1. Award #: DE-SC0004791
Recipient: University of Wyoming transferred to Utah State University, 0305 Old Main Hill, Logan, UT, 84322

2. Title: "X-ray Diffraction and Neutron Scattering Analysis of Natural and Synthetic Spider Silk Fibers"
PI: Randolph V. Lewis

3. Final Report Date: 11/11/2013 covering 07/01/2010 to 09/01/2013

4. DOE National Lab Collaborators: Argonne National Laboratory; Dr. Chris Benmore

5. Abstract: Spider silks have the potential to provide new bio-inspired materials for numerous applications in bioenergetics and products ranging from protective clothing to artificial ligaments and tendons. A number of spider silk genes have been cloned and sequenced by the Lewis laboratory revealing the basis for understanding the key elements of spider silk proteins with respect to their materials performance. In particular, specific amino acid motifs have been identified which have been conserved for over 125 million years in all spiders that use their silk to physically trap prey. The key element in taking the next step toward generating bio-based materials from spider silks will be to move from the current descriptive data to predictive knowledge.

Current efforts are focused on mimicking spider silk through synthetic proteins. In developing synthetic silk fibers, we first need to understand the complete secondary and tertiary structure of natural silk so that we can compare synthetic constructs to the natural material. Being able to compare the structure on a single fiber level is critical to the future of molecular directed mimic development because we can vary mechanical properties by different spinning methods. The new generation of synchrotron x-ray diffraction and neutron beamlines will allow, for the first time, determination of the molecular structure of silk fibers and synthetic mimics. We propose an exciting new collaborative research team working jointly between Argonne National Laboratory, Arizona State U. and the University of Wyoming to address the "characterization of synthetic and natural spider silk fibers using x-ray and neutron diffraction." Thus these new methodologies will provide understanding of current fibers and determine changes needed to produce fibers with specific properties.

The following specific aims are proposed:

- Synthesize spider silk fibers with molecular structures mimicking that of natural silks. Test the mechanic properties of these materials and compare them to natural silk fibers.
- Develop x-ray and neutron diffraction techniques to better determine the structure in amorphous and semicrystalline biopolymers, such as spider silk fibers.
- Combine mechanical testing and structural x-ray and neutron diffraction data to develop a molecular understanding of the structure-function relationship in spider silk materials.
- Elucidate the role water plays in spider silk fiber formation and structure. Emphasis will be placed on combined neutron and NMR studies.
- Use solid-state Nuclear Magnetic Resonance (NMR) to characterize synthetic and natural spider silk materials that show potential as a biomimetic material or bio-inspired polymer architecture.
- Develop EPSCoR student and postdoctoral training and exposure to national laboratory facilities.
- Further develop scientific outreach and chemical education programs and research.

Accomplishment of these objectives are will provide new methodologies to determine protein fiber structures and correlate these structures with mechanical properties and provide broad training in molecular structure analysis to students.

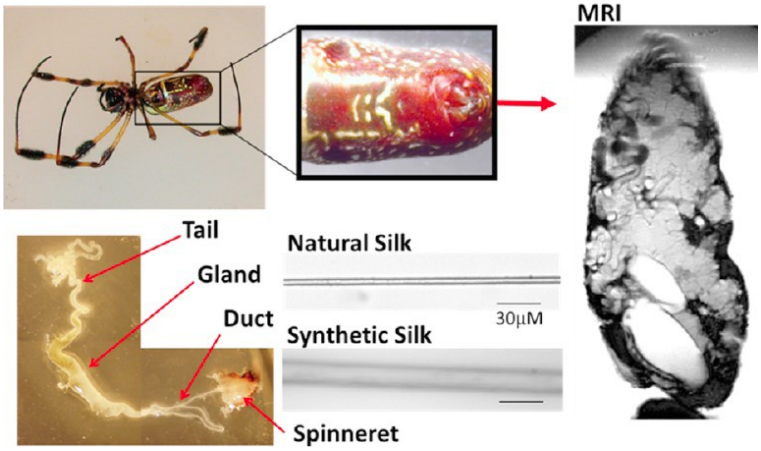
6. Accomplishments: Abstracts of papers listed to show accomplishments

