# Aqueous Processing and Fiber Spinning of Recombinant Spider Silks

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*Received August 15, 2001*

ABSTRACT: Spiders have captured the interest of scientists for many years because spider silks are among the toughest materials, having properties that surpass some man-made synthetic materials. Spinning recombinant silk to duplicate those properties has proved to be extremely difficult. This is the first known report of spinning recombinant silk fibers in an aqueous environment. The method seeks to keep the protein soluble throughout the process, not unlike the way the spider stores and spins silk. Recombinant silk proteins were produced by bacterial fermentation in which the cell pellets were lyophilized and lysed with organic acid. Silk protein was purified from the lysate by chromatography and processed in dilute denaturing buffer into a fiber spinning solution. Circular dichroism measurements of the silk solutions revealed an increase in $\beta$-sheet content as a function of time. Time-dependent self-association of silk protein was monitored in solution by dynamic light scattering. Furthermore, the observed increase in $\beta$-sheet content and self-association appear to be required for fiber formation. Recombinant silk fibers were 10−60 $\mu$m in diameter, water insoluble, and birefringent, indicating molecular orientation within the fiber.

## Introduction

Spiders produce up to seven different silks with a variety of functions and diverse properties ranging from the tough dragline to the elastic viscid capture silk. The structural fibers of the golden orb-weaver spider *Nephila clavipes* are extremely strong and flexible, absorbing impact energy from flying insects without breaking. Dragline silk fibers dissipate energy over a broad area and balance stiffness, strength and extensibility. These fibers are three times tougher than aramid fibers and five times stronger by weight than steel. Therefore, silk fibers possess the desirable mechanical properties for lightweight, high-performance fiber, and composite applications. Spiders produce these materials from an aqueous solution under ambient temperature and pressure, in contrast to hazardous solvents and harsh conditions needed to produce aramid fibers. The dragline fiber is predominantly proteineacous, comprised of greater than 80% glycine, alanine, and other short side chain amino acids[1] with crystalline regions of antiparallel $\beta$-pleated sheets.[2] Recently a number of techniques have been used for fiber structure analysis.[3] Molecular orientation within the dragline fiber is optically evident by birefringence.[4,5] Estimates of the crystallinity of the *N. clavipes* dragline fiber range from 10% based on synchrotron diffraction[6] to as high as 50−60% by Raman spectroscopy.[7] Modeling of spider silk fibers predict that formation of small $\beta$ sheet crystallites is an essential component to the high toughness properties of spider silk.[8,9] Simmons et al. determined that these $\beta$ sheet crystals are highly oriented along the axis of the fiber.[10]

Spiders are not capable of producing the quantities of silk necessary for fiber spinning studies. To address this problem recombinant spider silks have been expressed in *Escherichia coli*[11−14] and *Pichia pastoris*[15] in quantities sufficient for laboratory scale experiments. However, the purification and preparation of silk protein for fiber spinning have been particularly difficult due to its solubility characteristics and unique properties. A number of groups have attempted to spin silk protein fibers, most involving *Bombyx* silk[16−18] or regenerated spider silk.[19] Recombinant silk proteins have been spun from hexafluoro-2-propanol[20] or from dilute protein solutions in concentrated formic acid.[13] Here is the first reported spinning of recombinant silk fibers under aqueous conditions in a manner similar to that of the spider. Two recombinant silk proteins, NcDS and $[(SpI)_4/SpII)_1]_4$, were spun into fibers. NcDS is a 42.9 kDa protein from the C-terminus of *N. clavipes* major ampullate silk protein encoded by the NcDS cDNA clone described previously.[11] $[(SpI)_4/SpII)_1]_4$, a 55 kDa hybrid protein, combines consensus sequences from *N. clavipes* spidroin I and II.[14] Recombinant silk fibers from concentrated protein solutions were 10−60 $\mu$m in diameter, water insoluble, and birefringent, indicating molecular orientation within the fibers.

## Experimental Section

The NcDS cDNA was subcloned from pET21 (Novagen Inc., Madison, WI) into pET24 (Novagen) and expressed in BL21-(DE3) pLysS. $[(SpI)_4/SpII)_1]_4$ was cloned into pQE9 (QIAGEN, Valencia, CA) and expressed in SG13009(pREP4) or cloned into pET24 and expressed in BL21(DE3) pLysS.

