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Final Report: Developing Aciniform Spider Silk Biomaterials with Unique Structural Transitions and Properties

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a. REPORT	b. ABSTRACT	c. THIS PAGE			Gregory Holland
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RPPR Final Report
as of 20-Sep-2023

Agency Code: 21XD

Proposal Number: 75486BBH **Agreement Number: W911NF-20-1-0143**
INVESTIGATOR(S):

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Title: Developing Aciniform Spider Silk Biomaterials with Unique Structural Transitions and Properties
Begin Performance Period: 15-Jun-2020 **End Performance Period:** 14-Jun-2023
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Submitted By: Gregory Holland Email: gholland@sdsu.edu
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STEM Degrees: **STEM Participants:**

Major Goals: The major goals of the project are listed below (taken directly from the proposal):

(1) Characterize the mechanical properties of AC silk fibers (single fibers and fiber bundles) from various spider species. Measure mechanical properties of AC silk bundles in their native states and water-wetted (water-induced crosslinked) states. Correlate structural changes to any observed changes in mechanical properties.

(2) Apply and develop modern multinuclear, multidimensional MAS and dynamic nuclear polarization (DNP)-MAS SSNMR techniques to structurally characterize isotope-labeled AC spider silk fibers. Quantitatively correlate the secondary structures determined for the silks with the silk protein's primary amino acid sequence. Use SSNMR to investigate the silks when in contact with water to elucidate the origin of AC silk's unique hydration-induced alpha-helical coiled-coil to beta-sheet structural transition. This information will be used to develop models for the AC silk' s molecular architecture and to design new biomaterials based on AC silk. We already have published results related to this goal that provides the background for the proposed work.

(3) Perform XRD measurements at Argonne National Lab (ANL) on AC silk fibers and bundles to characterize molecular and hierarchical nanocrystalline structures formed in these materials. XRD will focus on pair-distribution functional (PDF) analysis for characterizing amorphous structures and distributions as well as a combination of wide and small angle x-ray scattering (WAXS and SAXS) for characterizing structures in the 1-100 nanometer length scale. XRD will inform on the degree of crystallinity and the organization of the beta-sheet / alpha-helical coiled-coil architecture, as well as its sensitivity to chemical and physical stimuli such as hydration induced cross-linking.

(4) Use 1H, 13C, and 15N two-dimensional (2D) and three-dimensional (3D) protein solution NMR to probe the AC silk protein conformational structure and dynamics in the gland fluid prior to fiber formation. Pulse field gradient (PFG) Diffusion NMR will be applied to measure silk protein diffusion to determine the extent of silk protein entanglement and oligomerization under native gland conditions and as a function of protein concentration.

(5) Conduct cryo-TEM experiments in collaboration with UCSD/NW to image AC silk proteins in the gland fluid and determine the extent of oligomerization. Use cryo-TEM methods to determine the size, shape/morphology and overall dimensionality of silk protein oligomers. Expose silk gland fluid to different biochemical triggers (pH and salts) to determine the processing conditions required for silk assembly.

(6) Molecular dynamics (MD) simulations will be used to generate structural models and inform interpretation of

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NMR, XRD and cryo-TEM biophysical characterization.

Accomplishments: A brief summary accomplishments for each goal is listed below:

(1) During the grant we conducted extensive mechanical testing of single spider silk fibers with a nano-mechanical fiber testing system (MTS) in our lab. We have done this for aciniform (AC), major ampullate (MA) and tubuliform (TU) egg case silks collected from different individuals for A. argentata spiders and other species and fiber types. For the A. argentata species, the results illustrate that while, MA is the strongest of the spider silks, AC silk is by far the toughest due to its significant extensibility (60%). It is also interesting to note that we have begun investigating TU silk mechanical properties that show it is significantly weaker and less tough than the other spider silks. We have compared MA mechanical data for two different Argiope species, another orb weaver (N. clavipes) and a cob weaver (L. hesperus). The results show that for MA silks from the three different species there are some notable variabilities particularly for the extensibility between species but, roughly speaking MA fibers from different spider species exhibit similar mechanical properties within error. We have also begun looking at the impact of water (fiber wetting) on spider silk mechanical properties. For MA silk, the fiber undergoes a supercontraction process where it swells in diameter and shrinks in length when wetted with water. This process results in a decrease in fiber strength and considerable increase in extensibility retaining the overall fiber toughness. We are particularly interested in AC silk's unique interaction with water where the fiber cross-links into a matted beta-sheet structure following a wet/dry treatment (see publication 2). We have begun to collect mechanical data while the AC silk fiber is wet and our preliminary results are shown in the final report. The wet AC silk fiber shows a significant increase in extensibility when wetted similar to MA silk but, without supercontraction. We are in the process of analyzing this data and reproducing this for other AC silk samples. We also have enough mechanical testing data for publication and have begun drafting a paper on this topic.

(2) We have made considerable headway in understanding the molecular architecture of AC silk fibers with magic angle spinning (MAS) SSNMR together with Alpha-Fold and molecular dynamics (MD) modeling approaches discussed in the final report. We have continued to isotope label AC silk and collect 2D SSNMR data. Throughout the grant, we produced AC silk from A. argentata spiders that is isotope enriched with 13C/15N-Pro, Leu, Ala and Val and a number of single isotope labeled silks including 13C/15N-Leu, 13C/15N-Thr, 13C/15N-Phe and collected a number of 2D MAS SSNMR spectra and extracted chemical shift data to characterize the secondary structure of the different amino acid residues. We are in the process of analyzing this data and drafting a manuscript. We have been able to completely assign in tabulate all 13C chemical shifts for the various labeled amino acids. Our initial results for Pro illustrate that it takes on an elastin-like type-II beta-turn conformation in AC silk fibers. This interpretation is similar to the Pro-containing region of the MaSp2 dragline spider silk protein and helps to explain the significant extensibility of AC silk fibers. While, the extensibility of AC silk is likely due to the predominant coiled-coil alpha-helical structure, contributions to extensibility from Pro-containing beta-turn regions are very likely.

(3) We have successfully collected preliminary fiber XRD at ANL and have analyzed the data. Initial interpretation is consistent with the as-spun AC fibers having a coiled-coil alpha-helical/beta-sheet superstructure. The data suggests the presence of both nanocrystalline beta-sheets and fiber-aligned alpha-helical structures from their weak reflections at 4.6 and 9 Å, respectively. A strong reflection in the ~30 Å range near the beamstop is suggestive of a coiled-coil higher-order architecture, but SAXS is required to further investigate this interpretation. During year 2 COVID prevented visits to ANL thus, additional data could not be collected. The ANL beam line shut down in year 3, unfortunately. We have access to new XRD capabilities at ASU are now being initiated for future work.

(4) We have made significant progress throughout the grant period on dissecting intact, unperturbed AC glands from A. aurantia spiders for solution NMR experiments. We have successfully isotope enriched the AC glands and collected 1D and 2D 1H/13C and 1H/15N HSQC NMR spectra. Four different samples have been prepared and data collected including for 13C/15N-Thr, 13C/15N-Leu, 13C/15N-Val and 13C/15N-Ala labeled AC silk proteins within glands. The 1H/13C HSQC for Leu labeled glands display a number of amino acid residue sites including Ala, Val, Thr, Leu, Gln and Der. Most notable is the methyl region of the spectrum (low ppm) where multiple structural environments are observed for Ala, Val and Leu. There are also at least three distinct structural environments observed for Ser in the alpha region of the spectrum. Our current hypothesis is that the AC silk protein will exist in the gland environment as a combination of alpha-helical (bead) and random coil (linker string) with no evidence of beta-sheet structure. This data is currently in the process of being analyzed and initially appears to be in line with our hypothesis. Understanding the state of the AC silk protein within in the gland (prior to fiber formation) is a critical step in understanding the spinning process.

(5) We have successfully collected NS-TEM and cryo-TEM on MA silk gland dope. This is a huge accomplishment, and we are comparing these structures to NMR data. We are in the process of collecting similar data on AC silk proteins but, so far have been unable to obtain high quality data. We plan to continue these measurements in future work and have a number of ideas regarding how we can improve the data.

(6) During the grant period we began using AlphaFold for AC silk protein structure prediction together with more traditional molecular dynamics (MD) simulations in GROMACS with coarse grain MARTINI force fields and atomistic force fields on shorter sequences. AlphaFold is an artificial intelligence (AI) program developed by DeepMind that can predict a given protein's 3D structure from only the primary amino acid sequence as an input. AlphaFold is very new only becoming available to the public in 2021 (Nature, 2021, 596, 583). Our initial predicted AC silk protein structures form AlphaFold are shown in the final report. The results are impressive and provide protein structure hypotheses to test experimentally via SSNMR for AC silk fibers and solution NMR for the protein structure within the gland. The predicted structure is consistent with the "beads-on-a-string" model where the bead is an alpha-helical bundle and the linker is a disordered random coil domain. The structures shown in the report are for the first ~1000 amino acids in the protein sequence for A. argentata AcSp1 that includes five bead domains connected by the linker regions. What is particularly important about having these structural models is that we can start to validate them with NMR both in solution (the gland state) and in final fibers. In addition, the predicted structures are good starting points for more refined coarse grained (CG) and atomistic MD simulations that we also pursued. It is shown in the final report that AlphaFold in combination with MD and NMR experiments provides a robust approach to determining the structure of AC silk proteins in solution and as solidified fibers. We are combining these simulations with simulation data for a publication and anticipate that this will be a powerful approach moving forward in our future work on AC silk structural biology and biomimetics.

Training Opportunities: Two PhD students were supported by the grant. PhD student, Dr. Dillan Stengel, successfully graduated in December 2021 with his PhD and worked on the project in year 1. A second PhD student, Hannah Johnson was partially supported in year 2 and 3 and is scheduled to graduate with her PhD in May 2023. One post-doc, Dr. Kevin Chalek, was partially supported on the grant and worked on the project in. years 2 and 3. All three individuals were extensively trained throughout the grant including solution and SSNMR, XRD, EM, MD and other simulation and data analysis methods as discussed in the final report.

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Results Dissemination: Talks:

1) Dillan Stengel, J. Bennett Addison, David Onofrei, and Gregory P. Holland "Water-induced ?-sheet Crosslinking of alpha-helical Spider Prey-wrapping Silk" ACS National Meeting, Spring 2021 Won Best Poster from the Division of Polymeric Materials: Science and Engineering

2) David Onofrei, Dillan Stengel, Hannah Johnson, Brittany Puzio and Gregory P. Holland "Spider Silks as Model Systems for the Design of Functional Protein-based Materials and Composites" AAAFM-UCLA International Conference, Summer 2021

3) Hannah R. Johnson, Katherine Adams, Christofer Layana, Salvador Vallejo, Gregory P. Holland "Informing Tunable Bio-composite Design with Fiber Formation in Spiders and Silkworms" AAAFM-UCLA International Conference, Summer 2021

4) Bennett Addison, Dillan Stengel, Gregory P. Holland, Ware “Selective 1D [13C-13C] Spin Diffusion Solid-state NMR to Probe Spatial Arrangements in Biomolecules” Experimental NMR Conference, Pacific Grove, CA (2021). [Held virtually due to the pandemic]

5) Gregory P. Holland “Developing Aciniform Spider Silk Biomaterials with Unique Structural Transitions and Properties” Seminar to the Army Research Labs (2021). [Invited, held virtually due to the pandemic]

6) Gregory P. Holland “Hierarchical Assembly of Spider Silk Proteins” Frontiers in Soft Matter and Macromolecular Networks, University of San Diego, San Diego, CA (2022). [Invited]

7) Gregory P. Holland “A Multimodal Biophysical Approach to Understand Biomaterials Formation” Biomaterials Seminar - University of Bayreuth, Germany (2022). [Invited]

8) Kevin Chalek, Dillan Stengel, Bennett Addison, Gregory P. Holland “Probing Cation-Pi Interactions in Spider Silk Fibers with Selective DARR Difference MAS SSNMR” Rocky Mountain Conference on Magnetic Resonance, Copper Mountain, CO (2022).

9) Kevin Chalek, Dillan Stengel, Bennett Addison, Gregory P. Holland “SSNMR Characterization of Aciniform Spider Silk Conformational Structure” Frontiers in Soft Matter and Macromolecular Networks, University of San Diego, San Diego, CA (2022).

10) Gregory P. Holland "Elucidating Spider Silk Structure and Assembly with NMR" North Jersey ACS NMR Topical Group, Held Virtually with attendees from around the world in the NMR community (2023). [Invited]

11) Julian E. Aldana, Kevin Chalek, David Onofrei and Gregory P. Holland "Predicting the Three-dimensional Structure of Spider Silk Proteins with AlphaFold and Molecular Dynamics Simulations" CSU Annual Biotechnology Symposium,

12) Gregory P. Holland "Combining Biophysical Methods to Understand Spider Silk Formation" Gordon Research Conference: Silk Proteins and the Transition to Biotechnologies, Bryant University, Smithfield, RI (2023). [Invited]

13) Kevin Chalek, Julian E. Aldana, Christian D. Lorenz, Gregory P. Holland "Combining Solid-state NMR and Molecular Dynamics Simulation to Characterize the Structure of Spider Silk Fibers" Gordon Research Conference: Silk Proteins and the Transition to Biotechnologies, Bryant University, Smithfield, RI (2023).

14) Gregory P. Holland "Combining Solution NMR and Modeling to Determine Structural Ensembles of Spider Silk Proteins" ACS Fall 2023 National Meeting, San Francisco, CA (2023). [Invited]

Papers:

1) Addison, J.B., Stengel, D., Bharadwaj, V.S., Happs, R.M., Doeppke, C., Wang, T., Bomble, Y.J., Holland, G.P., Harman-Ware, A.E. “Selective 1D 13C-13C Spin-Diffusion Solid-State NMR Methods to Probe Spatial Arrangements in Biopolymers Including Plant Cell Walls, Peptides and Spider Silk” J. Phys. Chem. B. 2020, 124, 9870-9883.

2) Stengel, D., Addison, J.B., Onofrei, D., Holland,G.P. “Water-induced beta-sheet Crosslinking of alpha-helical-rich Spider Prey-wrapping Silk” Adv. Funct. Mater. 2021, 31, 2170090. Made Cover Art

3) Chalek, K., Stengel, D., Addison, J.B, Holland, G.P. "Conformational Structure of Aciniform Spider Silk Determined by Solid-state NMR and Modeling" In Preparation (2023).

