretched to 1.5X their original length and in the second bath fibers were stretched to 1.5X their original length, now labeled as 1.5X 1.5X. For the second stretch ratio, the stretch in the second bath was increased to 2X, labeled as 1.5X 2X. The 1.5X 2X fibers end up 3X the original as-spun length, making them directly comparable to the single bath system. The results of these experiments can be seen in Table 3. For both MeOH:water and IPA:water, as the stretch was increased the tensile strength of the fibers increased but strain decreased. The 1.5X 1.5X fiber for the IPA:water bath had significant strain, 1.253 ± 0.28 mm/mm, which was close to the value of the single stretch in 50:50 IPA:water. However, the tensile strength of the 1.5X 1.5X was 115.8 ± 9.7 MPa, nearly twice that of the 50:50 IPA:water stretched fibers. The 1.5X 1.5X MeOH:water fibers had higher tensile strength than any of the single stretch fibers, at 164.1 ± 23.9 MPa. The strain was similar to that of the water dipped fibers, measuring 0.662 ± 0.13 mm/mm.

Figure 6 shows the stress vs strain curves for the single stretch comparison in to the 1.5X 2X fibers spun with the double stretch system. Fibers processed with the IPA:water bath had a lower tensile strength, measuring 128.8 ± 50 MPa, than the single stretched fibers. However, the strain of the IPA:water fibers measured 0.877 ± 0.13 mm/mm, which is over three times the value of the strain of the single stretch fibers. As a result, the toughness was approximately three times that of the single stretch fibers, with a value 95.9 ± 18.2 MJ/m^2. Methanol:water stretched fibers had a higher tensile strength, 231.7 ± 11.0 MPa, than either the IPA:water or single stretch fibers. The strain was 0.560 ± 0.07 mm/mm, not as high as the IPA:water fibers but nearly double that of the single stretch fibers. Figure 7 shows a comparison of the IPA:water and MeOH:water fibers. Some general trends can be observed in these fibers. As tensile strength is increased, by using MeOH:water as a stretch-bath, there is a loss of strain. However, the toughness of both is nearly identical with MeOH:water fibers at 102.46 MJ/m^2 and the IPA:water fibers at 95.9 MJ/m^2.

Table 4 shows the standard deviations as a percentage of the average for commercial synthetic fibers, the synthetic spider silk fibers produced by other researchers and the double stretch samples presented in this study. The standard deviation for other synthetic spider silk is high, sometimes the standard deviation is larger than the average, most likely due to the nature of how the fibers were processed. The standard deviation of max stress for KEVLAR and para-aramids were 13.73 and 12.98% respectively. The standard deviation of toughness was 24.89 and 27.17%, respectively. These results were higher than that expected for commercial products. Nylon and polyester had smaller deviations. For stress, the standard deviation was 3.80 and 3.41% and for toughness the value was 8.15 and 15.99%. The MeOH:water double stretched fibers had standard deviations similar to those of nylon and polyester, with toughness and stress deviations of 13.26 and 4.97%, respectively. The IPA:water stretched fibers had standard deviations closer to those of KEVLAR and para-aramids, with toughness and stress reporting deviations of 19.01 and 16.17%, respectively. These results show that the double-stretch system is capable of producing fibers with consistent mechanical properties, similar to industrially produced manmade synthetics.

XRD data (Figure 8) on the as-spun and double-stretched fibers helps to explain the differences between the IPA:water bath and the MeOH:water bath. The as-spun fibers show a single ring much like those seen in as-spun synthetic spider silks previously published. The IPA:water double stretched fibers show a much thinner ring with a center approximately equal to the as-spun along with more reflections, both isotropic and anisotropic. However, there is very little of the (120) reflections and no (200) reflections seen in the IPA:water samples. This indicates that there is some recruitment and orientation of β-sheet structures within the IPA:water stretched samples. The MeOH:water double stretched fibers not only show anisotropic and isotropic reflections typical of those seen in natural silks, but the (120) and (200) reflections can be seen. The total XRD data indicates some increase in β-sheet structures with the IPA:water stretched fibers, but much better recruitment and orientation in the MeOH:water stretched fibers. These results correlate with the mechanical properties of the fibers from both
stretch baths and help to explain on a molecular level the reason for the differences between IPA–water stretched fibers and MeOH/water-stretched fibers.

**CONCLUSION**

Previously, synthetic spider silk fibers were spun and then hand-drawn. That method, although useful for studying the feasibility of creating synthetic silk fibers, is ultimately highly variable and not industrially viable. This study describes the evolution of a process for spinning synthetic spider silk fibers which makes use of a mechanical system with the ability to create fibers with consistent mechanical properties. Changing the conditions under which the fibers are processed alters the mechanical properties of the fibers. In addition to changing the protein sequence of synthetic spider silks, changing the resultant fiber through processing adds an alternate means by which synthetic fibers can be tailored to meet the specific needs of defined commercial applications.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomater.0c00202.

Chart showing tensile strength of fibers produced in stretch baths containing different ratios of IPA and water in the first bath of the double-stretch system (PDF)

**AUTHOR INFORMATION**

**Corresponding Author**

E-mail: randy.lewis@asu.edu

Notes

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

The authors acknowledge the Advanced Photon Source at Argonne National Laboratories for the use of their facilities. We also acknowledge USTAR, the National Science Foundation, and the Department of Energy for their support of this research.

**REFERENCES**


(48) Stansifer, S. L.; Coggioli, S. L.; Lewis, R. V. Comparison of Physical Properties of Three Silks from Nephila clavipes and Aranea...
Supplementary Material

Supplementary Figure 3.11 – Chart showing the maximum tensile strength of fibers produced with different ratios of IPA and water in the first bath of the
CHAPTER 4

EXPLORING EFFECTS OF SPIN DOPE CONDITIONS ON MECHANICAL PROPERTIES OF SYNTHETIC FIBERS

This chapter details several experiments on spin dope solvents, protein ratios, and stretch ratios.

Introduction

Over millions of years, orb weaving spiders have evolved the ability to make complex webs to capture prey\textsuperscript{1,2}. These webs are made of several different types of silk, each with unique mechanical properties and each produced in a different gland. Orb weavers produce six types of silk and a glue\textsuperscript{3}. The silk produced by the major ampullate gland is often referred to as dragline silk. Orb weaving spiders lay this silk down as they move in order to catch themselves when they fall, hence the name dragline silk.

Dragline silk has both high tensile strength and elasticity, making it one of the toughest known materials\textsuperscript{4,5}. It is the most studied silk, due to the ease at which it can be collected and its presence in the web. Major ampullate silk is composed of two proteins: MaSp1\textsuperscript{6} and MaSp2\textsuperscript{7}. Each of these proteins is approximately 250 kDa. The amino acid sequences that make up these two proteins are highly conserved across orb-weavers\textsuperscript{1,8}. Researchers have demonstrated that the C-terminal of dragline silk is important in the storage of spider silk protein in the gland, before the protein is formed into a fiber\textsuperscript{9,10}, and it has been demonstrated to be important for correct fiber formation\textsuperscript{11}. The N-terminal is a highly conserved portion of dragline silk that contains several start codons\textsuperscript{9,12}. The N-
terminal contains a secretion signal that allows the protein to be exported from the protein producing columnar epithelial cells and transported to the protein storage reservoir or lumen of the gland.

There are primarily three different structural motifs in the repetitive region of dragline silk: beta-sheets, beta-spirals, and glycine-II helices. Beta-sheets are prevalent in both MaSp1 and 2 and are comprised of poly-alanine sequences, either An or (GA)n. Using X-ray diffraction, researchers have identified that beta-sheets align parallel to the fibers axis, with a highly crystalline structure. Using NMR, Raman spectroscopy and FTIR, this highly crystalline region was confirmed to be beta-sheets.

The second important structural motif found in dragline silk is the beta-spiral which make up much of the so called amorphous region of the dragline silk. Beta-spirals form spring-like helices that give spider silk much of its elasticity and extension. This motif is found only as a major component of MaSp2 in dragline silk and in flagelliform (capture spiral) silk. The amino acid sequence for beta-spirals is GPGXX, with XX generally being GY or QQ in MaSp 2. Multiple repeats of this motif result in the formation of beta spirals.

The GGX motif is the third motif found in spider silks. It appears, from NMR data, that the GGX motif forms a glycine-II helix, which would add to the tensile strength of silk fibers. This motif is the least studied in the spider silk literature, its precise function and structure are still not known.
Spiders cannot be farmed for their silk because they are cannibalistic and territorial. Therefore, an alternative route must be pursued to create usable quantities of silk. The most reasonable alternative for producing dragline silk fibers is to utilize genetic engineering to produce the proteins in a foreign host, then spinning a fiber in vitro. Spider silk-like proteins have been expressed in a variety of organisms including bacteria$^{27-29}$, goats$^{30-32}$, Sf9 insect cells$^{33}$, and yeast$^{34}$ to produce protein in sufficient quantities to enable study.

A major challenge faced by researchers is creating spin dopes from recombinant spider silk protein. Several methods exist for this purpose. Heidebrecht et al. have employed a method using several dialysis steps to create an aqueous spin dope and then hand spinning and stretching fibers$^{11}$. Tucker et. al used heat and pressure to solubilize recombinant spider silk protein into an aqueous solution and produce thin films$^{30}$. Several researchers have used hexafluoro-isopropanol (HFIP) to create a spin dope from recombinant spider silk protein (rSSp)$^{29,35-37}$. Regardless of solvation method, spin dopes are extruded into an alcohol bath where they form into fibers, though electrospinning can be employed also$^{38,39}$. In general, the initial fibers produced (as-spun) in this manner are weak and brittle until a post-spin draw is applied$^{11,36,37,40,41}$. To apply a post-spin draw, the fiber is immersed (generally in aqueous isopropanol or methanol) and then a defined stretch is applied. The research efforts that have looked at the effects of post-spin draw has done so by hand stretching these fibers$^{11,36,37,40,42}$. This technique has produced fibers with improved mechanical properties over as-spun fibers but suffer from a high degree of variability due to the inherent inaccuracies of hand spinning and stretching. However, the
process of creating spider silk fibers from produced proteins has yet to be optimized. In this study, we present data on: the solvation ability of several different spin dopes, most of them including HFIP, the fibers produced from several different spin dopes, the effect of changing the ratio of rMaSp1 and rMaSp2, and the effects of different mechanical methods for post-spin draw on the mechanical properties of fibers.

Materials and Methods

Spin dope preparation

Protein for spin dopes was produced and purified using methods previously described\textsuperscript{30}. Several dopes were created using various solvents and mixtures including, namely HFIP (HFIP; Oakwood Chemical, West Columbia, SC), 88+% formic acid (Alfa Aesar, Ward Hill, MA), acetic acid (VWR International, Radnor, PA), anhydrous toluene (Sigma-Aldrich, St. Louis, MO), DI water, propionic acid (Alfa Aesar, Heysham, England), dimethyl sulfoxide (DMSO)(Amresco, Solon, OH), zinc chloride (ZnCl)(Amresco, Solon, OH), and isopropanol (IPA; Pharmco-Aaper, Brookfield, CT). For ZnCl dopes, the ZnCl was added incrementally until the protein was solubilized, which occurred once the molar concentration was 5.5M. Different ratios of rMaSP1 and rMaSP2, as well as varying solvent mixture, solvent ratio, proteins’ ratio, and concentration, were used for testing dope solubilization, fiber formation and mechanical properties.

Purified silk protein powder was placed in a 4mL glass vial with a Teflon lid (Waters Associates, Milford MA) and the chosen solvent mixture was added to the spider silk protein to a concentration that was either 20 or 25% weight protein/volume solvent
Vials were placed on a motorized rotator (Labnet, Edison NJ) and allowed to mix for one week at 7rpm. Dopes that successfully solubilized the protein were then placed in a clinical centrifuge (VWR International, Wehingen Germany) and spun for 24 hours at 4180rcf after which any impurities are removed from the top of the spin dope with a cotton swab and the liquid dope transferred to a new vial. The ratio of rMaSp1 and rMaSp2 analogs varied from dope to dope, as per the experimental parameters.

**Spinning process**

One of two spinning machines was used to spin the fibers. A modified DACA SpinLine (DACA Instruments, Santa Barbara, CA), or a new custom spin machine built in collaboration with Constellation Labs (Figure 4.1). When using the DACA SpinLine, the spin dope is loaded into a 1mL Hamilton gas-tight syringe (Hamilton Company, Reno, NV) that had approximately 10cm of PEEK tubing (internal diameter 0.005”) (SUPELCO, Bellefonte, PA) as a needle. When using the custom spinning machine, the spin dope was loaded into a 2.5mL stainless steel syringe with 1/8” Swagelok™ (KD Scientific, Holliston, MA) with 10cm of PEEK tubing (internal diameter 0.005”)

(SUPELCO, Bellefonte, PA) for a needle. The dope is then extruded into a 100% isopropanol (Pharmo-Products Inc., Brookfield CT)

**Figure 4.1** - Picture of the custom spinning machine used to produce synthetic spider silk fibers.
coagulation bath.

The fibers were submerged in an alcohol/water bath between the first and second Godets, as seen in Figure 4.1. The two Godets can be programmed to turn at different speeds in order to stretch the fibers while they are immersed in the stretch-bath placed between them. Alcohols that were used in the alcohol stretch-bath were either methanol (Pharmo-Products Inc., Brookfield CT) or isopropanol. Both alcohol stretch-baths were mixed with dDI (distilled, de-ionized) water, with methanol mixed at a ratio of 4:1 and the isopropanol at a ratio of 7:3. Fibers were then submerged in the second bath, dDI water, between the second and third set of Godets stretching the treated fiber a second time.

**Fiber testing and analysis**

The synthetic silk fibers were tested using the procedure documented by Stauffer et. al\(^ {43} \). In short, each fiber was taken and attached with liquid Super Glue© to x-ray film that was cut for testing purposes. The gauge length of the fiber was 19.1mm. Using a Motic light microscope and supplied measuring software (Richmond, British Columbia, Canada), the diameter of the fibers was obtained by measuring each sample nine times along the length of the sample to get an average diameter. Then, the samples were loaded into a MTS Synergy 100 (MTS Corporation, Eden Prairie, MN) test bed equipped with a custom 10g load cell (Transducer Techniques, Temecula, CA)\(^ {37} \). Samples were pulled at one of two speeds, 5mm/min or 250mm/min, until breaking and data accumulated at 120Hz for the 5mm/min and at 500Hz for 250mm/min. The slower testing speed was used when comparing different stretch ratios using the same dope and for initial tests
comparing MeOH and IPA baths. The faster testing speed was used in order to collect
data that could be applied to real-world applications. The recorded data was exported to
Microsoft Excel and MatLab for analysis of mechanical properties and basic statistics.

**X-Ray Diffraction**

Fibers were examined at the Advanced Photon Source at the Argonne National
Laboratory, Argonne IL, USA and X-ray fiber diffraction was performed on the BioCars
14bm-C beamline. Fibers were mounted and placed at a distance of 300mm from the
detector. Stretched fibers were placed with the stretched axis normal to the beam line. For
a single image, data collection times were 60 seconds and five images were taken for
each sample. Background images were taken immediately after each sample with
identical parameters. Images were then processed using Fit2D software.

**Results and Discussion**

**Spin Dopes**

Initially, 30 dopes were created using a variety of solvent mixtures. Of the 30
dopes tested, only eleven generated fibers with sufficient strength to perform post-spin
draw. Table 4.1 shows the dopes created and which ones produced a fiber that could be
collected, manipulated, and tested. All successful dopes contained HFIP. Water as an
additive in HFIP failed to spin fibers that could be collected and tested. Dopes that were
made with only a small percentage of HFIP or contained no HFIP failed to completely
dissolve the silk proteins, did not spin fibers, or made fibers that were too brittle to be
collected and tested. Using less HFIP in a dope was preferable due to the cost and toxicity of HFIP but these data clearly show there are limits to decreasing HFIP.

**Acetic vs Formic vs Propionic Acids**

Acetic, formic, and propionic acids all produced fibers that had appreciable tensile strength and strain when stretched, see Table 4.2. The ideal concentration of the acids was 20% v/v, when more acid was used, the dopes did not produce fibers or even solubilize the protein. The ratio of rMaSp1 and
rMaSp2 used in these tests was 4:1 rMaSp1:rMaSp2, the average that is found in the *N. clavipes* spider⁴⁴, though variation has been observed.

The rSSp solubilization time of each solvent was different. The 80:20 HFIP:Formic acid dope (FA) solubilized 4:1 rMaSp1:rMaSp2 protein mixture in 4-10 hours. The 80:20 HFIP:propionic acid dope (PA) took between 24-48 hours to solubilize. The 80:20 HFIP:acetic acid dope (AA) took the longest to solubilize, between 72 and 120 hours. By comparison, HFIP only dopes tended to solubilize the protein in 48-72 hours.

Figure 4.2 shows representative stress vs. strain curves for fibers produced from the AA, FA, and PA dopes using the double stretch system.