**Fermentation.** First, 10 mL starter cultures were grown in 4xYT (per L: 32 g of tryptone, 20 g of yeast extract, 5 g of NaCl) with appropriate antibiotics to an $OD_{600} = 1.5-2$ and used to inoculate 5L fermentations. Growth of pQE$[(SpI)_4/SpII)_1]_4$ in SG13009(pREP4) was to an $OD_{600} = 4$ in 4xYT

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10.1021/ma011471o CCC: $22.00 
Published on Web 01/11/2002

**Table 1. Summary of the Preparation of Recombinant Silk Proteins into Spin Dopes[a]**

| protein | MW (Da) | lysis method | purification | purity % | spin buffer composition | spin dope concn (mg/mL) |
|---|---|---|---|---|---|---|
| NcDS | 42 970 | formic/GuCl[b] | Ni affinity | 50−89 | 1 M urea | 200−250 |
| $[(SPI)_4/(SpII)_1]_4$ | 55 731 | propionic/GuCl | Ni affinity | 85 | 160 mM urea | 65−125 |
| $[(SPI)_4/(SpII)_1]_4$ | 55 731 | propionic/GuCl | ion exchange | 42−90 | 160 mM urea, 10 or 100 mM glycine | 100−180 |

[a] All spin buffers contained 10 mM $NaH_2PO_4$, 1 mM Tris, and 20 mM NaCl and were adjusted to pH 5. The NcDS and ion exchange purified $[(SPI)_4/(SpII)_1]_4$ proteins were each spun into fibers a number of times. Only one sample of $[(SPI)_4/(SpII)_1]_4$ was affinity purified, and it was also able to be spun. [b] GuCl = guanidine-Cl.

medium with 100 $\mu$g/mL ampicillin and 25 $\mu$g/mL kanamycin. The pET24 constructs in BL21(DE3) pLysS were grown to an $OD_{600}$ = 15−20 in defined salts medium[21] with 30 $\mu$g/mL kanamycin and 34 $\mu$g/mL chloramphenicol. Expression was induced by addition of IPTG to a final concentration of 1 mM for 1−3 h. Cells were harvested by centrifugation and lyophilized for purification.

**Preparation of Spin Dopes. Lysis.** Lyophilized cell pellets were lysed with organic acid using 2 mL of propionic or 5 mL of formic acid per gram of cells. The mixture was diluted with distilled deionized water and 6 N guanidine hydrochloride to a final concentration of 2.3 N acid and 3 N guanidine hydrochloride. After being stirred for 1 h at ambient temperature, lysates were clarified by centrifugation, and the supernatant was purified by chromatography.

**Affinity Chromatography.** The supernatant was dialyzed extensively into 8 M urea, 50 mM $NaH_2PO_4$, 5 mM Tris, and 100 mM NaCl, at pH 8. Precipitated material was removed by centrifugation, the supernatant applied to a nickel−NTA resin (QIAGEN) at pH 8 washed at pH 7, and the recombinant protein eluted at pH 3. Eluent was dialyzed into 160 mM or 1 M urea containing 10 mM $NaH_2PO_4$, 1 mM Tris, and 20 mM NaCl, at pH 5, clarified by centrifugation and concentrated by ultrafiltration.

**Ion Exchange Chromatography.** The clarified supernatant was dialyzed into 2 M urea and 10 mM Tris, at pH 9.9 and applied to Q Sepharose FF (Amersham Pharmacia Biotech Inc., Piscataway, NJ) at pH 9.9. The flow through was collected and the column washed to recover the silk protein.

**Spin Dopes.** Purified protein solutions were dialyzed into 160 mM or 1 M urea containing 10 mM $NaH_2PO_4$, 1 mM Tris, and 20 mM NaCl, at pH 5. Glycine at a concentration of 10 or 100 mM was added to the ion exchange purified protein. The solution was centrifuged and concentrated by ultrafiltration into spin dopes containing up to 25% (w/v) protein. Quantitation of recombinant proteins in solution was determined spectrophotometrically at $A_{280}$ based on tyrosine content.[22] Purity was monitored by densitometry TotalLab (Madison, WI).

