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**Cell-Free Synthetic Biology & Biofabrication for Novel Silk Biomaterials  
Development**

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**06/25/2023  
Final Technical Report**

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# **AFOSR Final Report**

Award Number: FA2386-20-1-4079

Project Title: Cell-Free Synthetic Biology & Biofabrication for  
Novel Silk Biomaterials Development

PI: Hsuan-Chen Wu

Institution: National Taiwan University, Taiwan

Period of Performance: 09/30/2020~09/29/2021

AOARD Program Officer: Dr. Jeremy Knopp

# Section I: Basic Information

## Award Information

<b>Broad Agency Announcement</b>	FA9550-19-S-0003, Air Force Office of Scientific Research
<b>Award Number</b>	FA2386-20-1-4079
<b>Proposal Title</b>	Cell-Free Synthetic Biology & Biofabrication for Novel Silk Biomaterials Development
<b>Proposer Organizations</b>	National Taiwan University, Taiwan
<b>Principal Investigator; Technical Points of Contact (POC)</b>	Hsuan-Chen Wu, Ph.D. (Associate Professor) Department of Biochemical Science and Technology, National Taiwan University Telephone: 1-886-2-3366-4524 Email: hcwu7@ntu.edu.tw
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<b>Collaborators</b>	Ta-I Yang, Ph.D. Department of Chemical Engineering, Chung Yuan University, Taiwan
<b>Total Proposed Cost</b>	\$USD 100,000
<b>Period of Performance</b>	30 Sep 2020 to 29 Sep 2021
<b>Report Due Date</b>	28 Dec 2021

## Publications

1. Peer-reviewed article: Cell-free Synthetic biology towards recombinant spider silk production (Research in progress and manuscript in preparation; AOARD grant support will be acknowledged)
  - Established our proprietary cell-free protein expression platform (low cost and high efficiency)
  - Sequenced and constructed novel spider silk materials from golden orb-weaver *Nephila pilipes*
  - Probing the supramolecular interactions of multi-spider silk materials via the established cell-free system
2. Peer-reviewed article: Self-healable spider silk (Research in progress and manuscript in preparation; AOARD grant support will be acknowledged)
  - Discovered the novel characteristic of spider silk materials: repairability
  - Deciphering the molecular mechanisms for spider silk's self-healability

- Bioengineering of recombinant healable spider silk materials for application

## Patents and Technologies

1. Patent: Healable Silk Materials (In preparation)
  - First documented self-healability of spider silk materials
  - In the process of prior-art search for evaluating the patentability and claims drafting
2. Technologies: The world-leading technology for rapid (from cell-lysis to lyophilization) and low-cost recombinant spider silk materials purification

## Participants

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## Collaborators

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# Section 2: Final Report

## Abstract

Developing novel materials with tailored properties/functionalities remains a grand challenge. In nature, biology has offered enormous illustrations on precision molecular design and self-assembly that are incomparable to the conventional top-down design scheme. An emerging branch of research discipline to recapitulate or adapt the bioinspired material design and fabrication is called biomimicry. To succeed in biomimetic material fabrication, synthetic biology that synergizes engineering and biology has come to play a pivotal role. Synthetic biology (SynBio) enables programming of biological matters, such as proteins or polymers, in a fashion that “materials by design” can be readily achieved and render novel and yet desirable material properties through a bottom-up fabrication process. Our broad goal is to innovate the design, synthesis, and fabrication regimes of creating advanced biomaterials via the power of synthetic biology, which, in turn, could drastically change the landscape of developing future bioinspired materials. Taking the advantage of spider silk, the ultimate performance biomaterial, as our blueprint scaffold for engineering the next generation of silk materials. First, machine aided informatic analysis of silk gland transcriptomics (NGS) was established to offer entirely different perspectives in molecular design and functionality prediction. Second, the cell-free synthetic biology platform was performed in a facile and configurable setup (*in vitro*). This, in turn, achieved high content recombinant silk synthesis and *in-situ* probing of supra-hierarchical interactions and self-assembly of multi-spidroins. Last, the environmental adaptability of the novel silk materials is currently under evaluation. We envision the strategic platforms, once optimized, will not only expediate the pace for discovering novel biomaterials but also broaden the utility of synthetic biology in translational materials development. That can, in turn, lead to ultimate materials manufacturing in a sustainable and renewable manner.