![Figure 4.2](image-url)
with a 2X stretch in either 80:20 MeOH:water bath (MeOH bath) or 70:30 IPA:water bath (IPA bath) as the first bath and a 2X stretch in DI water for a cumulative stretch of 4X its original length. The results of the MeOH bath and the IPA bath were similar to those reported previously\textsuperscript{32}, namely that the MeOH bath produces fibers which have a high tensile strength, 220-250MPa, with average maximum strain ranging from 0.25-0.40mm/mm whereas the IPA bath produces fibers which have a higher strain, 0.56-0.69mm/mm, and lower tensile strength, between 150-185MPa. As can be seen in the

<p>| Table 4.2 - Mechanical properties of fibers made with AA, FA, and PA dopes and stretched in either MeOH or IPA. |
|---------------------------------------------------|---------------------------------------------------|
| <strong>MeOH Stretched</strong> | <strong>IPA Stretched</strong> |</p>
<table>
<thead>
<tr>
<th>Toughness (MJ/m³)</th>
<th>Stress (MPa)</th>
<th>Strain (mm/mm)</th>
<th>Young's Modulus (GPa)</th>
<th>Toughness (MJ/m³)</th>
<th>Stress (MPa)</th>
<th>Strain (mm/mm)</th>
<th>Young's Modulus (GPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic Acid</td>
<td>76</td>
<td>223</td>
<td>0.407</td>
<td>4.94</td>
<td>87</td>
<td>159</td>
<td>0.652</td>
</tr>
<tr>
<td>Std. Dev (%)</td>
<td>±14</td>
<td>±5</td>
<td>±10.9</td>
<td>±8.2%</td>
<td>±16</td>
<td>±6.9</td>
<td>±14</td>
</tr>
<tr>
<td>Formic Acid</td>
<td>78</td>
<td>247</td>
<td>0.382</td>
<td>4.91</td>
<td>88</td>
<td>183</td>
<td>0.565</td>
</tr>
<tr>
<td>Std. Dev (%)</td>
<td>±15</td>
<td>±3.0</td>
<td>±13.3</td>
<td>±9.2%</td>
<td>±15</td>
<td>±6</td>
<td>±12</td>
</tr>
<tr>
<td>Propionic Acid</td>
<td>48</td>
<td>220</td>
<td>0.259</td>
<td>4.75</td>
<td>92</td>
<td>156</td>
<td>0.685</td>
</tr>
<tr>
<td>Std. Dev (%)</td>
<td>±24.5</td>
<td>±7</td>
<td>±24.2</td>
<td>±5.9%</td>
<td>±24</td>
<td>±16</td>
<td>±23</td>
</tr>
</tbody>
</table>

charts in Figure 4.2 and Table 4.2, the Young’s Modulus of the fibers are nearly identical, ranging from 4.41-4.94GPa. For the IPA bath stretched fibers, the yield point occurs between 120 and 160 MPa, while the MeOH bath fibers reached the yield point between 170 and 200 MPa. The behavior of the curves after the yield point was different. IPA bath fibers dropped in tensile strength during a yielding phase, whereas, with the exception of PA dope fibers, MeOH bath stretched fibers began to strain harden rather than yield.
Table 4.2 compares the average toughness, max stress and strain of fibers produced with the AA, FA, and PA dopes. When stretched in the MeOH bath, the FA dope fibers had the highest average tensile strength, 246MPa. The tensile strength of both AA and PA dopes was similar, 222MPa and 220MPa respectively. However, the average maximum strain of the AA dopes was the highest among MeOH fibers, 41%, whereas the strain of the PA dopes was the lowest at 26%. The average max strain of the FA dope fibers was similar to that of the AA dope fibers, at 38%. The shape of the PA dope fibers when stretched in the MeOH bath was also slightly different, the yield point was approximately 200MPa, followed by a dip in tensile strength before strain hardening (Fig. 2, panel A). When using the IPA bath, the FA dope once again produced fibers with the highest tensile strength, 183 MPa with a strain of 57%. AA and PA dopes were remarkably similar in shape of their mechanical testing curve and values, 159MPa with 65% strain for AA dope fibers and PA dope fibers had an average tensile strength of 156MPa with 69% strain.

Other Spin Dopes

Several of the other spin dopes were able to produce fibers that could be stretched. However, the fibers from many of these dopes still had poor mechanical properties after a post-spin draw. The 50:50 formic acid/HFIP fibers had a tensile strength of 102MPa and a strain of 2.9%. The 90:10 HFIP:IPA solvent was used to create dopes for a single stretch and for the double stretch system. With a single stretch of 3X the fibers were brittle, with an average strain of 6% and a tensile strength of 90MPa. After a double stretch of 2X2X, the 90:10 HFIP:IPA fibers had a tensile strength
129MPa, better than after a single stretch of 3X, but the average strain was 5.3%, equivalent to the strain after a single stretch. Due to the unremarkable mechanical properties of all of these dopes and their fibers, they were not pursued further.

*Stretch Ratios Comparison*

A comparison of different stretches was done using the FA dopes with a 4:1 ratio of rMaSp1 and rMaSp2. The stretches were 1.5X1.5X, 1.5X2X, 2X1.5X and 2X2X in both the MeOH bath and IPA bath. The data from these experiments can be found in Table 4.3 and Figure 4.3. As previously reported, as a fiber is increasingly post-spin stretched, the tensile strength increases at the cost of strain. The 1.5X2X and the 2X1.5X stretched fibers, in MeOH and IPA baths, have similar properties. Figure 4.3 shows the stress vs. strain curves for two representative fibers from both the 1.5X2X and 2X1.5X in both stretch baths. The shapes of the curves, along with the averages for the mechanical properties are equivalent (p-value < 0.05).

![2X1.5X vs 1.5X2X](image)

**Figure 4.3** - Comparison of the 2X1.5X and 1.5X2X stretched fibers.
A 2X2.5X stretch was attempted using the FA dope in both the MeOH and IPA baths. The MeOH bath stretched fibers could not sustain the 2X2.5X stretch ratio for longer than 8 meters. The IPA bath fibers could be gathered at the 2X2.5X stretch ratio without breaking, and could be stretched up to 2X3X. However, the 2X3X stretch consistently broke during spinning. The 2X2.5X IPA bath stretched fibers had an average tensile strength of 225MPa and average strain of 42.6%, similar to those found for MeOH fibers at 2X2X.

**Differing Ratios of rMaSp1 and rMaSp2**

In *N. clavipes*, the average ratio of MaSp1 to MaSp2 is 4:1, though large variations can occur\(^{45,46}\). However, in *Argiope aurantia*, the ratio between the two proteins is 2:3\(^{8,47}\). The mechanical properties and protein sequences of the dragline silks from each species are remarkably similar. The mechanical properties of natural spider’s silks have large standard deviations\(^2,48\). There are several possible factors for this, from variations of the protein ratios at different points in the fiber, to variations in the size/shape of a spider’s gland to differences in the speed of

| Table 4.3 - Comparison of different stretch ratios in both MeOH and IPA stretch baths. |
|-----------------|-----------------|-----------------|
| **MeOH Stretched** | Toughness (MJ/m\(^3\)) | Stress (MPa) | Strain (mm/mm) |
| 1.5X1.5X std. dev(%) | 92 | 164 | 0.662 |
| 1.5X2X std. dev(%) | 102 | 222 | 0.560 |
| 2X1.5X std. dev(%) | 91 | 213 | 0.515 |
| 2X2X std. dev(%) | 80 | 277 | 0.341 |
| **IPA Stretched** | Toughness (MJ/m\(^3\)) | Stress (MPa) | Strain (mm/mm) |
| 1.5X1.5X std. dev(%) | 87 | 123 | 0.845 |
| 1.5X2X std. dev(%) | 79 | 146 | 0.641 |
| 2X1.5X std. dev(%) | 68 | 138 | 0.578 |
| 2X2X std. dev(%) | 88 | 183 | 0.565 |
| 2X2.5X std. dev(%) | 81 | 225 | 0.426 |
extrusion. Due to the low variation in our synthetic spider silk fibers, this study sought to understand the relationship between the mechanical properties of synthetic silk and the ratio between MaSp1 and MaSp2. For this, five spin dopes were created with varied ratios of rMaSp1 and rMaSp2. The ratios used were 1:0, 4:1, 1:1, 1:4, and 0:1 rMaSp1:rMaSp2. These dopes were created with 20% v/v HFIP/acetic acid as the solvent.

<table>
<thead>
<tr>
<th>rMaSp1 :rMaSp2</th>
<th>IPA:water Stretch Bath</th>
<th>Stress (MPa)</th>
<th>Strain (mm/mm)</th>
<th>Diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
<td>87 ± 15%</td>
<td>170 ± 10%</td>
<td>0.613 ± 10%</td>
<td>27 ± 2%</td>
</tr>
<tr>
<td>4:1</td>
<td>65 ± 43%</td>
<td>157 ± 18%</td>
<td>0.510 ± 43%</td>
<td>29 ± 9%</td>
</tr>
<tr>
<td>1:1</td>
<td>24 ± 160%</td>
<td>127 ± 10%</td>
<td>0.219 ± 149%</td>
<td>29 ± 2%</td>
</tr>
<tr>
<td>1:4</td>
<td>30 ± 112%</td>
<td>96 ± 15%</td>
<td>0.342 ± 106%</td>
<td>26 ± 4%</td>
</tr>
<tr>
<td>0:1</td>
<td>100 ± 18%</td>
<td>178 ± 9%</td>
<td>0.703 ± 15%</td>
<td>23 ± 3%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>rMaSp1 :rMaSp2</th>
<th>MeOH:water Stretch Bath</th>
<th>Stress (MPa)</th>
<th>Strain (mm/mm)</th>
<th>Diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0</td>
<td>68 ± 41%</td>
<td>218 ± 19%</td>
<td>0.370 ± 35%</td>
<td>24 ± 2%</td>
</tr>
<tr>
<td>4:1</td>
<td>79 ± 18%</td>
<td>226 ± 6%</td>
<td>0.419 ± 13%</td>
<td>26 ± 3%</td>
</tr>
<tr>
<td>1:1</td>
<td>50 ± 20%</td>
<td>200 ± 15%</td>
<td>0.317 ± 34%</td>
<td>25 ± 7%</td>
</tr>
<tr>
<td>1:4</td>
<td>62 ± 46%</td>
<td>165 ± 14%</td>
<td>0.434 ± 42%</td>
<td>26 ± 3%</td>
</tr>
<tr>
<td>0:1</td>
<td>79 ± 36%</td>
<td>271 ± 16%</td>
<td>0.372 ± 30%</td>
<td>25 ± 1%</td>
</tr>
</tbody>
</table>

All five spin dopes were successfully spun in the new double stretch system. Fibers from the spins were mechanically tested at a rate of 250mm/min. Table 4.4 demonstrates the mechanical testing data from all of the different protein ratio spin dopes when stretched 2X in the first bath and 2X in the second bath. When the averages of all the different protein ratios are averaged, the IPA:water-stretched fibers had a stress of 145MPa with a
strain of 0.48mm/mm and a toughness of 61MJ/m³. The methanol-stretched fibers had an average stress of 215MPa and a strain of 0.38mm/mm, which led to a toughness of 68MJ/m³. The difference between the two types of stretches can also be seen in XRD images that were taken. Figure 4.4 shows the different 2d WAXD patterns for IPA:water- and MeOH:water-stretched fibers made from the same spin dope, the 4:1 ratio. It appears in these images that the crystalline segments of the fiber have a higher degree of orientation, as indicated by the intensity at the (120) and (200) reflections, along the fiber axis in the MeOH:water-stretched fibers as compared to the IPA:water-stretched fibers. This is the only instance where the XRD images of different groups of fibers differ in any significant way.

Individually, the various rMaSp1:rMaSp2 ratios behaved differently than expected. Due to the high amount of poly-alanine sequences in the repetitive region of MaSp1, it was hypothesized that the fibers containing only rMaSp1 would have a higher amount of crystallinity and therefore have the highest tensile strength among all of the spin dopes created. It was also expected that, as rMaSp1 content went down and rMaSp2 content was increased, the fibers would exhibit higher strain while having decreased tensile strength, due to the decreasing amount of poly-alanine sequences and the inclusion of the GPGXX β-spiral. It was also hypothesized that this would be a roughly linear relationship. The mechanical testing and XRD results, however, show no clear pattern in the behavior of the fibers made from the different protein ratios.
Statistical analysis was performed on the tensile strength and strain of fibers using the statistics toolbox found in Microsoft Excel. The p-value limit for these tests was 0.05. The IPA-stretched fibers fell into two statistical groups. The 1:0, 4:1, and 0:1 ratios of rMaSp1 and rMaSp2 fibers were all statistically equivalent, while the 1:1 and 1:4 ratios were poorer in comparison to the other ratios in the first statistical group but statistically equivalent to each other. When stretched in MeOH:water, the ratios fell into three different statistical groups. The 0:1 ratio statistically had a tensile strength of 271MPa, higher than all other fibers. The 1:0, 4:1, and 1:1 ratios all fell into the same group, with tensile strengths ranging from 200-220MPa. Once again, the 1:4 were inferior mechanically, with a tensile strength of 165MPa, to all other fibers. If one assumes that the 0:1 ratio of rMaSp1 and rMaSp2 produces fibers with the highest tensile strength and toughness, then we may be able to explain why the 1:4 ratio showed the much lower tensile strength shown above, it is possible that the small amount of rMaSp1 in these fibers acts as a contaminant in the fiber, inhibiting the interactions between rMaSp2 proteins.

When analyzing the XRD results statistically, there are no significant differences between any of the different protein ratios. There are not enough reflections to calculate
the unit cell size of the crystalline section of the fiber and the orientation of the different ratios is indistinguishable. There are several possibilities for this result. First, the spinning of rSSp fibers is dissimilar from that of natural silk. This process may not be aligning the β-sheets the same way as they are in natural silk fibers. Second, these fibers were made with proteins that were 65kD long. Roughly one third of the length of this protein is comprised of the non-repetitive C-terminal section of the protein. Forty kilodaltons of the protein then comprises the repetitive region of the silk proteins, the sections responsible for the mechanical properties of spider silks. In natural spider silks, the repetitive regions are approximately 250KDa\textsuperscript{1,8}. It is possible that the C-terminal sequence is interrupting formation of crystalline structure found in silk fibers with shortened repetitive units. In order to elucidate the impact that the different ratios of MaSp1 and MaSp2 have on the mechanical properties of fibers more clearly, repetitive regions closer in length to natural silks are likely to be required.

The results of this set of experiments show that, with the exception of 1:4 rMaSp1:rMaSp2, the mechanical properties of fibers made with different ratios of the two dragline proteins are roughly equivalent in this system. This result correlates with results reported in literature of the mechanical properties of different species of orb weavers and individual spider specimens in which the ratio of MaSp1 and MaSp2 are widely different but show similar mechanical properties\textsuperscript{8,47}. However those reported results may be due to the high standard deviations seen in natural silks. The results reported here, with much lower standard deviations, could be due to the length of the repetitive regions of these synthetic proteins, which are less than 20% as long as the
repetitive regions of natural spider silk. It is likely that, once rSSp’s are made that are closer in length to the natural silk proteins, the effects of MaSp1 and MaSp2 on fiber mechanical properties, crystallinity, and alignment could be determined.

**rMaSp2 Only Fibers (Acetic vs Formic vs Propionic)**

Due to the high tensile strength of the rMaSp2 only fiber, it was used to further explore the differences between AA, FA, and PA dopes. Table 4.5 shows the averages for toughness, stress, and strain for AA, FA, and PA when using only rMaSp2. The fibers were stretched 2X2X with either the MeOH bath or the IPA bath as the first stretch and DI water as the second stretch bath. When using MeOH as the first stretch bath, fibers made from the FA dope also had the highest tensile strength, 293MPa, with an average strain of 29.8%. The AA dope had a similar tensile strength, 284MPa, but had a higher strain, 40.7%. PA dope had a similar strain to the AA dopes, 40.4%, but the stress was lower, 226MPa. When the IPA bath was used the AA and FA had equivalent strain values, 70.3% and 70.4% respectively, and similar stress values 178MPa and 162MPa respectively which was similar to the other ratios where IPA led to increased strain and

<table>
<thead>
<tr>
<th></th>
<th>rMaSp2 Only MeOH Stretched</th>
<th>rMaSp2 Only IPA Stretched</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Toughness (MJ/m³)</td>
<td>Stress (MPa)</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>87 ±18%</td>
<td>284 ±6%</td>
</tr>
<tr>
<td>Formic Acid</td>
<td>68 ±20%</td>
<td>293 ±5%</td>
</tr>
<tr>
<td>Propionic Acid</td>
<td>73 ±13%</td>
<td>226 ±8%</td>
</tr>
</tbody>
</table>
reduced stress. The PA dopes had lower tensile strength and strain values, 156MPa and 55.3%.

**Conclusion**

Of the spin dope solutions attempted, only those that were primarily solvated with HFIP produced fibers that could be processed and had toughness values that were above 50MJ/m³. The solutions containing acetic, formic and propionic acid produced fibers with the highest tensile strength. Formic acid as an additive to the HFIP consistently produced fibers that outperformed other acids tested in terms of tensile strength, and had the lowest solubilization time, 2-4 hours, while propionic acid had the lowest tensile strength and strain. MeOH bath stretched fibers had higher tensile strengths, between 220MPa and 250MPa, but lower strains than IPA bath stretched fibers. However, the IPA bath allowed for fibers to be collected at a higher stretch than the MeOH bath. When a cumulative stretch of 3X was reached in the double stretch system, regardless of the stretch order, the resulting FA 4:1 rMaSp1:rMaSp2 fibers had equivalent mechanical properties and stress vs. strain curve shapes. The different ratios of rMaSp1 and rMaSp2 had differing results than hypothesized. The rMaSp2 only fibers performed the best, with an average toughness of 79MJ/m³ when stretched in MeOH and 100MJ/m³ when stretched in IPA. The 1:4 and 1:1 ratio fibers performing significantly worse than the other ratios. When using rMaSp2 only, the same trends were seen in fibers when using either acetic, formic and propionic acid dopes, formic acid having the highest tensile strength and propionic acid having the lowest.
References


(8) Brooks, A. E.; Steinkraus, H. B.; Nelson, S. R.; Lewis, R. V. An investigation of the divergence of major ampullate silk fibers from *Nephi*a clavipes and *Ar<sub>g</sub>iop*e aurantia. *Biomacromolecules* 2005, 6 (6), 3095–3099 DOI: 10.1021/bm050421e.


(14) Sampath, S.; Isdebski, T.; Jenkins, J. E.; Ayon, J. V.; Henning, R. W.; Orgel, J. P. R. O.; Antipoa, O.; Yarger, J. L. X-ray diffraction study of nanocrystalline and


CHAPTER 5

APPARATUS AND METHODS FOR PRODUCING FIBERS FROM PROTEINS

The following chapter is a provisional patent application based on work done during this dissertation. It is presented here exactly as it was submitted to the US Patent office, therefore tables and figures are numbered without reference to the dissertation chapter.
APPARATUS AND METHODS FOR PRODUCING FIBERS FROM PROTEINS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 61/977,552 filed on April 9, 2014, the entirety of which is hereby incorporated by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

[0002] The present disclosure relates to methods and devices for preparing proteins into fibers, and is particularly useful for spinning recombinant silk proteins and enhancing the physical properties of the resultant fibers.

2. Description of the Related Art

[0003] Spider silks and other natural silks are proteinaceous fibers composed largely of non-essential amino acids. Orb-web spinning spiders have as many as seven sets of highly specialized glands that produce up to seven different types of silk. Each silk protein has a different amino acid composition, mechanical property, and function. The physical properties of a silk fiber are influenced by the amino acid sequence, spinning mechanism, and environmental conditions in which they are produced.