**Fiber Spinning.** Fibers were spun into a coagulation bath containing methanol and water. The coagulation of a small aliquot of spin dope was tested at several different methanol concentrations to determine the optimal percent methanol for the coagulation bath. Fibers were spun using a Harvard Apparatus Infusion/Withdrawal pump (Harvard Apparatus, Natick, MA) with a specialized microspinner (cavity volume 0.5 mL, 5 mm i.d.), and a 6 cm (0.125 mm i.d.) PEEK HPLC tubing (Sigma-Aldrich). As little as 25 $\mu$L of solution was spun, although 35−50 $\mu$L was more typical. Spin solutions were extruded into the coagulation bath at a rate of 2−10 $\mu$L/min. Fibers were removed from the bath for evaluation.

**Protein Characterization.** CD spectra were analyzed on an AVIV 60DS spectrophotometer from 260 to 182 nm using a 1 cm path length cell. Concentrated solutions (5−57 mg/mL) were diluted to 0.2−0.3 mg/mL as determined spectrophotometrically at $A_{280}$. Secondary structure was analyzed using the nonconstrained least squares algorithm supplied with the instrument. Dynamic light scattering (DLS) analysis using a DynaPro DLS (Protein Solutions Inc.) was used to compare the degree of self-association in protein solutions at different concentrations and temperatures. The apparent diffusion coefficient measured by the DLS ($D_{app}$ in Ficks, 1 F = $10^{-7}$ $cm^2/s$) is considered to be an empirical gauge of the size distribution of particles, roughly approximating a *z* average diffusion coefficient. The average size ($R_h$) was calculated from these values using the Stokes−Einstein relationship. A sample of ion exchange purified $[(SpI)_4/(SpII)_1]_4$ was generated by ultrafiltration to a concentration of 1.84 mg/mL. One portion was left concentrated and stored at 4 °C for 87 days. Another portion of the sample was diluted to 0.203 mg/mL and stored at 4 °C for 90 days, with analysis at 1, 12, and 90 days. The samples were then left at room temperature and reanalyzed periodically.

**Microscopy.** Fiber diameter and appearance were determined using an Optiphot2-pol polarizing microscope (Nikon Inc., Garden City, NY). Birefringence was observed under polarizing light with a first-order red plate. Fibers were coated 2× with gold/palladium and viewed on a CSM 950 SEM (Carl Zeiss, Jena, Germany) at 20 keV.

## Results and Discussion

Spiders have the amazing ability to spin high-performance fibers from an aqueous solution under ambient temperature and pressure. Spinning recombinant silk fibers under aqueous conditions described here is not unlike the way the spider spins silk. Recombinant silk protein was maintained in solution and concentrated by water removal to achieve a concentrated spin dope for extrusion through a long ductlike spinneret. The spin dope preparation methodology presented here is in contrast to other reports of spinning recombinant silk powder resolubilized in hexafluoro-2-propanol or formic acid.[13,20] In our experience, recombinant silk protein was extremely difficult to resolubilize after drying or precipitation. In fact, lyophilized protein was resistant to complete resolubilization by strong denaturants and solvents, such as guanidine hydrochloride and hexafluoro-2-propanol. Many of the aqueous based spin dopes prepared for fiber spinning have undergone gelation. Gel resolubilization was possible using formic acid, but the resulting solution was unstable, yielding a secondary gelation event at a concentration lower than that of the initial gelation.

**Fiber Spinning.** Fibers were spun from the NcDS and $[(SpI)_4/SpII)_1]_4$ proteins isolated with different acid lysis/chromatography methods (see Table 1). Purity of the spin dopes was measured by densitometry and was typically greater than 70% and as high as 92%.[23] Spin dopes with purity as low as 42% were spun into fibers that were capable of being drawn, were water insoluble and exhibited orientation. Fibers from the higher purity spin solutions were more robust and easier to remove from the bath without breaking. An attempt to spin *E. coli* lysate lacking recombinant spider silk protein did not produce intact fibers despite protein coagulation in the methanol bath. Furthermore, a spin solution containing a 24 kDa silk protein did not produce fibers (data not shown). We speculate that a 24 kDa silk protein is below the minimum MW required for fiber formation.