Keywords: Synthetic biology, spider silk, cell-free expression, healing

## Accomplishments

### ➤ Overall Objectives

The overall goal is to integrate bioengineering and bioinformatics to facilitate establishing the “design-build-test-learn” working model towards high-throughput facile design and synthesis of intelligent and novel silk biomaterials with structural programmability — self-repairing.

### ➤ Approaches

Bioinformatic tools: Next generation sequencing (NGS; second and third generations) with Sanger sequencing (first generation) to identify the native spider silk materials as the blueprints.

Computational algorithm (AI): Mapping of structural motifs and prediction of secondary structures from the newly identified spider silk genes.

Experimental tools: Synthetic biology and bio-industrial fermentation (5~20L) for molecular

construction and synthesizing functional spider silk materials; biomimetic purification and fabrication for efficient spider silk isolation and downstream material manufacturing at both lab and pilot scales.

## ➤ **Major Progresses and Results**

### (1) Identification and construction of novel spider silk gene sequences

In this part of research, we aim to explore and engineered novel spider silk materials. As a preliminary result, a novel N-terminal domain (NTD) of major ampullate spidroin (MaSp) silk proteins from *Nephila pilipes* was been successfully sequenced and identified (**Fig. 1**). Additionally, several new variants of MaSp were also identified. Consequently, we incorporated both the novel NTD motif into our library of silk backbones, including MaSp1 (high crystallinity), and MaSp2 (elasticity), MaSp3 (the major spidroin in dragline spider silk), and MaSp4. More efforts in sequencing and gene identification are currently underway and we anticipate, creating the sufficient collection of novel spider silk genes among varied species of spiders. This can, in turn, be serving as our proprietary big data collection for building up the machine learning algorithm/model to create future performance/functional biomaterials.

### (2) Building up in vitro protein production system via cell-free synthetic biology

The specific goal is to decipher the *in situ* of multi-monomeric interaction upon spidroin synthesis. Currently, there is lacking an available open system for probing the detailed molecular mechanism. Herein, we have successfully created an innovate platform, enabling the *in vitro* silk spidroin synthesis in the open configuration. In particular, a proprietary abiotic (cell-free) expression system has been established (**Fig. 2**). In this part of research, the cell-free system we created was able to carry out the biosynthesis of biomolecules, fluorescence proteins and silk spidroins, at a comparable productivity (~1mg/ml) to commercial kits yet with lower production cost (~1/5 based on our current estimation). Furthermore, our preliminary cell-free results suggested that spidroins were able to form multi-spidroin assembly, yielding micelle like structures (~200-800 nm at various synthesis conditions). This has been the first direct scientific evidence about the self-assembly so far. We believe this can not only reveal the secrets of molecular mechanism residing in spider silk materials and offer a perfect biomimetic template for advancing bioengineered biomaterials.

### (3) Fermentation and purification of multi-spidroin materials

Building upon the preliminary success of the cell-free system on spidroin synthesis and characterization, we further established the fermentation protocol and a unique spidroin purification procedure. The novel purification technology didn't involve any affinity, heat, and dialysis process. Additionally, the facile purification span (from cell-lysis to final purified silk protein) was within half day (3-5 hours) which is 2~5 fold more efficient than other commercial isolation protocols on the market. Our proprietary purification process awaits further optimization, yet the purity of the isolated silk materials could reach 90% based on PAGE analysis. Additionally, we are also currently developing the scale-up and streamlined version of our purification protocol in respond to future mass production.

### (4) Fabrication and functionality evaluation of recombinant silk materials

Besides the breakthrough of upstream production and purification, we have also primarily succeeded in manufacturing recombinant spider silk materials, biomimetic silk fiber spinning and scaffold fabrication. In particular, we have adopted the native spider silk spinning process, pH and ion assisted silk reorganization, to achieve the artificial spinning of our purified silk materials. In **Fig. X** the spun silk fibers could exhibit comparable toughness (energy absorbance capacity) to the native ones ( $180 \pm 8 \text{ MJ/m}^3$ ), which surpasses the body armor material Kevlar ( $\sim 50 \text{ MJ/m}^3$ ). More advanced biomimetic spinning processes are currently underway towards the ultimate silk fibers spinning and fabrication.