4) Onofrei, D., Botello, F., Holland, G.P. "Correlating the Mechanical Properties of Various Spider Silk Fibers with their Secondary Structures" In preparation (2023).

5) Johnson, H.R. and Holland, G.P. "Solution NMR of Native Acinform Spidroins Reveals Multi-domain Silk Protein Structure" In preparation (2023).

Honors and Awards: 1) Chalek, K. SDSU, 2nd Place Poster Award, Frontiers in Soft Matter and Macromolecular Networks, University of San Diego, San Diego, CA (2022)

2) Chalek, K.SDSU, Travel Award, Rocky Mountain Conference on Magnetic Resonance, Copper Mountain, CO (2022)

3) Holland, G.P. SDSU, College of Sciences Exceptional Service Award (2021)

4) Johnson, H.R. SDSU, University Graduate Fellowship (2021)

5) Stengel, D. PMSE Best Poster Award, Spring 2021 Virtual ACS National Meeting (2021)

Protocol Activity Status:

Technology Transfer: Patents:

WO2022150163A2-2022-07-14 titled "BIOMATERIALS AND BIOTEXTILES AND METHODS FOR MAKING SAME" published July 14, 2022; PCT Application

PARTICIPANTS:

Participant Type: PD/PI
Participant: Gregory P Holland
Person Months Worked: 1.00 **Funding Support:**
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National Academy Member: N

Participant Type: Postdoctoral (scholar, fellow or other postdoctoral position)
Participant: Kevin Chalek
Person Months Worked: 6.00 **Funding Support:**
Project Contribution:
National Academy Member: N

Participant Type: Staff Scientist (doctoral level)
Participant: David Onofrei
Person Months Worked: 1.00 **Funding Support:**
Project Contribution:
National Academy Member: N

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Participant Type: Graduate Student (research assistant)
Participant: Dillan Stengel
Person Months Worked: 12.00 **Funding Support:**
Project Contribution:
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Participant Type: Graduate Student (research assistant)
Participant: Hannah R. Johnson
Person Months Worked: 6.00 **Funding Support:**
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Article Title: Hydration-induced beta-sheet Crosslinking of alpha-helical-rich Spider Prey-wrapping Silk
Authors: Dillan Stengal, J. Bennett Addison, David Onofrei, Nha Uyen Huynh, George Youssef, Gregory P Hollar
Keywords: Spider Silk, Aciniform, NMR, Crosslinking, Coiled-coil
Abstract: Due to its moderate strength (~700 MPa) and impressive extensibility before breaking (~60-80%), orb-weaving spider aciniform (AC) prey-wrapping silks are actually the toughest of the spider silks but are remarkably understudied. Our previous results indicate that native AC silk fibers are an ?-helix rich coiled-coil / ?-sheet hybrid nanofiber, and that conversion of disordered or helical domains to ?-sheet aggregates is surprisingly minimal and overall ?-sheet content is low (~15%). In this work, we demonstrate through Scanning Electron Microscopy (SEM) that native AC silk fibers undergo matted cross-linking upon exposure to moisture that increases silk stiffness. The unique molecular mechanism of water-induced cross-linking is revealed with solid-state NMR (SSNMR) methods; water-induced morphological changes are correlated with an increase in AC silk protein ?-sheet content, and additionally we observe a minor unfolding of coiled-coil regions.
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Authors: Bennett Addison, Dillan Stengel, Vivek S. Bharadwaj, Renee M. Happs, Crissa Doeppke, Tuo Wang, Ya
Keywords: Fibers, Biomimetic Materials, Magnetic Properties, Biopolymers
Abstract: Two-dimensional (2D) and 3D through-space 13C–13C homonuclear spin-diffusion techniques are powerful solid-state nuclear magnetic resonance (NMR) tools for extracting structural information from 13C-enriched biomolecules, but necessarily long acquisition times restrict their applications. In this work, we explore the broad utility and underutilized power of a chemical shift-selective one-dimensional (1D) version of a 2D 13C–13C spin-diffusion solid-state NMR technique. The method, which is called 1D dipolar-assisted rotational resonance (DARR) difference, is applied to a variety of biomaterials including lignocellulosic plant cell walls, microcrystalline peptide fMLF, and black widow dragline spider silk. 1D 13C–13C spin-diffusion methods described here apply in select cases in which the 1D 13C solid-state NMR spectrum displays chemical shift-resolved moieties. This is analogous to the selective 1D nuclear Overhauser effect spectroscopy (NOESY) experiment utilized in liquid-state NMR.
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Article Title: Selective One-Dimensional ¹³C–¹³C Spin-Diffusion Solid-State Nuclear Magnetic Resonance Methods to Probe Spatial Arrangements in Biopolymers Including Plant Cell Walls, Peptides, and Spider Silk
Authors: Bennett Addison, Dillan Stengel, Vivek S. Bharadwaj, Renee M. Happs, Crissa Doeppke, Tuo Wang, Ya
Keywords: Materials Chemistry
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Publication Identifier Type: DOI Publication Identifier: 10.1002/adfm.202007161
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Date Submitted: Date Published: 1/4/21 12:00AM
Publication Location:
Article Title: Hydration?Induced ??Sheet Crosslinking of ??Helical?Rich Spider Prey?Wrapping Silk
Authors: Dillan Stengel, J. Bennett Addison, David Onofrei, Nha Uyen Huynh, George Youssef, Gregory P. Hollar
Keywords: Electrochemistry
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PATENTS:

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Patent Abstract:
Patent Number: 63.125,612
Patent Country: USA
Application Date: 16-Dec-2020 Application Status: 1
Date Issued: 15-Dec-2021

Partners

,

I certify that the information in the report is complete and accurate:
Signature: Gregory Holland
Signature Date: 9/14/23 4:26PM

Title: Developing Aciniform Spider Silk Biomaterials with Unique Structural Transitions and Properties

PI: *Gregory P. Holland, Department of Chemistry and Biochemistry, San Diego State University*

Grant: ARO BAA W911NF-17-S-002-03; **Final Report**

Major Goals

Statement of Objectives (Directly from Proposal)

AC silk has a number of novel properties that make it an extremely promising biomaterial including a toughness 50% greater than spider dragline silk and a novel fiber cross-linking property upon hydration/dehydration that we have discovered in our laboratory and plan to exploit for the design of a new class of biomaterials. Further, we believe that a better understanding of observed water-induced and strain-induced structural transformations should be of significant interest to polymer engineers and materials chemists; using water as a solvent to convert malleable, flexible and tough protein-based materials into rigid cross-linked sheets may prove instrumental to developing biomaterials tailored for ARO applications including protective clothing and other advanced textile needs and repairs (parachutes, tents, etc). Regardless of recent progress, significant questions remain unanswered regarding AC silks unique mechanical properties, molecular structure, higher-order architecture and assembly processes. Some questions include: does the globular/disordered "beads-on-a-string" model (**Figure 1**), proposed for recombinant AC

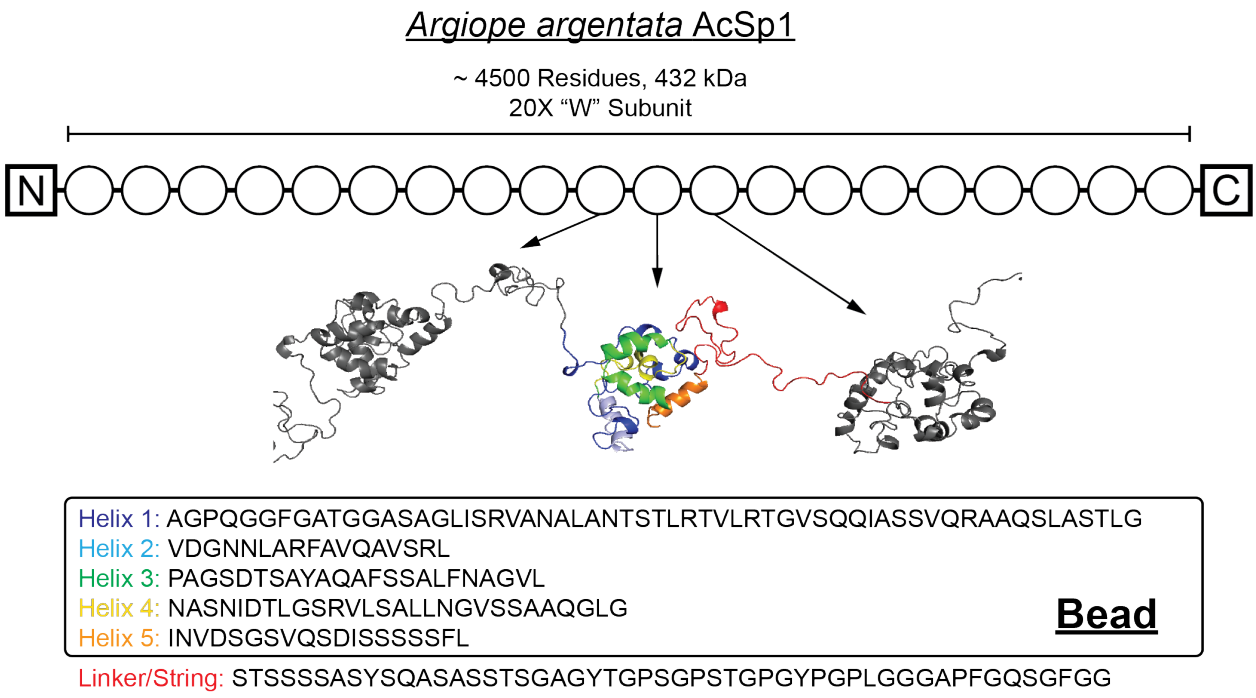

Figure 1. Schematic of *Argiope* AcSp1 prey wrapping silk. In the *A. argentata* species, there are 20 iterated repeats of the wrapping ("W") subunit. The solution NMR structure from a single *A. trifaciata* W subunit (PDB 2MU3) was used to create the theorized "beads-on-a-string" model hypothesized to occur in solution and colored to identify each area. The colored sequences are for *A. argentata* AcSp1.

protein, hold true for the native spinning dope? What differences exist between recombinant and native silk dopes? What is the higher-order spidroin assembly process for native AC silk protein when stored in the gland? Are there truly micellular-like superstructures in the native material when stored in the gland? What roles do shear, extension and biochemical changes play in conversion of soluble protein in the gland to insoluble fiber? Which regions of the protein participate in initial β-sheet aggregation upon fibrilization? How are α-helical coiled-coil superstructures organized within the fiber? What are the sizes, orientation, and percent total fiber composition of disordered, α-helical, and β-sheet nanostructures in the native wrapping silk? Can we understand detailed atomic-level structural changes that are induced upon water-wetting and mechanical stresses? Which regions of the protein are affected by water, and which regions are water-inaccessible? What are the changes to material mechanical properties upon water-induced crosslinking?

Developing new biomaterials based on AC silk relies on A) understanding the processing required to produce the silk fibers (the spinning process), B) a complete understanding of the silk protein structure on the molecular level, and C) better correlating the molecular protein structure to observed mechanical properties (tensile strength, extensibility and toughness). Therefore, this proposal aims to characterize the unique mechanical properties, molecular architectures and assembly process for AC spider silks. Through these studies we will provide a better understanding of the structure-function relationship between AC silk molecular protein architecture and their outstanding mechanical properties. Primary physical characterization methods will include mechanical testing, Nuclear Magnetic Resonance (NMR) spectroscopy (solution and solid-state), cryo- transmission electron microscopy (TEM) and synchrotron X-ray diffraction (XRD) in collaboration with Argonne National Labs (ANL). Additionally, Molecular Dynamics (MD) methods will be used to help interpret spectroscopic results and generate molecular-level models. This work embodies the Biomolecular Assembly and Organization thrust of the ARO Biochemistry Program where the focus is on: "understanding the molecular interactions and design rules that govern self-assembly of biomolecules into both naturally occurring biomolecular structures and designed architectures (ARO BAA W911NF-17-S-002-03)". *We aim to apply a combination of techniques to interrogate AC native fibers and gland fluid including solution, solid-state and diffusion-based NMR spectroscopies together with XRD at ANL and cryo-TEM at University of California San Diego (UCSD) and Northwestern University (NW). Our primary aim is to use the information gained as guidance for the design of a new class of biomaterials based on AC spider silk.* A list of specific goals that Prof. Holland plans to achieve in the proposed research is given below:

- Characterize the mechanical properties of AC silk fibers (single fibers and fiber bundles) from various spider species. Measure mechanical properties of AC silk bundles in their native states and water-wetted (water-induced crosslinked) states. Correlate structural changes to any observed changes in mechanical properties.
- Apply and develop modern multinuclear, multidimensional MAS and dynamic nuclear polarization (DNP)-MAS SSNMR techniques to structurally characterize isotope-labeled AC spider silk fibers. Quantitatively correlate the secondary structures determined for the silks with the silk protein's primary amino acid sequence. Use SSNMR to investigate the silks when in contact with water to elucidate the origin of AC silk's unique hydration-induced α-helical coiled-coil to β-sheet structural transition. This information will be used to develop models for the AC silk's molecular architecture and to design new biomaterials

based on AC silk. We already have published results related to this goal that provides the background for the proposed work.

- Perform XRD measurements at Argonne National Lab (ANL) on AC silk fibers and bundles to characterize molecular and hierarchical nanocrystalline structures formed in these materials. XRD will focus on pair-distribution functional (PDF) analysis for characterizing amorphous structures and distributions as well as a combination of wide and small angle x-ray scattering (WAXS and SAXS) for characterizing structures in the 1-100 nanometer length scale. XRD will inform on the degree of crystallinity and the organization of the β-sheet / α-helical coiled-coil architecture, as well as its sensitivity to chemical and physical stimuli such as hydration induced cross-linking.
- Use ^{1}H, ^{13}C, and ^{15}N two-dimensional (2D) and three-dimensional (3D) protein solution NMR to probe the AC silk protein conformational structure and dynamics in the gland fluid prior to fiber formation. Pulse field gradient (PFG) Diffusion NMR will be applied to measure silk protein diffusion to determine the extent of silk protein entanglement and oligomerization under native gland conditions and as a function of protein concentration.
- Conduct cryo-TEM experiments in collaboration with UCSD/NW to image AC silk proteins in the gland fluid and determine the extent of oligomerization. Use cryo-TEM methods to determine the size, shape/morphology and overall dimensionality of silk protein oligomers. Expose silk gland fluid to different biochemical triggers (pH and salts) to determine the processing conditions required for silk assembly.
- Molecular dynamics (MD) simulations will be used to generate structural models and inform interpretation of NMR, XRD and cryo-TEM biophysical characterization.

