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A Novel Expression: Spider Silk Synthesized for Mechanical Improvements With Kevlar

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A Novel Expression: Spider Silk Synthesized for Mechanical Improvements With Kevlar

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Abstract

This study examines spider silk proteins on the molecular level for the potential use of the components for a new mechanism. DNA on the molecular level is a fairly new innovation that has led to many researchers' abilities to synthesize and construct organic and inorganic materials. The resolution of design in synthetic material is used to create organic structures that allow for stronger use. A systematic review of previously collected protein in spider silk MaSp is analyzed and studied to assemble the necessary protein sequence for further synthesis. Key words such as amino acids, molecular analysis, and protein synthesis found in available spider silk proteins are used to find specific purifications and mechanisms for further production methods.

Acknowledgments

First and foremost I would like to give a huge thank you to Doctor Rosler. Right from the start Dr. Rosler was ecstatic to work on this project and her kindness as well as encouragement is what kept me going to finish. She has given me so much insight and an overall better understanding in not only molecular biology but genetics, cellular biology, and biochemistry. She is the reason I was aware this university has an Honors Program and encouraged me to join. I could not ask for a better professor at Johnson & Wales University, whose sole purpose is to see her students succeed. There have been multiple occasions where I have gone to Dr. Rosler for academic help as well as personal support. She is not only an amazing professor, but a great person and I would not have wanted to work on this project with anyone else.

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spider silk. And all of the ideas you have now with spider silk. Thank you for always listening and being patient.

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Chapter 1: Introduction to Spider Silk

Spider Silk and Challenges with Mass Production

Spider dragline silk is an extracellular fibrous protein that is exceptional in strength, toughness, and resistance to mechanical compression. Spider silk also has unprecedented molecular configurations including both hydrophobic and hydrophilic properties. This biological material is a major rival to Kevlar in which synthetic high-performance fibers are harmful to organisms due to their relation to plastic. Previously studied by researchers Zhang, Yang, Shao, and Hu there are universally low levels of silk protein expression found in heterologous systems of both prokaryotes and eukaryotes (Zhang *et al.*, 2010). With low regeneration of this protein, biospinning, a process also known as electrospinning in which an electric force is used to draw charged threads of polymer solutions to melt the fiber diameters to nanometers, is not an appropriate source ("*Electrospinning",* 2022*).* Spider silk is also compared to silk fibroin of the domestic silkworm. The speed at which the silk is spun is also detrimental in the tensile strength and ability in resistance. *A. pernyi* silk and spider dragline silk are remarkably similar in strain compared to stress ratios while spun through the same speed (Romer & Scheibel, 2008). As there is an increase in the spinning speed and tensile strength, there is a decrease in strain (Römer & Scheibel, 2008).

Molecular properties of Spider silk

Silk strength is not only determined by the spinning speed, but on the molecular level it is associated with the crystalline β-sheet structures, and elasticity is involved with the amorphous regions. Presented below in figure 1 are the beta sheets and amorphous matrix of natural spider silk.

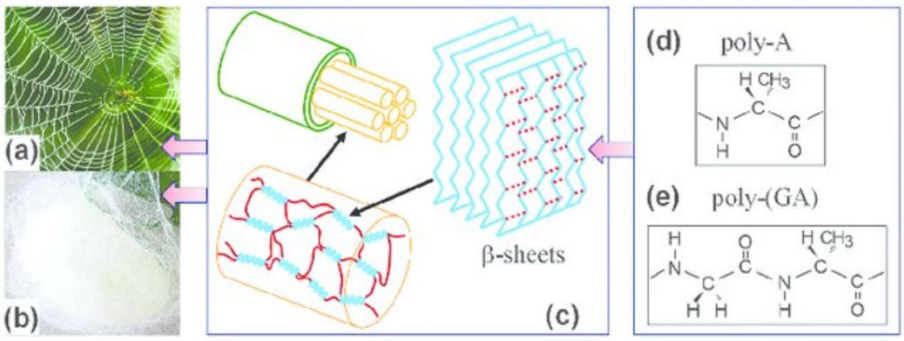


Figure 1. β-sheets and molecular configuration of the Poly-Ala tails

(A). spider silk spun into a web. (B). *Bombyx Mori* cocoon silk for comparison. (C) Natural silk proteins β-sheets morph numerous crystalline domains (blue) that are lodged in an amorphous matrix (red curves). Each β-sheet has multiple β-strands interlocked through hydrogen bonds (shown in red dashed lines). The β-sheets in different natural silks have different amino acid sequences. The β-sheets in the Nephila clavipes spider silk above (d), attribute repeated poly-Ala motifs, while those in the Bombyx mori silkworm silk are created by repeated (e) poly-GlyAla motifs (Zhang *et al.*, 2015).

This protein can assemble in three dimensions to build macroscopic structures and produce a transparent film similar to paper. The primary structure consists of nonpolar hydrophobic amino acids (glycine, alanine, and serine). This structure contains short polypeptide stretches of 10-15 repetitive amino acid sequences. The secondary structure portrays anti-parallel β-sheet conformations forming crystalline domains (Römer & Scheibel, 2008). This can also be seen in figure 1. More specifically, the proteins in spider silk are referred to as spidroins (MaSp1 and MaSp2). These have a core sequence, repetitive amino acid motifs,

and non-repetitive carboxyl- (C-) and amino (N-) terminal domains. These proteins have a higher molecular weight and are linked by cysteine bridges found in their termini. MaSp1/ADF4 are more hydrophobic proteins than MaSp2/ADF3. This reflects a hydrophobic/hydrophilic pair that allows the protein to have stronger mechanical properties (Kiseleva *et al*., 2020).

Dragline silk is also known for its proficiency (capability) in elasticity. The (GGX)n and (GGXX)n modules composed of 31-helices and β-turns provide for the elasticity of the fiber where the GPGXX motif forms these β-turns and creates a spiral that suggests the use in elastin. (GPGXX) stands for glycine (G), proline (P), glycine (G), and any amino acid (X)(X) which makes up the protein sequence. While strength is related to toughness, the difference is strength reflects capability and toughness resembles resilience. The toughness of dragline silk is comparable to energy stored in hydrogen bonded formed in the amorphous and crystalline phase. By breaking the hydrogen bonds in fiber stretching, which is irreversible in the amorphous phase, energy is dissipated. However, the crystalline phase is reversible, and energy is stored to use for elasticity (Römer & Scheibel, 2008).

Compared to Kevlar

On a molecular scale when compared to Kevlar, dragline silk surpasses toughness, durability, and elasticity. However, Kevlar is higher in density and strength. Attempts in 2008 with in vitro mass production of dragline silk have yet to be successful; artificial spinning led to worse mechanical properties than silk spun naturally (Römer & Scheibel, 2008). The issue with this was the difference in diameter in the silicon micro-spinners. The artificial silicon micro-spinnerets produced diameters that were ten times bigger than naturally occurring silks. There

also is a difference in production of silk depending on spinning speed. Silk produced at higher reeling speeds tend to have higher yield points but are less extensible and weaker than those spun at low speeds. In addition, resilience, ductility, and thread diameters depend on spinning speed and the temperature spun (Römer & Scheibel, 2008).

Nanotechnology

Nanotechnology, the use of matter on an atomic, molecular, and supramolecular scale for industrial purposes, can attach to silk-based material and be used to create macro-scale components with spider silk fibers serving as a matrix combined with the other functional elements (Lyddy, 2009). This nanotech is relatively new; however, it has great potential in learning more about the elements in spider silk and potentially creating a stronger material. In respect to this, natural polymers typically exhibit exceptional mechanical properties due to their highly ordered molecular structures. When used artificially, an isopropanol solution and nanoparticle coating fixed on the fibers can result in slightly improved mechanical properties. Researchers, Kiseleva, Kiselev, Nikolaeve, etc. have also found that blending spider-silk with silver (Ag) nanoparticles creates exceptional antimicrobial properties. In the synthesis of silver nanoparticles, spidroins were used as capping and stabilization molecules which resulted in hybrid solutions with antimicrobial activity against multiple multi-drug resistant clinical bacteria and anticoagulant and thrombolytic properties (Kiseleva *et al.,* 2020). When the spider silk is blended with zinc (Zn^{2+}), titanium (Ti^{4+}), and aluminum (Al^{3+}) in combination with water, it can improve overall toughness of the fibers. The nanoparticles magnetite (Fe_3O_4) provide well-defined and stable silk coatings (Kiseleva *et al*., 2020). This improvement occurs due to

hydrogen bonding interaction at oxide-sill interface. Hydrogen bonding is what causes the toughness and elasticity in spider silk (Kiseleva *et al.,* 2021).