[0004] Dragline spider silk is among the strongest known biomaterials. It is the silk used for the framework of a spider web and used to catch the spider if it falls. For example, the dragline silk of *A. diadematus* demonstrates high tensile strength (1.9 Gpa; ~15 gpd) approximately equivalent to that of steel (1.3 Gpa) and synthetic fibers such as aramid fibers (e.g., Kevlar®). Dragline silk is made of two proteins, Major Ampullate Spider Proteins 1 and 2 (MaSp1 and MaSp2).

[0005] The physical properties of dragline silk balance stiffness and strength, both in extension and compression, imparting the ability to dissipate kinetic energy without structural failure. Due to their desirable mechanical properties, proteinaceous fibers and silks may be desirable for new biomaterials, drug delivery, tendon and ligament
repair, as well as athletic gear, military applications, airbags, and tire cords among others.

[0006] The utility of silk proteins as "super filaments" has led to attempts to produce these silks in large quantities. Many of the creatures that produce proteinaceous fibers, however, cannot be farmed, for example spiders are territorial and cannibalistic. Methods of mass-producing synthetic silks have been developed including transgenic animals, plants, as well as recombinant techniques. Previous efforts at generating commercial fibers from silk proteins have been limited, with particular problems evident in maintaining stability, integrity, and workability of the fibers as well as scaling their production. The methods and apparatus disclosed herein offer innovative solutions to these problems culminating in the result of production of uniform and stable commercially viable quantities of silk fibers, including recombinant silk fibers.

SUMMARY OF THE INVENTION

[0007] In one aspect, an apparatus for producing silk fibers is disclosed and includes: an extruder; a first coagulation bath; an adjustable, mounting frame; a plurality of rollers located on the frame; a first stretch bath located between at least two rollers.

[0008] In some embodiments, the plurality of rollers includes a first set of rollers comprising at least three rollers, wherein each roller is located on the frame; and a second set of rollers comprising at least three rollers, wherein each roller is located on the frame. In some embodiments, the apparatus includes a second stretch bath.

[0009] In some embodiments, the apparatus includes one or more monitoring devices for inspecting properties of silk fibers passing by the one or more monitoring devices. In some embodiments, the apparatus includes a spool onto which fibers passing through the apparatus may be wound. In some embodiments, the apparatus includes one or more heat lamps.

[0010] In some embodiments, at least two rollers are immersed in a stretch bath. In some embodiments, each roller has a drum surface, and at least one roller has a v-shaped drum surface. In some embodiments, the apparatus includes a plurality of motors, each motor connected to a roller. In some embodiments, the apparatus
includes a spinning control capable of regulating the rotating speed of one roller relative to another roller.

[0011] In another aspect, a method of making a silk fiber is disclosed, and includes: extruding a spin dope comprising recombinant silk protein into a coagulation bath comprising an organic alcohol to form a silk fiber; winding the fiber through a plurality of adjustable rollers and a first stretch bath, wherein the a first set of rollers introduces the silk fiber into the stretch bath and a second set of rollers removes the silk fiber from the stretch bath, and wherein the rollers may be adjustably located relative to one another on an adjustable, mounting frame; stretching the silk fiber in the stretch bath by rotating one of the rollers faster than another roller.

[0012] In some embodiments, the plurality of rollers includes a first set three rollers and a second set of three rollers. In some embodiments, the silk protein is a recombinant spider silk protein. In some embodiments, the method includes stretching the silk fiber in a second stretch bath. In some embodiments, the method includes monitoring physical characteristics of the silk fiber. In some embodiments, the method includes collecting the fiber onto a spool. In some embodiments, the method includes heating the fiber with one or more heat lamps.

[0013] In some embodiments, the method includes using a spinning control capable of regulating the rotating speed of one roller relative to another roller. In some embodiments, the method includes air-stretching the silk fiber.

[0014] In some embodiments, the coagulation bath further includes a coagulating agent selected from an organic alcohol, high salt aqueous solution, and mixtures of the same. In some embodiments, the first stretch bath includes one or more of: an organic alcohol, aqueous salt, and mixtures of the same. In some embodiments, the second stretch bath comprises one or more of: an organic alcohol, aqueous salt, and mixtures of the same.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] Figure 1 shows a layout of components using one embodiment of the invention.

[0016] Figure 2 shows an alternate layout of components using one embodiment of the invention.
Figure 3 shows an alternate layout of components using one embodiment of the invention.

Figure 4 shows a v-shaped roller which may be used in some embodiments of the invention.

DETAILED DESCRIPTION

Definitions of Terms

In this specification and the claims that follow, singular forms such as “a,” “an,” and “the” include plural forms unless the context 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.

As used herein, the phrases “dope solution” or “spin dope” means any liquid mixture that contains silk protein and is amenable to extrusion for the formation of a biofilament or film casting. Dope solutions may also contain, in addition to protein monomers, higher order aggregates including, for example, dimers, trimers, and tetramers. Normally, dope solutions are aqueous solutions of between pH 4.0 and 12.0 and having less than 40% organics or chaotropic agents (w/v). In some embodiments, the dope solutions do not contain any organic solvents or chaotropic agents, yet may include additives to enhance preservation, stability, or workability of the solution. Dope solutions may be made by purifying and concentrating a biological fluid from a transgenic organism that expresses a recombinant silk protein. Suitable biological fluids include, for example, cell culture media, milk, urine, or blood from a transgenic mammal, cultured bacteria, and exudates or extracts from transgenic plants.
As used herein, the term “filament” means a fiber of indefinite length, ranging from microscopic length to lengths of a mile or greater. Silk is a natural filament, while nylon and polyester are synthetic filaments.

As used herein, the term “biofilament” means a filament created (e.g., spun) from a protein, including recombinantly produced silk proteins.

As used herein, the term “plasticizer” means a chemical added to polymers and resins to impart flexibility or stretchability, or a bonding agent that acts by solvent action on fibers. Water may act as a plasticizer, and a plasticizer means other substances which, owing to their intrinsic characteristics or by aiding in water retention, improve the ductility and plasticity of a fiber.

As used herein, the term “toughness” refers to the energy needed to break the fiber or filament. This is the area under the force elongation curve, sometimes referred to as “energy to break” or work to rupture.

As used herein, the term “elasticity” refers to the property of a body which tends to recover its original size and shape after deformation. Plasticity, deformation without recovery, is the opposite of elasticity. On a molecular configuration of the textile fiber, recoverable or elastic deformation is possible by stretching (reorientation) of inter-atomic and inter-molecular structural bonds. Conversely, breaking and re-forming of intermolecular bonds into new stabilized positions causes non-recoverable or plastic deformations.

As used herein, the term “extension” refers to an increase in length expressed as a percentage or fraction of the initial length.

As used herein, the term “fineness” means the mean diameter of a fiber (e.g., a biofilament), which is usually expressed in microns (micrometers).

As used herein, the term “micro fiber” means a filament having a fineness of less than 1 denier.

As used herein, the term “modulus” refers to the ratio of load to corresponding strain for a fiber, yarn, or fabric.

As used herein, the term “orientation” refers to the molecular structure of a filament or the arrangement of filaments within a thread or yarn, and describes the degree of parallelism of components relative to the main axis of the structure. A high degree of orientation in a thread or yarn is usually the result of a combing or
attenuating action of the filament assemblies. Orientation in a fiber is the result of shear flow elongation of molecules.

[0031] As used herein, the term “spinning” refers to the process of making filament or fiber by extrusion of a fiber forming substance, drawing, twisting, or winding fibrous substances.

[0032] As used herein, the term “tenacity” or “tensile strength” refers to the amount of weight a filament can bear before breaking. The maximum specific stress that is developed is usually in the filament, yarn or fabric by a tensile test to break the materials.

[0033] As used herein, the term “substantially pure” is meant substantially free from other biological molecules such as other proteins, lipids, carbohydrates, and nucleic acids. Typically, a dope solution is substantially pure when at least 60%, more preferably at least 75%, even more preferably 85%, most preferably 95%, or even 99% of the protein in solution is silk protein, on a wet weight or a dry weight basis. Further, a dope solution is substantially pure when proteins account for at least 60%, more preferably at least 75%, even more preferably 85%, most preferably 95%, or even 99% by weight of the organic molecules in solution.

Silk Proteins Suitable for Spinning

[0034] A variety of silk proteins can be used in the processes described herein. They include proteins from plant and animal sources, as well as recombinant and other cell culture source such as bacterial cultures. Such proteins may include sequences conventionally known for silk proteins (see for example, U.S. Patent No. 7,288,391, incorporated herein by reference in its entirety).

[0035] Biofilament proteins may be derived from conditioned media recovered from eukaryotic cell cultures, such as mammalian cell cultures, which have been engineered to produce the desired biofilaments as secreted proteins. Cell lines capable of producing the subject proteins can be obtained by cDNA cloning, or by the cloning of genomic DNA, or a fragment thereof, from a desired cell. Examples of mammalian cell lines useful for the practice of the invention include, but are not limited to BHK (baby hamster kidney cells), CHO (Chinese hamster ovary cells) and MAC-T (mammary epithelial cells from cows).
[0036] The biofilament proteins that may be spun into filaments may be from several recombinant sources. Examples of such proteins recombinantly expressed include those identified in U.S. Patent Application Nos. 61/707,571; 14/042,183; PCT/US2013/062722; 61/865,487; and 61/917,259 that are incorporated herein by reference in their entirety, including recombinantly produced major ampullate, minor ampullate, flagelliform, tubuliform, aggregate, aciniform and pyriform proteins. These proteins may be any type of biofilament proteins such as those produced by a variety of arachnids including, for example, Nephila clavipes, Araneus ssp. and A. diadematus. Also suitable for use in the invention are proteins produced by insects such as Bombyx mori. Dragline silk produced by the major ampullate gland of Nephila clavipes occurs naturally as a mixture of at least two proteins, designated as MaSpI and MaSpII. Similarly, dragline silk produced by A. diadematus is also composed of a mixture of two proteins, designated ADF-3 and ADF-4.

[0037] The biofilament proteins spun as described herein may be monomeric proteins, fragments thereof, or dimers, trimers, tetramers or other multimers of a monomeric protein. The biofilament proteins are encoded by nucleic acids, which can be joined to a variety of expression control elements, including tissue-specific animal or plant promoters, enhancers, secretory signal sequences and terminators. These expression control sequences, in addition to being adaptable to the expression of a variety of gene products, afford a level of control over the timing and extent of production.

[0038] Suitable proteins for spinning into filaments may be extracted from mixtures comprising biological fluids produced by transgenic animals, such as transgenic mammals, including goats. Such animals have been genetically modified to secrete a target biofilament in, for example, their milk or urine (see for example, U.S. Pat. No. 5,907,080; WO 99/47661 and U.S. patent publication Ser. No. 20010042255, all of which are incorporated herein by reference). The biological fluids produced by the transgenic animals may be purified, clarified, and concentrated, through such techniques as, for example, tangential flow filtration, salt-induced precipitation, acid precipitation, EDTA-induced precipitation, and chromatographic techniques, including expanded bed absorption chromatography (see for example U.S. patent application Ser. No. 10/341,097, entitled Recovery of

[0039] The biofilaments may originate from plant sources. Several methods are known in the art by which to engineer plant cells to produce and secrete a variety of heterologous polypeptides (see for example, Esaka et al., Phytochem. 28:2655 2658, 1989; Esaka et al., Physiologia Plantarum 92:90 96, 1994; and Esaka et al, Plant Cell Physiol. 36:441 446, 1995, and Li et al., Plant Physiol. 114:1103 1111). Transgenic plants have also been generated to produce spider silk (see for example Scheller et al., Nature Biotech. 19:573, 2001; PCT publication WO 01/94393 A2).

[0040] Exudates produced by whole plants or plant parts may be used. The plant portions can be intact and living plant structures. These plants materials may be a distinct plant structure, such as shoots, roots or leaves. Alternatively, the plant portions may be part or all of a plant organ or tissue, provided the material contains or produces the biofilament protein to be recovered.

[0041] Having been externalized by the plant or the plant portion, exudates are readily obtained by any conventional method, including intermittent or continuous bathing of the plant or plant portion (whether isolated or part of an intact plant) with fluids. Exudates can be obtained by contacting the plant or portion with an aqueous solution such as a growth medium or water. The fluid-exudate admixture may then be subjected to the purification methods of the present invention to obtain the desired biofilament protein. The proteins may be recovered directly from a collected exudate, such as a guttation fluid, or a plant or a portion thereof.

[0042] Extracts may be derived from any transgenic plant capable of producing a recombinant biofilament protein. Plant species representing different plant families, including, but not limited to, monocots such as ryegrass, alfalfa, turfgrass, celho grass, duckweed and wilgeon grass; dicots such as tobacco, tomato, rapeseed, azolla, floating rice, water hyacinth, and any of the flowering plants may be used. Other useful plant sources include aquatic plants capable of vegetative multiplication such as Lemna, and duckweeds that grow submerged in water, such as eelgrass and wilgeon grass. Water-based cultivation methods such as hydroponics or aeroponics are useful for growing the transgenic plants of interest, especially when the silk
protein is secreted from the plant's roots into the hydroponic medium from which the protein is recovered.

A. Spider silk proteins

Spider silk proteins are designated according to the gland or organ of the spider in which they are produced. Spider silks known to exist include major ampullate (MaSp), minor ampullate (MiSp), flagelliform (Flag), tubuliform, aggregate, aciniform, and pyriform spider silk proteins. Spider silk proteins derived from each organ are generally distinguishable from those derived from other synthetic organs by virtue of their physical and chemical properties. For example, major ampullate silk, or dragline silk, is extremely tough. Minor ampullate silk, used in web construction, has high tensile strength. An orb-web's capture spiral, in part composed of flagelliform silk, is elastic and can triple in length before breaking. Tubuliform silk is used in the outer layers of egg-sacs, whereas aciniform silk is involved in wrapping prey and pyriform silk is laid down as the attachment disk.

Sequencing of spider silk proteins has revealed that these proteins are dominated by iterations of four simple amino acid motifs: (1) polyalanine (Alaₙ); (2) alternating glycine and alanine (GlyAla)ₙ; (3) GlyGlyXaa; and (4) GlyProGly(Xaa)ₙ, where Xaa represents a small subset of amino acids, including Ala, Tyr, Leu and Gln (for example, in the case of the GlyProGlyXaaXaa motif, GlyProGlyGlnGln is the major form). Spider silk proteins may also contain spacers or linker regions comprising charged groups or other motifs, which separate the iterated peptide motifs into clusters or modules.

In some embodiments, biofilament proteins that can be used include recombinantly produced MaSpI and MaSpII proteins; minor ampullate spider silk proteins; flagelliform silks; and spider silk proteins described in any of U.S. Pat. Nos. 5,989,894; 5,728,810; 5,756,677; 5,733,771; 5,994,099; 7,057,023; and U.S. Provisional Patent Application No. 60/315,529 (all of which are incorporated herein by reference).

The sequences of the spider silk proteins may have amino acid inserts or terminal additions, so long as the protein retains the desired physical characteristics. Likewise, some of the amino acid sequences may be deleted from the protein so long as the protein retains the desired physical characteristics. Amino acid substitutions
may also be made in the sequences, so long as the protein possesses or retains the desired physical characteristics.

B. Mixtures of Sources and Alternative Proteins

In some embodiments, mixtures of biofilament proteins derived from synthetic and natural or sources may be used. The different proteins and polymers can be combined prior in the spin dope or combined post-extrusion. In some embodiments, fibers of animal or plant origin, such as wool, silk, collagen, and celluloses, or synthetic fibers such as polyolefin fibers, polyesters, polyamides (i.e., nylons), fibers from liquid crystalline polymers (e.g., aramids), polyoxymethylene, polycrylyics (i.e., polycrylonitrile), poly(phenylene sulfide), poly(vinyl alcohol), poly(ether ether ketone) (i.e., PEEK), poly[2,2′-(m-phenylene)-5,5′-bibenzimidazole] (i.e., PBI), poly(biylcolic acid), poly(glycolic acid-co-L-lactic acid, and poly(L-lactide), aromatic polyhydrazides, aromatic polyazomethines, aromatic polynimes, poly(butene-1), polycarbonate, polystyrene, and polytetrafluoroethylene may be used.

Silkworm silk proteins may also be prepared. Silkworm silk proteins include those from *Bombyx mori* including H-chain and L-chain proteins and recombinant versions thereof.

Spin Dope Preparation

Spin dopes may be created using 10-40% weight protein/volume solvent (w/v). Spin dopes may be created using a variety of solvents and mixtures. In some embodiments, the primary solvent is 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) which may be augmented with additives such as formic acid, propionic acid, anhydrous toluene, acetic acid, and isopropyl alcohol. In some embodiments, HFIP is the predominant constituent making up between 70 and 100% of the total volume of a spin dope. In some embodiments, organic acids can also be included, using up to 15% of each, in order to make a spin dope. Examples of suitable organic acids include formic acid, acetic acid, and propionic acid. In some embodiments, water alone or with various additives as described above can be used.

For example, spider dragline silk is composed of two proteins major ampullate silk protein 1 (MaSp1) and major ampullate silk protein 2 (MaSp2). Naturally, *Nephila clavipes* uses a ratio of 80% MaSp1 and 20% MaSp2. Shortened versions of these proteins can be used, generated by genetically altered goats. For the
creation of synthetic fibers, varying ratios of MaSp1-like and MaSp2-like protein can be used in spin dopes, from 0-100% of either can be used to make fibers with appreciable properties. Other components can be added to the spin dope for solvation, preservation, and to impart desirable physical characteristics.

[0051] To create the dopes, protein is placed in a glass vial. Solvents are then added, and the vials is placed on a motorized rotator and allowed to slowly mix. Formic acid dopes require approximately 12 hours to completely mix. Acetic acid dopes using 25-30% protein can take up to 3 days to completely dissolve. Once the protein is dissolved, impurities exist and can be removed by centrifugation. With the aqueous spin dopes heat and pressure are used to dissolve the protein (See Patent U.S. Patent Application No. 14/459,244, which is hereby incorporated by reference in its entirety, for details).

Fiber Spinning

[0052] Fibers can be spun by extruding a spin dope into a coagulation bath followed by passing one or more filaments through a series of rollers, one or more stretch baths, weaving and or winding components for collection and subsequent use. Illustrative apparatus and processes are shown in Figures 1-3. Similar elements are numbered with similar numbering between the drawings.

[0053] Referring to Figure 1, a system 1 for producing silk fibers is shown. The system includes a plunger 5 for forcing a spin dope solution through a spinneret 10 such as a spinneret plate. The spin dope solvent is removed from the extruded filament 30 as the extruded filament passes through an organic alcohol-containing coagulation bath 20.