1. Abstract:
The two Flag/MaSp 2 silk proteins produced recombinantly were based on the basic consensus repeat of the dragline silk spidroin 2 protein (MaSp 2) from the Nephila clavipes orb weaving spider. However, the prolinecontaining pentapeptides juxtaposed to the polyalanine segments resembled those found in the flagelliform silk protein (Flag) composing the web spiral: (GPGGX1 GPGGX2)2 with X1/X25A/A or Y/S. Fibers were formed from protein films in aqueous solutions or extruded from resolubilized protein dopes in organic conditions when the
Flag motif was (GPGGX1 GPGGX2)2 with X1/X25Y/S or A/A, respectively. Post-fiber processing involved similar drawing ratios (2–2.53) before or after water-treatment.
Structural (ssNMR and XRD) and morphological (SEM) changes in the fibers were compared to the mechanical properties of the fibers at each step. Nuclear magnetic resonance indicated that the fraction of b-sheet nanocrystals in the polyalanine regions formed upon extrusion, increased during stretching, and was maximized after water-treatment. X-ray diffraction showed that nanocrystallite orientation parallel to the fiber axis increased the ultimate strength and initial
stiffness of the fibers.Water furthered nanocrystal orientation and three-dimensional growth while plasticizing the amorphous regions, thus producing tougher fibers due to increased extensibility. These fibers were highly hygroscopic and had similar internal network organization, thus similar range of mechanical properties that depended on their diameters. The overall structure of the consensus repeat of the silk-like protein dictated the mechanical properties of the fibers while protein molecular weight limited these same properties. Subtle structural motif re-design impacted protein self-assembly mechanisms and requirements for fiber formation.
2. Abstract : Total x-ray scattering measurements of spider dragline silk fibers from *Nephila clavipes*, *Argiope aurantia* and *Latrodectus hesperus* all yield similar structure factors, with only small variations between the different species. X-ray scattering from fibers orientated perpendicular to the beam show a high degree of anisotropy, whereas along the fiber axis the diffraction pattern is less structured and isotropic. Differential pair distribution functions (PDF) obtained by integrating over wide sections of the equatorial and meridian planes indicate that, on average the majority (95%) of the atom-atom correlations do not extend beyond 1 nm. The local molecular orientations below 5 Å along the fiber axis are consistent with proteins in mixed orientations, which may be associated with the silks greater flexibility in this direction.

3. **Abstract:** Dragline silk from orb-weaving spiders is acopolymer of two large proteins, major ampullate spidroin 1(MaSp1) and 2 (MaSp2). The ratio of these proteins is known tohave a large variation across different species of orb-weavingspiders. NMR results from gland material of two different species of spiders, N. clavipes and A. aurantia, indicates that MaSp1 proteinsare more easily formed into β-sheet nanostructures, while MaSp2proteins form random coil and helical structures. To test if thisbehavior of natural silk proteins could be reproduced byrecombinantly produced spider silk mimic protein, recombinantMaSp1/MaSp2 mixed fibers as well as chimeric silk fibers fromMaSp1 and MaSp2 sequences in a single protein were producedbased on the variable ratio and conserved motifs of MaSp1 andMaSp2 in native silk fiber. Mechanical properties, solid-state NMR, and XRD results of tested synthetic fibers indicate the differing roles of MaSp1 and MaSp2 in the fiber and verify the importance of postspin stretching treatment in helping the fiber to form the proper spatial structure.