Microstructures

Microstructures on the molecular level can be used in designs for synthetic materials such as prosthetics or artificial muscles. Kevlar is a heat-resistant, synthetic, lightweight fiber that brings high tensile strength and improves protection in armor ("What is Kevlar?*",* 2022). This polyester is used in bullet-proof vests and also in most military and police uniforms for its protective characteristics. However, Kevlar has certain drawbacks, such as inflated cost, poor compressive strength, and high sensitivity to environmental factors. The proposal of obtaining protein synthesis from spider silk can be used to molecularly reconstruct fibers stronger than Kevlar.

This research proposal aims to synthesize *in vivo* bioengineered spider silk-like proteins into something that can act as a protective barrier. Researchers at the McKelvey School of Engineering at Washington University in St. Louis used synthesized polymerized proteins derived from microbes to produce a higher molecular weight in skeletal muscles that were spun into the fibers used as a material tougher than Kevlar (Bowen *et al*., 2021). The materials created in this study are nearly identical to proteins found in muscle cells and can be used biomedically for sutures and tissue engineering. With components this strong, there are endless possibilities to create a textile that can cover more than just the chest area. This research will address the question: is there a way to synthesize spider silk as an outer protective layer for Kevlar?

Chapter 2: Introduction to Kevlar

What is Kevlar?

Kevlar is a type of plastic-based, man-made fiber that is spun or woven into fabrics to achieve a high tensile strength. Molecularly, Kevlar is an aromatic polyamide, made from condensation of the amine (1, 4-phenylene-diamine) and the acid chloride (terephthaloyl chloride) ("All about Kevlar", 2020). Kevlar retains a rigid and tense state due to its molecular formation consisting of para-oriented benzene rings that lock together to form sheets via hydrogen bonds (figure 2). The hydrogen bonds are what gives this high-density material its strength as well as the intermolecular stacking between the aromatic groups. Kevlar, however, is an expensive material due to the difficulties of keeping the fibers water-insoluble in the concentrated sulphuric acid solution within the synthesis and spinning stage ("All About Kevlar", 2020).

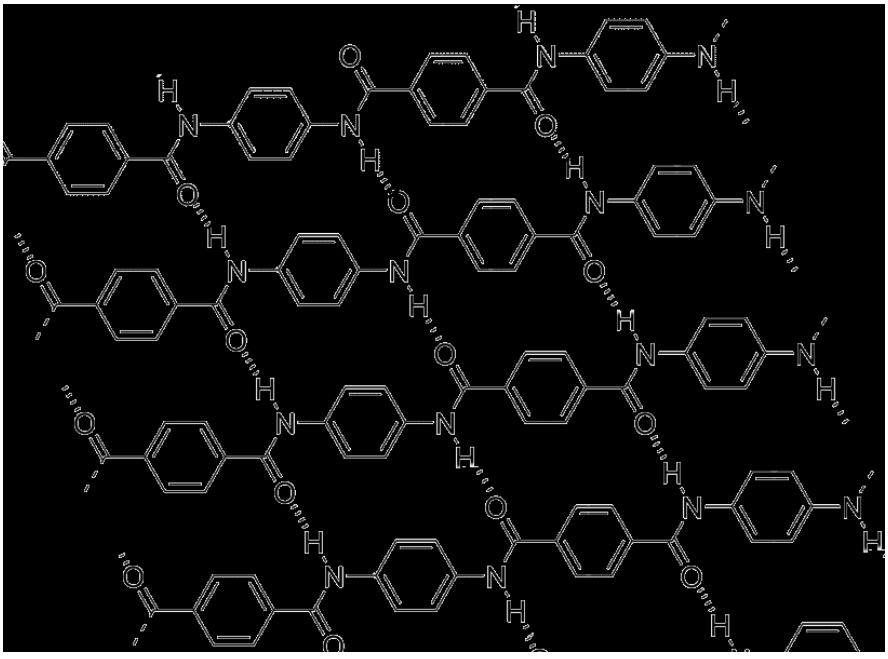


Figure 2. The Structure of Kevlar

The aromatic structure is held together through hydrogen bonding. The dashed lines shown are the hydrogen bonds from the Oxygen ions and the hydrogen ions. The hydrogen ions attach to the nitrogen ions and thus form the aromatic structure (Kabir, E. R. B., & Ferdous, E. N., 2022).

What is Kevlar used for?

This high-performance material allows suitability in the use of protective gear. Military and law enforcement communities benefit from the protection against ballistic projectiles and explosive fragmentation as well as being fire resistant. Protective vests are the most used for this material as they can be expended to protect against knives, needles, explosions, and bullets. It was also put into military helmets to protect against explosives and shrapnel and can absorb more than 20% kinetic energy than previous helmets. This material is also used in other aspects of protection and reinforcements such as airplane parts and suspension bridge cables ("Military apparel by Dupont: Military Protective Gear", 2022).

Molecular structure of Kevlar

Kevlar is a polymer composed of smaller arrangements of monomers. These molecules are arranged parallel to each other in a crystalline structure. This crystallinity contributes to its strength and rigidity. More specifically, it is a polyaromatic amide and are attached to other amide groups via hydrogen bonding (figure 3). ("What Makes Kevlar so Strong?", 2022).

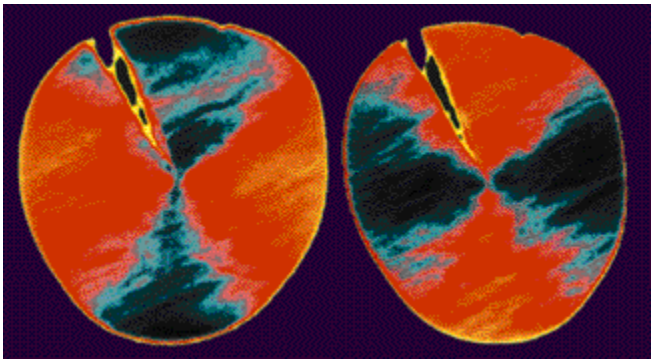


Figure 3. Aromatic Components of Kevlar Polymers

The image to the left shows a spoke-like orientation of the Kevlar polymers. There is a high degree of symmetry that allows for regularity in the internal structures of the fibers. The red and blue colors are the reflections of radiation that potentially indicate the strength of Kevlar fibers. ("What Makes Kevlar so Strong?", 2022).

The weight of spider line (dragline silk) is five times stronger than the weight of steel, two times more flexible than nylon, and ten times more elongated than Kevlar (Gu *et al*., 2020). Spider silk can withstand high temperatures of up to 300 C and temperatures as low as –40 C. Kevlar can withstand high temperatures at 450 C and low temperatures of –196 C ("Chemistry Structure", 2022). Kevlar can also withstand a larger range of temperatures than spider silk. Spider silk is biocompatible, and its major component is a protein that is non-toxic. It can absorb up to three times the amount of Kevlar, with a well-balanced strength including elasticity that outperforms synthetic fibers. Temperature is not the only statistic that shows the differences between Kevlar and spider silk. Fiber structure and molecular structure impact the durability, strength, toughness, and elasticity between these two polymers. Shown below in figure 4, depicted by researchers Kiseleva, Krivoshapkin, and Krivoshapkina, this image shows the differences between these three structures.