Last but not the least, we have also bioengineered our recombinant silk proteins as healable materials. Specifically, the silk material was cast into thin films and the resulting films were adhered to each other at the contact surface, with the supplementation of water and stress. The healed films were also to withstand the force upon stretching by the tensile tester. The location of the fracture of the films was constantly witnessed not at the pre-healed areas. In other words, the interfacial contact between the two healed films remained intact upon external disturbance. We believe the engineered silk materials, when properly tuned, can be served as an advanced repairable system with vast future potential in medical and industrial applications.

## Impacts

Perfectly aligned with materials genome initiative (MGI), we harness a biomimetic strategy toward advancing biomaterials discovery, design, and fabrication. Both computational and experimental methodologies are employed to achieve the grand goal. We envision such interdisciplinary work efforts synergistically expediate innovating silk biomaterials with unique characteristics and properties.

In brief, our preliminary success, such as the generation of novel spider silk materials with ultra-performance and unique healability, has led us to pursuing the next-phase research as well as application. We envision the advanced self-repairable silk biomaterials could bring significant benefits to the society in regard to wearable electronics & sensors, smart fabrics, functional coating, soft robotics, regenerative medicine, protective gears, and military equipment. Also, we anticipate our biological based production and fabrication approaches are aligned with the global trend toward green manufacturing and renewable economy per se.

# Technical Updates

## 1. Next generation sequencing of *Nephila pilipes* for silk gene collection (big data) and de novo construction of silk materials

- Generation of big data of spider silk materials
- Identification of novel silk sequences
- Construction for recombinant spider silk materials

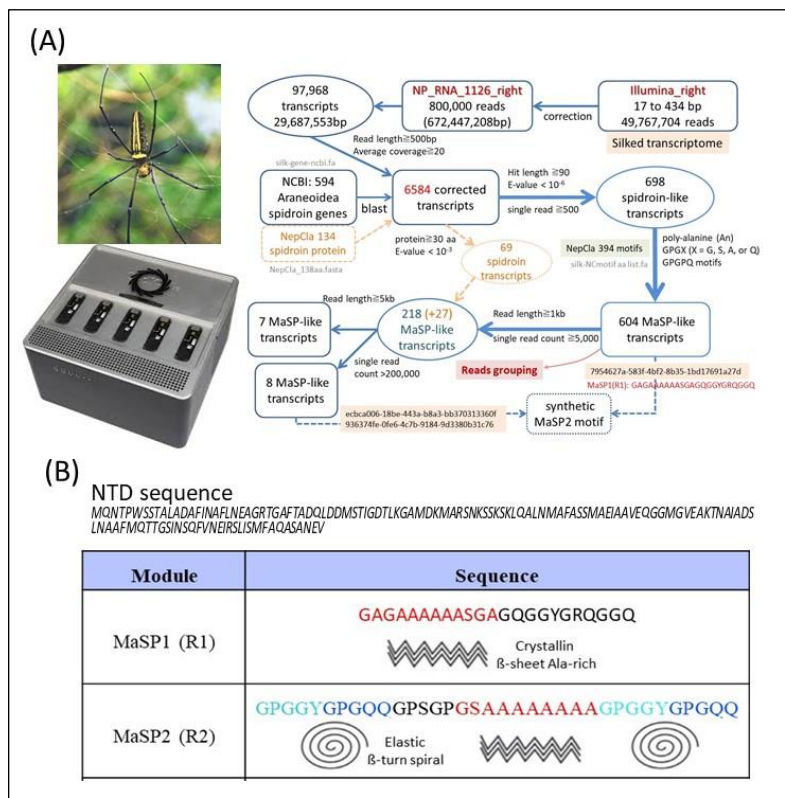


Figure 1. Data mining of spider silk genes from *Nephila pilipes*. (A) MaSp gland extraction and NGS flow chat for silk gene candidate identification. (B) Identification of novel silk genes and building blocks construction.

