E. COLI DERIVED SPIDER SILK MASP 1 AND MASP 2 PROTEINS AS CARBON FIBER PRECURSORS

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Utah State University
June 7, 2016

Project ID LM103

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Overview

Timeline
Start date: November 1, 2014
End date: October 30, 2016
Percent complete: 70%

Budget
• Total project funding
  – DOE: $1,490,744
  – Contractor share: $497,298
• Funding FY 2015: $997,758
• Funding FY 2016: $990,284

Barriers
□ 2.5.1. Lightweight Materials Technology (VTP MYPP 2011-2015)
  □ Performance: Match carbon fiber using spider silk instead of PAN

Partners
• U. of California, Riverside
• Oak Ridge Nat’l Laboratory
• Utah State University

Timeline
Barriers
Partners
# Relevance

## Overall Project Objective
Reduce the weight of vehicles thereby reducing greenhouse gas emissions and the dependence on foreign oil through the use of carbon fibers produced from spider silk protein fibers.

## Project Goals
- Maximize protein production via *E. coli* while maintaining full-length protein
- Develop a Scalable Fiber Spinning process
- Improve spider silk fiber mechanical properties
- Generate transgenic silkworms producing silk with much higher strength
- Determine optimal stabilization conditions for spider silk protein fibers for conversion to carbon fibers
- Conduct techno-economic analyses to estimate costs
### Milestones

**Recipient Name:** Randolph V. Lewis, Utah State University  
**Project Title:** Spider Silk MaSp1 and MaSp2 Proteins as Carbon Fiber Precursors

<table>
<thead>
<tr>
<th>Task #</th>
<th>Task Title</th>
<th>Milestone type</th>
<th>Milestone number</th>
<th>Milestone description</th>
<th>Milestone verification</th>
<th>Percent Completion</th>
<th>Expected Quarter</th>
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<tr>
<td>1</td>
<td>Fiber production</td>
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<td>Micro-structure</td>
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<td>Validation of sub models</td>
<td>Experimental Verification</td>
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<td>Q8</td>
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</table>
Approach/Strategy

- Create spider silk fibers with tensile strength of >750 MPa (Go/No Go with intermediate milestones) **Achieved Q4**

- Convert spider silk fibers to stabilized carbon fibers (Go/No GO, Q7 with intermediate milestones)

- Techno-economic analysis of estimated production costs (Final milestone Q8, with intermediate milestones)
Lego
Technical Accomplishments and Progress

- Create spider silk fibers with tensile strength of >750 Mpa

<table>
<thead>
<tr>
<th>Spools of bacterially produced spider silk protein, 350m of 8-fiber thread.</th>
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</table>

<table>
<thead>
<tr>
<th>As Spun</th>
<th>1.5x MeOH / 1.5x H2O</th>
<th>2x MeOH / 2x H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Max Stress (MPa)</td>
<td>14.3</td>
<td>164.1</td>
</tr>
<tr>
<td>Max Strain (%)</td>
<td>1.5</td>
<td>66</td>
</tr>
<tr>
<td>Toughness (MJ/m²)</td>
<td>0.092</td>
<td>92.3</td>
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<tr>
<td>Orientation Factor</td>
<td>NA</td>
<td>0.502</td>
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</table>

Post spin stretch of spider silk protein fibers with corresponding X-ray diffraction patterns showing increases crystallinity and orientation.

Electrospun spider silk protein fibers ranging from 100-350nm with corresponding tensile strengths and elongations. Note the non-linear behavior of both properties.

Comparison of natural and transgenic silkworm cocoons under UV light so the fluorescent probe attached to the spider silk protein can be used to identify the transgenic silkworms.

Stress-strain curves for control and transgenic silkworm silk. The samples are the same as described above in the table above. Note both the similar shapes and values for the different transgenic silkworm lines which is very similar to the variation in the controls.
Technical Accomplishments and Progress

- Convert spider silk fibers to stabilized carbon fibers (Go/No GO, Q7 with intermediate milestones)

**Conversion and Mechanical properties:**
Successful carbonization for both types of silk fibers

Feb. 2016 → first successful carbonization of “M4 fiber” (several carbon fibers have been produced since then)
March 2016 → first carbonization of “2015 fiber”