Accomplishments Under Goals (Year 1)

(1) Characterize the mechanical properties of AC silk fibers (single fibers and fiber bundles) from various spider species. Measure mechanical properties of AC silk bundles in their native states and water-wetted (water-induced crosslinked) states. Correlate structural changes to any observed changes in mechanical properties.

Accomplished: We have made a number of mechanical tensile measurements on single fibers and bundles of aciniform threads that show it is definitely the toughest of the spider silks. We have done this for aciniform silk collected from different individuals and for two different species of Argiope spiders. Water-induced cross-linked silks have proven difficult to mechanical test with a fiber tensile tester. However, through a new collaboration with George Youssef's Mechanics Lab in Mechanical Engineering we have been using AFM nano-indentation to determine the mechanical properties of water cross-linked aciniform mats. Some of this data is included in our recently published *Advanced Functional Materials* paper (**Table 1**).

Native Aciniform (as-spun)		***Aciniform wetted (1-day dry)***		***Aciniform wetted (2-day dry)***	
Material Stiffness (GPa)	***Std Dev (GPa)***	***Material Stiffness (GPa)***	***Std Dev (GPa)***	***Material Stiffness (GPa)***	***Std Dev (GPa)***
4.40	0.28	4.76	0.15	4.11	0.05

Table 1. Nano-indentation mechanical properties of native and water treated AC spider silks.

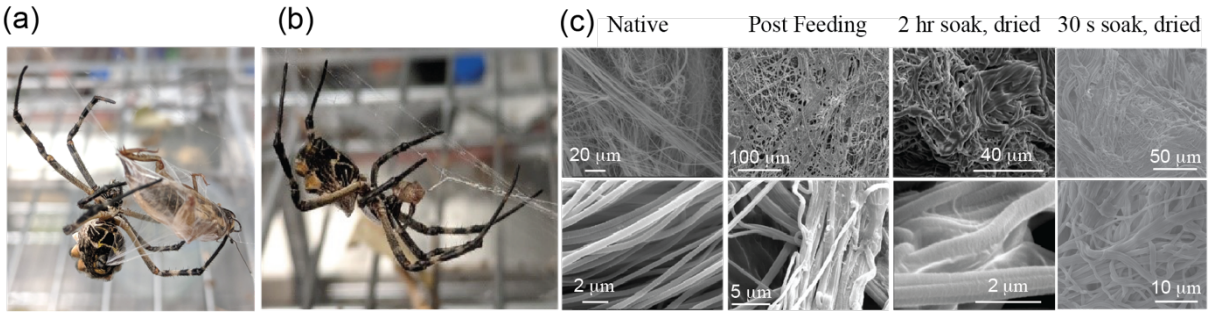

Figure 2. a), *A. argentata* spinning a cricket with AC silk. b), the same spider returning to eat its prey through EOD. c), SEM images comparing native prey-wrapping AC silk from *A. argentata* following feeding or water treatment where the silk was soaked for 2 hr or 30 s and then dried for one day.

(2) Apply and develop modern multinuclear, multidimensional MAS and dynamic nuclear polarization (DNP)-MAS SSNMR techniques to structurally characterize isotope-labeled AC spider silk fibers. Quantitatively correlate the secondary structures determined for the silks with the silk protein's primary amino acid sequence. Use SSNMR to investigate the silks when in contact with water to elucidate the origin of AC silk's unique hydration-induced alpha-helical coiled-coil to beta-sheet structural transition. This information will be used to develop models for the AC silk's molecular architecture and to design new biomaterials based on AC silk. We already have published results related to this goal that provides the background for the proposed work.

Accomplished: We have made considerable headway in understanding the molecular mechanism of the hydration-induced structural transition from α-helical coiled-coil to β-sheet matted cross-linked material. We demonstrated through Scanning Electron Microscopy (SEM) that native AC silk fibers undergo matted cross-linking upon exposure to moisture that increases silk stiffness (**Figure 2**). The unique molecular mechanism of water-induced cross-linking was revealed with solid-state NMR (SSNMR) methods; water-induced morphological changes are correlated with an increase in AC silk protein β-sheet content, and additionally we observe a minor unfolding of coiled-coil regions (**Figure 3 and 4**). Continued and increased β -sheet cross-linking is observed upon application of mechanical shear. We determine the size of these β-sheet domains to be 4-6 nm using WISE SSNMR (**Figure 5**). The observation that merely water treatment and shearing can be used to convert a protein-based material from a flexible/extensible α-helix-rich fiber to a rigid crossed-linked β-sheet mat is a novel observation that should provide new avenues in bioinspired materials design. This work is detailed in our recent article published in Adv. Funct. Mater.

(3) Perform XRD measurements at Argonne National Lab (ANL) on AC silk fibers and bundles to characterize molecular and hierarchical nanocrystalline structures formed in these materials. XRD will focus on pair-distribution functional (PDF) analysis for characterizing amorphous structures and distributions as well as a combination of wide and small angle x-ray scattering

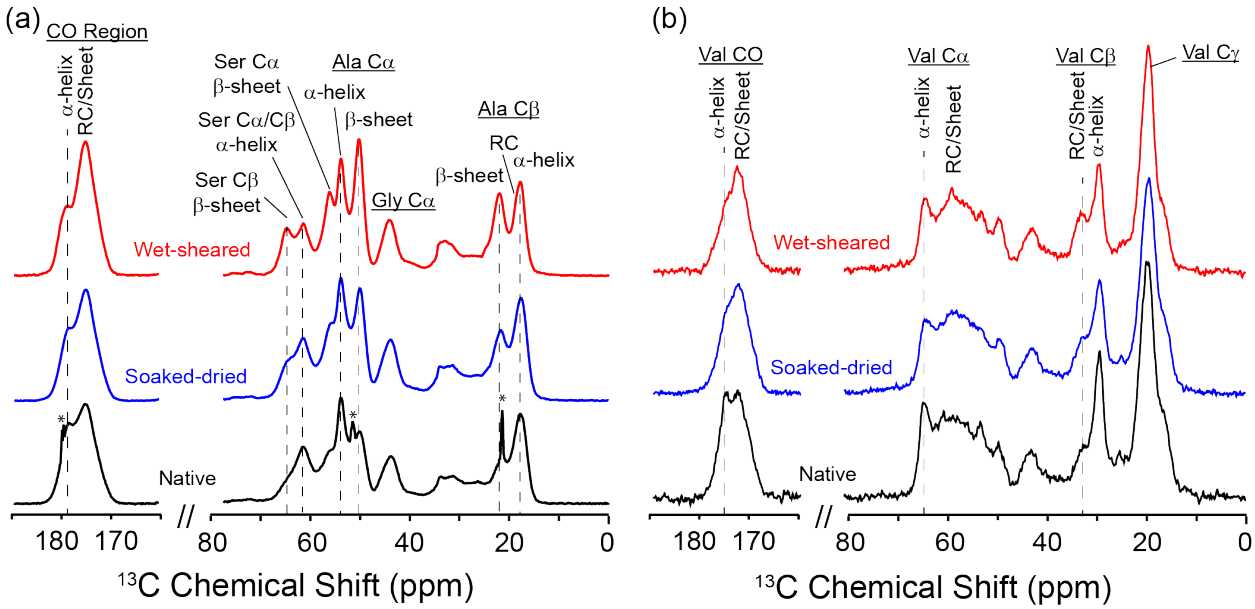

Figure 3. ^{13}C CP-MAS SSNMR spectra of *A. argentata* AC silk. a) AC silk labeled with ^{13}C-Ala. b) AC silk labeled with ^{13}C-Val. Peaks in a) with asterisks are from crystalline Ala contamination.

(WAXS and SAXS) for characterizing structures in the 1-100 nanometer length scale. XRD will inform on the degree of crystallinity and the organization of the beta-sheet / alpha-helical coiled-coil architecture, as well as its sensitivity to chemical and physical stimuli such as hydration induced cross-linking.

Accomplished: We have collected preliminary fiber XRD at ANL and are in the process of processing the data. Initial interpretation is consistent with the as-spun AC fibers having a coiled-coil α-helical superstructure. No b-sheet was observed which, is likely due to the low β-sheet content determined by SSNMR.

(4) Use ^{1}H, ^{13}C, and ^{15}N two-dimensional (2D) and three-dimensional (3D) protein solution NMR to probe the AC silk protein conformational structure and dynamics in the gland fluid prior to fiber formation. Pulse field gradient (PFG) Diffusion NMR will be applied to measure silk protein diffusion to determine the extent of silk protein entanglement and oligomerization under native gland conditions and as a function of protein concentration.

Accomplished: We are working on dissection and extraction of the AC gland for solution NMR and cryo-EM investigations. Unlike the major ampullate gland (MA), the AC gland is much more challenging to excise and remove from the spider unperturbed for ex situ analysis. We have been working with Prof. Cheryl Hayashi (AMNH) who is a leading expert in spider silk genomics and routinely obtains unperturbed AC glands to help us in this area. More attempts are being made this summer.

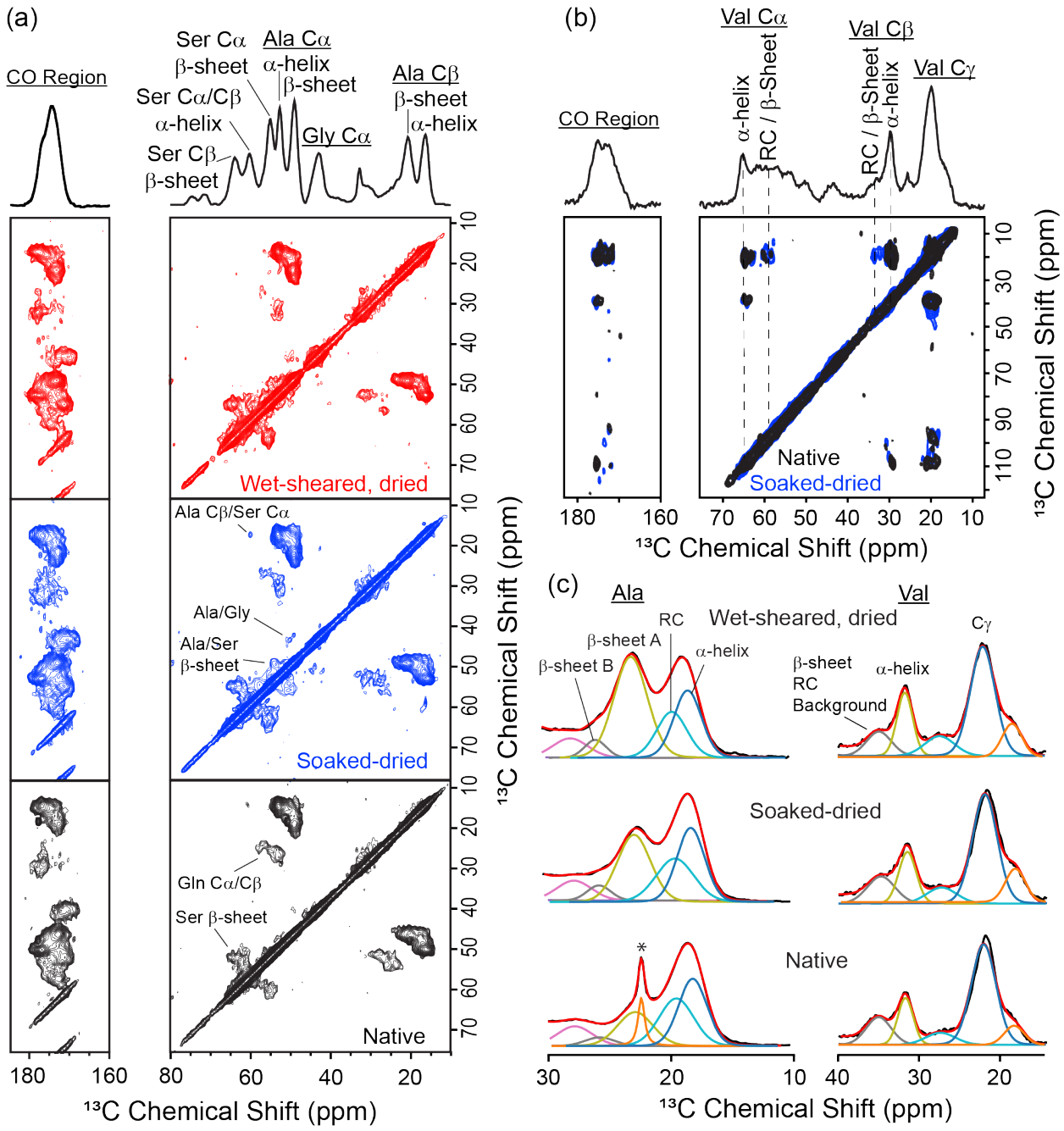

Figure 4. a), 2D ^{13}C-^{13}C DARR SSNMR spectra collected with a 100 ms mixing time for ^{13}C-Ala-labeled *A. argentata* AC silk after different treatments. b), the same as in a) but only ^{13}C-Val labeled. c), spectral deconvolutions of 1D ^{13}C CP-MAS data showing qualitative changes to the Ala Cβ (left) and Val Cβ (right) resonance after different treatments. Peak in c) with an asterisk are from crystalline Ala contamination. The fit parameters are summarized in Table 2re