2. The establishment of cell-free protein expression systems for spider silk spidroin production

- Proprietary cell-free expression platform with low reaction cost and high efficiency
- A pioneering approach for *in vitro* spider silk synthesis (no scientific documentation)
- An innovation in deciphering *in situ* hierarchical assembly/interaction of spidroins

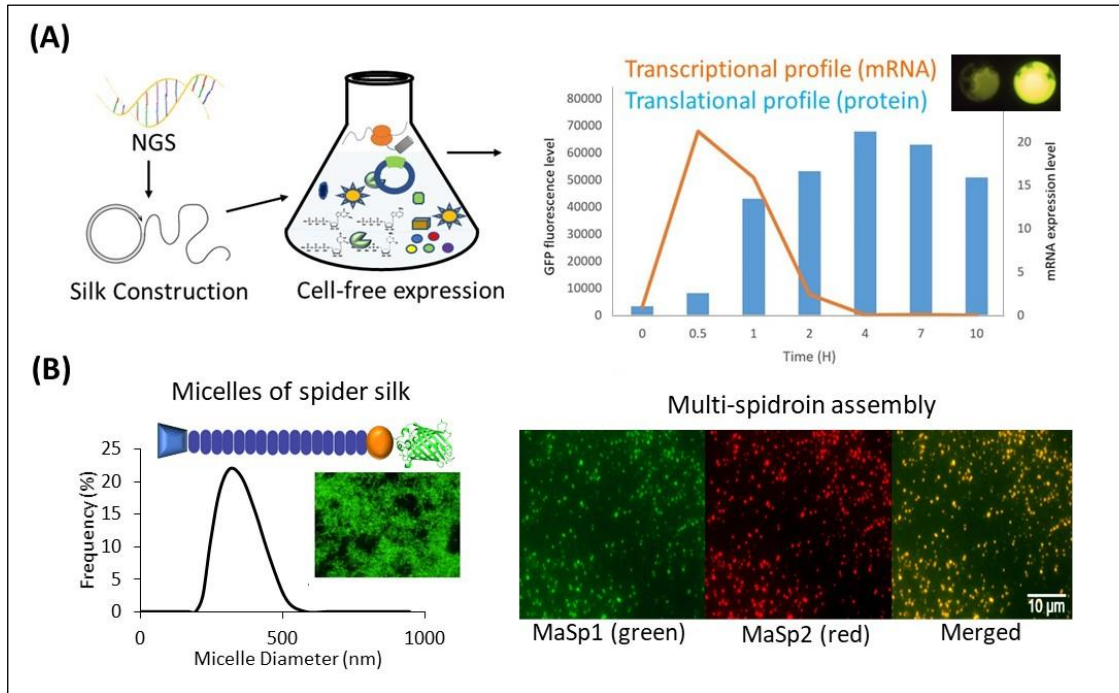


Figure 2. Cell free synthetic biology in spider silk spidroin production. (A) The schematic representation for *in vitro* transcription and translation procedures: Novel spidroin sequences from NGS, silk amplification/cloning, and synthesis in a test-tube. Evaluation and characterization of the cell-free system using green fluorescence protein (GFP) at the transcriptional and translational levels. (B) Cell-free production of spider silk spidroins and micellar formation upon synthesis. Multi-spidroin interaction/colocalization was witnessed in the co-synthesis experiment (MaSp1-GFP + MaSp2-mCherry).

### 3. Fermentation and advanced purification protocol of spidroins

- 5~20 L scale bioreactor production of spider silk spidroin
- Innovation in micellar like spidroin purification: tag-free, low cost, and high efficiency
- Biomimetic spinning and fabrication of silk materials
- Superior toughness of the recombinant spider silk fibers (180 MJ/m<sup>3</sup>; matching to the native ones)