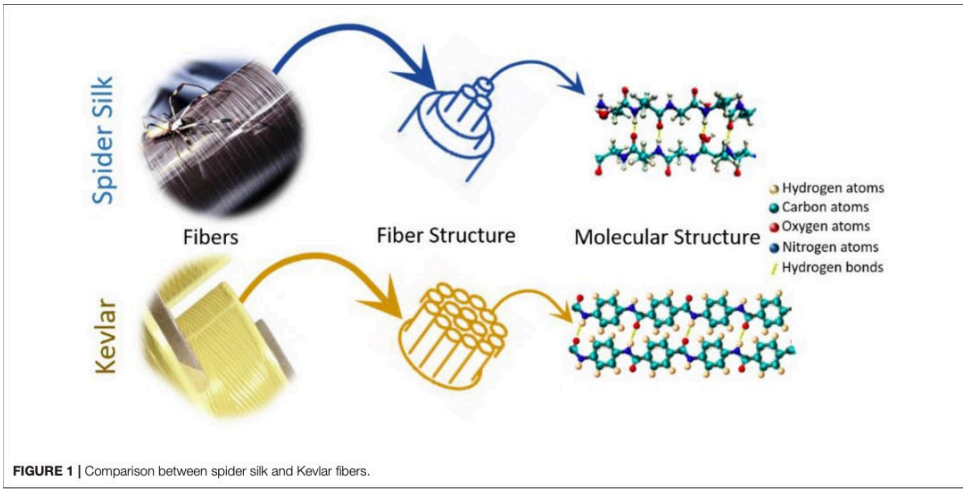


Figure 4. Comparison between Kevlar and Spider Silk

The fiber structure of Kevlar is arranged in parallel regions that are connected through hydrogen bonds. The spider silk protein is connected through hydrogen bonds parallel to each structure; however the structure is linear and not aromatic. (Kiseleva et al., 2020).

How is it like spider silk?

Like Kevlar, spider silk is arranged in parallel amorphous regions of β-sheet crystalline structures. Silk strength is attributed to crystalline β-sheet structures not positioning. The positioning reflects the support of the material. While spider silk is arranged in parallel structures, this reflects elasticity. The differences, as shown in figure 4, resemble Kevlar to arrange in aromatic rings while spider silk stays in a linear formation. The aromatic rings provide a more resilient strength while the linear formation allows elasticity (Kiseleva *et al*., 2020).

Chapter 3: Literature Review

Introduction

The following scholarly literature discusses ranges of research into the spider silk protein (MaSp) and the structural properties that allow for new studies in nanotechnology, host gene production, and mechanical engineering. Spider silk, more specifically dragline silk, is almost entirely composed of large proteins. These characterize the structure to compete if not outweigh Nylon and Kevlar synthetic fibers in a weight-to-weight ratio based on strength and tensile elasticity. Research scientists, Römer and Scheibel (2008) convey the structure-function relationship of highly repetitive spider silk spidroins and the conversion into fibers. The underlying proteins, MaSp, are produced in the ampullate glands which form the dragline silk that allows spiders to escape predators due to the high components in strength and elasticity. Further research into these proteins refer to the silk gene as Spidroin 1 or 2 (NCF-1/2) in reference to their sequence. To find more information on why these fibers are so strong in comparison to other textiles, researchers have discovered the molecular structures that contribute to gene expression.

Structural components

Proteins are composed of four distinct levels in structure. The spider silk protein consists of substantial amounts in hydrophobic amino acids (glycine or alanine) that form repetitive short polypeptide stretches in the primary structure (Römer & Scheibel, 2008). The amino acids in spider silk form α-helices and β-sheet structures in the core that are highly correlated with specific mechanical properties (Li *et al.,* 2021). The antiparallel β-sheet

nanocrystals contribute to mechanical strength due to their repetitive core domains. The proline-rich segments of the spidroin 2 repeats are found in a series of β-turns, while the polyalanine regions of spidroin 1 and NCF-2 form a β-sheet (Beckwitt *et al*., 1998). These sheets are from the poly-alanine sequences of MaSp1 and MaSp2 proteins zippered together on two sides of each polypeptide chain combining both hydrogen bonds and van der Waals interactions (Brooks *et al*., 2008). Both proteins are rich in proline and encode for crystal-forming poly-A blocks and repeat blocks of GPGXX (Rising et al*.,* 2005). Each block almost always starts with GGAGQGGY and ends with GQGAG prior to the poly-alanine region (Rising *et al*., 2005). The coding of specific nucleotides results in high or low amounts of specific amino acids in the protein. For nucleotides guanosine and cytosine found in the coding DNA sequence of the MaSp1 and MaSp2 genes, the abundance results in high amounts of alanine (A) and glycine (G) in the protein (Rising *et al.,* 2005). Guanosine and cytosine are important to the overall 3D structure of the spider proteins. Alanine and glycine amino acids contribute to the secondary structure of the protein which relays into the mechanics of spider silk: elasticity, strength, and toughness.

Hydrogen Bonding

The densely packed arrangement of this secondary structure in spider silk proteins makes it impenetrable to water. Additionally, it is more solid due to the added hydrogen bonds that form between regularly spaced polar amino acids (often Prolines, P). In these, the protein structure shows a specific hydrophobicity pattern with alternating hydrophobic and hydrophilic blocks to allow for phase separation during the spinning process (Römer & Scheibel, 2008). The

hydrophilic part of the pattern leads to the issue of sensitivity to humidity and water that causes super-contraction of the fibers, as shown in figure 5. Once these fibers get wet, the Hydrogen bonds that are predicted to form between the proteins become displaced with water-based hydrogen bonds instead. These hydrogen bond disruptions increase molecular mobility which in turn causes a decrease in fiber length by more than 50% (Li *et al*., 2021). The shortening of these fibers ensures the integrity and shape of the web.

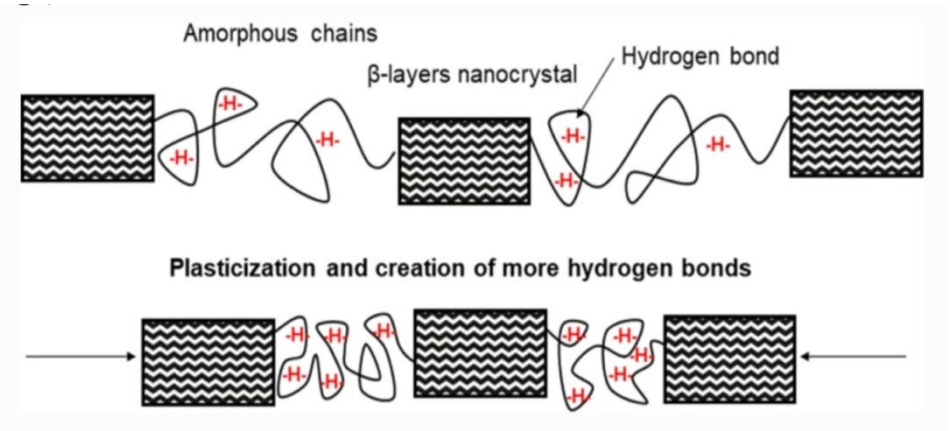


Figure 5. Effects of Supercontraction

The top image is showing natural spider dragline silk featuring the β-sheets and amorphous regions connecting them. The below image is showing what happens during supercontraction. The abundance of hydrogen bonding causes a shrinkage in the amorphous region between the β-sheets (Belbeoch *et al.,* 2021).

Determining why spider silk experiences a loss of structural integrity in the presence of humidity would allow for better designs in biomedical devices. Researchers Greco et al (2021) have discovered that multiple amino acid residues are a cause for super-contraction. The most known amino acid for this is proline, located in the repetitive regions of β-sheets (Greco *et al*., 2021). In comparison to native spun spider silk, the super-contraction is decreased to a considerable extent, reaching around 20% related to 45% in the artificial *Araneus diadematus* major ampullate silk. A major portion of this is due to the fact that artificial spinning is not as

successful as natural spinning and has shown worse mechanical properties than natural silk (Römer & Scheibel, 2008).