4. Abstract: Synchrotron X-ray micro-diffraction experiments were carried out on Nephila clavipes (NC) and Argiope aurantia (AA) major (MA) and minor ampullate (MiA) fibers that make up dragline spider silk. The diffraction patterns show a semi-crystalline structure with b-poly(L-alanine) nanocrystallites embedded in a partially oriented amorphous matrix. A superlattice reflection ‘S’ diffraction ring is

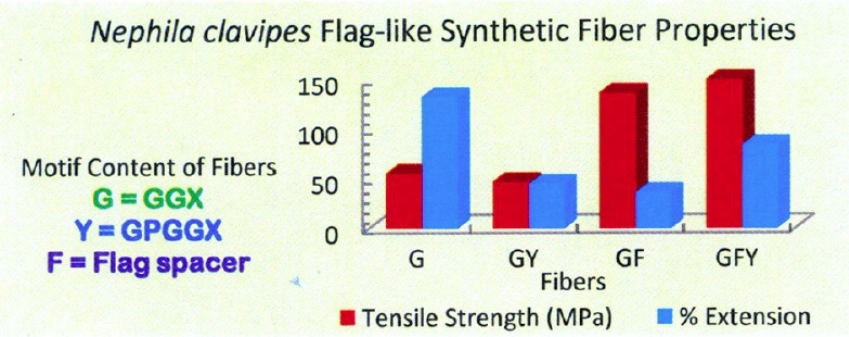
observed, which corresponds to a crystalline component larger in size and is poorly oriented, when compared to the b-poly(L-alanine) nanocrystallites that are commonly observed in dragline spider silks. Crystallite size, crystallinity and orientation about the fiber axis have been determined from the wide-angle X-ray diffraction (WAXD) patterns. In both NC and AA, the MiA silks are found to be more highly crystalline, when compared with the corresponding MA silks. Detailed analysis on the amorphous matrix shows considerable differences in the degree of order of the oriented amorphous component between the different silks studied and may play a crucial role in determining the mechanical properties of the silks.



5. Abstract: Adhesive silks spun by aquatic caddisfly (orderTrichoptera) larvae are used to build both intricate protectiveshelters and food harvesting nets underwater. In this study, weuse 13C and 31P solid-state NMR and wide angle X-raydiffraction (WAXD) as tools to elucidate molecular proteinstructure of caddisfly larval silk from the species Hesperophylaxconsimilis. Caddisfly larval silk is a fibroin protein basedbiopolymer containing mostly repetitive amino acid motifs.NMR and X-ray results provide strong supporting evidence fora structural model in which phosphorylated serine repeats (pSX)4 complex with divalent cations Ca2+ and Mg2+ to form rigid nanocrystalline β-sheet structures in caddisfly silk. 13C NMR data suggests that both phosphorylated serine and neighboring valine residues exist in a β-sheet conformation while glycine and leucine residues common in GGX repeats likely reside in random coil conformations. Additionally, 31P chemical shift anisotropy (CSA) analysis indicates that the phosphates on phosphoserine residues are doubly ionized, and are charge-stabilized by divalent cations. Positively charged arginine side chains also likely play a role in charge stabilization. Finally, WAXD results finds that the silk is at least 7–8% crystalline, with β-sheet interplane spacings of 3.7 and 4.5 Å.

6. Abstract: Flagelliform spider silk is the most extensible silk fiber produced by orb weaver spiders, though not as strong as the dragline silk of the spider. The motifs found in the core of the *Nephila clavipes* flagelliform Flag protein are: GGX, spacer, and GPGGX. Flag does not contain the polyalanine

motif known to provide the strength of dragline silk. To investigate the source of flagelliform fiber strength, four recombinant proteins were produced containing variations of the three core motifs of the *Nephila clavipes* flagelliform Flag protein that produces this type of fiber. The as-spun fibers were processed in 80% aqueous isopropanol using a standardized process for all four fiber types, which produced improved mechanical properties. Mechanical testing of the recombinant proteins determined that the GGX motif contributes extensibility and the spacer motif contributes strength to the recombinant fibers. Recombinant protein fibers containing the spacer motif were stronger than the proteins constructed without the spacer that contained only the GGX motif or the combination of the GGX and GPGGX motifs. The mechanical and structural X-ray diffraction analysis of the recombinant fibers provide data that suggests a functional role of the spacer motif that produces tensile strength though the spacer motif is not clearly defined structurally. These results indicate that the spacer is a primary contributor of strength with the GGX motif supplying mobility in the native *N. clavipes* Flag protein fibers of flagelliform silk fibers.