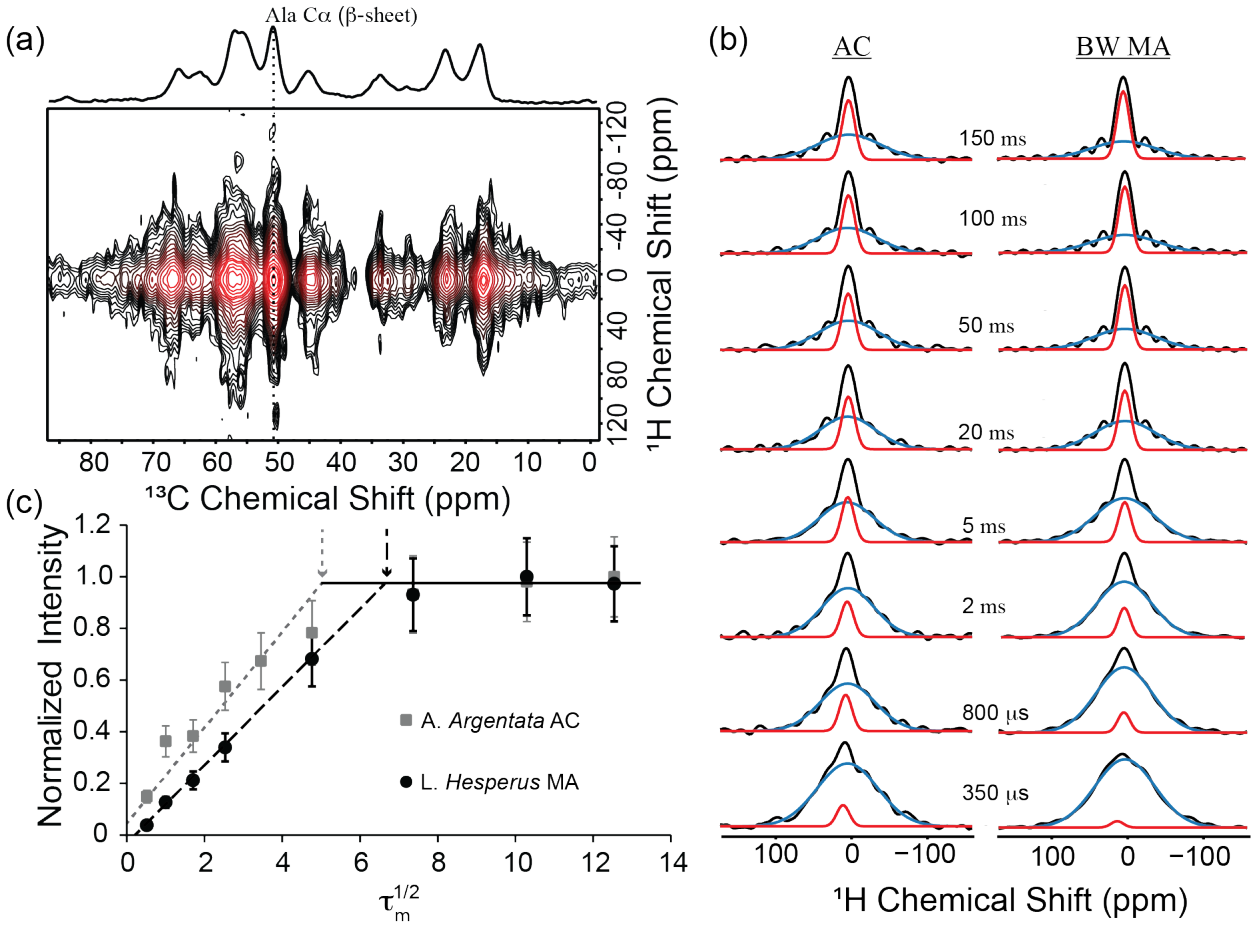

Figure 5. (**a**) A 2D $^{1}H/^{13}C$ WISE spectrum with a 50 ms spin-diffusion time for hydrated *A. argentata* AC silk. (**b**), deconvoluted fits of ^{1}H slices extracted at the Ala Cα β-sheet resonance in the WISE spectrum for AC silk and BW dragline. (**c**), build-up curves of narrow component (red) from deconvolutions for the two silks. The arrows indicate τ_m^* for each sample.

(5) Conduct cryo-TEM experiments in collaboration with UCSD/NW to image AC silk proteins in the gland fluid and determine the extent of oligomerization. Use cryo-TEM methods to determine the size, shape/morphology and overall dimensionality of silk protein oligomers. Expose silk gland fluid to different biochemical triggers (pH and salts) to determine the processing conditions required for silk assembly.

Accomplished: See accomplished in objective above.

(6) Molecular dynamics (MD) simulations will be used to generate structural models and inform interpretation of NMR, XRD and cryo-TEM biophysical characterization.

Accomplished: We have successfully run MD simulations on two different major ampullate silk proteins that are full length. This is a huge accomplishment, and we are comparing these structures to NMR data. We are now running the same simulations on the AC silk proteins and hope to have results soon. It should be stressed that MD is envisioned to provide a significant contribution to understanding all types of silk assembly and no MD simulations of full-length spider silk proteins have been conducted.

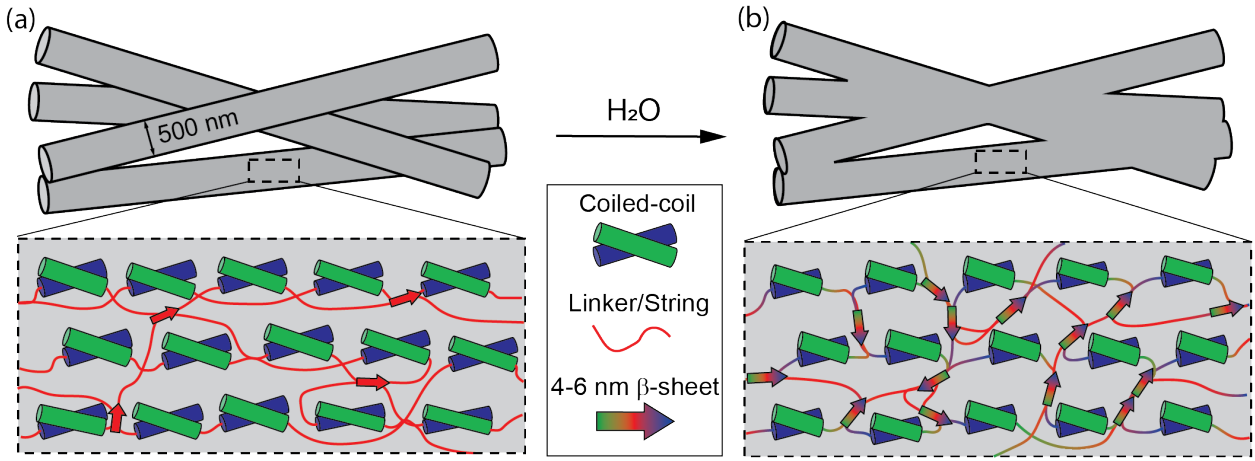

Figure 6. Proposed model of AC silks after contact with water. Helices 1-5 are blue and green cylinders that form α-helical coiled-coils. (**a**), before wetting there is little β-sheet content (~15%). (**b**), after water wetting some α-helical and RC structures convert to β-sheet, illustrated as shortened coiled-coils and the multi-colored arrows colored according to their origin from RC and/or helix structures.

Year 1 Summary

We have shown that prey-wrapping silks from the garden spider *A. argentata* undergo a remarkable cross-linking behavior when exposed to moisture that has not been observed in other silks to the best of our knowledge. Upon contact with water, SEM images reveal that individual silk fibers fuse together to form cross-linked fibrous sheets and mats. Comprehensive solid-state NMR data on ^{13}C-enriched wrapping silks has uncovered the molecular mechanism of inter-fiber cross-linking. Orthogonal isotopic enrichment schemes (Ala-labeling and Val-labeling) were chosen to understand these structural changes for Ala, Ser, Gly, and Val residues, shedding light on which regions of the primary protein sequence are most susceptible to water-induced β-sheet crosslinking. Overall, the data reveals that water-induced fiber cross-linking is driven by an increase in β-sheet protein secondary structure at the expense of disordered and loosely-structured α-helical motifs, whereas well-ordered coiled-coil structures remain in-tact for the most part. However, helical coiled-coil motifs also undergo partial α-to-β conversion with the addition of mechanical shear. This behavior is also seen in other structural protein-based biopolymers. For example, keratin has been shown to convert from α-to-β when stretched in the presence of moisture. Finally, WISE NMR data was used to estimate the β-sheet domain sizes and overall protein dynamics within water-hydrated wrapping silks. Even after water-induced crosslinking, β-sheet regions within AC silks are smaller (~4-6 nm) than those found in BW dragline fibers (~11 nm), suggesting that newly formed β–sheet structures are small and likely between two or more protein chains and potentially involving multiple fibers. **Figure 6** shows a proposed model to illustrate the structural changes of AC silks after water treatment. This data supports our prior conclusions that at a minimum there are three distinct domains in wrapping silks: (1) disordered regions that are easily plasticized by water, (2) rigid α-helices from highly-stable coiled-coil motifs, and (3) Ala and Ser-rich β-sheets are present but surprisingly minimal. Future work will further divulge the role of water in this process, turning a flexible silk fiber into a hardened matted sheet.

It is anticipated that the concept of utilizing water treatment to convert protein-based biomaterials from flexible and extensible, dominated by α-helical coiled-coil hierarchy, to rigid crossed-linked β-sheet assemblies, should provide new avenues in bioinspired material design.

The potential for this hydration-induced conformational switching behavior in a biomaterial are hypothesized to impact a broad range of fields including the Army that requires ever evolving functional materials. In the defense sector new novel materials based on the aciniform silk system could be utilized in advanced textiles for protective clothing, tents, parachutes and the repairs of such materials.

Accomplishments Under Goals (Year 2)

(1) Characterize the mechanical properties of AC silk fibers (single fiber s and fiber bundles) from various spider species. Measure mechanical properties of AC silk bundles in their native states and water-wetted (water-induced crosslinked) states. Correlate structural changes to any observed changes in mechanical properties.

Accomplished: In year 2, we have continued mechanical testing of single spider silk fibers with a nano-mechanical fiber testing system (MTS). We have done this for AC, major ampullate (MA) and tubuliform (TU) egg case silks collected from different individuals for *A. argentata* spiders (**Figure 7**) and other species and fiber types (**Table 2**). For the *A. argentata* species, the results illustrate that while, MA is the strongest of the spider silks, AC silk is by far the toughest due to its significant extensibility (60%) (**Table 2**). It is also interesting to note that we have begun investigating TU silk mechanical properties that show it is significantly weaker and less tough than the other spider silks. We have compared MA mechanical data for two different *Argiope* species, another orb weaver (*N. clavipes*) and a cob weaver (*L. hesperus*). The results show that for MA silks from the three different species there are some notable variabilities particularly for the extensibility between species but, roughly speaking MA fibers from different spider species exhibit similar mechanical properties within error. We have also begun looking at the impact of water (fiber wetting) on spider silk mechanical properties. For MA silk, the fiber undergoes a supercontraction process where it swells in diameter and shrinks in length when wetted with water. This process results in a decrease in fiber strength and considerable increase in extensibility retaining the overall fiber toughness (see **Table 2**, results for *L. hesperus*^{SC}). We are particularly interested in AC silk's unique interaction with water where the fiber cross-links into a matted β-sheet structure following a wet/dry treatment (**see publication 2**). We have begun to collect mechanical data while the AC silk fiber is wet and our preliminary results are shown in **Figure 8**. The wet AC silk fiber shows a significant increase in extensibility when wetted similar to MA silk but, without supercontraction. We are in the process of analyzing this data and reproducing this for other AC silk samples. We also have enough mechanical testing data for publication and have begun drafting a paper on this topic.

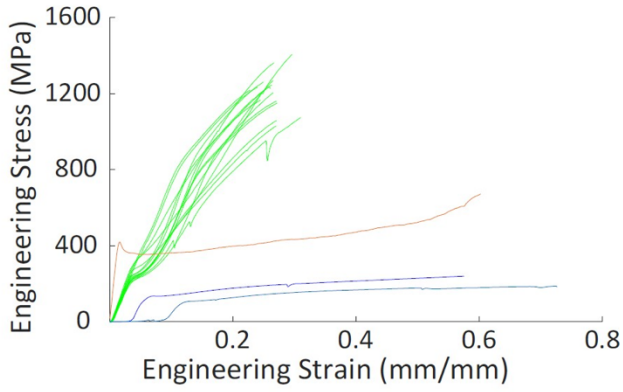

Figure 7. Engineering Stress/Strain curves for *A. Argentata* MA (green), AC (orange) and TU (blue) spider silks. Mechanical data was collected with a Keysight UTM T150 single fiber mechanical testing system.

Table 2. Mechanical Properties of Native and Water Treated Spider Silk Fibers for Different Species and Fiber Types. SC Indicates the Fiber was Supercontracted in Water.

Species	Strength (MPa)	Extensibility(mm/mm)	Toughness (MJ/m^2)	Fiber Diameter (μm)
L. hesperus(MA) N = 24	1008±267/1280±284	34±4%/29±4%	164±82	2.54±0.15
L. hesperus(MA^{sc}) N = 3	602±92/919±99	53±8/42±7%	153±36	3.43±0.3
N. clavipes(MA) N = 28	1146±188/1340±194	17±3%/16±3%	113±33	3.59±0.2
A. argentata(MA) N = 15	1204±104/1524±106	27±2%/24±2%	177±15	1.84±0.84
Acin N=3	650	60%	230*	0.7
A. aurantia(TB) N = 4	212±27/318±35	65±7%/50±7	100.79±1.38	6.42±0.26
L. hesperus(TB) N = 3	314±35/448±41	43±15%/36±14%	118.42±	4.31±12

Water-induced cross-linked AC silks that have been wet/dried have proven difficult to mechanical test with the MTS fiber tensile tester. We are in the process of developing an approach to conduct these measurements on fiber bundles. In addition, through a new collaboration with George Youssef's Mechanics Lab, Mechanical Engineering at SDSU, we have been using AFM nano-indentation to determine the mechanical properties of water cross-linked AC silk mats that have been dried (**reported in publication 2 and report 1**) and this effort will be continued in year 3.

(2) Apply and develop modern multinuclear, multidimensional MAS and dynamic nuclear polarization (DNP)-MAS SSNMR techniques to structurally characterize isotope-labeled AC spider silk fibers. Quantitatively correlate the secondary structures determined for the silks with the silk protein's primary amino acid sequence. Use SSNMR to investigate the silks when in contact with water to elucidate the origin of AC silk's unique hydration-induced α-helical coiled-coil to β-sheet structural transition. This information will be used to develop models for the AC silk's molecular architecture and to design new biomaterials based on AC silk. We already have published results related to this goal that provides the background for the proposed work.