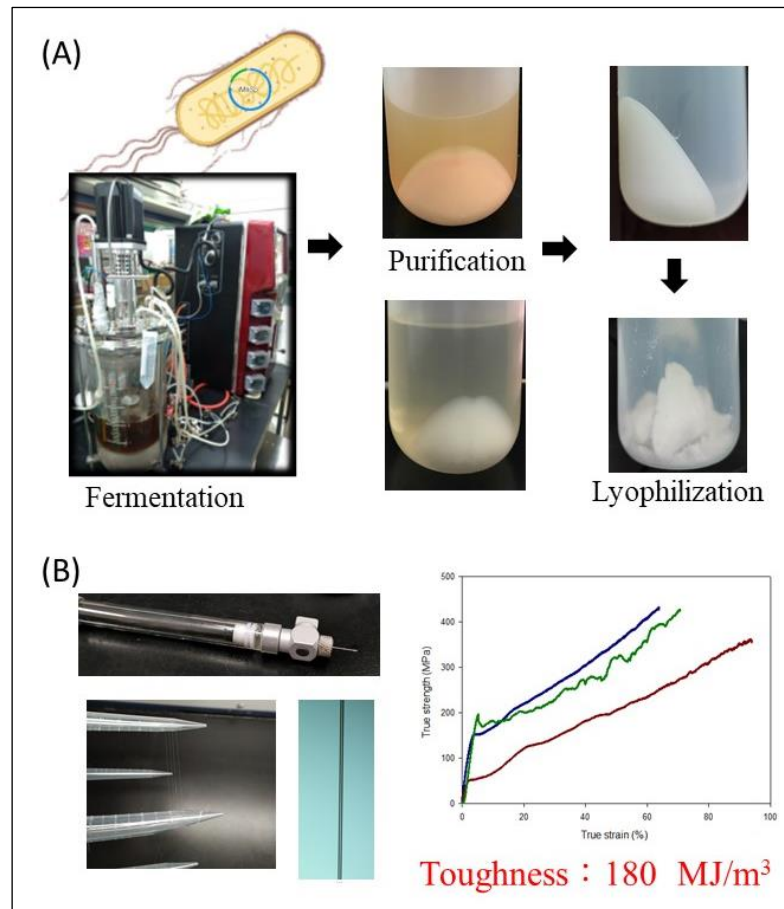


Figure 3. Production, purification and fabrication of recombinant spider silk materials. (A) Spider silk production by fermentation. An innovative tag-free and facile purification process was developed to expediate the downstream silk material manufacturing. (B) Wet spinning of recombinant spider silk spidroin, yielding strong and elastic artificial silk fibers (comparable toughness to the native spider silk fibers)

#### 4. Fabrication of healable spider silk materials

- Repairability as a novel feature of recombinant spider silk materials
- Bioengineering the future performance biomaterial

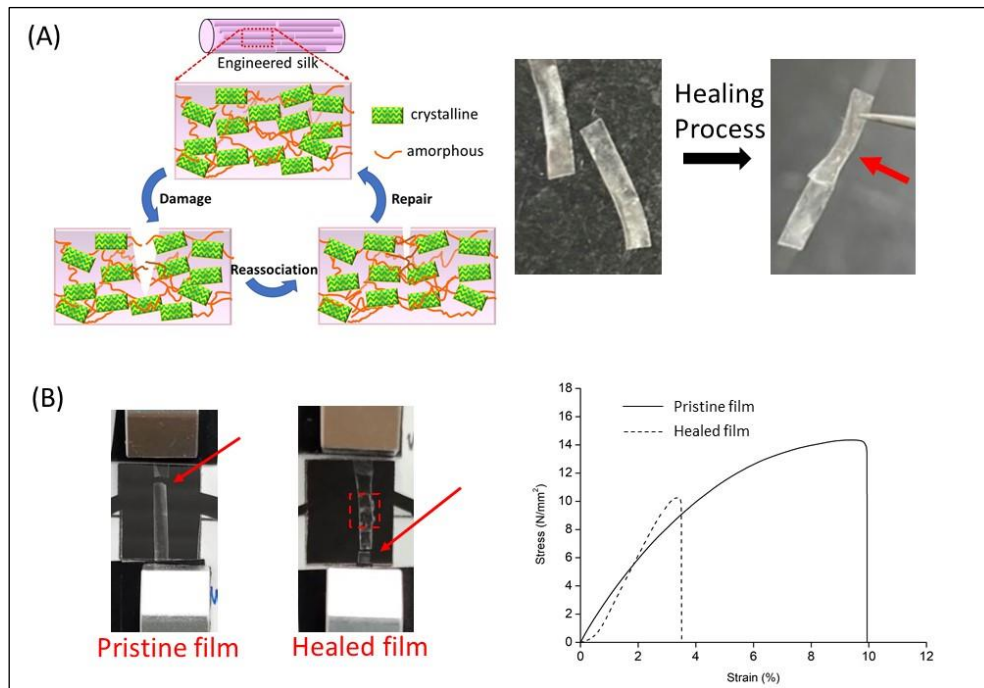


Figure 4. Manufacturing and characterization of repairable recombinant spider silk materials. (A) Schematic of the proposed molecular hypothesis/mechanism on healable recombinant spider silk materials. Two separate recombinant spider silk films were bond together after the healing process. (B) Mechanical/tensile test on the healed spider silk films (compared to the pristine films). The healed region remained intact after the tensile challenge, indicating notable healing capability of the engineered spider silk materials.