Silk Assembly

A successful silk assembly depends directly on extending, aligning, and packaging individual silk proteins that flow inside the spinning duct. Many artificial spinning ducts have not been successful in obtaining relevant proteins expression. The reason they have found this to occur is while using silicon micro-spinnerets, as a mimic of a wet-spinning process, several meters of the spider silk fibers could be produced; however, the diameter was up to ten times larger than naturally occurring silks (Römer & Scheibel, 2008). The speed at which the spider silk can be produced in bio-spinning correlates to the tendency of tensile strength and strain at breaking energy. There is a decrease in these breaking energies with increasing speed of manufacturing done in the ampullae gland (Zhang *et al.,* 2010). High intensity spinning causes the breakage of the amide bonds and the wreckage of the molecular conformation that changes the crystalline structure. Silk strength is wildly attributed to crystalline β-sheet structures (Figure 6), due to the aligned parallel fiber axis and resultant hydrogen bonding that occurs (Zhang *et al*., 2010). The incorporation of nanotechnology defeats this prospect by integrating inorganic materials with the interrelations of the fibers to create a more efficient toughness in hybrid spider silk fibers (Kiseleva *et al.,* 2020).

Nanotechnology

Hybrid versions of spider silk have been proven to have increases in toughness, strength, and elasticity in addition to an eco-friendly nanoparticle synthesis process. In research to create a spider silk fiber-based hybrid material with increased toughness, researchers Kiseleva, Krivoshapkin, and Krivoshapkina at Laboratory of Solution Chemistry in St. Petersburg Russia, have emphasized a template for deposition of zinc (Zn), titanium (Ti) and aluminum (Al) by multiple pulsed vapor phase infiltration (Kiseleva et al., 2020 Hybrid material). Long exposure to water vapor results in the breakage of inner hydrogen bonds in the silk fibers, the metal ions bind to these breakage sites forming metal-coordinated/ covalent bonds to increase the toughness. Carbon nanotubes have also been used for fabricating tough but lightweight and flexible, conductive spider silk hybrid fibers (Kiseleva et al., 2020 Hybrid Silk). The production of spider silk fibers reinforced by carbon nanotubes have shown higher yields in mechanical properties than synthetic polymeric high-performance fibers like Kevlar. Three-dimensional carbon materials also have great application projections as electrode materials for supercapacitors due to their advantages of high electrical conductivity and fast electrical diffusion (Gu et al., 2020). The composition of spider silk is not limited to proteins and can be expanded to the structure of the webs themselves. This structure can be compared and further synthesized to similar materials, like Kevlar or steel.

Synthesized material

Previous discussion of the many properties of spider silk lend itself as protective material similar to that of Kevlar, the man-made, bulletproof material, that has taken advantage of science as a protective barrier for human life. However, incorporating these types

of Kevlar-like textiles for mass production is cost effective and highly non-eco-friendly. Being able to synthesize textiles like spider silk that is stronger and more elastic than Kevlar, can lead to a lot of biomechanics that will better suffice protection. Scientist Essaidi (2015) implemented a bulletproof matrix composed of spider silk proteins produced by transgenic goat milk, into human skin cells via grafting (results not peer-reviewed) (2.6g 329 m/s). Transgenic goat milk has been developed to express recombinant spidroins capable of production and secretion of these genes in this host organism. Goats that express this protein in their milk tended to have lower yields until CRISPR-Cas9 was used to increase the purity and quantity. The alpha-s2-casein gene was replaced with the MaSp1 gene and an average maximum stress of 21-73 MPa (megapascal) was produced (Ramezaniaghdam *et al.*, 2022). Overall, spider protein production in goat milk is similar to the outcomes in *Bombyx mori* as a host for cell transgenics.

Many organisms have been studied for the mass production of this spider dragline silk or as a recombinant alternative. The goal in using mammalian cell lines is to produce secretions through protein-rich milk via mammary glands in larger volumes (Whittall *et al.,* 2021). However, the production of recombinant fibers through this process results in tensile strengths below that of native dragline silk. The limitations found in harvesting natural dragline silks correspond to the physical nature and behavioral implications found in this organism. Authors Ramezaniaghdam et al (2022) write that the cost for natural dragline silk production from one million *N. madagascariensis* spiders will require 70 working individuals at over $500,000.00 and only make 3.4 meters in textiles. This estimation does not even account for the cannibalistic nature of most spiders which makes them unsuitable for livestock breeding. An alternative is the use of insect cell lines for *in vivo* expression of the silk genes from the silkworm (*Bombyx*

mori). Silkworm genes are an ideal gene candidate as silkworms have been manufacturing silk for over thousands of years and their assembly processes do not rely on artificial spinning following expression. The use of an insect expression system rather than a mammalian one is more advantageous because of the similarities between the phylogenetic class as well as the comparable amino acid sequences between *Bombyx mori* and spider silk.

Molecular Techniques

Recent studies have been conducted with the use of newer molecular techniques that allow for successful genome editing. Researchers Xu, Dong, Yu, and others used transcription activator-like effector nucleases (TALENs) to target gene mutagenesis of the FibH gene in *B. mori* and incorporate the spider silk gene (MaSp1) in place (Xu et al., 2018). Through incorporation of the FibH promoter to control the MaSp1 expression, the results indicated a transformation efficiency of 7.8-12.9%, however, this technique provided a more stable expression system for the integration into the cells. TALENs also has produced less off-target sites than the use in CRISPR-Cas9. Insect cell lines have been known to be closely approximate to the cytosolic environment of native spider cells and thus provide an environment suited to produce recombinant spider silks. The cell line Sf9, first derived from the fall armyworm (*Spodoptera frugiperda*), has been used as a host in the baculovirus system for recombinant spidroins (Whittall *et al.,* 2021). The assembly of these spidroins within insect lines has not yet been employed but could be a great candidate for mass production in future studies (Whittall *et al*., 2021).

Chapter 4: Methods

Proposed DNA Sequence

The proposed novel MaSp-Kevlar protein sequence consists of head-to-tail arrangements of the GPGXX motifs to form the β-turn spiral of elastin (Figure 6). GPGXX motifs are found in major ampullate silk and contribute to the amino acid arrangements of elastin. Proline residues found in the GPGXX motif contribute to the retractive forces after stretching which occurs from the hydrogen bonds breaking through extension (Rising *et al.,* 2005). The major ampullate gland secretes the MaSp1 and MaSp2 proteins that are held together through three to five disulfide bonds. The transcript for these genes has an abundance of G and C nucleotides that results in a lot of glycine and alanine as well as a preference of A and T as the third nucleotide (Rising *et al*., 2005). Each block of the gene begins with GGAGQGGY and ends with GQGAG before the start of the poly-alanine region. In this project, we will use the construct GGAGQGGYGGPGGSGQGGPGGY-GQG-(A). Each section encodes a different region along the gene MaSp. The β-turn is encoded by GGPGGS and GPGGY in between a spacer, GQG. The poly-A tail will follow another spacer, GQG, and is shown in Figure 6A.