Solvent composition and extrusion conditions for spinning NcDS and $[(SpI)_4/SpII)_1]_4$ were determined empirically. The NcDS protein was isolated by formic acid lysis and affinity chromatography, processed into

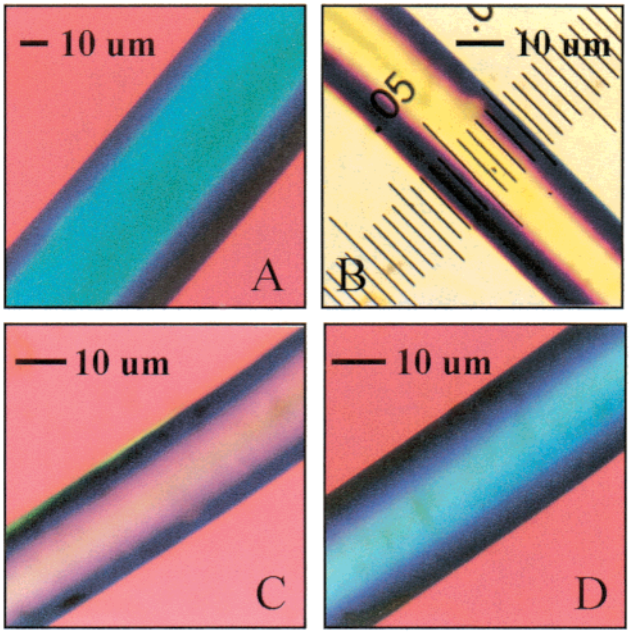


**Figure 1.** Light microscopy of recombinant silk fibers. (A) 62 $\mu$m NcDS fiber from a 25% protein solution shows birefringence under polarizing light with a first-order red plate. (B−D) pQE[$(SPI)_4/(SpII)_1]_4$ fibers. (B) 24 $\mu$m fiber spun from 6.5% protein solution is shown under regular light microscopy. (C) Under polarizing light with a first-order red plate, the 6.5% fiber has weak birefringence. (D) The 12.5% fiber has a diameter of 31 $\mu$m and shows more birefringence than the 6.5% fiber.

a 25% (w/v) spin dope and spun into 60 $\mu$m diameter fibers. Under polarizing light with a first-order retardation plate the fibers had a uniform blue color on a red background (Figure 1A), indicative of orientation along the axis of the fiber. Interestingly, the spin solution was initially incapable of fiber formation and only produced fibers after aging for 5 days at room temperature.

The $[(SpI)_4/SpII)_1]_4$ protein was purified by two different methods and both successfully spun into fibers. Affinity chromatography purified $[(SpI)_4/SpII)_1]_4$ from a formic acid lysate yielded spin solutions as high as 12.5% (w/v). Fibers spun from 6.5 and 12.5% (w/v) solutions had mean diameters of 24 and 31 $\mu$m, respectively. Fibers were removed from the bath and were water insoluble. Microscopic examination of the fibers revealed regions of the surface that were generally smooth and defect free (Figure 1B). Fibers produced from the 6.5% dope showed weak birefringence with the first-order red plate (Figure 1C) relative to the 12.5% fiber (Figure 1D). The use of birefringence to characterize orientation of natural spider silk fibers has been previously described.[4,5] Enhanced birefringence in fibers spun from higher concentration spin dopes is consistent with the anticipated increase in molecular orientation. Because these fibers were not drawn the orientation may be the result of shear occurring as the solution is forced through the ductlike spinneret.

A 21.5% (w/v) spin dope of $[(SpI)_4/SpII)_1]_4$ purified by ion exchange chromatography was spun into fibers with diameters ranging from 10.2 to 15.8 $\mu$m. These fibers were drawn 2.5 times their original length in the coagulation bath. The fibers were water insoluble, birefringent, and brittle after removal from the bath. When observed by SEM, the fiber had a smooth non-

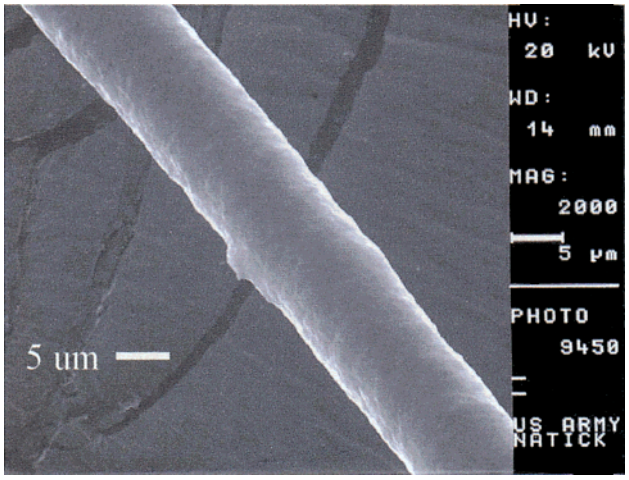


**Figure 2.** Scanning electron micrograph of a $[(SPI)_4/(SpII)_1]_4$ fiber spun from a 21.5% spin solution. The fiber was drawn to 2.5× its original length.

descript surface (Figure 2), and the interior appeared to be solid (data not shown).