[0054] The extruded filament 30 is oriented by stretching and extrusion. In some embodiments, the filaments are first extruded into a coagulation bath through an air gap. The air gap allows the filaments to undergo some stretching on the order of two to three times the strain (2-3-fold extension), which produces a high degree of molecular orientation which is sustained as the filament immerses into the coagulation bath. Alternatively and as shown in Figure 1, the extruded filament 30 passes through a needle or tubing 15 and into coagulation bath 20.

[0055] A single or multiple fibers are guided to a series of rollers. For example, a first set of rollers 40 having three rollers enable further orientation of the fiber for
introduction into a bath, such as stretch bath 50. The fiber passes through the bath and exits by passing around a second set of rollers 60. The second set of rollers guide the fiber to a second stretch bath 70. The fiber 30 passes through the second bath 70 and around a third set of rollers 80 to a winding spool 90.

[0056] The number of rollers can vary depending on the number of stretch (whether air or bath stretch) that are desired. As shown in Figure 2, an alternative arrangement of rollers is shown. Particularly, fiber 130 can be guided through a stretch bath 170 with rollers 183 and 185 that are immersed in the bath 170. In embodiments, immersion of rollers may be complete or partial.

[0057] The number of stretch baths can vary. For example, as shown in Figure 3, a system 200 is shown with a single stretch bath 250 through which fiber 230 passes between a first set of rollers 240 to a second set of rollers 260.

Stretch Baths.

[0058] As explained above, silk fibers are stretched in stretch baths between rollers operated at various speeds. In some embodiments, the stretch baths are aqueous that contain water, typically deionized water, and may optionally include other components. Optional components include organic alcohols and salts and mixtures of the same. Suitable alcohols include methanol, ethanol, n-propanol, isopropyl alcohol, n-butanol, sec-butanol, and tert-butyl alcohol and mixtures of the same. Suitable salts include ammonium sulfate, sodium sulfate, potassium sulfate and other highly charged salts. Other suitable salts include the alkaline, alkaline earth, and ammonium nitrate and phosphate salts and mixtures of the same.

[0059] In some embodiments, the aqueous, stretch bath consists of deionized water. In some embodiments, the aqueous, stretch bath consists of a saline solution. In some embodiments, the aqueous, stretch bath consists of a miscible mixture of water and methanol. In some embodiments, the aqueous, stretch bath consists of a miscible mixture of water and ethanol. In some embodiments, the aqueous, stretch bath consists of a miscible mixture of water and n-propanol. In some embodiments, the aqueous, stretch bath consists of a miscible mixture of water and isopropanol. In some embodiments, the aqueous, stretch bath consists of a miscible mixture of water and n-butanol. In some embodiments, the aqueous, stretch bath consists of a miscible
mixture of water and sec-butanol. In some embodiments, the aqueous, stretch bath consists of a miscible mixture of water and tert-butanol.

[0060] In some embodiments, the proportion of alcohol present is less than or about 90% (for example 10% water, volume/volume). In some embodiments, the proportion is less than or about 80%. In some embodiments, the proportion is less than or about 70%. In some embodiments, the proportion is less than or about 60%. In some embodiments, the proportion is less than or about 50%. In some embodiments, the proportion is less than or about 40%. In some embodiments, the proportion is less than or about 35%. In some embodiments, the proportion is less than or about 30%. In some embodiments, the proportion is less than or about 25%. In some embodiments, the proportion is less than or about 20%. In some embodiments, the proportion is less than or about 15%. In some embodiments, the proportion is less than or about 10%. In some embodiments, the proportion is less than or about 5%. In some embodiments, the proportion is less than or about 1%.

[0061] The amount of alcohol or salt present in each aqueous, stretch bath may vary between baths. In some embodiments, the proportions of organic alcohol and/or salt in each stretch bath are the same. In some embodiments having a plurality of stretch baths, the baths may have varying proportions of organic alcohol and/or salt in some or all of the baths.

[0062] In some embodiments, the stretch baths may consist of any of the aforementioned organic alcohols and mixtures of the same and be substantially free of water.

[0063] In some embodiments, the stretch baths are heated. In some embodiments, the fibers are heated under a heat lamp before entering a stretch bath. In some embodiments, the fibers are heated under a heat lamp after exiting a stretch bath.

**Stretching**

[0064] Stretching of the fibers is achieved by rotating one or more rollers at a faster rate than a roller located closer to the coagulation bath. In some embodiments, the roller speed is operated by electronic means such as a spinning control and a computer. In some embodiments, the computer control may include a graphical interface that displays and allows a user to adjust the spinning rate of any individual roller or group of rollers relative to another roller or group of rollers.
[0065] In some embodiments, the spinning control receives information from monitoring devices which characterize one or more physical properties of the silk fibers to adjust the speed of one or more rollers for further stretching, reduced stretching, and selective stretching. For example, if the desired physical property can be adjusted by increasing the desired stretch in a single bath, the spinning control may only increase the relative spin rates of rollers located at the end of a downstream bath (relative to other baths). In so doing, the stretching modification via a different spin rate is selectively tuned on only stretch bath.

[0066] In some embodiments, spinning speeds can be controlled between 0.1 mm/min to 1 m/min. In some embodiments, spinning speeds can be controlled between 0.1 mm/min to 10 m/min. In some embodiments, spinning speeds can be controlled between 0.1 mm/min to 5 m/min. In some embodiments, spinning speeds can be controlled between 0.1 mm/min to 2 m/min.

[0067] In some embodiments, the systems can include components that track tension measurements in the fibers. In such systems, the spinning control can dynamically respond to changes measured tensions in segments of the path of the fiber for quality control of the physical properties of the silk fiber.

[0068] In some embodiments, the fiber is stretched more than its original length in the coagulation bath. In some embodiments, the fiber is stretched between more than its original length in the coagulation bath and up to about 20 times that length. In some embodiments, the fiber is stretched between more than its original length in the coagulation bath and up to about 15 times that length. In some embodiments, the fiber is stretched between more than its original length in the coagulation bath and up to about 10 times that length. In some embodiments, the fiber is stretched between more than its original length in the coagulation bath and up to about 5 times that length. In some embodiments, the fiber is stretched between more than its original length in the coagulation bath and up to about 4 times that length. In some embodiments, the fiber is stretched between more than its original length in the coagulation bath and up to about 2.5 times that length. In some embodiments, the fiber is stretched between more than its original length in the coagulation bath and up to about 2.25 times that length.
length. In some embodiments, the fiber is stretched between more than its original length in the coagulation bath and up to about 2 times that length. In some embodiments, the fiber is stretched more than its original length in the coagulation bath.

[0069] In some embodiments, a set of two or more rollers is referred to as a godet.

**Fiber Monitoring**

[0070] Fibers prepared using the apparatus and its variants can also be characterized in real time monitoring. For example, the apparatus may also include a microscope for capturing real time images of the shape, orientation, thickness, and other observable properties. Other spectroscopic monitors may also be employed such as Raman and FTIR spectrometers.

**Fiber Weaving and Bundling**

[0071] In some embodiments, the systems may include other fiber processing components such as for weaving and winding the fibers into yarns, fabrics, and other materials. A variety of textile weaving, twisting, and other handling can be applied to fibers using conventionally known components.

[0072] In some embodiments, some or all of the rollers may be shaped to guide the fibers along a specified path. For example, a roller 1000 is shown in Figure 4 that has drum surface 1005. The drum surface 1005 may be v-shaped so that the fiber passes over the roller 1000 at point 1010 providing for finer control of the fiber location as it passes around (or past) the roller.

**Examples**

[0073] For testing, each fiber is taken and mounted a stiff flat material like cardboard or used X-ray film with liquid Super Glue™. The diameter of the fibers is obtained by measuring each sample nine times along the length of sample using a light microscope. Fiber samples are fastened to a test bed equipped with a custom 10g load cell (Transducer Techniques, Temecula, CA). Samples are pulled at either a quasi-static rate of 5 mm/min or at a quasi-dynamic rate of 1000 mm/min until breaking. Data is exported and analyzed to obtain mechanical properties. Average stress is reported in units of MPa; average strain in units of mm/mm; toughness in units of MPa.
All baths were kept at room temperature unless otherwise specified. A blue PEEK tubing was used for extrusion needle (0.010" ID) unless otherwise specified.

Fibers were processed quasi-static or quasi-dynamic speeds. Quasi-static speeds are relatively slow, i.e. at a rate of 0.5 mm per minute. Quasi-dynamic speeds are relatively fast, i.e. at a rate of 250-1000 mm per minute. Where no stretching takes place, all rollers and winding spool were run at substantially identical speeds such that the resulting fiber was not stretched a stretch bath or at any point. In some examples, one or more rollers downstream from other rollers are turned a faster rates to stretch a fiber, e.g. a "1.5x" stretch is accomplished by turning downstream rollers at a speed equal to about 150% of the speed of an upstream roller, i.e. resulting in a fiber stretch factor of 1.5. Similarly, a "2x" stretch is accomplished by turning downstream rollers at a speed equal to about 200% of the speed of an upstream roller, i.e. resulting in a fiber stretch factor of 2.

Example 1

Dope was prepared using an 80:20 ratio of MaSp1 and MaSp2 at a concentration of 25% w/v (399.1 mg of MaSp1 and 101.2 mg of MaSp2) in an 80:20 ratio of HFIP and formic acid (1600 μL of HFIP and 400 μL of formic acid). A spinning line was set up using the apparatus configuration shown in Figure 1. Two, 24" stretch baths were used. The first bath was filled with a 4:1 (80:20) ratio of methanol and deionized water (1600 mL methanol and 400 mL of DI water). The second bath was filled with deionized water only. The physical characteristics of the fibers using these parameters are shown in Table 1.

Table 1

<table>
<thead>
<tr>
<th>Stretching</th>
<th>No stretch</th>
<th>1.5x each bath</th>
<th>2x each bath</th>
</tr>
</thead>
<tbody>
<tr>
<td>Speed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average Stress (MPa)</td>
<td>14.3</td>
<td>164.1</td>
<td>257.7</td>
</tr>
<tr>
<td>Average Strain (mm/mm)</td>
<td>0.015</td>
<td>0.662</td>
<td>0.401</td>
</tr>
<tr>
<td>Toughness (MPa)</td>
<td>0.092</td>
<td>92.3</td>
<td>84.4</td>
</tr>
</tbody>
</table>

Example 2
Dope was prepared using a 1:1 ratio of MaSp1 and MaSp2 at a concentration of 25% w/v (399.1 mg of MaSp1 and 101.2 mg of MaSp2) in an 80:20 ratio of HFIP and formic acid (1600 µL of HFIP and 400 µL of formic acid). A spinline was set up using the apparatus configuration shown in Figure 1. Two, 24” stretch baths were used. The first bath was filled with a 4:1 (70:30) ratio of isopropanol (IPA) and deionized water (1600 mL IPA and 400mL of DI water). The second bath was filled with deionized water only. The physical characteristics of the resulting fibers are shown in Table 2.

Table 2

<table>
<thead>
<tr>
<th>Stretching</th>
<th>1.5x each bath</th>
<th>2x each bath</th>
</tr>
</thead>
<tbody>
<tr>
<td>Speed</td>
<td>Quasi-static</td>
<td>Quasi-static</td>
</tr>
<tr>
<td>Aver Max Stress</td>
<td>157.5</td>
<td>240.5</td>
</tr>
<tr>
<td>Average Max Strain</td>
<td>1.01</td>
<td>0.407</td>
</tr>
<tr>
<td>Toughness</td>
<td>132.2</td>
<td>81.1</td>
</tr>
</tbody>
</table>

Example 3

Dope was prepared using a 100% MaSp2 at a concentration of 25% w/v (500.7 mg MaSp2) in an 80:20 ratio of HFIP and acetic acid (1600 µL of HFIP and 400 µL of acetic acid). A spinline was set up using the apparatus configuration shown in Figure 1. Two, 24” stretch baths were used. The first bath was filled with a 4:1 (70:30) ratio of isopropanol and deionized water (1600 mL IPA and 400mL of DI water). The second bath was filled with deionized water only. The physical characteristics of the fibers using these parameters are shown in Table 3.

Table 3
### Table 4

<table>
<thead>
<tr>
<th>Stretching</th>
<th>2x each bath</th>
<th>2x each bath</th>
<th>2.5x each bath</th>
<th>2.5x each bath</th>
</tr>
</thead>
<tbody>
<tr>
<td>Speed</td>
<td>Quasi-static</td>
<td>Quasi-dynamic</td>
<td>Quasi-static</td>
<td>Quasi-dynamic</td>
</tr>
<tr>
<td>Average Max Stress</td>
<td>231.7</td>
<td>290.1</td>
<td>288.5</td>
<td>347.6</td>
</tr>
<tr>
<td>Average Max Strain</td>
<td>0.3</td>
<td>0.35</td>
<td>0.22</td>
<td>0.24</td>
</tr>
<tr>
<td>Toughness</td>
<td>56.2</td>
<td>80.6</td>
<td>49.6</td>
<td>65.9</td>
</tr>
</tbody>
</table>

**Example 4**

Dope was prepared using an 80:20 ratio of MaSp1 and MaSp2 at a concentration of 25% w/v (400.2 mg of MaSp1 and 101.2 mg of MaSp2) in an 90:10 ratio of HFIP and propionic acid (1800 μL of HFIP and 200 μL of propionic acid). A spinline was set up using the apparatus configuration shown in Figure 1. Two, 24" stretch baths were used. The first bath was filled with a 4:1 (80:20) ratio of methanol and deionized water (1600 mL methanol and 400 mL of DI water). The second bath was filled with deionized water only. The physical characteristics of the fibers using these parameters are shown in Table 4.

**Example 5**

Dope was prepared using 100% MaSp1 of 20% w/v (499.4 mg of MaSp1) in an 80:20 ratio of HFIP and formic acid (2000 μL of HFIP and 500 μL of formic acid). A spinline was set up using the apparatus configuration shown in Figure 1. Two, 24" stretch baths were used. The first bath was filled with a 7:4 (70:30) ratio of
isopropanol and deionized water (1400 mL IOPA and 600 mL of DI water). The second bath was filled with deionized water only. The physical characteristics of the fibers using these parameters are shown in Table 5.

Table 5

<table>
<thead>
<tr>
<th></th>
<th>1.5x each bath</th>
<th>1.5x each bath</th>
<th>2x each bath</th>
<th>2x each bath</th>
</tr>
</thead>
<tbody>
<tr>
<td>Speed</td>
<td>Quasi-static</td>
<td>Quasi-dynamic</td>
<td>Quasi-static</td>
<td>Quasi-dynamic</td>
</tr>
<tr>
<td>Average Max Stress</td>
<td>104.8</td>
<td>142.7</td>
<td>145.2</td>
<td>192.5</td>
</tr>
<tr>
<td>Average Max Strain</td>
<td>0.78</td>
<td>0.76</td>
<td>0.36</td>
<td>0.44</td>
</tr>
<tr>
<td>Toughness</td>
<td>63.7</td>
<td>95.2</td>
<td>44.1</td>
<td>74.8</td>
</tr>
</tbody>
</table>

Example 6

Dope was prepared using a 20:80 ratio of MaSp1 and MaSp2 at a concentration of 25% w/v (103 mg of MaSp1 and 399.3 mg of MaSp2) in an 80:20 ratio of HFIP and formic acid (2000 μL of HFIP and 500 μL of formic acid). A spinline was set up using the apparatus configuration shown in Figure 1. Two, 24” stretch baths were used. The first bath was filled with a 4:1 (80:20) ratio of methanol and deionized water (1600 mL methanol and 400 mL of DI water). The second bath was filled with deionized water only. The physical characteristics of the fibers using these parameters are shown in Table 6.
### Table 6

<table>
<thead>
<tr>
<th>Stretching</th>
<th>1.5x each bath</th>
<th>1.5x each bath</th>
<th>2x each bath</th>
<th>2x each bath</th>
</tr>
</thead>
<tbody>
<tr>
<td>Speed</td>
<td>Quasi-static</td>
<td>Quasi-dynamic</td>
<td>Quasi-static</td>
<td>Quasi-dynamic</td>
</tr>
<tr>
<td>Average Max Stress</td>
<td>136.4</td>
<td>164.8</td>
<td>182.5</td>
<td>215.9</td>
</tr>
<tr>
<td>Average Max Strain</td>
<td>0.68</td>
<td>0.82</td>
<td>0.25</td>
<td>0.2</td>
</tr>
<tr>
<td>Toughness</td>
<td>77.6</td>
<td>110.3</td>
<td>37.3</td>
<td>34.1</td>
</tr>
</tbody>
</table>

**Example 7**

Dope was prepared using an 80:20 ratio of MaSp1 and MaSp2 at a concentration of 20% w/v (320 mg of MaSp1 and 80.6 mg of MaSp2) in an 70:15:15 ratio of HFIP, formic acid, and acetic acid (1400 µL of HFIP, 300 µL of formic acid, and 300 µL of acetic acid). A spinline was set up using the apparatus configuration shown in Figure 1. Two, 24” stretch baths were used. The first bath was filled with a 4:1 (80:20) ratio of methanol and deionized water (1600 mL methanol and 400 mL of DI water). The second bath was filled with deionized water only. The physical characteristics of the fibers using these parameters are shown in Table 7.

### Table 7

<table>
<thead>
<tr>
<th>Stretching</th>
<th>1.5x each bath</th>
<th>2x each bath</th>
</tr>
</thead>
<tbody>
<tr>
<td>Speed</td>
<td>Quasi-static</td>
<td>Quasi-static</td>
</tr>
<tr>
<td>Average Max Stress</td>
<td>147.3</td>
<td>207.5</td>
</tr>
<tr>
<td>Average Max Strain</td>
<td>0.55</td>
<td>0.32</td>
</tr>
<tr>
<td>Toughness</td>
<td>71.2</td>
<td>58.5</td>
</tr>
</tbody>
</table>

**Example 8**

Dope was prepared using an 80:20 ratio of MaSp1 and MaSp2 at a concentration of 20% w/v (320.8 mg of MaSp1 and 80.8 mg of MaSp2) in an 80:20 ratio of HFIP and formic acid (1600 µL of HFIP and 400 µL of formic acid). A spinline was set up using the apparatus configuration shown in Figure 1. Two, 36”
stretch baths were used. The first bath was filled with a 2M ammonium sulfate in deionized water (264.28 mg (NH₄)₂SO₄ in 2 L of DI water) and kept at a temperature of 62°C. The second bath was filled with 1:1 ratio of isopropanol and deionized water (1 L each). The physical characteristics of the fibers using these parameters are shown in Table 8.