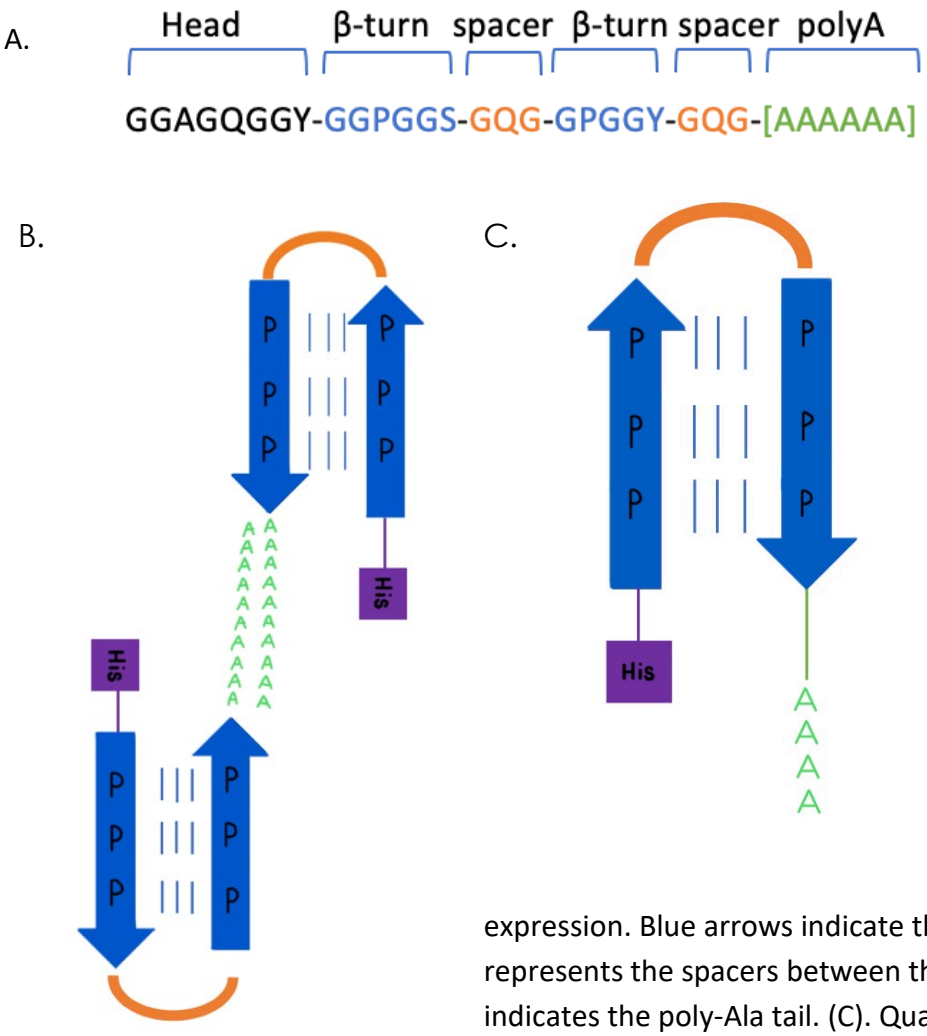


Figure 6. Generated construct of MaSp-Kevlar protein sequence

(A). Construct for the proposed MaSp-Kevlar protein sequence featuring the head of the sequence, β-turns, spacers, and poly-A tail. (B). Tertiary structure of MaSp protein expression. Blue arrows indicate the β-turns, orange represents the spacers between the turns, and green indicates the poly-Ala tail. (C). Quaternary structure of the MaSp protein sequence. Poly-Ala tails are connected through hydrophobic interference. The addition of HisX6 could be used to direct quaternary structure into new angles to improve strength.

Nanoparticle Additives

The addition of nanoparticles, materials with dimensions in the nano scale (under 100 nm), in spider silk templates can insure strength and high-performance (Murthy, 2017). Spider silk can serve as a template for nanoparticle coating due to its mechanical properties that are not altered to nanoparticle assembly. The addition of metal nanoparticles leads to antimicrobial

additives, extra tough fibers, and vapor sensors. In this proposed synthesis, spider silks contract while exposed to polar environments and in the case, there is bonding with gold, the yields are lower due to the covalent bonds formed (Kiseleva *et al.,* 2020). Figure 5 resembles this phenomenon with the addition of different metals in between the β-sheets where previous hydrogen bonding occurred.

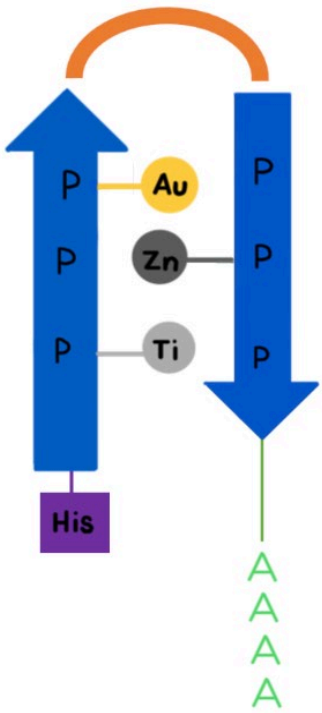


Figure 7. Tertiary structure of MaSp-Kevlar protein with metal nanoparticles

β-sheets of MaSp protein with infusion of metal nanoparticles (Au, Ti, and Zn). The fusion of the nanoparticles is formed by the breakage of the hydrogen bonds and insertion of covalent bonds formed between the proline and the metals. (orientation of his tag) – charged beads

Reverse translation

Reverse translation is the process of using a table of amino acid codons to predict the specific nucleotide sequences that encode for the organism being used ("Reverse translation", 2022*)*. In this study, the selected organism is that of the baculovirus Sf9 insect cells and will be used as the collection of amino acids. The predicted sequences are typically used for the design of PCR primers for the detection of DNA fragments that might encode for the desired gene product (*Reverse translation).* In this study, the procedure of reverse translation is used for the

proposed MaSp-Kevlar sequence that will be acquired through further analysis and expression. The discovered gene sequence is found in Figure 8 below. Each RE (restriction enzyme) site is marked followed by the reverse translation sequence, poly-A tail, and a different encoding RE site. These specific codons are accounted for Sf9 insect cells codon preference using the GenScript online database ("GenScript", 2022).

Transient Gene Expression

Transient gene expression is the expression of genes in a brief time after plasmid DNA encoding an expression cassette that has been introduced into the cell. Transgenic expression does not fuse with the host cell DNA and results in the loss of the vector it was planted in after several cell replication cycles (Cai *et al.,* 2010). This technique is used to study short-term gene expression. The expression of the MASP1 gene is what will to be determined from this expression.

Expression Vector

The baculovirus expression vector system is a way to get insect cells to make proteins for the selected proteins of interest. A baculovirus has circular genomes that allow for greater infection outcomes. The baculovirus vector is used to get the selected markers/ protein into the cell for expression. The vector will be controlled by the hsp promoter which will express the gene inserted. The cells Sf9 are used for expression; however transfection needs to occur to get this into the cells themselves (Lee *et al.*, 2000).

In the vector used for this project, the selectable marker for this will include a poly-adenylated EGFP (enhanced green fluorescent protein gene) under the control of a constitutively active polyhedrin promoter. EGFP (enhanced green fluorescent protein) is used as a reporter protein that monitors the peptide bonding of the gene of interest. This selectable marker will show a stronger fluorescence than a regular GFP promoter. The use in GFP was originally discovered by Douglas Prasher in 1987 for his idea of reporting protein expression in a cell by somehow linking it to a specific protein (Zimmer, 2022). Further along the vector, the tagged Kevlar-like spider silk gene will be under the control of the heat shock 70 promoter (hsp 70) and contain a fused hexahistidine (His-6) tag with a SV40 3' poly-A tail. Normally, the heat shock proteins' role in cells is to facilitate the restoration of normal function inducing targeted gene transcription of gene products that refold denatured proteins and to help fold newly synthesized proteins (S.L. Yu MB, A., & BChir). In this system however, when the SF9 cells are heat shocked (37ºC induction for 1 hour), the induced heat shock proteins will also bind to the recombinant hsp 70 promoter and induce the spider gene expression. The SV40 poly-A tail is used for the proper termination of transcription activity in eukaryotes (Li, S. P., et al). This system will allow the expression of the protein but also the termination after that expression because it is encoding at the 3' end. The His-6 tag is used for protein purification discussed later.