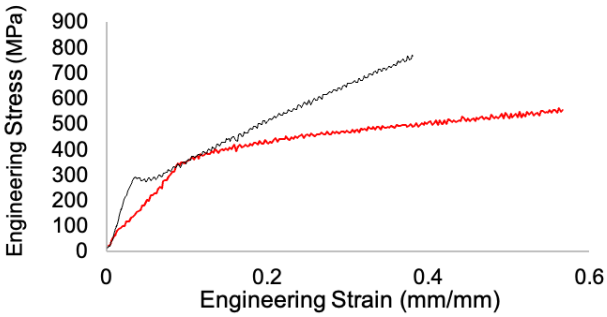

Figure 8. Engineering Stress/Strain curves for *A. Argentata* AC silk dry (black) and wet (red). Mechanical data was collected with a Keysight UTM T150 single fiber mechanical testing system.

<u>Accomplished:</u> We have made considerable headway in understanding the molecular architecture of AC silk fibers with magic angle spinning (MAS) SSNMR together with Alpha-

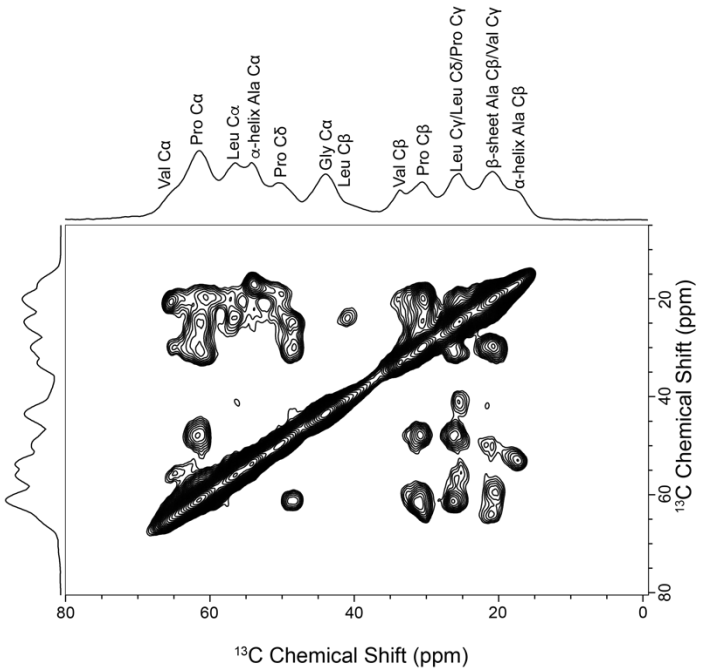

Figure 9. 2D ^{13}C-^{13}C MAS SSNMR DARR spectrum for $^{13}C/^{15}N$-Pro, Leu, Ala and Val isotope labeled *A. argentata* AC silk. The spectrum was collected with a short mixing time of 100 ms highlighting intra-residue amino acid contacts.

Fold and molecular dynamics (MD) modeling approaches discussed below. We have continued to isotope label AC silk and collect 2D SSNMR data. In year two, we have produced AC silk from *A. argentata* spiders that is isotope enriched with $^{13}C/^{15}N$-Pro, Leu, Ala and Val and collected a number of 2D MAS SSNMR spectra and extracted chemical shift data to characterize the secondary structure of the different amino acid residues. The DARR ^{13}C-^{13}C MAS SSNMR spectrum for this sample collected with a short 100 ms missing time is shown in **Figure 9**. This mixing time establishes intra-residue contacts allowing for complete assignment of the ^{13}C chemical shifts that can be used for secondary structure characterization. We are in the process of analyzing this data and drafting a manuscript. We have been able to completely assign in tabulate all ^{13}C chemical shifts for the four labeled amino acids. Our initial results for Pro illustrate that it takes on an elastin-like type-II β-turn conformation (**see Table 3**) in AC silk fibers. This interpretation is similar to the Pro-containing region of the MaSp2 dragline spider silk protein (*Chem. Commun.* 2010, 46, 6714) and helps to explain the significant extensibility of AC silk fibers. While, the extensibility of AC silk is likely due to the predominant coiled-coil α-helical structure, contributions to extensibility from Pro-containing regions are very likely.

We are analyzing and fitting our SSNMR data to extract the quantitative fraction of specific amino acids in α-helical, random coil (RC) and β-sheet structures for isotope enriched AC silk fibers. We then can correlate these secondary structure quantities with the primary amino acid sequence (**Figure 1**) and compare/validate with our published structural model for AC silk (*Chem. Commun.* 2018, 54, 10746). This structure work is also being compared with modeling approaches discussed below. Quantification for Leu in different secondary structures is shown in **Figure 10**. The results show that Leu is predominantly in an α-helical secondary

Table 3. ^{13}C SSNMR Chemical Shifts (ppm) for Pro in *A. argentata* AC silk and Secondary Structures for Biopolymers.

Residue	Chemical Shifts	Elastin	Collagen	Random Coil
Pro Cα	60.7	60.0	58.2	61.9
Pro Cβ	30.1	29.9	29.1	30.6
Pro Cγ	25.7	24.6	24.1	25.6
Pro Cδ	47.6	48.2	47.1	48.3
Pro CO	173.9	171.8	173.9	174.1

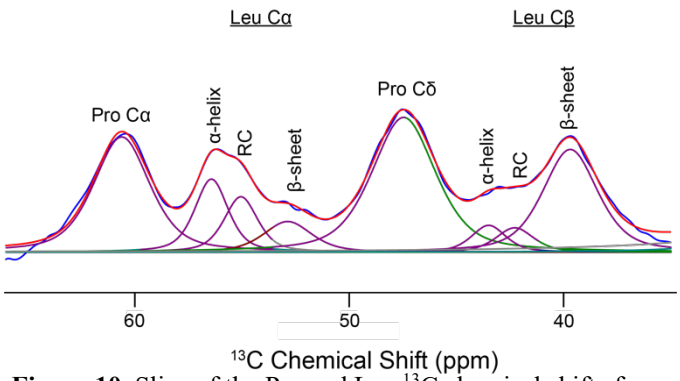

Figure 10. Slice of the Pro and Leu ^{13}C chemical shifts from 2D ^{13}C-^{13}C MAS SSNMR DARR spectrum. The slice was fit to extract the fraction of Leu in α-helical, random coil (RC) and β-sheet fractions. The results indicate that Leu is predominantly α-helical (~60%) with RC and β-sheet fractions 20% each.

structure (~60%) with ~20% each β-sheet and RC conformations. This secondary structure quantification roughly agrees with the model shown in **Figure 1** where 89% of Leu is located in the α-helical-rich bead region for the AcSp1 silk protein. However, it also indicates that it is likely the bead is not completely α-helical (particularly for Leu) since 40% of Leu is determined of be in RC (~20%) and β-sheet (~20%) structures. Another interesting result that we have obtained from our SSNMR data is that the Gly CO ^{13}C chemical shift aligns with a predominantly β-sheet structure. This is in line with our interpretation that the linker region forms the β-sheet assemblies in AC silk fibers since Gly abundance is significantly higher in this region compared the to the α-helical bead where Gly abundance is considerably lower (**see Figure 1**). We have begun comparing our secondary structure quantifications with models determined using the AlphaFold tool (discussed below in simulation section). We will continue to compare our SSNMR secondary structure quantification data in year 3 to further refine our model for AC silk fiber structure based on primary amino acid sequence and modeling.

We have produced additional AC silk fibers that are isotopically enriched in Phe and conducted some initial quantifications of secondary structure from SSNMR data. By slicing through the aromatic ring ^{13}C resonance in the ^{13}C-^{13}C 2D DARR spectrum (**Figure 11**) we are able to resolve and extract the C_α and C_β chemical shifts for Phe. This result reveals two clearly resolved resonances that correspond to Phe in α-helical and β-sheet secondary structures. Our initial quantification results indicates that Phe exhibits a roughly 50/50 population of α-helix/β-sheet secondary structure. This result agrees very well with the prediction from the primary amino acid sequence and "α-helical bead on a linker string model" where the ratio of Phe in the two regions are 50/50 (**Figure 1**). This is consistent with the linker forming the β-sheet structure in AC silk fibers and further corroborates our

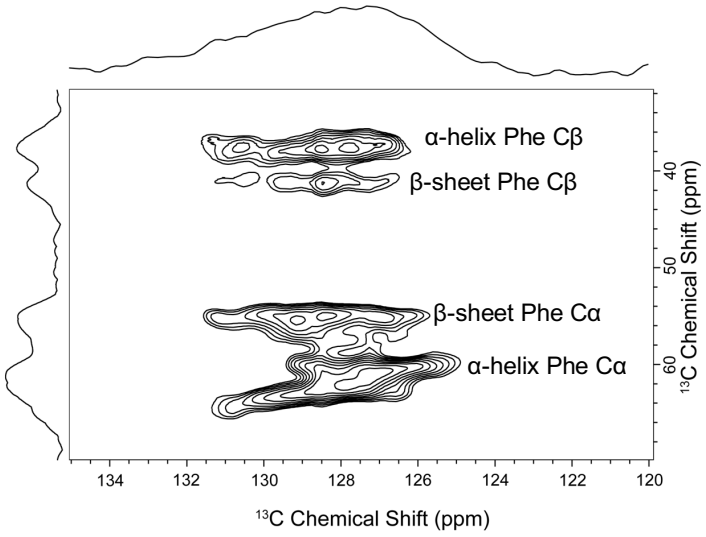

Figure 11. 2D ^{13}C-^{13}C MAS SSNMR DARR spectrum of Phe isotope enriched AC spider silk fibers. The projection on the y-axis is from a slice through the Phe aromatic ring ^{13}C resonance. The results indicate that Phe is predominantly α-~60%) with RC and β-sheet fractions 20% each.

structural model for AC silk structure. We have also collected 2D through-bond double quantum single quantum (DQ/SQ) INADEQUATE SSNMR data on isotope enriched AC silk fibers to further resolve resonances and confirm our DARR data and interpretation. The INADEQUATE experiment displays improved resolution by doubling the frequency of the indirect dimension and allows for improved resolution for the CO resonances. We are in the process of analyzing this data and combining it with our DARR data for a SSNMR manuscript on AC silk fiber structure. Through these experiments we are building significantly on our evolving original structural model (**Figure 1**).

(3) Perform XRD measurements at Argonne National Lab (ANL) on AC silk fibers and bundles to characterize molecular and hierarchical nanocrystalline structures formed in these materials. XRD will focus on pair-distribution functional (PDF) analysis for characterizing amorphous structures and distributions as well as a combination of wide and small angle x-ray scattering (WAXS and SAXS) for characterizing structures in the 1-100 nanometer length scale. XRD will inform on the degree of crystallinity and the organization of the β-sheet / α-helical coiled-coil architecture, as well as its sensitivity to chemical and physical stimuli such as hydration induced cross-linking.

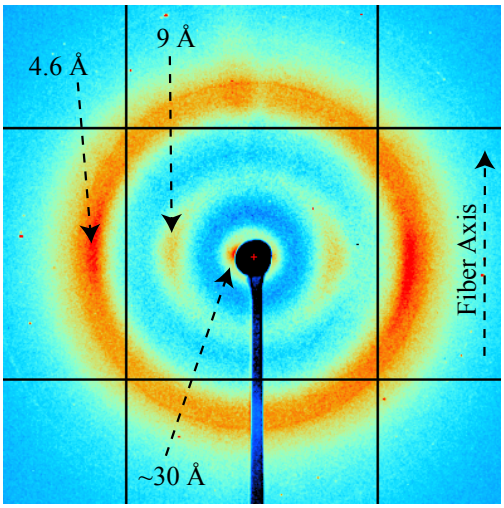

Figure 12. WAXS image of an *A. aurantia* AC native AC silk bundle collected at ANL BioCARS (sector 14 ID-B). Strong XRD reflections are indicated with arrows in the figure.

Accomplished: We have successfully collected preliminary fiber XRD at ANL and are in the process of analyzing the data (**see Figure 12**). Initial interpretation is consistent with the as-spun AC fibers having a coiled-coil α-helical superstructure. The data suggests the presence of both nanocrystalline β-sheets and fiber-aligned α-helical structures from their weak reflections at 4.6 and 9 Å, respectively. A strong reflection in the ~30 Å range near the beamstop is suggestive of a coiled-coil higher-order architecture, but SAXS is required to further investigate this interpretation. During year 2 COVID prevented visits to ANL thus, additional data could not be collected. We now have access to ANL and measurements will continue in year 3. We also have access to new XRD capabilities at ASU that will also be explored in year 3.

(4) Use ^{1}H, ^{13}C, and ^{15}N two-dimensional (2D) and three-dimensional (3D) protein solution NMR to probe the AC silk protein conformational structure and dynamics in the gland fluid prior to fiber formation. Pulse field gradient (PFG) Diffusion NMR will be applied to measure silk protein diffusion to determine the extent of silk protein entanglement and oligomerization under native gland conditions and as a function of protein concentration.

Accomplished: We have made significant progress on dissecting intact, unperturbed AC glands from *A. aurantia* spiders for solution NMR experiments (**see Figure 13**). We have successfully isotope enriched the AC glands and collected 1D and 2D $^{1}H/^{13}C$ and $^{1}H/^{15}N$ HSQC NMR spectra. Four different samples have been prepared and data collected including for $^{13}C/^{15}N$-Thr, $^{13}C/^{15}N$-Leu, $^{13}C/^{15}N$-Val and $^{13}C/^{15}N$-Ala labeled AC silk proteins within glands. The $^{1}H/^{13}C$ HSQC for

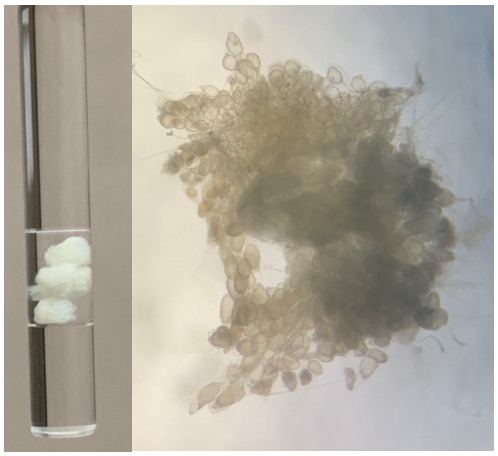

Figure 13. Picture of successfully dissected intact *A. aurantia* AC silk glands (right) and four glands loaded in a solution NMR tube (left) for spectroscopic characterization. The AC silk gland is an assembly of 100's of tiny sacs that are completely intact and closed to maintain the glandular AC silk proteins in a near native state.