A number of variables affects the nascent fiber, including pump speed, coagulation bath composition, and spin dope properties. Methanol concentration of the coagulation bath was important because it determined the speed of fiber coagulation and the ability to draw fibers in the bath. Ideally the fiber was translucent as it was spun into the bath and did not coagulate immediately. These fibers were mildly tacky and capable of being drawn in the bath with a maximum draw ratio of 2.5. When the methanol content was too high, rapid coagulation occurred, precluding fiber draw. Determination of mechanical properties by tensile testing was unsuccessful due to the small size and fragility of the fibers.

**Silk Solution Characterization.** Conformational changes in $[(SpI)_4/SpII)_1]_4$ spin dopes were observed by CD. The current CD algorithms are not especially reliable for calculating the $\beta$ sheet structure, since they are based on datasets from globular proteins. However, they are useful for detecting conformational changes occurring in a given sample. Secondary structure was affected by buffer composition and temperature when the solutions were stored over a period of time. At 30 °C, solution storage in 160 mM urea buffer had increased $\beta$ sheet structure relative to 1 M urea (Figure 3). The 1 M urea buffer presumably disrupted hydrogen bonding and therefore $\beta$ sheet formation. This effect was overcome by elevated temperature, however. After several days at 30 °C, a silk solution in 1 M urea buffer exhibited an increase in $\beta$ sheet content with a decrease in random coil (Table 2), while storage at 4 °C showed virtually no change in 2° structure (data not shown). In general, the $\beta$ turn and $\alpha$ helix contents did not change significantly.

Other structural changes apart from $\beta$ sheet formation appeared to occur in solution and affect fiber behavior. Fiber formation of the 25% NcDS spin dope was time dependent but was not attributable to a significant 2° structure change. Furthermore, recombinant silk solutions have been observed to undergo gel formation with prolonged incubations. These gels are unsuitable for extrusion. However, spin dopes stored at 4 °C or spin dopes containing lower protein concentrations are far less likely to form gels. These observations suggest that self-association of proteins is required for

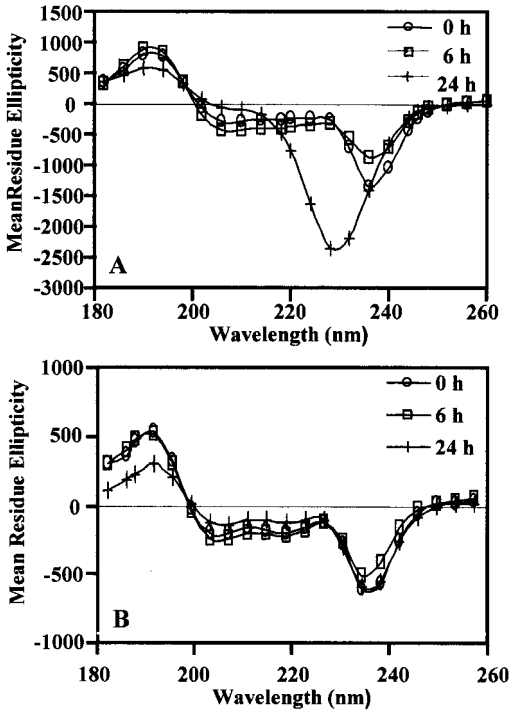


**Figure 3.** CD spectra of $[(SPI)_4/(SpII)_1]_4$ silk solutions showing the effect of buffer composition on secondary structure. Samples were aged in 160 mM and 1 M urea containing 10 mM $NaH_2PO_4$, 1 mM Tris, and 20 mM NaCl, at pH 5 at 30 °C. In 160 mM (A), the $\beta$ sheet increased from 39% to 67%, while the change was less pronounced in 1 M (B), which increased from 26% to 33%.