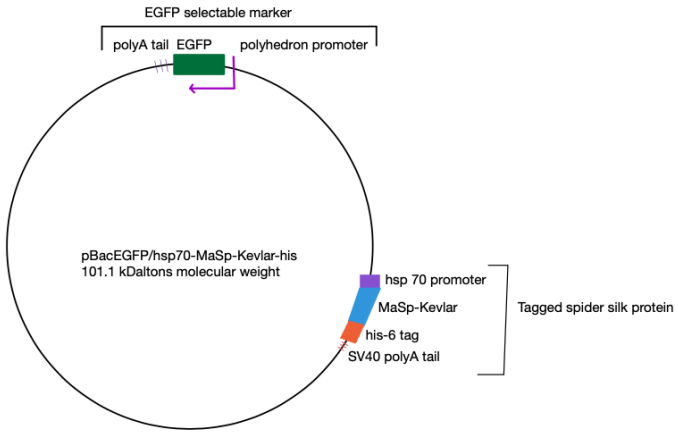


Figure 8. pBacEGFP/hsp70-MaSp-Kevlar-His construct

The construct shown above will inoculate SF9 cells. It is composed of two gene loci: a selectable marker containing a poly-A tail, EGFP, and a polyhedron promoter, and a tagged spider silk protein that includes an hsp70 promoter, the MASP-Kevlar gene, his-6 tag, and a SV40 poly-A tail (redo to MaSp-Kevlar). Molecular weight for the proposed sequence was collected from the Expasy website (*Compute pI/MW).* (B). Reverse translation sequence. The red outlined sequence represents the RE sites (EcoR1 and BamH1). Black lettering stands for the head of the sequence, blue for β-sheets, orange for spacers, and green for the poly-A tail.

MaSp-Kevlar DNA sequence

5'
GAATTCGGTGGTGCTGGTCAGGGTGGTTACGGTGGTCCC
GGTGGTTCCGGTCAGGGTGGTCCCGGTGGTTACGGTCA
GGGTGCTGCTGCTGCTGCTGCTGGATCC
3'

Subcloning

Subcloning is a technique used to get a particular DNA sequence and its resultant protein expressed in a cell. This technique will be used to get the protein MaSp-Kevlar expressed in Sf9 insect cells. The genetic instruction of the protein MASP1 will be engineered into the donor vector that is shown above. It is then taken into the bacmid prep where the vector with the selectable markers is combined with the baculovirus DNA. The next step is transposition. This process takes segments of a chromosome and relocates them throughout the genome ("Transposition", 2022). What will happen with the donor vector and the bacmid is the combination together to produce the desired recombinant bacmid DNA.

Transfection

Transfection is the process of artificially imposing DNA/RNA into cells in a process other than viral infection. The bacmid is then inserted into the cell from transfection and then infection begins (20-96 hours). The polyhedron gene on the baculovirus allows for replication and survival after a host's death. This also allows us to produce working proteins in insects. On the vector synthesized for this project, the MaSp-Kevlar gene will be in place of this polyhedral gene, in purpose for expression in the cells. (Lee et al., 2000). Typically, there is an overabundance in production of the polyhedron and in replacement of this, there will be an overabundance of production of the MaSp-Kevlar protein.

FACs Sorting and Analysis

To purify high concentrations of the MaSp-Kevlar protein and separate them from endogenous Sf9 proteins, protein purification using the C-terminal His-tag will be employed from EGFP+ cells. Protein purification is the process of obtaining the protein of interest in four main steps: cell lysing, protein binding to a matrix, washing, and elution (*Protein purification Methods)*. In this proposed study, EGFP cells will be identified and sorted via a FACs sorter (Bigfoot Spectral Cell Sorter) from Thermo Fischer Scientific. The cell sorter works by obtaining the EGFP+ (green) cells and collecting them for further analysis, while dumping the EGFP- (nongreen) cells (Mack *et al.,* 2014). The EGFP+ cells are then heat shocked at 37ºC for 48-96 hours in order to drive expression of the MaSp-Kevlar construct. This process is shown in Figure 9. Heat shocking allows for the restoration of normal functions by refolding denatured proteins

into new synthesized ones. It also allows for the entry of the construct DNA into the cytosol possible.

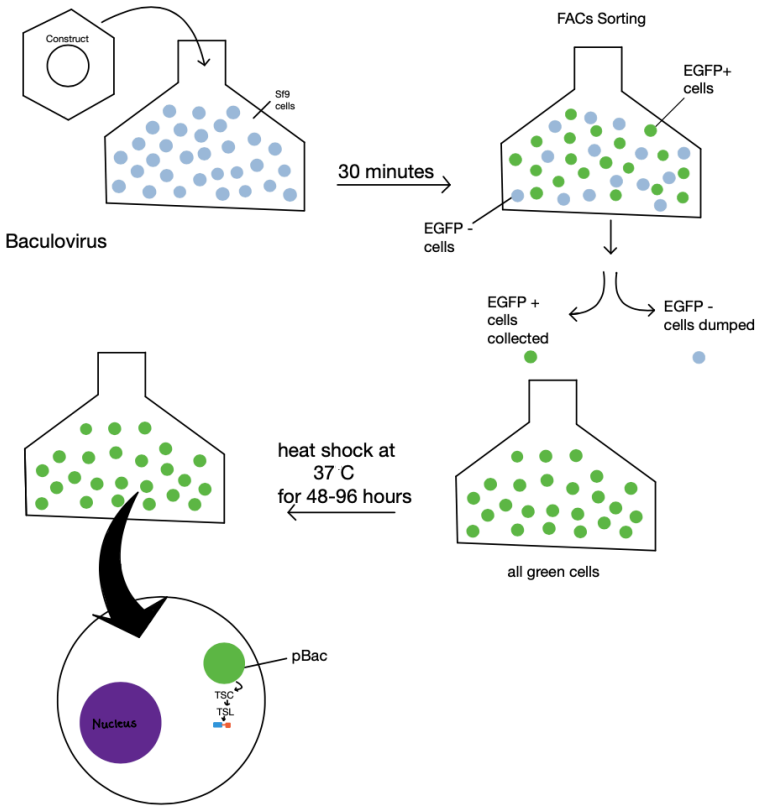


Figure 9. FACs sorting and Analysis

The donor plasmid is inserted and run through steps for incubation and purification. The amount of time for incubation is about 30 minutes. The cells are displayed in positive (EGFP+) or negative (EGFP-) markers that will light up green or stay the same color. The FACs sorter dumps the negative cells and then heat shocks the solution of cells for 48-96 hours at 37 C. The last image is a magnified version of the cells showing the nucleus (purple), the pBac positive cell (green) and the attached MaSp-Kevlar-His protein that underwent transcription and translation.

Cell Collection

Following centrifugation of the EGFP+ cells that have been heat shocked for 48-96 hours, cell lysates will be prepared. Centrifuging is the process of collecting the cells and using them for further purification. Following resuspension in buffer, cells will be lysed with sonication to break cell membranes in the presence of protease inhibitors to ensure endogenous protease activity does not hamper our MaSp-Kevlar production (Wang et al., 2015). Cell debris will then be pelleted via centrifugation and proteins will be collected in the lysate and resuspended in buffer.

Protein Purification

The purified proteins have hexahistidine (His(6)) tag proteins attached that can be removed through further purification. Figure 10 shows the process of removal. The proteins are washed through a chromatography flask with nickel (Ni^{+2}) metal ions attached to the walls. Ni^{+2} ions attach to histidine in an octahedral coordination as Histidine behaves as a tridentate ligand coordinating the carboxyl group (Valenti *et al*., 2006). Once the proteins have gone through this chromatography, the His tags attach to the Ni^{+2} and the rest of the proteins that do not have His attached elute off. Once the only proteins left are the His tagged proteins, these are now washed in imidazole solution to attach the Ni^{+2} in replacement of the His tagged proteins. His proteins are now washed and eluted from the solution.