Leu labeled glands is shown in **Figure 14**. A number of amino acid residue sites are resolved for Ala (A), Val (V), Thr (T), Leu (L), Gln (Q) and Ser (S). Most notable is the methyl region of the spectrum (low ppm) where multiple structural environments are observed for Ala β, Val γ and Leu δ. There are also two distinct structural environments observed for Ser β. Our current hypothesis is that the AC silk protein will exist in the gland environment as a combination of α-helical (bead) and random coil (linker string) with no evidence of β-sheet structure. This data is currently in the process of being analyzed and initially appears to be in line with our hypothesis.

We have also begun collecting $^{1}H/^{15}N$ HSQC NMR spectra for isotope enriched AC glands and data for $^{13}C/^{15}N$-Thr enriched samples is shown in **Figure 15**. Gly (G), Thr (T) and Ser (S) resonances are observed. For each amino acid multiple structural sites are present, three for Gly, three for Ser and a broad multi-component amide resonance for Thr. The observation of multiple environments is similar to observations made in the ^{13}C HSQC spectrum for the Leu enriched sample (**Figure 14**) and indicates that each amino acids is present in multiple structural environments. It is also interesting to mention that there is considerable variability in some of the linewidths of the resonances (e.g. Thr is very broad in the ^{1}H dimension). The variability in linewidths between resonances shows that there is a distribution in dynamics between the sites with broad resonances indicating more restricted motions (or rapid exchange processes for amide protons) and sharp resonance point to rapid dynamics on NMR timescales.

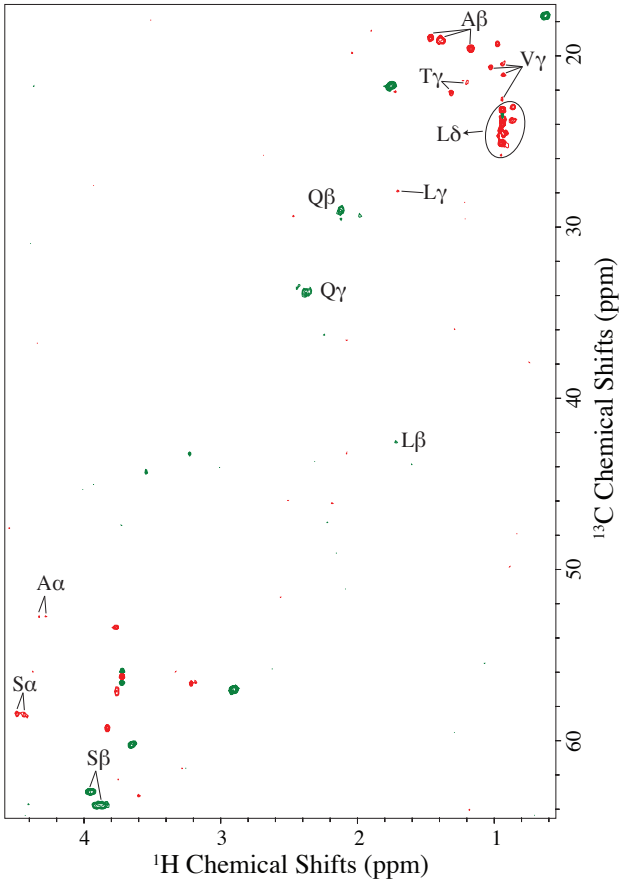

Figure 14. $^{1}H/^{13}C$ HSQC solution NMR spectrum of $^{13}C/^{15}N$-Leu enriched intact AC glands dissected from *A. aurantia* spiders.

In order to delve a bit deeper into the molecular structure and dynamics for the AC silk proteins in the gland environment ^{13}C solution NMR spectra were collected and closely inspected. ^{13}C solution NMR spectra for $^{13}C/^{15}N$-Ala and $^{13}C/^{15}N$-Val isotope enriched AC glands dissected for *A. aurantia* spiders is shown in **Figure 16**. When

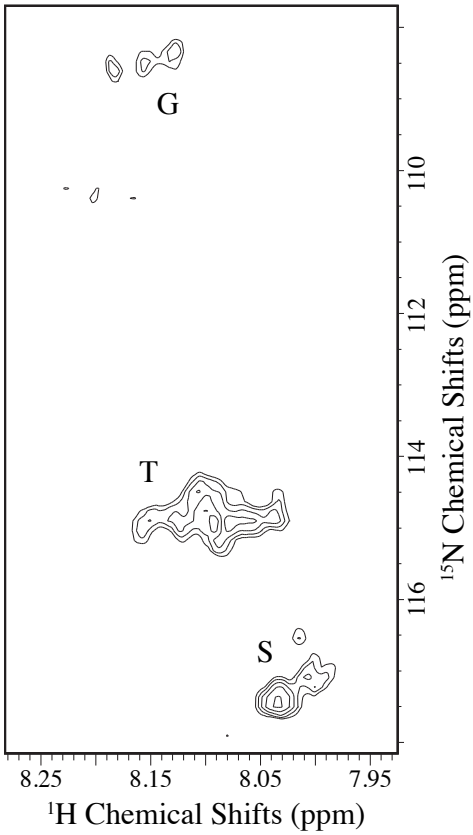

Figure 15. ^{1}H/^{15}N HSQC solution NMR spectrum of ^{13}C/^{15}N-Leu enriched intact AC glands dissected from *A. aurantia* spiders.

comparing both spectra clear contributions from broad and sharp resonances for both Ala and Val are observed consistent with slow and rapid backbone dynamics, respectively. Interestingly, the broad components of the Cα and Cβ resonances for both Val and Ala are aligned with α-helical secondary structures while, the sharp components are indicative of random coil environments. This is consistent with an interpretation where the bead is a well-structured α-helical bundle leading to slow molecular reorientations and broad resonance lines and random coil environments from the linker regions that are highly flexible yielding sharp resonance lines. This interpretation will require 3D protein solution NMR experiments to assign the different environments to see if the backbone-walk amino acid assignment is consistent with this. It is also noteworthy that there are very sharp resonance components that are consistent with small molecules such as individual amino acids that are present in the gland.

Overall, these solution NMR AC gland results are quite promising and it should be possible to conduct backbone assignments and advanced structural characterization leading to a model for AC silk protein structure in the gland environment. The initial starting structure in the gland is extremely intriguing as little to no information is available regarding to the AC silk protein structure within the native gland environment with no information regarding the mechanism of AC silk spinning. Information gained will provide the required information to begin understanding the spinning mechanism. In addition, comparisons can be made between the protein structure in the gland and the structural model we are producing based on SSNMR for AC silk fibers (discussed above).

(5) Conduct cryo-TEM experiments in collaboration with UCSD/NW to image AC silk proteins in the gland fluid and determine the extent of oligomerization. Use cryo-TEM methods to determine the size, shape/morphology and overall dimensionality of silk protein oligomers. Expose silk gland fluid to different

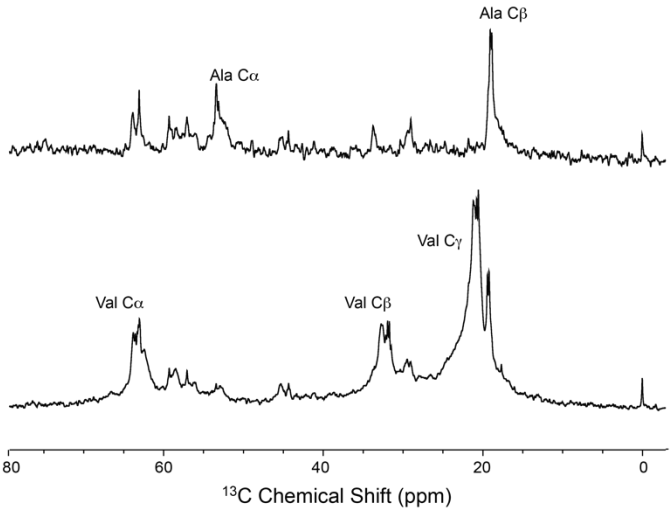

Figure 16. ^{13}C solution NMR spectrum of ^{13}C/^{15}N-Ala (top) and ^{13}C/^{15}N-Val (bottom) enriched intact AC glands dissected from *A. aurantia* spiders.

biochemical triggers (pH and salts) to determine the processing conditions required for silk assembly.

Accomplished: We have successfully collected cryo-TEM on MA silk gland dope. This is a huge accomplishment, and we are comparing these structures to NMR data. We are in the process of collecting similar data on AC silk proteins but, so far have been unable to obtain high quality data. We plan to continue these measurements in year 3.

(6) Molecular dynamics (MD) simulations will be used to generate structural models and inform interpretation of NMR, XRD and cryo-TEM biophysical characterization.

Accomplished: In year 2 we began using AlphaFold for AC silk protein structure prediction. AlphaFold is an artificial intelligence (AI) program develop ed by DeepMind that can predict a given protein's 3D structure from only the primary amino acid sequence as an input. AlphaFold is very new only becoming available to the public in 2021 (*Nature*, 2021, 596, 583). Our initial predicted AC silk protein structures form AlphaFold are shown in **Figure 17.** The results are impressive and provide protein structure hypotheses to test experimentally via SSNMR for AC silk fibers and solution NMR for the protein structure within the gland. The predicted structure is consistent with the "beads-on-a-string" model (**Figure 1**) where the bead is an α-helical bundle and the linker is a disordered random coil domain. The structures in **Figure 17** are for the first ~1000 amino acids in the protein sequence for *A. argentata* AcSp1 that includes five bead domains connected by the linker regions. What is particularly important about having these structural models is that we can start to validate them with NMR. In addition, the predicted structures are good starting points for more refined coarse grained (CG) and atomistic molecular dynamics (MD) simulations that we are currently pursuing. It is anticipated that AlphaFold in combination with MD and NMR experiments will provide a robust approach to determining the structure of AC silk proteins in solution and as solidified fibers and will be a focus of year 3 efforts.

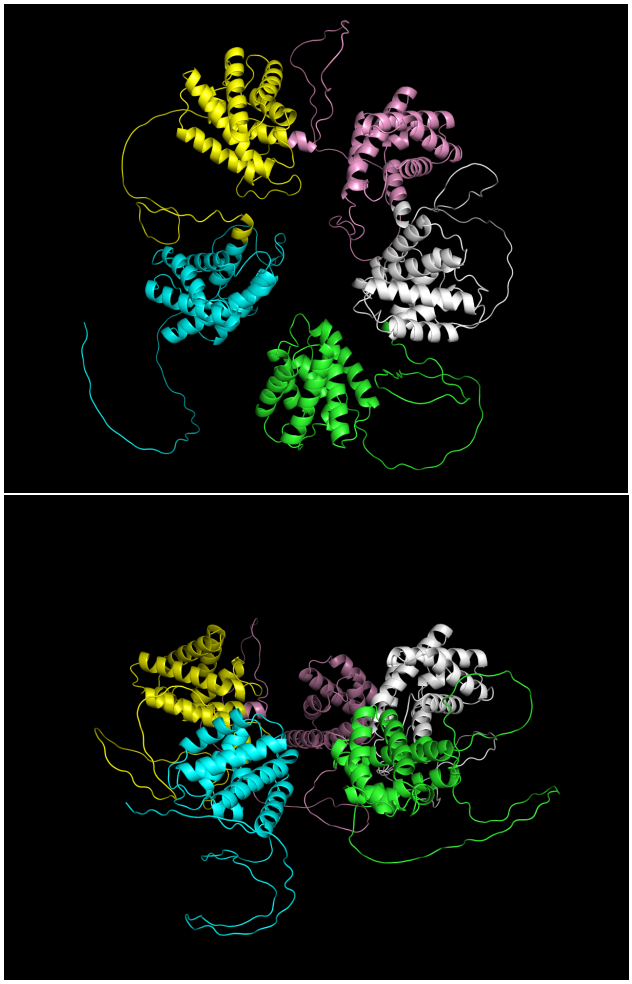

Figure 17. 3D protein structure prediction for *A. argentata* AcSp1 from AlphaFold. The structure was predicted from the first ~1000 amino acids which includes 5 beads connected by the linker regions. Each bead is comprised of a five α-helical bundle separated by a flexible random coil disordered linker region. Two different views are shown (top and bottom)

Year 2 Summary

We have made considerable progress in mechanically testing single spider silk fibers using our MTS with a focus on AC silk fibers both natively spun and following water wetting and have enough data for a publication (Goal 1). We have successfully obtained data on wet/dry AC fibers and have begun a collaboration with George Youssef's lab in Mechanical Engineering to conduct AFM nano-indentation on cross-linked AC silk mats following wet/dry processing that is ongoing. We made significant headway on SSNMR secondary structural characterization of AC silk fibers to continue to build a hierarchical model for AC silk fiber structure (Goal 2). A number of isotope enriched AC silk fibers have been produced and SSNMR data collected. We are in the process of analyzing the data and putting together a manuscript on the topic. We have preliminary XRD data on AC silk fiber bundles that we have analyzed indicating a dominant α-helical coiled-coil superstructure with a minor β-sheet component consistent with our structural model from SSNMR (Goal 3). The COVID pandemic prevented us from collecting additional XRD data at ANL in year 2 but, we have access now and this XRD work will continue in year 3. We also have access to XRD capabilities at ASU and this avenue is also being explored. Significant headway has been made in collecting solution NMR data on isotope enriched proteins within the AC silk gland with four unique labeling strategies accomplished and 2D solution NMR data collected (Goal 4). Our initial interpretation is consistent with the "beads-on-a-string" model with no β-sheet content for the AC silk protein in the gland. Evidence for α-helical bundles and random coil string domains was observed. This is a big accomplishment as nothing is known about the state of the AC silk protein in the gland or the subsequent spinning process and this is a big first step towards beginning to understand the starting state of the AC silk protein. Attempts have been made on cryo-TEM of AC silk proteins extracted from the gland but, have been unsuccessful thus far (Goal 5). This effort will continue in year 3. Lastly, we have made significant progress towards the simulation aspect of AC silk proteins (Goal 6). A newly available AI/ML computational approach is being used (AlphaFold) to predict the structure of AC silk proteins. AlphaFold has allowed us to predict the 3D structure of *A. argentata* AcSp1 which was not previously available and we are working towards comparing this structure with NMR data to validate the model. The importance of AlphaFold as a tool in this research cannot be underestimated as it provides a strong hypothetical structural model of the protein about which NMR experiments can be designed and data compared to confirm the model. It has also provided us with solid starting 3D structures of the AC silk protein to conduct CG and atomistic MD simulations.