**Table 2. Effect of Storage Temperature on Percent Secondary Structure as Determined by CD Analysis of $[(SPI)_4/(SpII)_1]_4$ Solution Aged at 30 °C in 1 M Urea, 10 mM $NaH_2PO_4$, 1 mM Tris, 20 mM NaCl, and pH 5[a]**

| time (days) | $\beta$ sheet | random coil | $\alpha$ helix | $\beta$ turn |
|---|---|---|---|---|
| 0 | 24.3 | 51.6 | 0.5 | 23.6 |
| 3 | 52.8 | 38.9 | 1.2 | 7.1 |
| 5 | 69.6 | 9.4 | 4.1 | 16.9 |
| 7 | 66.3 | 6.9 | 6.4 | 20.4 |

[a] $\beta$ sheet content increased significantly with a corresponding decrease in random coil, while the $\beta$ turn and $\alpha$ helix content had little change.

extrusion of a fiber but that continued self-association results in a 3-D network of protein (i.e., a gel) incapable of producing fibers.

To investigate this possibility, dynamic light scattering (DLS)[24,25] was used as an empirical measure of the average particle size of a recombinant protein during storage in 2 M urea, 10 mM Tris, and 10% ethanol, at pH 3 (Figure 4). The increase in radius with time confirms that association is strongly promoted by elevated temperature and protein concentrations. These data also indicated that low concentrations of protein do not associate significantly for up to 90 days at 4 °C in this solvent; therefore, a stock solution can be stored for extended periods. Other experiments (not shown) indicated that particle size increased during the first processing step in which the protein was concentrated and conditions at that step may need to be adjusted to improve storage stability. Still other experiments demonstrated that at high protein concentrations (>10 mg/mL) particles are formed that are too large for characterization by DLS and that dilution does not result in the dissolution of the larger particles. It is quite possible that these larger particles are an incipient stage to gel

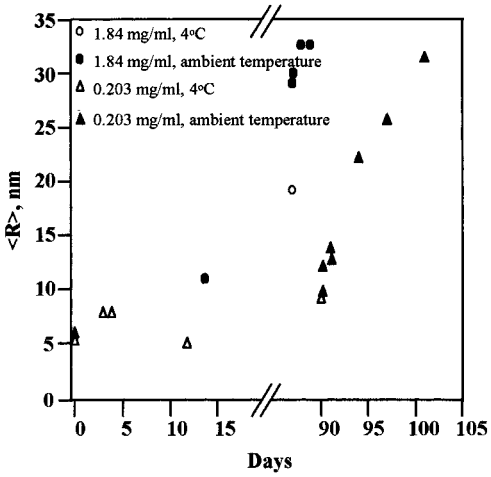


**Figure 4.** Average size of soluble spider dragline protein $[(SpI)_4/(SpII)_1]_4$ monitored by DLS at concentrations of either 1.84 mg/mL (circles) or 0.203 mg/mL (triangles) and stored at either 4 °C (open symbols) or ambient temperature (closed symbols) in 2 M urea, 10 mM Tris, and 10% ethanol, at pH 3. The size of the monomeric protein is expected to be approximately 3−4 nm, suggesting that the protein may have undergone some association prior to or during purification.

formation. Identifying the molecular interactions occurring in solution and subsequently controlling them will be important for spinning fibers with consistent behavior and desired properties.

## Conclusion

Here described is the first known report of an aqueous method for spinning recombinant silk fibers. The method is environmentally friendly and has been used to spin recombinant silk fibers with mechanical properties similar to natural spider silk.[26] The fibers are 10−60 $\mu$m in diameter, exhibit molecular orientation, and are water insoluble. Silk protein can be maintained in a spin dope at a high concentration in preparation for fiber spinning. An increase in $\beta$ sheet content and self-association was observed when the spin dopes were prepared. These molecular events are at least in part responsible for fiber formation. Understanding the molecular interactions occurring in solution will be necessary to control solubility and spinning parameters. Although silk fibers can be spun under environmentally friendly conditions, a lack of recombinant material has hampered efforts of obtaining quantitative fiber characterization (e.g., mechanical properties). A number of variables are important during protein processing and spinning fibers. Ideally these variables would be correlated with fiber properties to identify those most relevant and to optimize the method. Research is underway to address the protein production issue[26] which will enable us to quantitatively evaluate the potential of recombinant spider silk fibers.

**Acknowledgment.** The authors wish to thank Steve Fossey, Robert Stote, Ron Segars, and Jean Herbert (Natick Soldier Center) for their valuable input.

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MA011471O