Table 8

<table>
<thead>
<tr>
<th>Stretching</th>
<th>1.5x each bath</th>
<th>1.5x first bath / 2x second bath</th>
</tr>
</thead>
<tbody>
<tr>
<td>Speed</td>
<td>Quasi-static</td>
<td>Quasi-static</td>
</tr>
<tr>
<td>Average Max Stress</td>
<td>118.3</td>
<td>243.6</td>
</tr>
<tr>
<td>Average Max Strain</td>
<td>0.09</td>
<td>0.21</td>
</tr>
<tr>
<td>Toughness</td>
<td>9.5</td>
<td>44</td>
</tr>
</tbody>
</table>

[0092] Example 9

[0093] Dope was prepared using an 80:20 ratio of MaSp1 and MaSp2 at a concentration of 20% w/v (320.1 mg of MaSp1 and 80 mg of MaSp2) in an 80:20 ratio of HFIP and formic acid (1600 μL of HFIP and 400 μL of formic acid). A spinline was set up using the apparatus configuration shown in Figure 1. Two, 16" stretch baths were used with heat lamps located 20 cm above the span between the second and third roller of each set of rollers maintaining an air temperature of 50°C at 20 cm away from the lamp (the position of the fiber). The first bath was filled with a 2M ammonium sulfate in deionized water (264.28 mg (NH₄)₂SO₄ in 2 L of DI water). The second bath was filled with deionized water only. The physical characteristics of the fibers using these parameters are shown in Table 9.

Table 9

<table>
<thead>
<tr>
<th>Stretching</th>
<th>2x bath 1 / 1.5x bath 2</th>
<th>2x each bath</th>
</tr>
</thead>
<tbody>
<tr>
<td>Speed</td>
<td>Quasi-static</td>
<td>Quasi-static</td>
</tr>
<tr>
<td>Average Max Stress</td>
<td>189.7</td>
<td>203.1</td>
</tr>
<tr>
<td>Average Max Strain</td>
<td>0.19</td>
<td>0.22</td>
</tr>
<tr>
<td>Toughness</td>
<td>33.1</td>
<td>38.8</td>
</tr>
</tbody>
</table>
Example 10

Dope was prepared using an 80:20 ratio of MaSp1 and MaSp2 at a concentration of 20% w/v (320.1 mg of MaSp1 and 80 mg of MaSp2) in an 80:20 ratio of HFIP and formic acid (1600 μL of HFIP and 400 μL of formic acid). A spinline was set up using the apparatus configuration shown in Figure 2. Two, 16" baths were used. The first bath was filled with a 2M ammonium sulfate in deionized water (264.28 mg (NH₄)₂SO₄ in 2 L of DI water). The second bath was filled with deionized water only. The physical characteristics of the fibers using these parameters are shown in Table 10.

Table 10

<table>
<thead>
<tr>
<th>Stretching</th>
<th>No stretch</th>
<th>2x first bath / 1x second bath</th>
<th>3x first bath / 1x second bath</th>
<th>4x first bath / 1x second bath</th>
</tr>
</thead>
<tbody>
<tr>
<td>Speed</td>
<td>Quasi-static</td>
<td>Quasi-static</td>
<td>Quasi-static</td>
<td>Quasi-static</td>
</tr>
<tr>
<td>Average Max Stress</td>
<td>77.6</td>
<td>109.8</td>
<td>145.5</td>
<td>173.4</td>
</tr>
<tr>
<td>Average Max Strain</td>
<td>0.03</td>
<td>0.6</td>
<td>0.55</td>
<td>0.41</td>
</tr>
<tr>
<td>Toughness</td>
<td>1.32</td>
<td>54.8</td>
<td>65.9</td>
<td>60.6</td>
</tr>
</tbody>
</table>

Example 11

Dope was prepared using an 80:20 ratio of MaSp1 and MaSp2 at a concentration of 20% w/v (640.6 mg of MaSp1 and 159.9 mg of MaSp2) in an 80:20 ratio of HFIP and formic acid (3200 μL of HFIP and 800 μL of formic acid). A spinline was set up using the apparatus configuration shown in Figure 2. Two, 16" baths were used. A heat lamp was located 20 cm above fiber between the second bath and a winder maintaining a temperature of about 50 °C at the location of the fiber. The first bath was filled with an 80:20 mixture of isopropanol and water (1600 mL of IPA and 400 mL of deionized water), and the second bath was filled with a 10:90 ratio of isopropanol and water (200 mL of IPA and 1800 mL of deionized water). The physical characteristics of the fibers using these parameters are shown in Table 11.
Table 11

<table>
<thead>
<tr>
<th>Stretching</th>
<th>No stretch</th>
<th>2x first bath / 1x second bath</th>
<th>3x first bath / 1x second bath</th>
</tr>
</thead>
<tbody>
<tr>
<td>Speed</td>
<td>Quasi-static</td>
<td>Quasi-static</td>
<td>Quasi-static</td>
</tr>
<tr>
<td>Average Max Stress</td>
<td>84.17</td>
<td>108.9</td>
<td>141.1</td>
</tr>
<tr>
<td>Average Max Strain</td>
<td>0.02</td>
<td>0.34</td>
<td>0.66</td>
</tr>
<tr>
<td>Toughness</td>
<td>0.84</td>
<td>34.4</td>
<td>74.6</td>
</tr>
</tbody>
</table>

Example 12

Dope was prepared using an 80:20 ratio of MaSp1 and MaSp2 at a concentration of 20% w/v (640.6 mg of MaSp1 and 159.9 mg of MaSp2) in an 80:20 ratio of HFIP and formic acid (3200 μL of HFIP and 800 μL of formic acid). A spinline was set up using the apparatus configuration shown in Figure 3. A single, 16" bath was used. The first bath was filled with a 50:50 mixture of isopropanol and deionized water (1L each), and the second bath was filled with a 10:90 ratio of isopropanol and water (200 mL of IPA and 1800 mL of deionized water). The physical characteristics of the fibers using these parameters are shown in Table 12.

Table 12

<table>
<thead>
<tr>
<th>Stretching</th>
<th>1.5x</th>
<th>2.5x</th>
<th>3.5x</th>
</tr>
</thead>
<tbody>
<tr>
<td>Speed</td>
<td>Quasi-static</td>
<td>Quasi-static</td>
<td>Quasi-static</td>
</tr>
<tr>
<td>Average Max Stress</td>
<td>51.7</td>
<td>59.3</td>
<td>84.3</td>
</tr>
<tr>
<td>Average Max Strain</td>
<td>1.53</td>
<td>1.43</td>
<td>0.44</td>
</tr>
<tr>
<td>Toughness</td>
<td>62.7</td>
<td>61.6</td>
<td>31</td>
</tr>
</tbody>
</table>
CLAIMS

What is claimed is:

1. An apparatus for producing silk fibers, comprising:
   an extruder;
   a first coagulation bath comprising a coagulating agent selected from
   the group consisting of an organic alcohol, high salt aqueous solution, and mixtures of
   the same;
   an adjustable, mounting frame;
   a plurality of rollers located on the frame; and
   a first stretch bath located between at least two rollers and comprising
   an organic alcohol, an aqueous salt, or mixtures of the same.

2. The apparatus of claim 1, wherein the plurality of rollers comprises:
   a first set of rollers comprising at least three rollers, wherein each
   roller is located on the frame; and
   a second set of rollers comprising at least three rollers, wherein each
   roller is located on the frame.

3. The apparatus of claim 1, further comprising a second stretch bath
   comprising an organic alcohol, an aqueous salt, or mixtures of the same.

4. The apparatus of claim 1, further comprising one or more monitoring
   devices for inspecting properties of silk fibers passing by the one or more monitoring
   devices.

5. The apparatus of claim 1, further comprising a spool onto which fibers
   passing through the apparatus may be wound.

6. The apparatus of claim 1, further comprising one or more heat lamps.

7. The apparatus of claim 1, wherein at least two rollers are immersed in
   the first stretch bath.
8. The apparatus of claim 1, wherein each roller has a drum surface, and at least one roller has a v-shaped drum surface.

9. The apparatus of claim 1, further comprising a plurality of motors, each motor connected to a roller.

10. The apparatus of claim 1, further comprising a spinning control capable of regulating the rotating speed of one roller relative to another roller.

11. A method of making a silk fiber, comprising:
extruding a spin dope comprising recombinant spider silk protein into a coagulation bath comprising an organic alcohol to form a silk fiber;
wind the fiber through a plurality of adjustable rollers and a first stretch bath, wherein the a first set of rollers introduces the silk fiber into the stretch bath and a second set of rollers removes the silk fiber from the stretch bath, and wherein the rollers may be adjustably located relative to one another on an adjustable mounting frame;
stretching the silk fiber in the stretch bath by rotating one of the rollers faster than another roller.

12. The method of claim 11, wherein the plurality of rollers comprises a first set three rollers and a second set of three rollers.

13. The method of claim 11, further comprising stretching the silk fiber in a second stretch bath.

14. The method of claim 11, further comprising monitoring physical characteristics of the silk fiber.

15. The method of claim 11, further comprising collecting the fiber onto a spool.

16. The method of claim 11, further comprising heating the fiber with one or more heat lamps.
17. The method of claim 11, wherein each roller has a drum surface, and at least one roller has a v-shaped drum surface.

18. The method of claim 11, further comprising using a spinning control capable of regulating the rotating speed of one roller relative to another roller.

19. The method of claim 11, further comprising air-stretching the silk fiber.
ABSTRACT

Methods and apparatuses for preparing protein fibers (biofilaments) from recombinant biofilament proteins are disclosed. The methods are particularly useful for spinning fibers of spider silk or silkworm silk proteins from recombinant sources and may be used to spin such fibers for use in the manufacture of industrial and commercial products.
Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections.

Inventor(s)

Randolph V. Lewis, Nibley, UT;
Justin A. Jones, Nibley, UT;
Cameron G. Copeland, Logan, UT;

Applicant(s)

Randolph V. Lewis, Nibley, UT;
Justin A. Jones, Nibley, UT;
Cameron G. Copeland, Logan, UT;

Assignment For Published Patent Application

Utah State University, North Logan, UT

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application claims benefit of 61/977,552 04/09/2014

Foreign Applications for which priority is claimed (You may be eligible to benefit from the Patent Prosecution Highway program at the USPTO. Please see http://www.uspto.gov for more information.) - None.

Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

If Required, Foreign Filing License Granted: 04/20/2015

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is US 14/881,951

Projected Publication Date: 10/15/2015

Non-Publication Request: No
PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process simplifies the filing of patent applications on the same invention in member countries, but does not result in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).
LICENSE FOR FOREIGN FILING UNDER
Title 35, United States Code, Section 184
Title 37, Code of Federal Regulations, 5.11 & 5.15

**GRANTED**

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related application(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR Parts 730-774); the Office of Foreign Assets Control, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

**NOT GRANTED**

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may file the application pursuant to 37 CFR 5.15(b).

---

**SelectUSA**

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop technology, manufacture products, deliver services, and grow your business, visit [http://www.SelectUSA.gov](http://www.SelectUSA.gov) or call +1-202-482-6800.
CHAPTER 6
CURRENT PROJECTS, FUTURE WORK, ENGINEERING CRITERIA, AND CONCLUSIONS

This chapter details plans for finishing immediate projects and collaborations, ideas for what direction the research could go in the future and conclusions based on the aims and achievements of the research presented in this dissertation.

Current/Future Work

Multi-Fiber Spinning

As mentioned in Chapter 2, a 24-fiber spinning head has been designed and will be produced by the Space Dynamics Laboratory at Utah State University. The current design for the head is seen in Figure 6.1. The design is similar to the one produced by Rad Cam Inc. but with several key differences. First, rather than using metal tubing that has been sweated into the head, the holes are being manufactured into the piece. Channels are being made, as can be seen in the schematic, by serial reduction in diameter. Second, the internal volume is being reduced. This is being done in an attempt to even the pressure across the entire head. To accomplish this, the bottom half is being

Figure 6.1 - Schematic of the new multi-fiber spinning head design.
given a conical shape. Third, the inlet for the system is no longer being designed with chromatography fittings. Syringes that use the Luer lock system were acquired, along with connector parts and tubing, negating the need for the multiple plumbing pieces that have been used previously. Additionally, this new standardized plumbing eliminates the multiple diameter changes in the system, which is expected to further even the pressure. This new design should allow for the simultaneous spinning of 24 fibers. This level of production can produce the yarn needed for the creation of multiple prototypes.

*Composites*

Composite materials are used in several applications, from automobiles to satellites. This is due to their strength and their relatively low weight. There is a push in the industry to create composites that are made of “green” alternatives. Now that hundreds of meters of synthetic silk can be spun into yarns, composites made with synthetic spider silk fibers and epoxy could be easily made. With the strength of silk and the “green” nature of the material, these composites have the potential to replace synthetic polymers in modern composite materials. Thomas Fronk, a professor in the Mechanical Engineering department at USU, has years of experience in composites and is excited at the prospect of using synthetic spider silk to create novel composite materials. Additionally, Troy Munro, a PhD student under Dr. Heng Ban in the Multiscale Thermophysical Laboratory at USU, has expressed interest in characterizing the thermal properties of these composites. With these collaborators, spider silk composites will be designed, constructed, and characterized.
Bacterially Produced Protein Spinning

Most of the bacterial-based proteins are being devoted to the development of aqueous spin dopes. Aqueous dopes use much lower protein concentrations for spinning than the HFIP method. These aqueous dopes are proving to be a monumental achievement for silk-based biomaterials, in addition to fibers forming films, coatings, and gels. However, the fibers created from these dopes have yet to match the mechanical properties of fibers created from dopes produced using HFIP. One reason for this could be the configuration of the protein in the heated aqueous dope solution. Another reason could be the viscosity of the aqueous spin dopes. Currently, the viscosity of aqueous spin dopes is close to or exactly that of water, whereas the HFIP produced spin dopes are much more viscous due to their higher concentration of protein. Whatever the reason, the fibers coming out of the extruder tend to be fragile, breaking even if the bath is lightly disturbed. In a laboratory setting, this issue can be overcome, but if the intention is to commercially produce fibers with this method, then a method or additive needs to be found that will allow for more robust spinning. In the meantime, creating dopes using the methods described in this dissertation can be used to help discover which chimeric proteins can produce fibers with the desired properties. Also, by applying similar techniques to a variety of different constructs, a better understanding of how the different gene motifs behave can be achieved.

Conclusions

This research project had two aims:
• Aim #1: Create a mechanical process that can spin synthetic spider silk fibers with consistent mechanical properties.

• Aim #2: Understand how processing parameters affect the mechanical properties of synthetic spider fibers.

As outlined in the introduction of this dissertation, the process for creating synthetic spider silk when I began this project was crude. Synthetic spider silk had been created by several people but the fibers were weak and brittle unless stretched. The stretching was performed by hand, a tedious and time consuming method which could only produce small amounts (10-20cm) of synthetic silk with reasonable mechanical properties at a time. The mechanical process I developed, detailed in Chapters Two and Three, increased the mechanical properties of synthetic spider silks over those processed by hand and allows for hundreds of meters of synthetic silk to be made at a time. Figure 6.2 shows a comparison of fibers that were stretched by hand at the beginning of this research project.

![Figure 6.2 - Comparison of (A) hand-drawn fibers that were produced and (B) mechanically stretched fibers as part of this research.](image-url)
and fibers produced using the mechanical process that is the topic of this dissertation. The process I developed is the first of its kind to produce synthetic spider silk in a mechanical method, without the need for tedious hand-stretching techniques. The synthetic silk fibers created had consistent properties with as good or better standard deviations than currently-used industrial polymers. A patent for this process has been submitted and its development is the topic of a publication. With this, I believe that Aim #1 has been satisfied.

While fine-tuning the system, it was discovered that different post-spin treatments had large effects on the mechanical properties of synthetic fibers. It was believed that by changing spider silk proteins at the genetic level, tunable fibers could be created, where the properties of the fibers are changed based on which protein motifs are used. My results show that a second level of customization and control is possible. By changing the processing method used to create synthetic spider silk, the mechanical properties can be altered. This approach of creating tunable silk fibers by changing the genetic code for the protein and the spinning process allows for the creation of biomaterials for a wider number of applications. To further aid in the customization of creating tunable fibers, a custom spinning machine was designed, built, and tested. I believe the custom spinning machine, along with its software, could be sold to other universities and research groups looking to create synthetic fibers, be they from silk, cellulose, synthetic polymers, etc. This could add another source of revenue to a spider silk company.
Engineering Design Criteria and Feedback

In order to accomplish the second aim of this dissertation, several key aspects of the spinning process needed be identified and the criteria for their success outlined. The parameters for these processes were tested multiple times in several different iterations until the best condition was found. Originally, the fiber spinning and post-spin draws were done on the DACA SpinLine. However, this system had several limitations, as explained in Chapter Two. A new system was required to fully explore and optimize the spinning process and the details of the engineering design of the system are explained in detail in Chapter Two. To summarize, this machine provided variable bath positions and sizes, precise stretching conditions, and software that allowed for a number of adjustments to be made during the spinning of fibers. Using the customized DACA SpinLine and, later, the new custom spinning machine, several experiments were performed in an attempt to understand and optimize the spinning process. The key aspects of spinning that were identified are: spin dopes, the coagulation bath, the stretching process, and scaling-up to multiple fibers. Presented below are summaries of the results from many of these experiments. For a fuller list see the tables provided in the Appendices A-C of this dissertation. All of these accomplishments fulfill the second aim of this research dissertation.

1) Spin Dope – The criteria for a successful spin dope were: solubilization of protein, production of process-able fibers and fibers created from dopes with mechanical properties at least as good as current published data. Chapter Four details the results from using 30 different spin dope solutions. To summarize,
for successful fiber formation, HFIP needed to be the majority of the solution. Formic, acetic and propionic acid produced fibers which performed better than all other additives. The concentration of protein in the spin dopes was also tested. From 5-35% w/v the protein would dissolve and could form a fiber in the coagulation bath. Below 15% w/v the fibers were difficult to process using the post spin double stretch system. Fibers with the best mechanical properties and ease of use were produced when using a 25% w/v spin dope. Additionally, the ratio of different spider silk proteins was tested, and the results can be found in Chapter Four. The 4:1 ratio of rMaSp1 and rMaSp2 and the rMaSp2 only ratios consistently produced fibers with the best tensile strength and elongation.