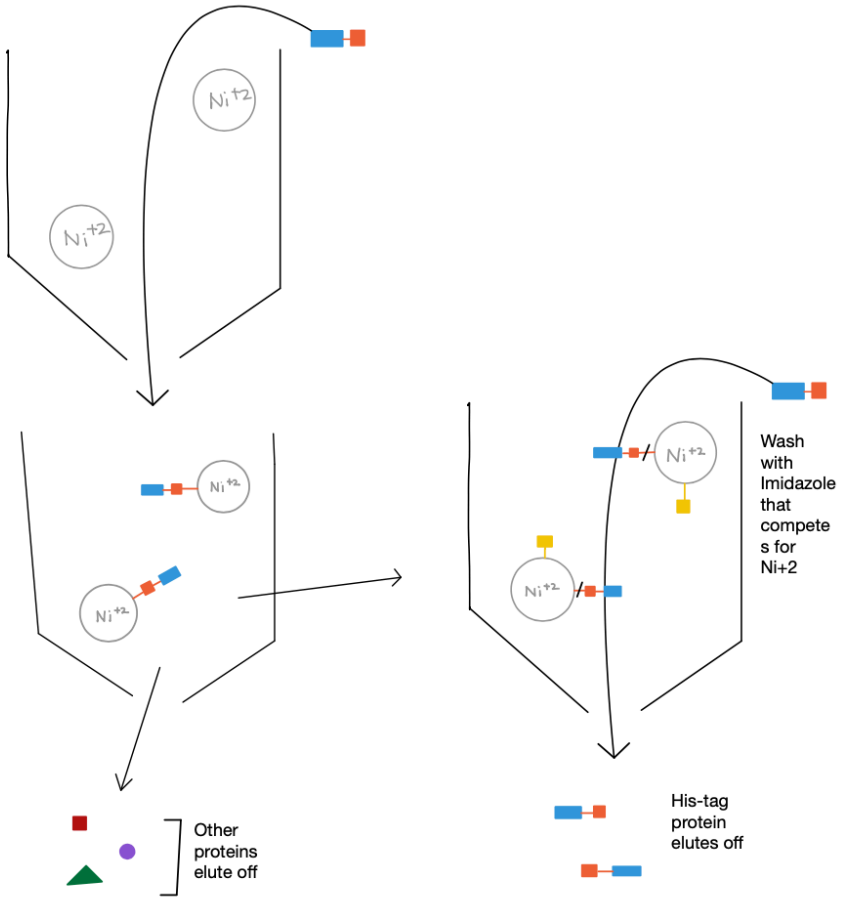


Figure 10. Protein Purification on Ni^{+2} column

The first image is the incubation of the His-tagged proteins and metal Ni^{+2} ions. After the first tube of solution the Histidine binds with the Ni^{+2} and other proteins elute off. The third step shows the addition of imidazole where the Ni^{+2} binds, and the histidine elutes off with the purified protein.

Assays

The gene expression assays replace previous assays that measure gene expression of one gene or a group of genes (Kirby *et al.,* 2007). The expression assay used in this proposal will be conducted using the same analysis as researcher Michaela Hugie in *Expression Systems for Synthetic Spider Silk Protein Purification* (Hugie, 2019).

Tensile Strength Test

Following expression assays, tensile strength tests are conducted. These tests are destructive test processes that gain information on tensile strength, yield strength, and ductility of the material. The main outcome for a tensile test is a stress-strain curve that gives the relationship between strain and engineering stress (Farhat, 2021).

Young's Modulus for Elasticity

Young's Modulus is a common measurement scale for elasticity. It measures the rate of change of strain as a function of stress and represents the slope of a stress-strain curve ("Modulus of elasticity testing", 2022). It will be used in this project to measure the strength and elasticity of the MaSp-Kevlar protein structure obtained from protein purification. Notices for an increase in strength, elasticity, and toughness will be documented and compared to values observed in other spider silk proteins (Römer & Scheibel, 2008).

Chapter 5: Future Applications

Hybrid Materials

Advancements in genetic engineering and nanotechnology have given rise to the production of hybrid, biomaterials. These technologies have the potential to overcome the routine material for bullet proof textiles and other significantly strong fabrics. Fabric coating, carbon nanoparticles, and other hybrid organic techniques allow for the impending production in newer fabrics (Kiseleva *et al*., 2021).

3D Printing

Other forms of production in spider silk spinning can be accomplished artificially through 3D printing or incubation baths with motorized wheels (Kiseleva et al., 2020; Greco et al., 2020). Incubation in deionized water (dH_2O) or phosphate buffered saline (PBS) showed that the protein fibers did not dissolve in incubation times of >5 hours (Greco *et al*., 2020). After the incubation bath these fibers were placed onto a motorized wheel with diapositive slide frames attached where they were then air dried and tested for characterization mechanics. 3D printing can influence the production of frameworks with complex geometries in spider silk that is typically difficult to fabricate with traditional techniques (Kiseleva *et al*., 2020). In 3D printing, a liquid-solid transition occurs due to physical or chemical cross-linkers to transform it into hydrogel. Using this technique, the production of spider silk can create different frameworks with shapes distinct from those of natural fibers which can be extremely favorable in manufacturing (Kiseleva *et al*., 2020).

Cellulose nanofibers have great mechanical properties and are composed of printer-friendly materials that include shear thinning behavior and structural stability when 3D printed (Huang *et al*., 2020). Cellulose nanofibers are a reinforcement material with low density and high biocompatibility. Bacterial cellulose (BC) is used in hydrogels that reinforce 3D printing and give a more compact result in the fibers shown in Figure 11. Hydrogels are three-dimensional networks of hydrophilic polymers that can swell and hold large amounts of water while maintaining its structure (Bahram *et al*., 2016). Using spider silk proteins with the composed hydrogel in (Huang *et al*., 2020), we can synthesize the protein into other materials, such as Kevlar, and potentially can molecularly merge the constructs together.

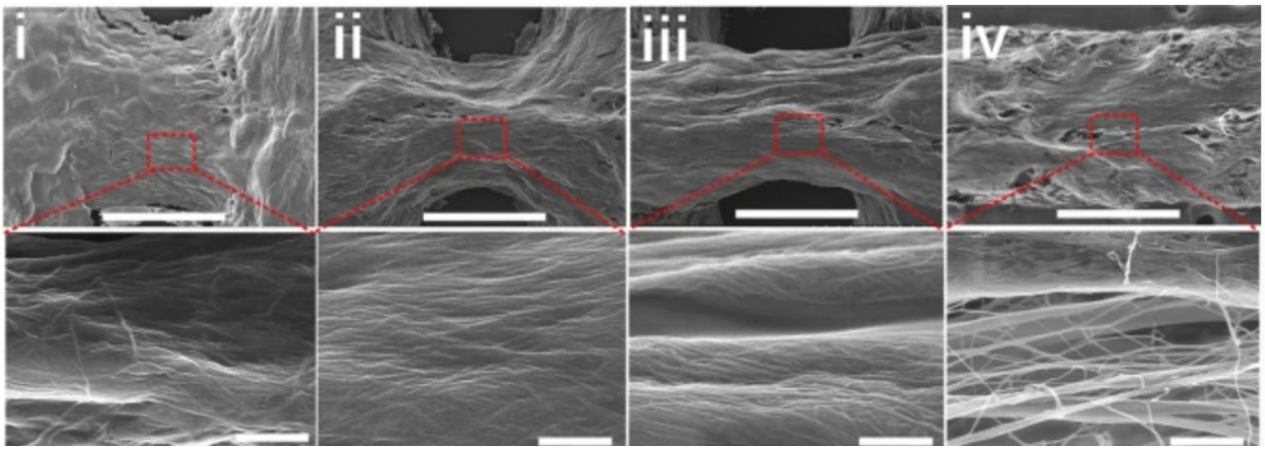


Figure 11. Morphology of printed silk lines after freeze-drying
The printed lines were smooth and uniform with higher concentrations of oxidized bacterial cellulose (OBC), which contributes to the retention of the shape. These nanofibrils were bonded together through Silk fibroin (SF) hydrogel (i-iii). Without the SF hydrogel, the OBC fibers were incompact (iv) (Huang *et al.,* 2020).