Accomplishments Under Goals (Year 3)

(1) Characterize the mechanical properties of AC silk fibers (single fiber s and fiber bundles) from various spider species. Measure mechanical properties of AC silk bundles in their native states and water-wetted (water-induced crosslinked) states. Correlate structural changes to any observed changes in mechanical properties.

Accomplished: We have spent year 3 analyzing the mechanical data collected in years 2 and 3 (details above in year 2). The results are now in manuscript form and we anticipate submitting the manuscript this Fall.

(2) Apply and develop modern multinuclear, multidimensional MAS and dynamic nuclear polarization (DNP)-MAS SSNMR techniques to structurally characterize isotope-labeled AC

spider silk fibers. Quantitatively correlate the secondary structures determined for the silks with the silk protein's primary amino acid sequence. Use SSNMR to investigate the silks when in contact with water to elucidate the origin of AC silk's unique hydration-induced α-helical coiled-coil to β-sheet structural transition. This information will be used to develop models for the AC silk's molecular architecture and to design new biomaterials based on AC silk. We already have published results related to this goal that provides the background for the proposed work.

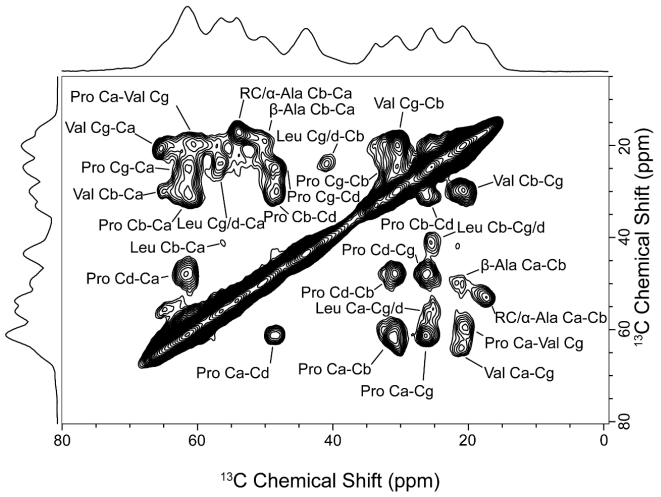

Figure 18. 2D ^{13}C-^{13}C MAS SSNMR DARR spectrum for $^{13}C/^{15}N$-Pro, Leu, Ala and Val (VALP) isotope labeled *A. argentata* AC silk. The spectrum was collected with a short mixing time of 100 ms. Complete intra-residue amino acid contacts assignment is shown in the Figure.

Accomplished: In year 3, we continued our SSNMR secondary structure characterization of isotopically ($^{13}C/^{15}N$) enriched AC spider silk fibers. Throughout the grant, we produced AC silks from *A. argentata* spiders that were isotope enriched with $^{13}C/^{15}N$-Val, Ala, Leu, and Pro (VALP) and a number of single isotope labeled silks including $^{13}C/^{15}N$-Leu, $^{13}C/^{15}N$-Thr, $^{13}C/^{15}N$-Phe and collected a number of 2D MAS SSNMR spectra and extracted chemical shift data to characterize the secondary structure of the different amino acid residues. We are in the process of analyzing this data and drafting a manuscript on this topic to be submitted this Fall. For the VALP sample we have completely assigned the ^{13}C-^{13}C 2D DARR MAS spectrum which establishes through-space correlations via the dipolar couplings and establishes intra-residue assignments (**Figure 18**). The results show that Val, Ala and Leu are predominantly in an α-helical/random coil conformation while, Pro takes on a type II β-turn secondary structure.

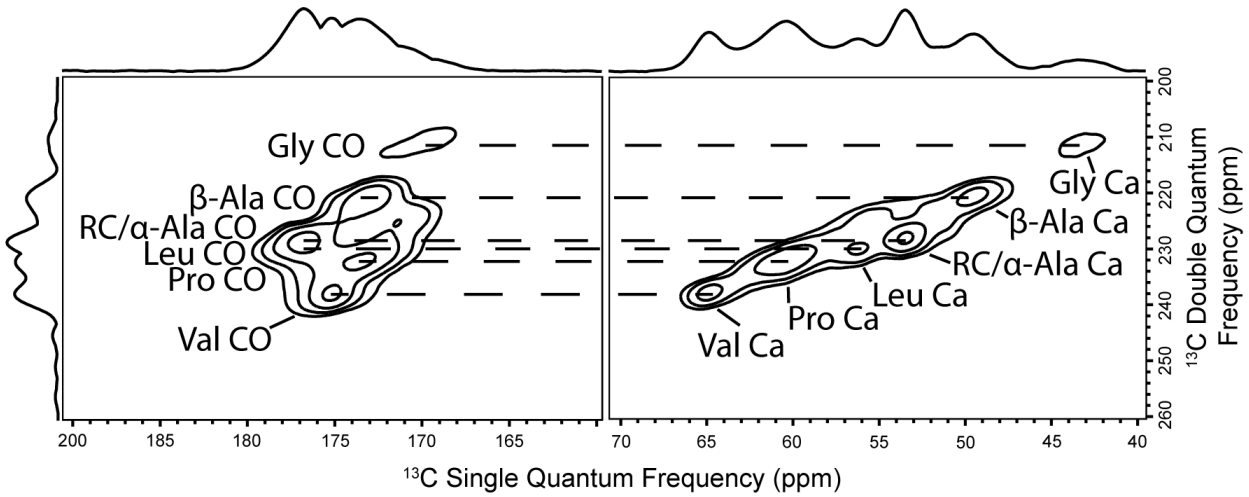

Figure 19. 2D ^{13}C-^{13}C DQ/SQ INADEQUATE spectrum for $^{13}C/^{15}N$-Pro, Leu, Ala and Val (VALP) isotope labeled *A. argentata* AC silk. The spectrum was collected with 30 kHz MAS. Through-bond Cα–CO correlations are highlighted. This experiment allows for extraction of the CO chemical shift for each amino acid site.

We have also collected ^{13}C MAS double quantum/single quantum (DQ/SQ) INADEQUATE through-bond correlation experiments that allow us to extract the carbonyl (C=O) chemical shifts (**Figure 19**) and confirm the assignments from the DARR. This INADEQUTE experiment improves the resolution by doubling the frequency in the indirect dimension allowing us to extract the CO chemical shift for each residue that confirms our secondary structure assignment from the DARR experiment and further provides information regarding Gly. The results for Gly are quire informative and show that although Ala, Val and Leu are dominated by an α–helical conformation and some random coil structure, Gly is predominantly in a β–sheet structure (see **Table 4**). This is quite an interesting result. The β-sheet content of AC silk is quite low (see reference 2) but, Gly seems to dominate in these nanocrystalline structures. This agrees with our hypothesis from AlphaFold and simulations that indicates disordered linker regions in solution and SSNMR shows that these Gly-rich regions are converted to β-sheet structures in AC silk fibers (see discussion in simulations section below).

We have also collected and analyzed ^{13}C MAS SSNMR data for single labeled AC spider silk that were labeled with $^{13}C/^{15}N$-Thr and $^{13}C/^{15}N$ Leu. The data for Thr is shown in **Figure 20**. As can be seen from the spectra the labeling is highly selective for Thr and the ^{13}C chemical shift results are summarized in the **Table 4** illustrating that Thr is neither in the β-sheet or α-helical domain and agrees with a random coil conformation. This is rationalized further in the sections below in our AlphaFold structure prediction where Thr is located at the ends of the α-helical domains and in the linker regions where Thr appears to be unstructured in the fiber form.

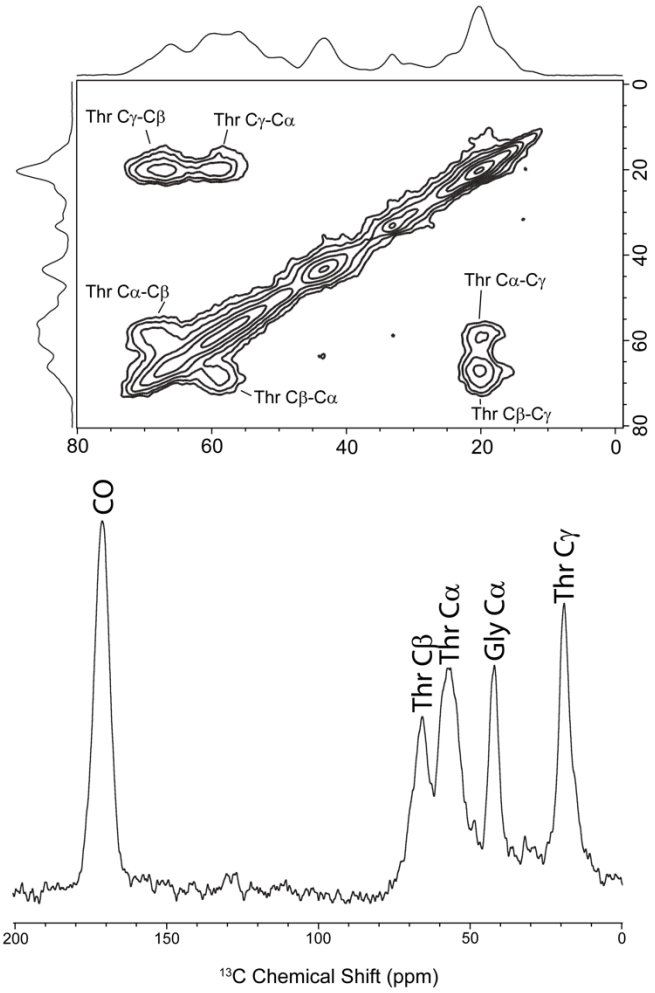

Figure 20. 2D ^{13}C-^{13}C MAS SSNMR DARR spectrum for $^{13}C/^{15}N$-Thr isotope labeled *A. argentata* AC silk. The spectrum was collected with a short mixing time of 100 ms. Complete intra-residue amino acid contacts assignment is shown in the figure. The 1D ^{13}C CP-MAS SSNMR spectrum is shown below.

Our results for single labeled $^{13}C/^{15}N$-Leu AC spider silk are shown in **Figure 21**. Leu is not very soluble in aqueous solutions and as a result the labeling is not as high compared to some of our other samples as can be seen from the lower S/N. However, the data is sufficient to extract the ^{13}C chemical shifts and we have collected sufficient 2D data (not shown) to confirm our assignment. The chemical shift results are shown in **Table 4** and indicate that Leu is undoubtedly in a predominant α-helical

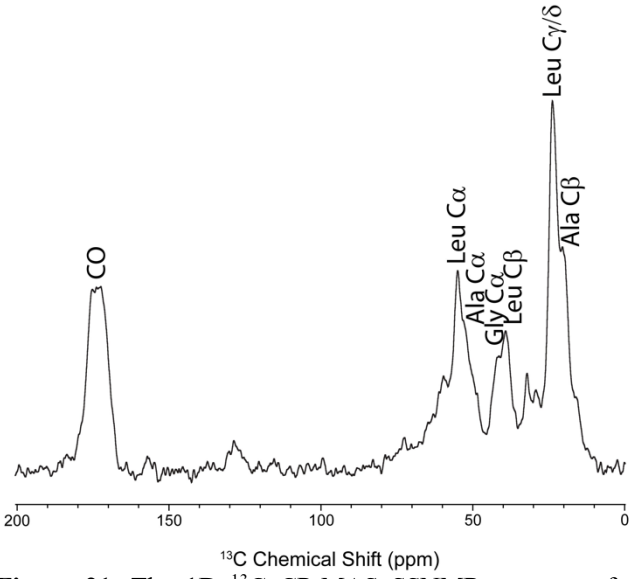

Figure 21. The 1D ^{13}C CP-MAS SSNMR spectrum for $^{13}C/^{15}N$-Leu isotope labeled *A. argentata* AC silk.

Table 4. ^{13}C SSNMR Chemical Shifts (ppm) for Gly, Leu, Thr in *A. argentata* AC Silk and Secondary Structures for Biopolymers.

Residue	Chemical Shifts	β-sheet	Random Coil	α-helix
Gly Cα	43.5	43.2	43.3	44.7
Gly CO	170.1	170.1	171.8	173.3
Leu Cα	54.9	51.8	52.8	55.3
Leu Cβ	39.2	42.2	40.3	39.5
Thr Cα	59.2	59.1	59.5	63.4
Thr Cβ	67.0	68.4	67.4	66.2
Thr CO	172.6	171.6	172.5	173.4

structure. This again agrees with our AlphaFold structure predictions where Leu is highly represented in very stable α-helical coiled-coil structures (see below).

(3) Perform XRD measurements at Argonne National Lab (ANL) on AC silk fibers and bundles to characterize molecular and hierarchical nanocrystalline structures formed in these materials. XRD will focus on pair-distribution functional (PDF) analysis for characterizing amorphous structures and distributions as well as a combination of wide and small angle x-ray scattering (WAXS and SAXS) for characterizing structures in the 1-100 nanometer length scale. XRD will inform on the degree of crystallinity and the organization of the β-sheet / α-helical coiled-coil architecture, as well as its sensitivity to chemical and physical stimuli such as hydration induced cross-linking.

Accomplished: We have successfully collected preliminary fiber XRD at ANL and have analyzed the data. Initial interpretation is consistent with the as-spun AC fibers having a coiled-coil α-helical/β-sheet superstructure. The data suggests the presence of both nanocrystalline β-sheets and fiber-aligned α-helical structures from their weak reflections at 4.6 and 9 Å, respectively. A strong reflection in the ~30 Å range near the beamstop is suggestive of a coiled-coil higher-order architecture, but SAXS is required to further investigate this interpretation. During year 2 COVID prevented visits to ANL thus, additional data could not be collected. The ANL beam line shut down in year 3, unfortunately. We have access to new XRD capabilities at ASU that are now being initiated for continuation of this work.

(4) Use ^{1}H, ^{13}C, and ^{15}N two-dimensional (2D) and three-dimensional (3D) protein solution NMR to probe the AC silk protein conformational structure and dynamics in the gland fluid prior to fiber formation. Pulse field gradient (PFG) Diffusion NMR will be applied to measure silk protein diffusion to determine the extent of silk protein entanglement and oligomerization under native gland conditions and as a function of protein concentration.