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Data type</th>
<th>Diameter (µm)</th>
<th>Peak Stress (ksi)</th>
<th>Modulus (mpsi)</th>
<th>Strain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M4 Prec</td>
<td>Mono filament</td>
<td>28.42 (1.18)</td>
<td>24.6 (1.7)</td>
<td>1.1 (0.0)</td>
<td>17.73 (6.17)</td>
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<tr>
<td>Fib. 2015</td>
<td>Bundle: raw data</td>
<td>50.55 (18.64)</td>
<td>14.8 (14.6)</td>
<td>4.1 (3.6)</td>
<td>0.34 (5.68)</td>
</tr>
<tr>
<td>M4 0006</td>
<td>Bundle: raw data</td>
<td>52.5 (8.4)</td>
<td>23.5 (9.6)</td>
<td>0.7 (0.8)</td>
<td>2.40 (1.14)</td>
</tr>
<tr>
<td>M4 0009</td>
<td>Bundle: C5 reassessed*</td>
<td>25.46 (0.09)</td>
<td>20.7 (20.7)</td>
<td>0.9 (0.9)</td>
<td>1.39 (0.59)</td>
</tr>
<tr>
<td>M4 0010</td>
<td>Bundle: C5 reassessed*</td>
<td>25.46 (0.09)</td>
<td>20.7 (20.7)</td>
<td>0.9 (0.9)</td>
<td>1.39 (0.59)</td>
</tr>
<tr>
<td>M4 0011</td>
<td>Bundle: raw data</td>
<td>54.8 (11.1)</td>
<td>25.9 (12.3)</td>
<td>0.8 (0.5)</td>
<td>4.49 (1.66)</td>
</tr>
<tr>
<td>M4 0012</td>
<td>Bundle: C5 reassessed*</td>
<td>25.46 (0.09)</td>
<td>20.7 (20.7)</td>
<td>0.9 (0.9)</td>
<td>1.39 (0.59)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>87.8 (4.6)</td>
<td>33.1 (7.0)</td>
<td>0.6 (0.1)</td>
<td>5.27 (1.59)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>111.1 (6.8)</td>
<td>33.1 (7.0)</td>
<td>0.6 (0.1)</td>
<td>5.27 (1.59)</td>
</tr>
</tbody>
</table>

Properties of precursor and carbon fiber:
- Average fiber diameter is around 9 µm (this value is not firm)*
- Carbon fibers from M4 potential of 100 ksi tensile strength
- Elastic Modulus of 4.1 Misi has been achieved on initial trials.
- Fused filaments have been observed. Common issue in fiber process. Solution: silicon based finishing application on precursor

* Cross Section (CS) of single fiber are measured from SEM images ≤ 9 µm. In calculations of bundle surface area, single fiber diameter assumed to be 9 µm and bundles consist of 8 fused filaments.

Stretch Study of Fiber 2015 and Fiber M4 (Jan 2016)

- M4 showed higher yield than previous fiber
- Stretching window has been identified between $T_y$ and $T_f$ for both materials at the beginning of the process
- New precursor Fiber M4 has shown better stretch characteristics during process. (This potentially leads to higher mechanical performance)
  - Fiber 2015 : Max 4.6%
  - Fiber M4 (2016) : Max 25.6%

→ M4 Fibers can be stretched typically up to 20% during process (carbonized batched obtained on Apr 5th, 2016)

**Crosslinking agents and suggested synthetic pathways for obtaining crosslinking of the MaSp fibers are selected based on the type and concentration of reactive groups.** (acidic, basic and hydroxy groups) present in MaSp1&2 molecular chains.

**Potential Crosslinking chemicals:**
- N-Hydroxysuccinimide (NHS) ester, Resorcinol diglycidyl ether (Di-epoxides)
- Carboxylate moiety reacting with -COOH
- Hydroxy reacting with -OH
- 4,4-methylene bis phenyl disocyanate (MDI)
- Resorcinol Diglycidyl Ether
- 2-heptabutyldihydrazide
- 4,4’-methylene bis phenyl disocyanate

DOE June 7, 2016
Technical Accomplishments and Progress

• Techno-economic analysis of estimated production costs (Final milestone Q8, with intermediate milestones)

Mass Balances to Create System Models for Analysis

Process System Model

- Protein Production
- Traditional Media Fermentation
- Heat Induction
- Minimal Media
- Glycerol Based Media

Foundational Inputs

- Centrifuge & Homogenize
- Affinity Chromatography
- Flocculation
- Filtration & Washing
- Lyophilization
- Spray Dry
- Drying
- Extrusion & Stretching

Critical Feedback

- TEA/LCA

Process Optimization & the Economic Benefits

- Pilot Plant
- Add Heat Induction
- Add Glycerol Alternative
- Add Minimal Media
- Add Flocculation 2.5 g/l of Protein