Artificial Spinning

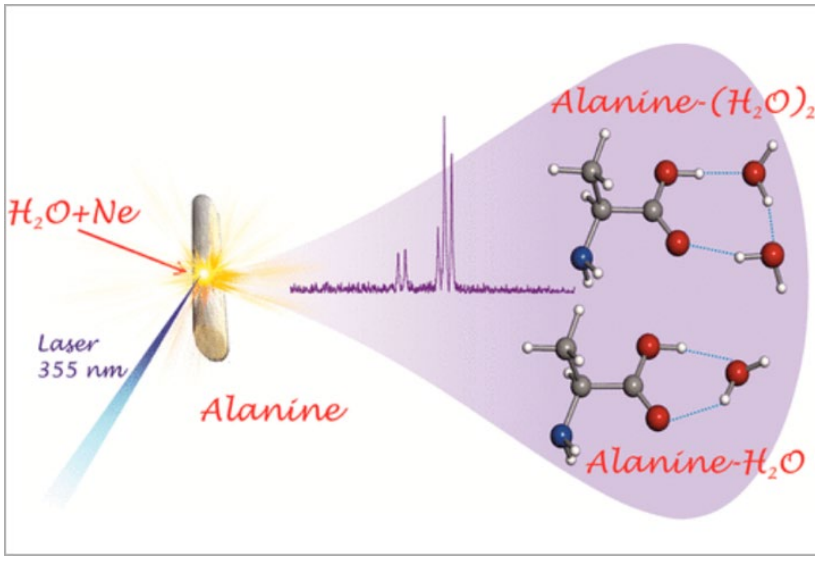
Hybrid materials built upon spider silk protein can be used to create stronger, tougher, and more elastic components for future studies (Kiseleva *et al.*, 2021). Artificial spinning allows

for cheaper and more efficient production of the spider protein without the limitations in natural farming of spiders. Due to their predatory and solitary nature, spiders are cannibalistic and have caused limitations in production that are highly cost- ineffective.

The use of spider silk as a fiber coating is a relatively new idea. In previous works, researchers Kiseleva and others have studied the deposition of zinc (Zn), titanium (Ti), and aluminum (Al) to increase the toughness in the spider silk strand (Kiseleva et al., 2020). Hydrogen bonding to other inorganic molecules can result in stronger bonds and tougher breakage. Due to the metals already encased and bonded to the spider silk proteins, these materials can be coated over different synthetic materials such as Nylon or Kevlar.

Certain hydrophilic processes with attaching a synthetic material to a hybrid material need to take notice. The poly-(Alanine) tail in spider silk consists of the amino acid alanine and can undergo hydrogen bonding to form a six-membered cycle (H_2O) or an eight-membered cycle (H_2O_2) shown in Figure 12 (Vaquero *et al.,* 2014).

Figure 12: Alanine and Water Complexes



Water molecules binding to carboxylic acid groups of alanine acting as both proton donors and acceptors. Alanine-H_2O are held together via hydrogen bonds and form a six-membered structure (bottom right). Alanine-$(H_2O)_2$ create three intermolecular hydrogen bonds and form a larger eight-membered ring (top right) (Vaquero *et al*., 2014).

Considering Kevlar is a polyaramid molecule that forms hydrogen bonds in a trans-formation (linear), the strength is unparalleled. These hydrogen bonds can potentially attach to the alanine amino acids found in the poly-A tail, shown in Figure 13, in spider silk proteins to act as a tougher material coating for better elasticity and strength.

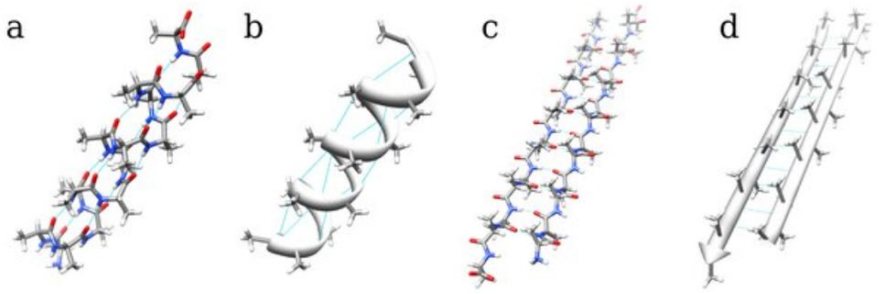


Figure 13. Secondary Structure Types

(a) α-helix in full atom representation. Side chains of alanine. (B) anti-parallel β-sheet in full-atom (c) and cartoon (d) representation. Protruding sticks are the poly-(Alanine) tails. Hydrogen atoms shown in white. Hydrogen bonds in blue. Direction from N to C-terminal are represented with arrowheads (Sikosek, 2012).

Carbon Nanomaterials

Carbon nanomaterials are tough but lightweight and flexible (Kiseleva et al., 2020). They are typically composed in nanotubes that can be accomplished through water-based and dry-coating methods. These carbon nanotubes have molecules that bind together through van der Waals forces and can develop ultra-high strength with low weight (Berger 2021). Incorporation with this material, spider silk proteins can be synthesized with the nanotubes through reinforcements in the molecular β-sheets of the protein. Different formations in the β-sheets of the protein contribute to different areas of strength and flexibility. In Kevlar, the trans formation of the polyaramids provides the most strength because it is linearly formed and the

fully extended chains pack more flawlessly into the crystalline form that makes up the fiber ("Aramids", 2022).

Biomedical research

Spider silk has been used since the ancient times in Greece and Rome to stop the bleeding of battle wounds as a coverage or poultice. It was also used as an astringent, styptic and a febrifuge. In modern times, further uses for spider silk have proven useful (Salehi *et al.,* 2020). Advances in technology have allowed for the synthesis of bone and cartilage regeneration, skin and wound regeneration, and also heart muscle and nerve fiber regeneration. Different mechanisms can be used to accomplish these newer endeavors such as the use in hydrogels, nonwoven meshes, and films/coatings (Salehi *et al.,* 2020). The proposed MaSp-Kevlar sequence can be further synthesized through electrospinning and perform 2D meshes that contain various fiber orientations. Fibrous non-woven meshes are known to support adhesion in fibers of range 700 nm (Salehi *et al.,* 2020). Hydrogels are used typically in 3D printing and create molecular networks with water content above 95%. Spider silk proteins β-sheet nanofibrils self-assemble through mechanism of nucleation-aggregation and compress to hydrogel formations. Hydrogels have also been combined with living cells to generate hierarchical tissue-like structures (Salehi *et al.,* 2020).

Bone is composed of both inorganic and organic materials with collagen as an organic substrate. What collagen lacks in mechanical stability, spider silk thrives in. Using the proposed MaSp-Kevlar protein and bone sialoprotein fusion, cell attachment can occur to form adhesions of human mesenchymal stem cells (Salehi et al., 2020). The addition of the silica domain in induced biosilicification to the C-terminal end showed higher precipitation and promoted

differentiation of bone marrow. Fusing with bone cells and cartilage are not the only capable biosynthesis tried for spider silk. Cardiomyocytes have also shown good interaction in contact with charged spider silks. Selectivity occurs in cell-binding due to cellular differences (Salehi *et al*., 2020). Spider silk can also be used for wound dressings. Salehi *et al*. (2020) presented that the wound-healing rate on second-degree burns in rats was increasingly higher than those of just collagen cells. Spider silk can increase the initiation of vascularization and re-epithelialization at the site of injury. Comparably, peripheral nerve regeneration can also be affected by spider silk implication. Implantation of biodegradable and biocompatible scaffolds as nerve guidance conduits can increase regeneration after neural injuries (Salehi *et al*., 2020). Since spider silk is made of proteins, it is highly biodegradable and can be used for decellularized vein grafts where cellular adhesion and migration of cell bodies can extend neurite forming ganglion-like structures (Salehi et al., 2020).

Chapter 6: Conclusion

The goal of this thesis was to determine how protective materials could be made biologically to increase availability and decrease cost. This research application is designed to synthesize and purify a novel Kevlar-like protein (MaSp-Kevlar) that can be mass produced and manipulated into fabrics. To accomplish this, background research into existing biological materials was determined to be an impressive material that fit the criteria for flexibility, durability, and adaptability. Using a heat shock promoter driven over-expression system that includes the MaSp-Kevlar sequence in insect (Sf9) cells, one could generate and purify spider-silk Kevlar-like proteins in a reliable and cost-efficient manner.

Artificial spinning is also a newer generation of protein synthesis in comparison to natural spinning. With comparisons to better synthesis than using other host systems, artificial spinning is a more reliable and cost-effective tool. Future agendas in the aspect for biomaterials relays the components of potential fabric coating and nanomaterials. Further analysis can be done to determine if fabric coating on Kevlar can be accomplished.

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