Accomplished: We have made significant progress throughout the grant period on dissecting intact, unperturbed AC glands from *A. aurantia* spiders for solution NMR experiments. We have successfully isotope enriched the AC glands and collected 1D and 2D $^{1}H/^{13}C$ and $^{1}H/^{15}N$ HSQC NMR spectra (discussed in year 2). Four different samples have been prepared and data collected including for $^{13}C/^{15}N$-Thr, $^{13}C/^{15}N$-Leu, $^{13}C/^{15}N$-Val and $^{13}C/^{15}N$-Ala labeled AC silk proteins within glands. The $^{1}H/^{13}C$ HSQC for Leu labeled glands display a number of amino acid residue sites including Ala, Val, Thr, Leu, Gln and Ser. Most notable is the methyl region of the spectrum (low ppm) where multiple structural environments are observed for Ala, Val and Leu. There are also at least three distinct structural environments observed for Ser in the alpha region of the ^{13}C-HSQC spectrum. Our current hypothesis is that the AC silk protein will exist in the gland environment as a combination of α-helical (bead) and random coil (linker string) with no evidence of β-sheet structure in the gland environment. This data is currently in the process of being analyzed and initially appears to be in line with our hypothesis we are putting this data together for a manuscript. Understanding the state of the AC silk protein within in the gland (prior to fiber formation) is a critical step in understanding the spinning process.

(5) Conduct cryo-TEM experiments in collaboration with UCSD/NW to image AC silk proteins in the gland fluid and determine the extent of oligomerization. Use cryo-TEM methods to determine the size, shape/morphology and overall dimensionality of silk protein oligomers. Expose silk gland fluid to different biochemical triggers (pH and salts) to determine the processing conditions required for silk assembly.

Accomplished: We have successfully collected NS-TEM and cryo-TEM on MA silk gland dope. This is a huge accomplishment, and we are comparing these structures to NMR data. We are in the process of collecting similar data on AC silk proteins but, so far have been unable to obtain high quality data. We plan to continue these measurements in future work and have a number of ideas regarding how we can improve the data.

(6) Molecular dynamics (MD) simulations will be used to generate structural models and inform interpretation of NMR, XRD and cryo-TEM biophysical characterization.

Accomplished: During the grant period we began using AlphaFold for AC silk protein structure prediction together with more traditional molecular dynamics (MD) simulations in GROMACS with coarse grain MARTINI force fields and atomistic force fields on shorter sequences. AlphaFold is an artificial intelligence (AI) program developed by DeepMind that can predict a given protein's 3D structure from only the primary amino acid sequence as an input. AlphaFold is very new only becoming available to the public in 2021 (Nature, 2021, 596, 583). Our initial predicted AC silk protein structures form AlphaFold are shown in the **Figure 22**. The results are impressive and provide protein structure hypotheses to test experimentally via SSNMR for AC silk fibers and solution NMR for the protein structure within the gland. The predicted structure is consistent with the "beads-on-a-string" model where the bead is an α-helical bundle and the linker is a disordered random coil domain. The structures shown **Figure 22** are from AlphaFold predictions conducted on the first ~1000 amino acids in the protein sequence for *A. argentata* AcSp1 that includes five bead domains connected by the linker regions. What is particularly important about having these structural models is that we are validating them with NMR both in solution (the gland state) and in final fibers (as discussed above). In addition, the predicted

structures are good starting points for more refined coarse grained (CG) and atomistic MD simulations that we also pursuing. It is shown here that AlphaFold in combination with MD and NMR experiments provides a robust approach to determining the structure of AC silk proteins in solution and as solidified fibers. We are combining these simulations with SSNMR data (above) for a publication and anticipate submitting this Fall and show that this will be a powerful approach moving forward in our future work on AC silk structural biology and biomimetic studies.

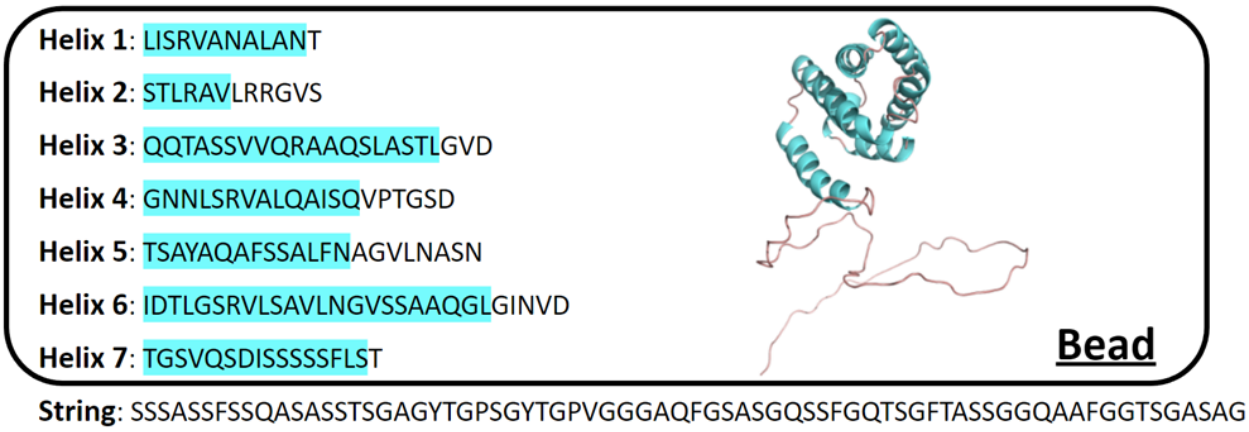

Figure 22. AlphaFold predicted structure for one bead and one string region for AcSp showing the regions that form well-defined α-helical bundles. High confidence α-helical structures are highlighted in blue in the amino acid sequence. The string region forms a disordered random coil structure in solution however, we know from our SSNMR studies that portions of this string form β-sheet structure in AC silk fibers. The β-sheet structures include Ser, Ala and Gly while, Thr appears to maintain disordered random coil structures.

Year 3 Summary

We have made considerable progress in mechanically testing single spider silk fibers using our MTS with a focus on AC silk fibers both natively spun and following water wetting and have a working draft that we put together in year 3 and plan to submit this Fall (Goal 1). We made significant headway on SSNMR secondary structural characterization of AC silk fibers to continue to build a hierarchical model for AC silk fiber structure together with AlphaFold and MD structural models and have a working draft to be submitted this Fall (Goal 2, Goal 6). A number of isotope enriched AC silk fibers have been produced and SSNMR data collected that are included in this manuscript. We have preliminary XRD data on AC silk fiber bundles that we have analyzed indicating a dominant α-helical coiled-coil superstructure with a minor β-sheet component consistent with our structural model from SSNMR (Goal 3). The ANL beamline shutdown prevented us from collecting additional XRD data at ANL in year 3 but, we have access now at ASU and this XRD work will continue in future work. Significant headway has been made in collecting solution NMR data on isotope enriched proteins within the AC silk gland with four unique labeling strategies accomplished and 2D solution NMR data collected (Goal 4). Our initial interpretation is consistent with the "beads-on-a-string" model with no β-sheet content for the AC silk protein in the gland. Evidence for α-helical bundles and random coil string domains was observed. This is a big accomplishment as nothing is known about the state of the AC silk protein in the gland or the subsequent spinning process and this is a big first step towards beginning to understand the starting state of the AC silk protein in solution. Attempts have been made on cryo-TEM of AC silk proteins extracted from the gland but, have been unsuccessful thus far (Goal 5). This effort will continue in future work and we have some new ideas along these lines. Lastly, we have made significant progress towards the simulation aspect of AC silk proteins (Goal 6). A newly

available AI/ML computational approach is being used (AlphaFold) to predict the structure of AC silk proteins. AlphaFold has allowed us to predict the 3D structure of *A. argentata* AcSp1 which was not previously available and we have compared this structural model with NMR data to validate the model. The importance of AlphaFold as a tool in this research cannot be underestimated as it provides a strong hypothetical structural model of the protein about which NMR experiments can be designed and data compared to confirm the model for the protein both in solution (the gland) and in the fiber form. It has also provided us with solid starting 3D structures of the AC silk protein to conduct CG and atomistic MD simulations.

Publications

1) Addison, J.B., Stengel, D., Bharadwaj, V.S., Happs, R.M., Doeppke, C., Wang, T., Bomble, Y.J., Holland, G.P**.,** Harman-Ware, A.E. "Selective 1D ^{13}C-^{13}C Spin-Diffusion Solid-State NMR Methods to Probe Spatial Arrangements in Biopolymers Including Plant Cell Walls, Peptides and Spider Silk" *J. Phys. Chem. B.* **2020**, *124*, 9870-9883.

2) Stengel, D., Addison, J.B., Onofrei, D., Holland,G.P**. "**Water-induced β-sheet Crosslinking of α-helical-rich Spider Prey-wrapping Silk" *Adv. Funct. Mater.* **2021**, *31*, 2170090. **Made Cover Art**

3) Chalek, K., Stengel, D., Addison, J.B, Holland, G.P. "Conformational Structure of Aciniform Spider Silk Determined by Solid-state NMR and Modeling" In Preparation (2023).

4) Onofrei, D., Botello, F., Holland, G.P. "Correlating the Mechanical Properties of Various Spider Silk Fibers with their Secondary Structures" In preparation (2023).

5) Johnson, H.R. and Holland, G.P. "Solution NMR of Native Acinform Spidroins Reveals Multi-domain Silk Protein Structure" In preparation (2023).

Results Dissemination as Talks

1) Dillan Stengel, J. Bennett Addison, David Onofrei, and Gregory P. Holland "Water-induced β-sheet Crosslinking of alpha-helical Spider Prey-wrapping Silk" ACS National Meeting, Spring 2021 ***Won Best Poster from the Division of Polymeric Materials: Science and Engineering***

2) David Onofrei, Dillan Stengel, Hannah Johnson, Brittany Puzio and Gregory P. Holland "Spider Silks as Model Systems for the Design of Functional Protein-based Materials and Composites" AAAFM-UCLA International Conference, Summer 2021

3) Hannah R. Johnson, Katherine Adams, Christofer Layana, Salvador Vallejo, Gregory P. Holland "Informing Tunable Bio-composite Design with Fiber Formation in Spiders and Silkworms" AAAFM-UCLA International Conference, Summer 2021

4) Bennett Addison, Dillan Stengel, Gregory P. Holland, Ware "Selective 1D [^{13}C-^{13}C] Spin Diffusion Solid-state NMR to Probe Spatial Arrangements in Biomolecules" Experimental NMR Conference, Pacific Grove, CA (2021). [Held virtually due to the pandemic]

5) Gregory P. Holland “Developing Aciniform Spider Silk Biomaterials with Unique Structural Transitions and Properties” Seminar to the Army Research Labs (2021). [***Invited***, held virtually due to the pandemic]

6) Gregory P. Holland “Hierarchical Assembly of Spider Silk Proteins” Frontiers in Soft Matter and Macromolecular Networks, University of San Diego, San Diego, CA (2022). [***Invited]***

7) Gregory P. Holland “A Multimodal Biophysical Approach to Understand Biomaterials Formation” Biomaterials Seminar - University of Bayreuth, Germany (2022). [***Invited***]

8) Kevin Chalek, Dillan Stengel, Bennett Addison, Gregory P. Holland **“**Probing Cation-Pi Interactions in Spider Silk Fibers with Selective DARR Difference MAS SSNMR” Rocky Mountain Conference on Magnetic Resonance, Copper Mountain, CO (2022).

9) Kevin Chalek, Dillan Stengel, Bennett Addison, Gregory P. Holland **“**SSNMR Characterization of Aciniform Spider Silk Conformational Structure” Frontiers in Soft Matter and Macromolecular Networks, University of San Diego, San Diego, CA (2022).

10) Gregory P. Holland "Elucidating Spider Silk Structure and Assembly with NMR" North Jersey ACS NMR Topical Group, Held Virtually with attendees from around the world in the NMR community (2023). [Invited]

11) Julian E. Aldana, Kevin Chalek, David Onofrei and Gregory P. Holland "Predicting the Three-dimensional Structure of Spider Silk Proteins with AlphaFold and Molecular Dynamics Simulations" CSU Annual Biotechnology Symposium,

12) Gregory P. Holland "Combining Biophysical Methods to Understand Spider Silk Formation" Gordon Research Conference: Silk Proteins and the Transition to Biotechnologies, Bryant University, Smithfield, RI (2023). [Invited]

13) Kevin Chalek, Julian E. Aldana, Christian D. Lorenz, Gregory P. Holland "Combining Solid-state NMR and Molecular Dynamics Simulation to Characterize the Structure of Spider Silk Fibers" Gordon Research Conference: Silk Proteins and the Transition to Biotechnologies, Bryant University, Smithfield, RI (2023).

14) Gregory P. Holland "Combining Solution NMR and Modeling to Determine Structural Ensembles of Spider Silk Proteins" ACS Fall 2023 National Meeting, San Francisco, CA (2023). [Invited]

Honors and Awards

1) Chalek, K. SDSU, 2nd Place Poster Award, Frontiers in Soft Matter and Macromolecular Networks, University of San Diego, San Diego, CA (2022)

2) Chalek, K.SDSU, Travel Award, Rocky Mountain Conference on Magnetic Resonance, Copper Mountain, CO (2022)

3) Holland, G.P. SDSU, College of Sciences Exceptional Service Award (2021)

4) Johnson, H.R. SDSU, University Graduate Fellowship (2021)

5) Stengel, D. PMSE Best Poster Award, Spring 2021 Virtual ACS Meeting (2021)

Training Opportunities

Two PhD students were supported. PhD student, Dr. Dillan Stengel, successfully graduated in December 2021 with his PhD. A second PhD student, Hannah Johnson was partially supported and one post-doc, Dr. Kevin Chalek was partially supported on the grant. All three individuals were extensively trained through out the grant including solution and SSNMR, XRD, EM, MD and other simulation and data analysis methods.

Technology Transfer

WO2022150163A2-2022-07-14 titled "BIOMATERIALS AND BIOTEXTILES AND METHODS FOR MAKING SAME" published July 14, 2022; PCT Application

Participants Name

Holland, Gregory (PI); Chalek, Kevin (PD); Onofrei, David (Doctoral Level Research Scientist); Hannah Johnson (Graduate Student); Dillan Stengel (Graduate Student)

Role

PD/PI

Postdoctoral (scholar, fellow or other postdoctoral position)

Staff Scientist (doctoral level)

Graduate (PhD) Students (2)

Person Months

One month for the PI and Staff Scientist per year of the grant. 1 year (12 months) for the each of the graduate students and 6 moths in year 2 and 3 for the PD.