7. Abstract: Spider silk is a biomaterial with incredible mechanical properties, and as a result, there are various potential applications for it. Recent research has focused on producing synthetic spider silk fibers with the same impressive mechanical properties as the native fibers. For this study, three proteins based on the *Argiope aurantia* Major ampullate Spidroin 2 (MaSp2) consensus repeat sequence were expressed, purified and spun into fibers. A number of post-spin draw conditions were tested to determine the effect of each condition on the mechanical properties of the fiber. In all cases, post-spin stretching improved the mechanical properties of the fibers. Aqueous isopropanol was the most effective solution for increasing extensibility, while other solutions worked best for each fiber type for increasing tensile strength. The strain values of the stretched fibers correlated with the amount of β-spiral protein sequence. Structural analysis, including X-ray diffraction and Raman spectroscopy, surprisingly showed little change in the initial as-spun fibers compared with the post-spin stretched fibers in contrast to other studies.

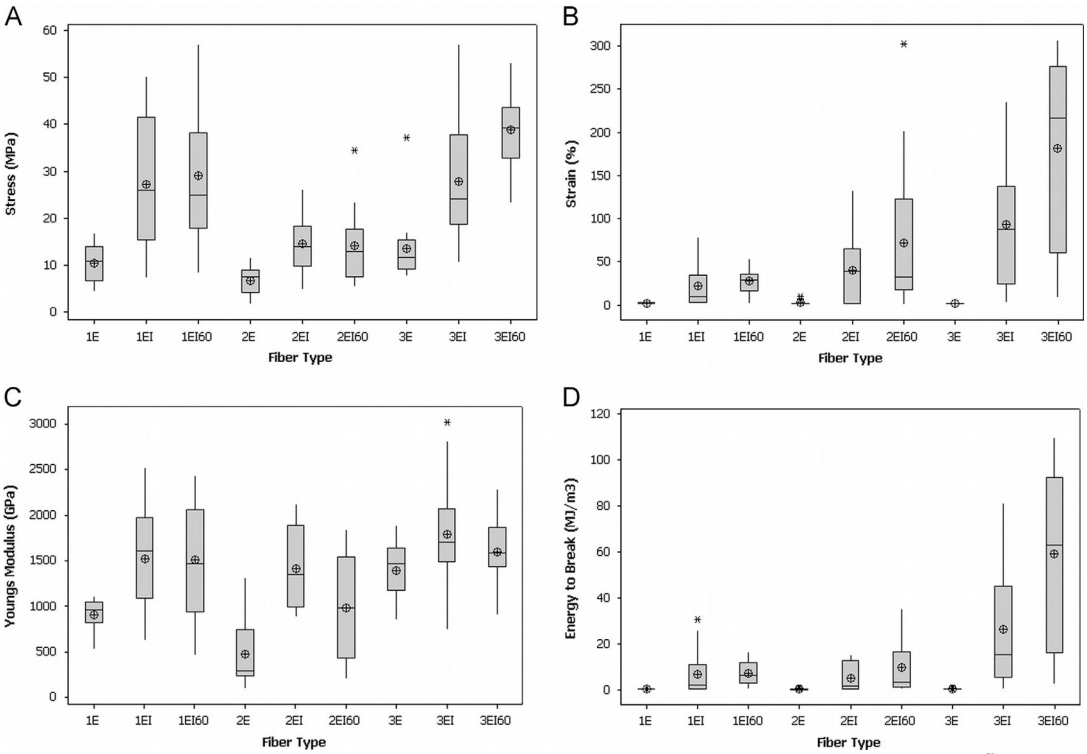


Fig. 3 – _Average mechanicalpropertiesforas-spun,IPApost-spinstretchedandIPAat60 1C post-spinstretched fibers. Boxplots showtherangeofvaluesofall fibers withinatreatmentgroupforstress(A),strain(B),Young'smodulus(C)and energy tobreak(D).Theboxesshowthemiddle50%ofthedataalsocalledtheinterquartilerange.Thewhiskersextending from thetopandbottomofeachboxshowtherestofthedata.Anydatapointfartherthan1.5timesawayfromthe interquartilerangewasconsideredanoutlieranddenotedwithanasterisk.Thecirclewithcrosshairsindicatesthemean and thehorizontallineacrosseachboxrepresentsthemedian.Eachtreatmentgrouphad nZ14.