- Price $ kg⁻¹

- Costs are driven most significantly by the material consumption, specifically by purification
- Research is being focused towards reducing material consumption
- Process optimization can significantly reduce the cost of production

Sale Price Estimate as a Function of Protein Expression

- Protein yield has the most direct impact on sale price
- Increasing yield has little effect on cost, but significantly reduces the sale price estimate
- Other high yielding recombinant operations have yields above 20 g/l

DOE June 7, 2016
Response to Previous Year Reviewer’s Comments

This project was not presented at the 2015 Annual Merit Review.
### Partners and Collaborators

- **Dr. Cheryl Hayashi, U. of California, Riverside, co-PI.**
  Gene sequences and comparisons for spider silk protein gene choices to produce.

- **Drs. Soydan Ozcan and Felix L. Paulauskas, ORNL co-PIs.**
  Spider silk fiber conversion to carbon fiber and analyses of those fibers.

- **Dr. Jeff Yarger, Arizona State University, collaborator.**
  NMR, Raman and X-ray diffraction.

- **Argonne National Laboratory, facilities.**
  X-ray diffraction facility
Remaining Challenges and Barriers

- Convert spider silk fibers to stabilized carbon fibers (Go/No Go, Q7)
- Further improve the strength of the spider silk fibers
- Increase spider silk protein production to drive costs down
Proposed Future Work

Based on the three remaining challenges the following is the future work.

• Convert spider silk fibers to stabilized carbon fibers (Go/No GO, Q7)
  o Optimize the oxidation process with regard to the temperature ramping, final temperature and time of heating.
  o Test crosslinking agents to better stabilize the proteins.
  o Use different spider silk proteins with higher carbon content.

• Further improve the strength of the spider silk fibers
  o Introduce the multi-fiber spinning head (24 fibers).
  o Determine the effects of photo-crosslinking of the proteins during spinning.
  o Improve spinning conditions via additives as well altering spinning physical conditions
  o Breed top silkworms and induce partial knockout of silkworm silk gene

• Increase spider silk protein production to drive costs down
  o Generate higher cell densities by optimizing carbon feed rate
  o Use higher induction levels to increase protein production/ unit of bacteria
  o Add additional antibiotic at induction to prevent loss of resistance
Summary

• Maximize protein production via *E. coli* while maintaining full-length protein
  o Protein production has gone from 0.5g/L to as high as 4.0 g/L
  o Purification process developed with 17-fold lower costs

• Develop a Scalable Fiber Spinning process
  o Up to 1000m of 8 fiber thread has been spun
  o Moving to a 24 fiber thread spinning head

• Improve spider silk fiber mechanical properties
  o Improved from 200 MPa to over 400 MPa

• Generate transgenic silkworms producing silk with much higher strength
  o Improved from 600 MPa to over 900 MPa with stable transmission

• Determine optimal stabilization conditions for spider silk protein fibers for conversion to carbon fibers
  o In process

• Conduct techno-economic analyses to estimate costs
  o Nearly complete for the fiber production prior to conversion to carbon fibers
## Special Mechanical Properties of Spider Silks

<table>
<thead>
<tr>
<th>Material</th>
<th>Strength (MPa)</th>
<th>Strain (%)</th>
<th>Toughness (KJ/kg)</th>
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</thead>
<tbody>
<tr>
<td>Dragline silk</td>
<td>4000</td>
<td>35</td>
<td>400</td>
</tr>
<tr>
<td>Minor Ampullate silk</td>
<td>1000</td>
<td>5</td>
<td>30</td>
</tr>
<tr>
<td>Flagelliform</td>
<td>1000</td>
<td>&gt;200</td>
<td>400</td>
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<tr>
<td>Tubiliform silk</td>
<td>1000</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td><em>Bombyx mori</em> silk</td>
<td>600</td>
<td>20</td>
<td>60</td>
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</table>

aData from Gosline, Lewis, Altman
## Production Methods

<table>
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<tr>
<th>System</th>
<th>Protein Yield per Year</th>
<th>Production Time</th>
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</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>12 kg per run</td>
<td>2-4 months</td>
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<tr>
<td>Goats</td>
<td>18 kg per goat</td>
<td>1-2 years</td>
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<tr>
<td>Alfalfa</td>
<td>218 kg per acre</td>
<td>4-5 years</td>
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<tr>
<td>Silkworm</td>
<td>??</td>
<td>2 years</td>
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