2) **Coagulation Bath** – The criteria for the coagulation bath was to generate proper fiber formation so the fiber then could be pulled from the coagulation bath and processed through the rest of spinning process. In all, four different coagulation baths were attempted. IPA, MeOH, 50:50 IPA:MeOH, and 70:30 IPA:MeOH. The IPA only bath was found to be best for all dopes produced with the recombinant silk protein produced by Utah State University’s transgenic goat herd. For recombinant silk proteins based on the sequence of flagelliform silk produced in bacteria, it was found that the MeOH bath, which made the silk fibers from the goat proteins too brittle to successfully process, helped to stabilize the fibers and allow them to be processed without breaking and thus was superior to IPA.
3) **Stretching and Stretch Baths** – The design criteria of stretch ratios and stretch bath compositions was to maximize tensile strength, elongation or toughness (depending on the need and application). Chapter Three covers the development of the stretching process and how it evolved from a single bath process to a two bath system with different bath compositions for each bath. Chapter Three also covered some of the early stretch bath compositions that were attempted. Table 6.1 shows the results from many of the different stretch bath positions that were attempted with the double-stretch system. Though many different stretch baths can be used to create synthetic spider silk fibers, ultimately the 70:30 IPA:water bath and the 80:20 MeOH bath followed by

---

**Table 6.1 - The average max stress and strain of fibers processed using different stretch bath compositions.**

<table>
<thead>
<tr>
<th>Bath 1</th>
<th>Bath 2</th>
<th>Max Strain (MPa)</th>
<th>Max Strain (mm/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80:20 MeOH:Water</td>
<td>Water</td>
<td>247</td>
<td>0.382</td>
</tr>
<tr>
<td>70:30 IPA:Water</td>
<td>Water</td>
<td>183</td>
<td>0.565</td>
</tr>
<tr>
<td>60:40 IPA:Water</td>
<td>Water</td>
<td>161</td>
<td>0.238</td>
</tr>
<tr>
<td>50:50 IPA:Water</td>
<td>Water</td>
<td>122</td>
<td>0.354</td>
</tr>
<tr>
<td>2M Ammonium Sulfate</td>
<td>Water</td>
<td>203</td>
<td>0.224</td>
</tr>
<tr>
<td>2M Ammonium Sulfate</td>
<td>50:50 IPA:Water</td>
<td>141</td>
<td>0.333</td>
</tr>
<tr>
<td>60°C 2M Ammonium Sulfate</td>
<td>50:50 IPA:Water</td>
<td>244</td>
<td>0.209</td>
</tr>
<tr>
<td>80:20 MeOH:Water</td>
<td>26mM Rhodamine B</td>
<td>230</td>
<td>0.348</td>
</tr>
<tr>
<td>80:20 MeOH:Water</td>
<td>90:10 Water:IPA</td>
<td>170</td>
<td>0.345</td>
</tr>
<tr>
<td>70:30 IPA:Water</td>
<td>1M KPO₄₃</td>
<td>218</td>
<td>0.299</td>
</tr>
<tr>
<td>80:20 MeOH:Water</td>
<td>1M KPO₄₃</td>
<td>137</td>
<td>0.588</td>
</tr>
<tr>
<td>1M KPO₄₃</td>
<td>Water</td>
<td>Failed</td>
<td>Failed</td>
</tr>
<tr>
<td>35:35:30 IPA:MeOH:Water</td>
<td>Water</td>
<td>192</td>
<td>0.478</td>
</tr>
<tr>
<td>80:20 MeOH:Water</td>
<td>20% w/v ZnCl</td>
<td>Failed</td>
<td>Failed</td>
</tr>
<tr>
<td>0°C 80:20 MeOH:Water</td>
<td>Water</td>
<td>Failed</td>
<td>Failed</td>
</tr>
<tr>
<td>0°C 70:30 IPA:Water</td>
<td>Water</td>
<td>Failed</td>
<td>Failed</td>
</tr>
</tbody>
</table>
water stretches were found to provide fibers with the best max stress and max strain, while also being the easiest and most cost-effective to use. Different stretch ratios were found to be possible and the amount of stretch the fibers experienced during the system were directly related to the mechanical properties of the fibers, as shown in Chapters Three and Four. Additionally, during the development of the two-stretch and multiple-fiber-spinning processes, several different bath lengths and the possible addition of heat lamps to the process were all tested. Heat lamps did not add any significant strength or elongation to the fibers, but did allow fibers from the less concentrated dopes (15% w/v) to be manipulated more easily due to better drying. As for bath lengths, the optimal length for single fiber production was 24 inches, but for multiple fiber spinning a longer length, 36 inches, was optimal.

1) **Multi-Fiber Spinning** – Criteria for the formation of multiple fibers at a time was to allow the thread to be created without fusing the individual fibers or sticking to the spool and to maintain or exceed the mechanical properties seen when spinning one fiber. Chapter Two explains the changes that had to be made to the double-stretch system to accommodate the spinning of multiple fibers simultaneously. In short, a longer stretch bath was needed to allow for more time for the fibers to stretch and more time to dry was needed as well as heat lamps and a small fan to aid drying and prevent fusion of individual fibers.
The work contained in this dissertation was done using recombinant spider silk proteins produced in transgenic goats. Originally, it was expected that the system developed would be used to produce synthetic fibers from different recombinant spider silk proteins being developed by other researchers in the Lewis Spider Silk Lab. Unfortunately, very few of these other fibers were produced using the process developed. This was an issue of availability. Sufficient quantities of bacterial, plant, or new goat-derived proteins have rarely been available for testing spinning procedures. Some bacteria-based protein fibers have been made on the new spinning system. The FLYS4T construct, a custom-made chimeric protein with motifs from flagelliform and dragline silk gene sequences, was spun using the 8-fiber spinning system. However, most of these spins have not been able to continuously spin enough fiber to be collected and tested. The spin dopes created from these bacterial-based proteins often behave like spin dopes created from goat protein that have a high salt concentration or contamination. This suggests that the current level of purity from bacteria-based proteins is not high enough. One experiment was run where a custom MaSp2 construct was dissolved in a spin dope both before and after an additional purification step. The dope made before this step could not be spun whereas the dope made with further purified protein was able to be spun and collected. For further work in fibers, a higher level of purity than is currently available needs to be achieved.

Due to unavailability of different spider silk proteins, focus was shifted to the spinning of multiple fibers at once, in an attempt to make the process more commercially viable. The attempts of this endeavor are largely detailed in Chapter Six and a new design
was outlined above. In short, these endeavors have been successful. Currently, eight fibers can be spun simultaneously. Synthetic spider silk yarn is currently being produced with the intention of making a glove prototype, in collaboration with the knitting lab at Drexel University. This technology is also being used to create yarns for use in composites, thermal testing, and further characterization experiments. These exciting developments hold the possibility for several future collaborations and innovations.

One of the eventual goals of synthetic spider silk fibers is to create fibers with the same properties as natural spider dragline silk. I believe that one of the biggest reasons that synthetic spider silk has yet to achieve the same mechanical properties of natural spider silk is the length of the protein. Whether the molecular model where poly-alanine sections of the protein fold in on themselves or the model that these sections pair up with similar sections from other protein chains are used, a longer protein will facilitate the creation of larger crystalline regions in the fiber. Currently several proteins are being produced by the Lewis laboratory that are double and triple the size of the proteins produced in transgenic goats. I look forward to seeing the results from fibers made using the same procedures as the goat-derived protein and comparing the results. I believe that this test will help the development of this technology for commercial use. In combination with the advances made in spinning synthetic spider silk outlined in this dissertation, these new fibers will be used in several applications.
References

APPENDICES
APPENDIX A

RESULTS FROM HAND-DRAWN EXPERIMENTS
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Diped in Field for 10 seconds</td>
<td>9.96</td>
<td>1.02</td>
<td>6.94</td>
<td>1.12</td>
<td>5.08</td>
<td>0.12</td>
<td>3.94</td>
<td>0.12</td>
<td>2.06</td>
<td>0.12</td>
<td>1.00</td>
<td>0.12</td>
<td>0.96</td>
<td>0.12</td>
<td>0.94</td>
<td>0.12</td>
<td>0.92</td>
<td>0.12</td>
<td>0.90</td>
<td>0.12</td>
<td>0.88</td>
<td>0.12</td>
</tr>
<tr>
<td>Diped in Field for 10 seconds</td>
<td>9.94</td>
<td>1.01</td>
<td>6.92</td>
<td>1.10</td>
<td>5.06</td>
<td>0.10</td>
<td>3.92</td>
<td>0.10</td>
<td>2.04</td>
<td>0.10</td>
<td>1.00</td>
<td>0.10</td>
<td>0.96</td>
<td>0.10</td>
<td>0.94</td>
<td>0.10</td>
<td>0.92</td>
<td>0.10</td>
<td>0.90</td>
<td>0.10</td>
<td>0.88</td>
<td>0.10</td>
</tr>
<tr>
<td>Diped in Field for 10 seconds</td>
<td>9.92</td>
<td>1.00</td>
<td>6.90</td>
<td>1.08</td>
<td>5.04</td>
<td>0.08</td>
<td>3.90</td>
<td>0.08</td>
<td>2.02</td>
<td>0.08</td>
<td>1.00</td>
<td>0.08</td>
<td>0.96</td>
<td>0.08</td>
<td>0.94</td>
<td>0.08</td>
<td>0.92</td>
<td>0.08</td>
<td>0.90</td>
<td>0.08</td>
<td>0.88</td>
<td>0.08</td>
</tr>
<tr>
<td>Diped in Field for 10 seconds</td>
<td>9.90</td>
<td>0.99</td>
<td>6.88</td>
<td>1.06</td>
<td>5.02</td>
<td>0.06</td>
<td>3.88</td>
<td>0.06</td>
<td>2.00</td>
<td>0.06</td>
<td>1.00</td>
<td>0.06</td>
<td>0.96</td>
<td>0.06</td>
<td>0.94</td>
<td>0.06</td>
<td>0.92</td>
<td>0.06</td>
<td>0.90</td>
<td>0.06</td>
<td>0.88</td>
<td>0.06</td>
</tr>
<tr>
<td>Diped in Field for 10 seconds</td>
<td>9.88</td>
<td>0.98</td>
<td>6.86</td>
<td>1.04</td>
<td>5.00</td>
<td>0.04</td>
<td>3.86</td>
<td>0.04</td>
<td>1.98</td>
<td>0.04</td>
<td>0.98</td>
<td>0.04</td>
<td>0.94</td>
<td>0.04</td>
<td>0.92</td>
<td>0.04</td>
<td>0.90</td>
<td>0.04</td>
<td>0.88</td>
<td>0.04</td>
<td>0.86</td>
<td>0.04</td>
</tr>
<tr>
<td>Diped in Field for 10 seconds</td>
<td>9.86</td>
<td>0.97</td>
<td>6.84</td>
<td>1.02</td>
<td>4.98</td>
<td>0.02</td>
<td>3.84</td>
<td>0.02</td>
<td>1.96</td>
<td>0.02</td>
<td>0.96</td>
<td>0.02</td>
<td>0.92</td>
<td>0.02</td>
<td>0.90</td>
<td>0.02</td>
<td>0.88</td>
<td>0.02</td>
<td>0.86</td>
<td>0.02</td>
<td>0.84</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Table 1A - Results from hand standards after experiments.
APPENDIX B

RESULTS FROM EXPERIMENTS PERFORMED ON SINGLE STRETCH MECHANICAL SYSTEM
<table>
<thead>
<tr>
<th>Time</th>
<th>0%</th>
<th>2%</th>
<th>6%</th>
<th>10%</th>
<th>13%</th>
<th>16%</th>
<th>20%</th>
<th>24%</th>
<th>26%</th>
<th>28%</th>
<th>30%</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:30</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:31</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:32</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:33</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:34</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:35</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:36</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:37</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:38</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:39</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:40</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:41</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:42</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:43</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:44</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:45</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:46</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:47</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:48</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:49</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:50</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:51</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:52</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:53</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:54</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:55</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:56</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:57</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:58</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
<tr>
<td>16:59</td>
<td>32%</td>
<td>34%</td>
<td>36%</td>
<td>38%</td>
<td>40%</td>
<td>42%</td>
<td>44%</td>
<td>46%</td>
<td>48%</td>
<td>50%</td>
<td>52%</td>
</tr>
</tbody>
</table>

**Notes:**
- Values in this table are approximate.
- Time entries are in 10-minute increments.
- The table includes data for the period from 16:30 to 16:59.

**Table Title:** Results from single mechanical stress tests on materials.

**Columns:**
- Time
- 0%
- 2%
- 6%
- 10%
- 13%
- 16%
- 20%
- 24%
- 26%
- 28%
- 30%
- 31%
- 32%
- 33%
- 34%
- 35%
- 36%
- 37%
- 38%
- 39%
- 40%
- 41%
- 42%
- 43%
- 44%
- 45%
- 46%
- 47%
- 48%
- 49%
- 50%
- 51%
- 52%
- 53%
- 54%
- 55%
- 56%
- 57%
- 58%
- 59%

**Rows:**
- Each row represents a specific time interval from 16:30 to 16:59.
- Values in each row indicate the percentage of stress experienced by the materials.

**Legend:**
- 0%: Initial stress level
- 2%: Stress level after 2 minutes
- 6%: Stress level after 6 minutes
- 10%: Stress level after 10 minutes
- 13%: Stress level after 13 minutes
- 16%: Stress level after 16 minutes
- 20%: Stress level after 20 minutes
- 24%: Stress level after 24 minutes
- 26%: Stress level after 26 minutes
- 28%: Stress level after 28 minutes
- 30%: Stress level after 30 minutes
- 31%: Stress level after 31 minutes
- 32%: Stress level after 32 minutes
- 33%: Stress level after 33 minutes
- 34%: Stress level after 34 minutes
- 35%: Stress level after 35 minutes
- 36%: Stress level after 36 minutes
- 37%: Stress level after 37 minutes
- 38%: Stress level after 38 minutes
- 39%: Stress level after 39 minutes
- 40%: Stress level after 40 minutes
- 41%: Stress level after 41 minutes
- 42%: Stress level after 42 minutes
- 43%: Stress level after 43 minutes
- 44%: Stress level after 44 minutes
- 45%: Stress level after 45 minutes
- 46%: Stress level after 46 minutes
- 47%: Stress level after 47 minutes
- 48%: Stress level after 48 minutes
- 49%: Stress level after 49 minutes
- 50%: Stress level after 50 minutes
- 51%: Stress level after 51 minutes
- 52%: Stress level after 52 minutes
- 53%: Stress level after 53 minutes
- 54%: Stress level after 54 minutes
- 55%: Stress level after 55 minutes
- 56%: Stress level after 56 minutes
- 57%: Stress level after 57 minutes
- 58%: Stress level after 58 minutes
- 59%: Stress level after 59 minutes
<table>
<thead>
<tr>
<th>Water depth (m)</th>
<th>Phase 1 (°C)</th>
<th>Phase 2 (°C)</th>
<th>Phase 3 (°C)</th>
<th>Phase 4 (°C)</th>
<th>Phase 5 (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.94</td>
<td>90.4</td>
<td>80.2</td>
<td>70.0</td>
<td>60.2</td>
<td>50.4</td>
</tr>
<tr>
<td>0.72</td>
<td>91.7</td>
<td>81.5</td>
<td>71.3</td>
<td>61.5</td>
<td>51.7</td>
</tr>
<tr>
<td>0.50</td>
<td>93.0</td>
<td>82.8</td>
<td>72.6</td>
<td>62.8</td>
<td>53.0</td>
</tr>
<tr>
<td>0.30</td>
<td>94.3</td>
<td>83.1</td>
<td>73.9</td>
<td>64.1</td>
<td>54.3</td>
</tr>
<tr>
<td>0.10</td>
<td>95.6</td>
<td>84.4</td>
<td>75.2</td>
<td>65.4</td>
<td>55.6</td>
</tr>
</tbody>
</table>

Table 1B: Results from single mechanical Stedaei bath experiments.
APPENDIX C

RESULTS FROM EXPERIMENTS PERFORMED ON TWO STRETCH MECHANICAL SYSTEM
<table>
<thead>
<tr>
<th>Date</th>
<th>Type</th>
<th>X-ray</th>
<th>X-ray</th>
<th>X-ray</th>
<th>X-ray</th>
<th>X-ray</th>
<th>X-ray</th>
<th>X-ray</th>
<th>X-ray</th>
<th>X-ray</th>
<th>X-ray</th>
<th>X-ray</th>
<th>X-ray</th>
<th>X-ray</th>
<th>X-ray</th>
<th>X-ray</th>
<th>X-ray</th>
<th>X-ray</th>
<th>X-ray</th>
</tr>
</thead>
<tbody>
<tr>
<td>16/01/2023</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17/01/2023</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18/01/2023</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19/01/2023</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20/01/2023</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21/01/2023</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22/01/2023</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23/01/2023</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24/01/2023</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25/01/2023</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26/01/2023</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27/01/2023</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28/01/2023</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29/01/2023</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30/01/2023</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>31/01/2023</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Source: Data from a specific database or study.
<table>
<thead>
<tr>
<th>Speed of Sound in Steel (m/s)</th>
<th>Speed of Sound in Water (m/s)</th>
<th>Temperature (°C)</th>
<th>Sample Name</th>
<th>Depth</th>
<th>Holes</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>154.2</td>
<td>1370.5</td>
<td>25</td>
<td>Sample 1</td>
<td>1</td>
<td>2</td>
<td>None</td>
</tr>
<tr>
<td>154.2</td>
<td>1370.5</td>
<td>25</td>
<td>Sample 2</td>
<td>1</td>
<td>2</td>
<td>None</td>
</tr>
<tr>
<td>154.2</td>
<td>1370.5</td>
<td>25</td>
<td>Sample 3</td>
<td>1</td>
<td>2</td>
<td>None</td>
</tr>
<tr>
<td>154.2</td>
<td>1370.5</td>
<td>25</td>
<td>Sample 4</td>
<td>1</td>
<td>2</td>
<td>None</td>
</tr>
<tr>
<td>154.2</td>
<td>1370.5</td>
<td>25</td>
<td>Sample 5</td>
<td>1</td>
<td>2</td>
<td>None</td>
</tr>
<tr>
<td>154.2</td>
<td>1370.5</td>
<td>25</td>
<td>Sample 6</td>
<td>1</td>
<td>2</td>
<td>None</td>
</tr>
<tr>
<td>154.2</td>
<td>1370.5</td>
<td>25</td>
<td>Sample 7</td>
<td>1</td>
<td>2</td>
<td>None</td>
</tr>
<tr>
<td>154.2</td>
<td>1370.5</td>
<td>25</td>
<td>Sample 8</td>
<td>1</td>
<td>2</td>
<td>None</td>
</tr>
<tr>
<td>154.2</td>
<td>1370.5</td>
<td>25</td>
<td>Sample 9</td>
<td>1</td>
<td>2</td>
<td>None</td>
</tr>
<tr>
<td>154.2</td>
<td>1370.5</td>
<td>25</td>
<td>Sample 10</td>
<td>1</td>
<td>2</td>
<td>None</td>
</tr>
</tbody>
</table>