7. Papers

1. Combining Flagelliform and Dragline Spider Silk Motifs to Produce Tunable Synthetic Biopolymer Fibers

Florence Teule´,[1] Bennett Addison,[2] Alyssa R. Cooper,[1] Joel Ayon,[2] Robert W. Henning,[3] Chris J. Benmore,[4,5] Gregory P. Holland,[2] Jeffery L. Yarger,[2,5] Randolph V. Lewis[1]

Biopolymers 97 (6): 419-431 (2012)

2. Total X-Ray Scattering of Spider Dragline Silk

C.J. Benmore, T. Izdebski and J.L. Yarger

PHYSICAL REVIEW LETTERS 108, 178102 (2012)

3. Reproducing Natural Spider Silks' Co-Polymer Behavior in Synthetic Silk Mimics

Bo An, Janelle E. Jenkins, Sujatha Sampath, Gregory P. Holland, Mike Hinman, Jeffery L.Yarger and Randolph Lewis,

Biomacromolecules *13* (12), 3938–3948 (2012)

4. X-ray diffraction study of nanocrystalline and amorphous structure within major and minor ampullate dragline spider silks

Sujatha Sampath, Thomas Isdebski, Janelle E. Jenkins, Joel V. Ayon, Robert W. Henning, Joseph P. R. O. Orgel, Olga Antipoa and Jeffery L. Yarger

Soft Matter 8, 2713-2722 (2012

5. β-Sheet Nanocrystalline Domains Formed from Phosphorylated Serine-Rich Motifs in Caddisfly Larval Silk: A Solid State NMR and XRD Study
J. Bennett Addison, Nicholas N. Ashton, Warner S. Weber, Russell J. Stewart, Gregory P. Holland, and Jeffery L. Yarger
Biomacromolecules 14, 1140−1148 (2013)

6. *Nephila clavipes* Flagelliform Silk-like GGX Motifs Contribute to Extensibility and Spacer Motifs Contribute to Strength in Synthetic Spider Silk Fibers
Sherry L. Adrianos, Florence Teulé, Michael B. Hinman, Justin A. Jones, Warner S. Weber, Jeffery L. Yarger, Randolph V. Lewis
Biomacromolecules 14: 1751-1760 2013 (2013)

7. Effects of different post-spin stretching conditions on the mechanical properties of synthetic spider silk fibers
Amy E. Albertson, Florence Teulé, Warner Weber, Jeffery L. Yarger, Randolph V. Lewis,
(in press) Journal of the Mechanical Behavior of Biomedical Materials

To be submitted: We expect to submit an additional 2 papers based on data collected during this grant.

8. Personnel:
Yr. 1: Bo An, graduate student (25% till May when he graduated),Yang Liu (100% starting this summer) and Florence Teule, research scientist (50%) and three other graduate students supported on other funds (materials and beam time).
Yr. 2: Sherry Adrianos, graduate student, and Florence Teule, research scientist (50%) and three other graduate students supported on other funds (materials and beam time).
Yr. 3: Sherry Adrianos (graduate student who finished her PhD this summer) and Mike Hinman, research scientist (50%) and three other graduate students supported on other funds (materials and beam time).

9. Other Support: None of these overlap but all will use the methods developed by this grant.

NSF, Collaborative Research: Biomimetic Nanostructured Materials Based on Synthetic Spider Silk, 2013-2016, (co-PI). This grant is to use electro-spinning to generate nano-scale fibers from spider silk proteins.
NSF, PFI to Develop Spider Silk Products, 2013-2015, (PI). This grant is to collaborate with 3 companies to help them develop products based on spider silk fibers and films.
ONR, Adhesive Spider Silks for Naval Applications, 2013-2015 (PI). This grant is to assess the use of bacterially expressed spider piriform silk fibers for adhesive uses.

10. Cost Status:
$600,000 was awarded but the final $15,000 payment was not made so the actual funds were $585,000. The matching was all covered combined at USU and UW.