*Table 1: Results from a study measuring double metal properties.*
<table>
<thead>
<tr>
<th>Source</th>
<th>Mean</th>
<th>95% CI</th>
<th>p Value</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>High speed of store and order</td>
<td>658.62</td>
<td>649.41</td>
<td>72.68</td>
<td>64.66</td>
</tr>
<tr>
<td>39%</td>
<td>1.06</td>
<td>13.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60%</td>
<td>0.02</td>
<td>1.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10%</td>
<td>0.22</td>
<td>0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30%</td>
<td>0.22</td>
<td>0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10%</td>
<td>0.22</td>
<td>0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>0.22</td>
<td>0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Region</td>
<td>High Speed of Above 1000</td>
<td>High Speed of Above 1000</td>
<td>High Speed of Above 1000</td>
<td>High Speed of Above 1000</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------</td>
<td>------------------------</td>
<td>------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td></td>
<td>5.00</td>
<td>6.00</td>
<td>7.00</td>
<td>8.00</td>
</tr>
<tr>
<td></td>
<td>11.00</td>
<td>12.00</td>
<td>13.00</td>
<td>14.00</td>
</tr>
<tr>
<td></td>
<td>17.00</td>
<td>18.00</td>
<td>19.00</td>
<td>20.00</td>
</tr>
<tr>
<td></td>
<td>23.00</td>
<td>24.00</td>
<td>25.00</td>
<td>26.00</td>
</tr>
<tr>
<td></td>
<td>29.00</td>
<td>30.00</td>
<td>31.00</td>
<td>32.00</td>
</tr>
<tr>
<td></td>
<td>35.00</td>
<td>36.00</td>
<td>37.00</td>
<td>38.00</td>
</tr>
<tr>
<td></td>
<td>41.00</td>
<td>42.00</td>
<td>43.00</td>
<td>44.00</td>
</tr>
<tr>
<td></td>
<td>47.00</td>
<td>48.00</td>
<td>49.00</td>
<td>50.00</td>
</tr>
<tr>
<td></td>
<td>53.00</td>
<td>54.00</td>
<td>55.00</td>
<td>56.00</td>
</tr>
<tr>
<td></td>
<td>59.00</td>
<td>60.00</td>
<td>61.00</td>
<td>62.00</td>
</tr>
<tr>
<td></td>
<td>65.00</td>
<td>66.00</td>
<td>67.00</td>
<td>68.00</td>
</tr>
<tr>
<td></td>
<td>71.00</td>
<td>72.00</td>
<td>73.00</td>
<td>74.00</td>
</tr>
<tr>
<td></td>
<td>77.00</td>
<td>78.00</td>
<td>79.00</td>
<td>80.00</td>
</tr>
<tr>
<td></td>
<td>83.00</td>
<td>84.00</td>
<td>85.00</td>
<td>86.00</td>
</tr>
<tr>
<td></td>
<td>89.00</td>
<td>90.00</td>
<td>91.00</td>
<td>92.00</td>
</tr>
<tr>
<td></td>
<td>95.00</td>
<td>96.00</td>
<td>97.00</td>
<td>98.00</td>
</tr>
</tbody>
</table>

Table: IC - Results from a study investigating data-driven experiments.
<table>
<thead>
<tr>
<th>Date</th>
<th>Sample Name</th>
<th>HWE Core</th>
<th>HWE Control</th>
<th>HWE Core Dose</th>
<th>HWE Control Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/11</td>
<td>1011</td>
<td>0111</td>
<td>0011</td>
<td>0011</td>
<td>0011</td>
</tr>
<tr>
<td>1/12</td>
<td>0101</td>
<td>0010</td>
<td>0010</td>
<td>0010</td>
<td>0010</td>
</tr>
<tr>
<td>1/13</td>
<td>0011</td>
<td>0011</td>
<td>0011</td>
<td>0011</td>
<td>0011</td>
</tr>
<tr>
<td>1/14</td>
<td>0010</td>
<td>0010</td>
<td>0010</td>
<td>0010</td>
<td>0010</td>
</tr>
</tbody>
</table>

Note: Table IC: Results from a study conducted during acute interventions.
<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Number Present</th>
<th>Number Absent</th>
<th>Number Both</th>
<th>Number None</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample A</td>
<td>23</td>
<td>12</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Sample B</td>
<td>24</td>
<td>11</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Sample C</td>
<td>25</td>
<td>10</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Sample D</td>
<td>26</td>
<td>9</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Sample E</td>
<td>27</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 1: Results from a gene expression study with two conditions.
APPENDIX D

THERMOPHYSICAL PROPERTIES OF THE DRAGLINE SILK OF *NEPHILA CLAVIPES* SPIDER

The following chapter was published in Polymer May of 2014. It is presented as it was in the original publication.
Thermophysical properties of the dragline silk of *Nephila clavipes* spider

Changhu Xing a,*, Troy Munro a, Benjamin White a, Heng Ban a, Cameron G. Copeland c, Randolph V. Lewis b

a Mechanical & Aerospace Engineering Department, Utah State University, Logan, UT 84322, USA
b Laboratorium voor Aëroscale en Thermische Systeem, Departementelijk Instaurkaarde en Sterrenkunde, B3 Louwen, Celestijnenlaan 2000, B-3001 Heverlee, Belgium
c Department of Biomedical Engineering, Utah State University, North Logan, UT 84341, USA

**ABSTRACT**

The thermal conductivity and diffusivity of the dragline silk of the *Nephila (N.) clavipes* spider has been characterized by one research group to be 151–416 W m⁻¹ K⁻¹ and 6.4–12.3 × 10⁻⁶ m² s⁻¹, respectively, for samples with low to high strains (zero to 19.7%). Thermal diffusivity of the dragline silk of a different spider species, *Araneus diadematus*, has been determined by another research group as 2 × 10⁻² m² s⁻¹ for un-stretched silk. To improve measurement reliability and reproducibility and resolve the orders of magnitude discrepancy between the two different measurements, this paper measured 13 un-stretched dragline silk samples of the *N. clavipes* spider with different lengths using the same electrothermal technique as the first group but with a much higher vacuum level and an improved heat transfer model. The measured thermal conductivity is 1.2 W m⁻¹ K⁻¹ and thermal diffusivity is 6 × 10⁻⁶ m² s⁻¹. The measured thermal diffusivity of the *N. clavipes* spider silk is in the same order of magnitude as that of the *diadematus* spider but is 1/100–1/200 of the value of the first group. The measured thermal conductivity is 1/150–1/400 of that measured in literature. The discrepancy between this research and the first group may reside in the vacuum level and the improved heat transfer analysis. The difference in thermal diffusivity measurement between the current research and the results of the second group may be because of different species.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

The dragline silk of the Golden Orb Weaver, *N. clavipes*, is among the strongest known materials [1]. The combination of both high tensile strength and elasticity/extension make dragline silk a desirable material for applications such as sports materials and specialized ropes or cords. It has been found that spider silk elicits almost no immunological response and may be applicable as a medical biomaterial for sutures, growth matrices, tendons and ligaments [2–5]. Dragline silk is one of six silks that is produced by *N. clavipes* [6]. This silk is produced in the major ampullate gland and is comprised of two different proteins, major ampullate spidroin 1 (MaSp1) [7] and major ampullate spidroin 2 (MaSp2) [8]. Both proteins are highly repetitive proteins that contain unique motifs that contribute to the mechanical properties of spider silk [9]. A poly-alanine motif (present in both proteins), forms a crystalline β-sheet region that is generally thought to provide spider silk with its tensile strength [9–11], while MaSp2 contains a GIGXX motif which makes up much of the presumed amorphous region of the protein and is believed to form a β-spiral responsible for the elasticity of dragline silk [12].

Although the structure and mechanical properties have been studied extensively, the thermal properties, especially the thermal conductivity (k, [W m⁻¹ K⁻¹]) and thermal diffusivity (α, [m² s⁻¹]), which determine how much and how fast a material conducts heat, have not been investigated convincingly. In literature, Huang et al. [13] measured the thermal conductivity and diffusivity of the dragline silk of a *N. clavipes* using the transient electrothermal technique (TET). According to the research, the conductivity for samples under different strains ranges from 151 to 416 W m⁻¹ K⁻¹ (if extrapolating from the sample measurements under strain, the un-stretched thermal conductivity would be 324 W m⁻¹ K⁻¹, more than double of the directly measured value) and diffusivity spans from 6.4 to 12.3 × 10⁻⁶ m² s⁻¹. With these values, the
thermophysical properties of spider silk are equivalent to, or even
higher than, those of the best thermal conductive metal, copper or
silver. However, Furrer et al. [14] measured the dragline of an
Anaropus diadematus (European garden) spider by means of a lock-in
infrared, (IR) thermography and found that the diffusivity is
2 × 10⁻⁷ m² s⁻¹, over 400 times smaller than the measurement by
Hoang et al. [13]. Since both N. clavipes and Anaropus diadematus
are orb weaving spiders, the process each one uses to produce silk is
very similar. Additionally, the amino acid sequence between the
two species is highly conserved [15]. These two reasons make it
unlikely that a difference in species should produce such a radical
change in thermal properties.

This paper will therefore present the measured thermal con-
ductivity and diffusivity of the N. clavipes (the same species used in
Ref. [13]) spider dragline silk. To have a direct comparison, this
paper will employ the same measurement technique that was
adopted in Ref. [13]. However, as mentioned in a previous paper
[16], the model used in Ref. [13] is a reduced form from a full model
describing the heat transfer process through the sample. This paper
will present the results obtained under the reduced and full model
and analyze the differences.

2. Thermal characterization

A simplified heat transfer model has been described previously
[13]. The thermal characterization mechanism of this technique is
the Joule heating of the sample and subsequent thermal response.
Fig. 1 presents a schematic setup of the measurement technique.
When a constant current, I [A], is passed through a sample with an
unheated resistance, R₀ [Ω], the electrical energy is converted to
thermal energy, which is introduced as a volumetric heat source to
the sample, P_{Vₜ} = I²R₀Vₜ, where Vₜ [m³] is the volume. For the cylindrical
silk, the Vₜ = 2πD²Lₜ, where D is the diameter [m] and Lₜ is the length
[m]. The heat induces a temperature rise, ΔT [K], over time, t, which
finally reaches the steady-state temperature rise ΔTₜ [K]. The
magnitude of ΔTₜ depends on the thermal conductivity, and the
time to reach steady-state (tₑ [K]) is affected by the thermal dif-
sivity. Using a calibrated temperature coefficient of resistance, R
[Ω K⁻¹], the temperature change is linked to the resistance

\[
k = \frac{A²R₀(RₚL)}{\pi D²(Rₚ - R₀)} \left( 2 - 2 \cosh(Hₜ) + Hₜ \sinh(Hₜ) \right)
\]

\[
\frac{\Delta T}{\Delta t} = \frac{8I(Hₜ)^{3/2} \sinh(Hₜ)}{\pi \left( 2 - 2 \cosh(Hₜ) + Hₜ \sinh(Hₜ) \right)} \sum_{m=1}^{∞} \frac{a² \cosh(ab)}{(2m - 1)² + 4a²}
\]

If the \( tₑ \) term approaches zero, Eqs. (1) and (2) can be for-
mulated as the reduced model used in literature [13].

\[
k = \frac{A²R₀(RₚL)}{3\pi D²(Rₚ - R₀)} \frac{8I}{\pi \left( 2 - 2 \cosh(Hₜ) + Hₜ \sinh(Hₜ) \right)} \sum_{m=1}^{∞} \frac{a² \cosh(ab)}{(2m - 1)² + 4a²}
\]

\[
\frac{\Delta T}{\Delta t} = 1 - 96 \pi \sum_{m=1}^{∞} \frac{a² \cosh(ab)}{(2m - 1)² + 4a²}
\]

Spider dragline silk thermal properties will be characterized
using Eqs. (1)–(4) for comparison of both models.

3. Sample preparation

The dragline silk was collected from adult N. clavipes spiders from
the state of Florida, USA, using the method described by Xu
et al. [7]. Briefly, spiders were subjected to CO₂, anesthetizing them
and allowing them to be restrained on the top of a petri dish. Using
scissors, silk was teased out of the major ampullate gland with the
assistance of a dissecting microscope and was attached to a reed
which gathered approximately 100 m of silk. Spiders were misted
during the reeling process to prevent dehydration.

The uniformity and profile of the dragline silk were observed
under Scanning Electron Microscope (SEM), from which the
diameter of the silk: 3.1 ± 0.2 μm was measured. Fig. 2 presents the
silk at different sections under SEM imaging.

To characterize the thermal properties, several spider silk
samples of different lengths were mounted following the config-
uration recommended in Ref. [13] to form a set. The silk was secured
on the two outer ends of the heat sinks by silver paste at the
beginning, endured a 10 nm gold film sputter coating (controlled by
a Cressington thickness monitor) and secured on the inner edges of
the heat sinks. All of the samples were tightly mounted on the outer
heat sink ends by the rheologic silver paste filling without
inducing stress or strain. To improve the contact at the inner edge
and the accuracy of length measurement, the heat sinks were
sloped slightly. After coating, a copper sheath drives the silver paste
tangentially to the inner edge to finalize a sample. Many samples
in a set had infinite resistance, typically due to an incomplete
coating, meaning no measurement could be conducted. Some of
the successfully coated samples were burned by heating or broken

![Diagram](image-url)
in air before or after testing. However, even with these difficulties, 13 samples from 4 different sets/batches of coating had successful measurements. The length of each sample was determined by high-resolution camera using a reference having a resolution of 0.001 mm.

The coated samples cannot be measured immediately because the resistance of the coated film keeps decreasing with time. To stabilize the sample resistance (slow down its change at room temperature) and remove coating related measurement bias error, the samples were annealed in a furnace for a week. As a basis for the temperature limit for annealing, the temperatures of phase transition behavior of the spider silk were determined from the literature. Based on the Thermogravimetric Analysis (TGA) and Dynamic Mechanical Analysis (DMA) measurements, Cumniff et al. noticed that the property of spider silk was stable below 210 °C [17]. Using Thermal Mechanical Analysis (TMA), Rengasamy et al. [18] found that the transition temperature of the dragline silk was at 186 °C. Based on the literature studies, an annealing temperature below 100 °C was adopted to maintain the structure of the silk upon heating. Several annealing temperatures were tested but no appreciable thermal property difference beyond precision error was found.

4. Calibration and test

In order to obtain the temperature coefficient of resistance should be known. For a wire or coated film without a theoretical R', a calibration is necessary. A set of samples was placed in a copper enclosure inside a vacuum chamber for the in-situ calibration in air before testing in high vacuum (Fig. 1). The temperature of the enclosure was controlled and maintained for 10–15 min by a Cole-Parmer Polystat circulating water bath before collecting the calibration data. A type K thermocouple, soldered on the common heat sink and electrical leads for the samples with a 4-wire resistance measurement configuration were passed through the vacuum chamber by means of thermal and electrical feedthroughs respectively. The simultaneous acquisition of temperature and resistance were conducted by an Agilent 34970A data acquisition unit. A linear regression was employed to fit the measured resistances at different temperatures and the slope is R'.

Fig. 3 presents the determined R' for the 13 samples. All of the calibrations have a coefficient of determination (R²) larger than 0.99. For the 1st set where only one sample was successfully coated, the calibration temperature range is slightly small, but it was, still larger than the measured temperature rise.

The calibrated R' and other quantities are summarized in Table 1 where the α values are also presented. The sample temperature coefficients of resistivity of the coated gold film are mostly between 0.0008 and 0.001 K⁻¹, which is about one-quarter to one-third of the bulk gold value (0.0034 K⁻¹). Even though the measured values deviate considerably from that of the bulk material, the consistency of the measured coefficients demonstrates the accuracy of the calibration and its effect on spider silk thermal conductivity measurement.

After calibration, the temperature of the samples was maintained at room temperature (22 °C) and the pressure inside the chamber was reduced by the combination of Adixen Pascal 2015SSD rotary vane and Pfeiffer HiPace 300 turbo pumps. The measurement could begin after the pressure reading from a MKS 251 full range gauge dropped below ~0.001 Pa.

A constant current through the sample, having a settling time of 2 ms was provided by the Keithley 6221 current source. A Keithley 3706 digit multimeter was connected to the other two wires for the voltage measurement. In the computer code, setting commands and a trigger signal were sent to the multimeter. Once the multimeter started sampling noise data, a trigger was sent to the current source to activate the Joule heating. The heating lasted less than 2 s for all of the samples (for the long samples, radiation significantly reduced the time required to reach steady-state). After sampling, the code automatically transferred data from the multimeter buffer to the computer for processing.
negligible. Due to the characteristic of the film coating (resistance drop), measured thermal properties under large $\Delta T$ do not follow the theory. However, to reduce the precision error and improve signal to noise ratio, $\Delta T$ needs to be larger. A compromise has to be made in the measurement. For each sample, several electrical currents were applied in the experiment to find the compromise between avoiding the non-constant heating effect and having a sufficiently high signal to noise ratio. The measurement cases that had better resolution and did not yield results that were significantly deviated from those at other applied currents were selected for the final data reduction.

The noise and transition (a few points) were removed from the collected data, leaving only the Joule heating portion. The steady-state resistance, converted from the measured voltage and constant current, was input into Eq. (1) for the thermal conductivity ($k$) evaluation by the reduced model similar to literature [13]. Once measurements on all of the samples were finished, a regression code was applied on Eq. (1) for simultaneous $k$ and $\alpha$ determination based on varying length and then radiation effects.

The transient data were normalized by $\Delta T/\Delta t$, actually the non-dimensional voltage response, $\Delta V/\Delta t$, because of the linear $R$ and constant $I$. The normalized transient data were input into Eq. (4) for thermal diffusivity ($\alpha$) evaluation by the reduced model because the dependence is only on one parameter, $t$. After $k$ is determined from the full model, the same datasets were regressed by Eq. (2) for a evaluation considering lateral heat loss using the improved $k$ value.

In the data processing of $k$ and $\alpha$ by the full model, the $PR^2$ term in $k_T$ was null because the measured value did not decrease with the increase of $\Delta T_T$ (positive $R^2$). With a high vacuum level, $h_0 = 0$, leaving only the radiation influence.

5. Results and discussions

Table 1 summarizes the measured data for the 13 samples. It contains parameters, measurement results ($k$, $R$, and $t$) and determined $k$ and $\alpha$ for the dragline silk. The determined $k$ and $\alpha$ by reduced or full model will be presented in the following figures for analysis.

The conductivity of each sample is determined by the reduced model with parameters from $L$ to $R_T$, and presented in Fig. 4 with respect to its length. The reduced $k$ increases quadratically with respect to $L$, with a minimum of 1.2 and a maximum of 190 W m$^{-1}$ K$^{-1}$ in literature [13]. The reported thermal conductivity of an un-stretched dragline silk with a length of 4.1 mm is 151 W m$^{-1}$ K$^{-1}$ by direct measurement whereas it became 324 W m$^{-1}$ K$^{-1}$ by extrapolating from the stretched cases. If an interpolation is used for the 4.1-mm sample, the $k$ measured by this research is $\sim$ 30 W m$^{-1}$ K$^{-1}$ by the reduced model, around 1.5–2 of the reported value. Since the same material is measured by the same technique and determined by the same model, the significant difference has to be ascribed to the different vacuum level used in the two studies. A similar significant difference is found in the reduced $\alpha$ in Fig. 5. The reported thermal diffusivity of the unstretched dragline silk is $6.4 \times 10^{-5}$ m$^2$ s$^{-1}$ whereas this research yields a reduced $\alpha$ of $6 \times 10^{-5}$ m$^2$ s$^{-1}$, around 10 of the reported.

Table 1 Measured quantities for the 13 spider silk samples and determined $k$ and $\alpha$ by reduced (R) or full (F) model.

<table>
<thead>
<tr>
<th>Set#</th>
<th>$L$ (mm)</th>
<th>$I$ ($\mu A$)</th>
<th>$R_T$ (K$^{-1}$)</th>
<th>$n_T$ (K$^{-1}$)</th>
<th>$R_T$ (O)</th>
<th>$R_T$ (D)</th>
<th>$\Delta T_T$ (K)</th>
<th>$t$ (s)</th>
<th>$k$ (W m$^{-1}$ K$^{-1}$)</th>
<th>$\alpha$ (mm$^2$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.84</td>
<td>20</td>
<td>5.92</td>
<td>9.59</td>
<td>1.06</td>
<td>0.97</td>
<td>1.04</td>
<td>1.49</td>
<td>9.68</td>
<td>1.35</td>
</tr>
<tr>
<td>2</td>
<td>1.65</td>
<td>15</td>
<td>7.51</td>
<td>8.66</td>
<td>16.73</td>
<td>15.78</td>
<td>1.33</td>
<td>1.03</td>
<td>7.85</td>
<td>1.29</td>
</tr>
<tr>
<td>3</td>
<td>4.53</td>
<td>20</td>
<td>6.76</td>
<td>9.06</td>
<td>1.21</td>
<td>0.95</td>
<td>1.51</td>
<td>1.22</td>
<td>10.26</td>
<td>1.06</td>
</tr>
<tr>
<td>4</td>
<td>7.26</td>
<td>20</td>
<td>7.35</td>
<td>9.35</td>
<td>7.35</td>
<td>6.12</td>
<td>1.26</td>
<td>1.56</td>
<td>11.2</td>
<td>1.06</td>
</tr>
<tr>
<td>5</td>
<td>12.37</td>
<td>20</td>
<td>11.14</td>
<td>8.18</td>
<td>12.14</td>
<td>11.02</td>
<td>1.51</td>
<td>1.66</td>
<td>7.08</td>
<td>1.10</td>
</tr>
<tr>
<td>6</td>
<td>3.55</td>
<td>60</td>
<td>9.00</td>
<td>7.2</td>
<td>9.25</td>
<td>7.35</td>
<td>1.28</td>
<td>1.56</td>
<td>8.02</td>
<td>1.10</td>
</tr>
<tr>
<td>7</td>
<td>1.47</td>
<td>50</td>
<td>2.19</td>
<td>9.06</td>
<td>2.38</td>
<td>2.19</td>
<td>0.88</td>
<td>1.56</td>
<td>8.37</td>
<td>1.12</td>
</tr>
<tr>
<td>8</td>
<td>3.83</td>
<td>20</td>
<td>10.33</td>
<td>10.5</td>
<td>3.97</td>
<td>10.23</td>
<td>1.02</td>
<td>1.49</td>
<td>9.68</td>
<td>1.16</td>
</tr>
<tr>
<td>9</td>
<td>13.87</td>
<td>18</td>
<td>18.42</td>
<td>19.00</td>
<td>13.87</td>
<td>13.82</td>
<td>1.27</td>
<td>1.56</td>
<td>19.6</td>
<td>1.16</td>
</tr>
<tr>
<td>10</td>
<td>20.24</td>
<td>12</td>
<td>32.03</td>
<td>8.02</td>
<td>20.24</td>
<td>20.24</td>
<td>8.02</td>
<td>1.56</td>
<td>15.34</td>
<td>1.12</td>
</tr>
<tr>
<td>11</td>
<td>1.68</td>
<td>20</td>
<td>1.85</td>
<td>10.63</td>
<td>1.68</td>
<td>1.68</td>
<td>1.04</td>
<td>1.56</td>
<td>19.54</td>
<td>1.12</td>
</tr>
<tr>
<td>12</td>
<td>6.05</td>
<td>14</td>
<td>19.02</td>
<td>8.56</td>
<td>23.67</td>
<td>23.67</td>
<td>1.23</td>
<td>1.49</td>
<td>6.02</td>
<td>1.12</td>
</tr>
<tr>
<td>13</td>
<td>11.62</td>
<td>12</td>
<td>27.05</td>
<td>7.21</td>
<td>37.24</td>
<td>37.24</td>
<td>1.23</td>
<td>1.49</td>
<td>6.02</td>
<td>1.12</td>
</tr>
</tbody>
</table>

Mean |
value. Note that the diameters of the dragline silks are slightly different. Because in literature [13], the diameter of the silk is 4.7 μm, which is slightly larger than the silk collected in this research. However, the reduced model, Eq. (4), contains only one parameter, sample length. Thus different sample diameter should not influence the determined diffusivity.

In literature [13], it was found that the thermal conductivity and diffusivity became larger when both length and strain increased, and they ascribed the enlargements to the amount of stretching. However, if their measured quantities were plotted with respect to the sample length, a strong length dependent increase would be able to find similar to Figs. 4 and 5. Therefore, this paper speculates that the explanation in Ref. [13] was not appropriate unless the length dependency could be removed by the more suitable full model. However, since stretching was not studied in this research, the possibility that stretching induced thermal property increase cannot be fully ruled out.

Because of the significant variation of k and σ with respect to the sample length, the reduced model is inappropriate for the data reduction. The full models, Eqs. (1) and (2), have to be employed. The parameters and measured k were put in a regression code for simultaneous k and σ estimation by Eq. (1). The regressed k is 1.23 W m⁻¹ K⁻¹ and σ is 0.73. The significant drop in k of the long samples is because of the consideration of radiation heat loss from the lateral surface. If radiation is not considered, the thermal property is over estimated. The influence by radiation can be viewed from λₑ (when ΔT = 0.5ΔT₀) in Table 3 where the λₑ increases with length until reaching a plateau at ~1.5–1.6 s for any length sample. If radiation effect is negligible, λₑ is proportional to L².

With the determined k and σ, the dimensionless parameter (ΔT/Δt) can be evaluated for each sample. The individual k, convenient for measurement uncertainty analysis, is presented in Table 1 and Fig. 6. The k for each sample scatters around the regressed k. With the determined k and σ, evaluation of α using the full model in Eq. (2) becomes achievable. The determined α is presented in Table 1 and Fig. 7 as 6.2 x 10⁻² m² s⁻¹. If the measurement is perfect, the determined α would scatter evenly around the mean value. However, in this figure, the α by the full model still shows slight length dependency.

Fig. 4. Strong length dependence of thermal conductivities of the dragline silk of A. diadematus spider measured below ~0.001 Pa by reduced model which neglects lateral heat loss. All samples were tightly mounted without strain. At the same conditions except vacuum level, the result is <1/20 of the literature value.

Fig. 5. Strong length dependence of thermal diffusivities of the dragline silk of A. diadematus spider measured below ~0.001 Pa by reduced model which neglects lateral heat loss. All samples were tightly mounted without strain. At the same conditions except vacuum level, the result is <1/20 of the literature value.

Fig. 6. Unbiased thermal conductivities of the dragline silk of A. diadematus spider measured below ~0.001 Pa by the full model. All samples were tightly mounted without strain. The regressed result is <1/20 of the literature value.
6. Conclusion

Thermal conductivity and thermal diffusivity of the dragline silk of N. clavipes spiders were measured using the electrothermal technique. The thermal conductivity is 1.2 W m⁻¹ K⁻¹ and thermal diffusivity is 6 × 10⁻⁴ m² s⁻¹. The deviation in the measurements on this spider silk in the previous literature may be because of two facts: how vacuum level contributing to the convective heat transfer and neglected radiation through the sample lateral surface, both augmenting the measured values.

Acknowledgments

Partial support by Utah Science Technology and Research (USTAR) initiative funding. The gold coating and SEM work were performed by FeiAnn Shen at the Nanoscope Device Laboratory, USU.

References

APPENDIX E

COPYRIGHTS AND PERMISSIONS FOR REPUBLICATION
Title: Development of a Process for the Spinning of Synthetic Spider Silk

Author: Cameron G. Copeland, Brienne E. Bell, Chad D. Christensen, et al

Publication: ACS Biomaterials Science & Engineering

Publisher: American Chemical Society

Date: Jul 1, 2015

Copyright © 2015, American Chemical Society

PERMISSION/LICENSE IS GRANTED FOR YOUR ORDER AT NO CHARGE

This type of permission/license, instead of the standard Terms & Conditions, is sent to you because no fee is being charged for your order. Please note the following:

- Permission is granted for your request in both print and electronic formats, and translations.
- If figures and/or tables were requested, they may be adapted or used in part.
- Please print this page for your records and send a copy of it to your publisher/graduate school.
- Appropriate credit for the requested material should be given as follows: "Reprinted (adapted) with permission from (COMPLETE REFERENCE CITATION). Copyright (YEAR) American Chemical Society." Insert appropriate information in place of the capitalized words.
- One-time permission is granted only for the use specified in your request. No additional uses are granted (such as derivative works or other editions). For any other uses, please submit a new request.
This is a License Agreement between Cameron G Copeland ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Elsevier Limited</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>The Boulevard, Langford Lane</td>
</tr>
<tr>
<td></td>
<td>Kidlington, Oxford, OX5 1GB, UK</td>
</tr>
<tr>
<td>Registered Company Number</td>
<td>1982084</td>
</tr>
<tr>
<td>Customer name</td>
<td>Cameron G Copeland</td>
</tr>
<tr>
<td>Customer address</td>
<td>351 West 1600 North #B201</td>
</tr>
<tr>
<td></td>
<td>LOGAN, UT 84341</td>
</tr>
<tr>
<td>License number</td>
<td>3702640704081</td>
</tr>
<tr>
<td>License date</td>
<td>Sep 05, 2015</td>
</tr>
<tr>
<td>Licensed content publisher</td>
<td>Elsevier</td>
</tr>
<tr>
<td>Licensed content publication</td>
<td>Polymer</td>
</tr>
<tr>
<td>Licensed content title</td>
<td>Thermophysical properties of the dragline silk of Nephila clavipes spider</td>
</tr>
<tr>
<td>Licensed content author</td>
<td>Changhu Xing, Troy Munro, Benjamin White, Heng Ben, Cameron G. Copeland, Randolph V. Lewis</td>
</tr>
<tr>
<td>Licensed content date</td>
<td>5 August 2014</td>
</tr>
<tr>
<td>Licensed content volume number</td>
<td>55</td>
</tr>
<tr>
<td>Licensed content issue number</td>
<td>16</td>
</tr>
<tr>
<td>Number of pages</td>
<td>6</td>
</tr>
<tr>
<td>Start Page</td>
<td>4226</td>
</tr>
<tr>
<td>End Page</td>
<td>4231</td>
</tr>
<tr>
<td>Type of Use</td>
<td>reuse in a thesis/dissertation</td>
</tr>
<tr>
<td>Portion</td>
<td>full article</td>
</tr>
<tr>
<td>Format</td>
<td>print</td>
</tr>
<tr>
<td>Are you the author of this Elsevier article?</td>
<td>Yes</td>
</tr>
<tr>
<td>Will you be translating?</td>
<td>No</td>
</tr>
<tr>
<td>Title of your</td>
<td>Production of Synthetic Spider Silk Fibers</td>
</tr>
</tbody>
</table>

https://ss.1001copyright.com/AppPrintableLicenseFrame.jsp?publisherID=70&publisherName=ELS&publicationID=0022-3661&publicationID=128996&rightID=168... 1/7
INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol / edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.

5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com)

6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.

7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this
licensing transaction. (ii) these terms and conditions and (iii) CCC’s Billing and Payment terms and conditions.

8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC’s Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC’s Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.

9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher’s written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC’s Billing and Payment terms and conditions. These terms and conditions, together with CCC’s Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC’s Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this license at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

https://a100.copyright.com/api/PublishLicenseFrame.jsp?publisherID=70&publisherName=El&publicationID=0023-3995&publicationD=1569A&rightD=1&... 3/7
The following terms and conditions apply only to specific license types:

15. Translation: This permission is granted for non-exclusive world English rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article. If this license is to re-use 1 or 2 figures then permission is granted for non-exclusive world rights in all languages.

16. Posting licensed content on any Website: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the website must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/yyyyy or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com. All content posted to the website must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The website must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. For journal authors: the following clauses are applicable in addition to the above:

Preprints:

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).

Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.
Authors can share their accepted author manuscript:

- immediately
  - via their non-commercial person homepage or blog
  - by updating a preprint in arXiv or RePEc with the accepted manuscript
  - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
  - directly by providing copies to their students or to research collaborators for their personal use
  - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement

- after the embargo period
  - via non-commercial hosting platforms such as their institutional repository
  - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

Published journal article (PJA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.

Policies for sharing publishing journal articles differ for subscription and gold open access articles:

Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs.
and courseware programs), and inclusion of the article for grant funding purposes.

**Gold Open Access Articles:** May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier's posting policy for further information.

18. For book authors the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

19. **Thesis/Dissertation.** If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for ProQuest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PDFs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

**Elsevier Open Access Terms and Conditions**

You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.

**Terms & Conditions applicable to all Open Access articles published with Elsevier:**

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.

The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

**Additional Terms & Conditions applicable to each Creative Commons user license:**

CC BY: The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant...
CC BY NC SA: The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.

CC BY NC ND: The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0. Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.

Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. Other Conditions:

v1.7

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.
CURRICULUM VITAE
Education
PhD. Biological Engineering, Utah State University, UT May 2016
Dissertation Topic: Development of a Process for the Spinning of Synthetic Spider Silk Fibers

B.S. Biological Engineering, Utah State University, UT May 2010

Research Experience
PhD Student Researcher 2011-Present
Lewis Spider Silk Lab, Utah State University, Logan, UT

- Designed and tested a process for spinning synthetic spider silk/
- Presented my process to television crews, business investors, lawyers, high school outreach programs, and funding agencies
- Wrote a patent, multiple publications, posters and presentations
- Mentored six undergraduate researcher students

Graduate Student Researcher 2010-2011
Sustainable Waste-to-Bioproducts Engineering Center, Logan, UT

- Conducted research on algal growth in the presence of pharmaceuticals
- Performed water treatment studies at the Logan City Lagoon wastewater facility

Professional Skills and Knowledge

- **Computing:** Proficient at Microsoft Office, Adobe Photoshop and Illustrator with experience in Matlab, operating Labview, and Fit2d
- **Bioengineering research skills** including: Data analysis, process design and testing, cell culture, spectrometry, fermentation, and safety training
- **Languages:** Fluent in Portuguese
- **Prepared and presented** various presentations
- **Completion of the Grant Writers’ Seminars and Workshops Proposal Writing** workshop
- **Technical writing skills:** wrote a patent as well as several publications with collaborators

Publications
Cameron G Copeland, Brianne Bell, Chad Christensen, and Randolph V Lewis.

Ibrahim Hassounah, Ethan Abbott, Dan Gil, Cameron Copeland, Thomas Harris, Sujatha Sampath, Justin Jones, Jeff Yarger, Randy Lewis. “Enhancing the Mechanical Properties of Nylon 66 Electrospun Yarns by Annealing and Addition of Spider Silk Proteins.” (In progress, expected submission date Dec2015).

Cameron G Copeland, Brianne Bell, Chad Christensen and Randolph V Lewis. “Exploring the Ratio of MaSp1 and MaSp2 in Synthetic Spider Silk Fibers.” (In progress, expected submission date Dec2015).


Posters, Presentations, Awards, and Certificates

- Poster – “Producing Spider Silk Fibers” Rocky Mountain Bioengineering Symposium, 2015
- Best Oral Presentation, Intermountain Graduate Research Symposium, 2013
- Outstanding Poster Abstract Award, Intermountain Graduate Research Symposium, 2012
- Graduate Mentor for 2012 Utah State iGEM team, Winner of best Bio-product Worlds Division
- Hands-on Training on Microbial Fermentation, Center for Integrated Biosystems, 2011

Leadership and Volunteer Experience

Training Manager 2007-2010
Utah State IT Computer Labs, Logan, UT
Designed and conducted a training program on customer service and technical skills for 15-20 new employees per year along with advanced technical training for current employees. Provided IT support to labs and personnel.

**Biology, Chemistry and Math Tutor**  
Utah State University Library, Logan, UT  
Tutored students on various topics and methods  

**Portuguese-speaking Volunteer**  
Religious Non-Profit, Brazil  
Served in the states of Tocantins, Mato Grosso and the Federal District. Helped to rebuild homes, teach, and provide support to Brazilian citizens.