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Combining flagelliform and dragline spider silk motifs to produce tunable synthetic biopolymer fibers

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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 proline-containing pentaptides juxtaposed to the polyalanine segments resembled those found in the flagelliform silk protein (Flag) composing the web spiral: (GPGGX₁GPGGX₂)₂ with X₁/X₂=A/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 (GPGGX₁GPGGX₂)₂ with X₁/X₂ = Y/S or A/A, respectively. Post fiber processing involved similar drawing ratios (2–2.5×) 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. NMR indicated that the fraction of β-sheet nanocrystals in the polyalanine regions formed upon extrusion, increased during stretching, and was maximized after water-treatment. XRD 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 redesign impacted protein self-assembly mechanisms and requirements for fiber formation.

Keywords

synthetic silk fibers; mechanical properties; post-spinning; ssNMR; XRD; structure function; dragline; flagelliform

INTRODUCTION

Spider dragline and flagelliform capture silks from orb weaving spiders are extremely tough fibers because, unlike most natural and man-made materials, they combine high levels of both strength and extensibility, in opposite fashion.^{1–3} Indeed, dragline silk is extremely strong (4 GPa) and has moderate extensibility (35%) compared to the weaker flagelliform silk (1 GPa) which is the most extensible of all silks (200%). These mechanical properties originate from the modular nature of their extremely large and highly repetitive proteins (spidroins) that are composed of basic repeats built with very specific combinations of key structural amino acid motifs.^{3–8} The strongest major ampullate or dragline silk is made of two proteins, major ampullate spidroins 1 and 2 (MaSp 1⁵ and MaSp 2⁶), which are based on basic repeats that combine respectively, (GA)_n or (A)_n crystalline β -sheet-forming domains with (GGX)_n and/or (GPGXX)_n that form disordered, flexible helical and turn-like structures. The extremely elastic flagelliform silk on the other hand, only contains one single very large protein (Flag) which has a basic repeat composed of an very long (GPGGX)_{43–62} sequences embedded between shorter (GGX)_n and ‘spacer’ sequences.^{4, 7–8} Spacer sequences are very conserved sequences that are characterized by the absence of glycine and alanine amino acids (G and A).

Correlations between the primary sequences of these silk proteins and the different mechanical properties of these two types of silks,^{3, 4} supported by in fiber structural analyses, revealed the secondary structures of these different motifs. The (GPGXX)_n motifs form type II β -turn structures⁹ and act as molecular nanosprings^{4,10} providing elasticity to both silks. The (GGX)_n motifs are predicted to form 3₁ helical conformations.¹¹ Finally, the (A)_n/(GA)_n motifs, which form β -sheet nanocrystals^{11–16} that orient preferentially parallel to the fiber axis, account in large part for the extreme strength and toughness of dragline silk.

Several structural studies showed that in the highly chemically regulated environment of the major ampullate silk gland, the very concentrated (50% w/v),¹⁶ but yet soluble, MaSp proteins undergo rapid irreversible structural changes in their polyalanine regions (β -sheet formation) that promote their self-aggregation, which is necessary to initiate fiber formation.^{17–20} Further evidence for this *in vivo* controlled process points toward the pivotal role of their C-termini in initiating the β -sheet driven assembly mechanism by either recruiting chaperones to help with the necessary structural transitions,²¹ and/or participate in disulfide crosslinks to promote a favorable alignment of the MaSp proteins.^{22–25} Additionally, dragline silk fiber formation requires both MaSp 1 and 2 proteins.^{26–27} While it is clear that for dragline silk the crystallization of the polyalanine regions seems to be the driving force for self-assembly of both MaSp proteins into a fiber, the absence of such sequences in the Flag protein suggests a different mode for fiber assembly. Recent Raman spectroscopy data showed the highly disordered state of the Flag protein in solution inside the gland, and its unique lack of structural reorganization and alignment upon spinning compared to that occurring during the spinning of dragline silk.²⁸ Studies of mini recombinant Flag proteins (eFlag), with or without native C-terminus sequences, in solution, also suggest the potential role of the C-terminus sequence in crosslinking the Flag proteins *in vivo*, thus providing a means for their *in vivo* polymerization into a fiber.²⁹ However, for the capture silk, whether or not other structural motifs in the repetitive domain also participate in the Flag self-assembly process is yet unknown, and only suggestions of such involvement were made for the spacer region.⁴

From studies on dragline silk spinning, it is clear that during the complex multi-step *in vivo* silk spinning, both repetitive and flanking non-repetitive regions of the proteins are crucial to rapidly trigger proper protein aggregation, nucleation, and processing leading to fiber

formation. The main reason is that the *in vivo* silk protein processing relies on rapid protein structural transitions and concentration occurring in the spinning duct, as well as simultaneous water removal.^{17–20} In the absence of a finely-tuned spinning process like that of the spider, in a synthetic production scheme that deals with shorter synthetic recombinant silk-like proteins, silk protein sequence engineering and successive adequate fiber post-spinning processes are critical to optimize mechanical properties. The recent engineering of bacterial strains that are better equipped to produce longer repetitive silk proteins³⁰ may eventually help circumvent some of the limitations of this system.

Artificial spinning of spider silk fibers may one day become the source of high performance fibers that have a tremendous potential for applications. Several studies report the extrusion or artificial spinning of regenerated spider silks and recombinantly produced native-like or synthetic versions of dragline MaSp 1 or MaSp 2 proteins into single fibers.^{30–34} While all of these studies agreed that the mechanical properties of the synthetic fibers were inferior to those of dragline silk fibers despite different spinning and post-spinning processes, very few of these provided any or sufficient detailed structural information on the fibers to show the changes resulting from a specific processing.^{31–32} Therefore, a full and very basic understanding of structure-function relationships in artificial silk fibers, that defines the basic requirements for protein aggregation and fiber formation, is sorely lacking. Additionally, none of these studies focused on flagelliform silk structural motifs, though this silk clearly has the best attributes for elasticity and thus should also be of interest for fiber engineering.

Water is a well-known natural plasticizer of native silk proteins that greatly affects mechanical properties of the fibers.^{35–37} However, water plasticize flagelliform and dragline silks differently since flagelliform silk becomes much more elastic than dragline silk when wet.³⁸ These results show further evidence towards the importance of the primary structure of the Flag protein sequence that contains a high number of β -turn forming motifs. They also hint to the importance of the different nature of the Flag proline-containing pentapeptide motif compared to that seen in MaSp 2, *i.e.* (GPGGX)_n with X= A,V,Y,S,T *vs.* (GPGX₁X₂)_n with X₁X₂= QQ, or GY, and their potential additional role in the superior extensibility of flagelliform silk compared to dragline silk. Thus, the Flag protein sequence definitely provides a slightly different pool of alternative types of elastic motifs worthy of testing in artificial silk fibers.

To address this structure function issue, we designed two recombinant proteins with consensus repeats based on that of dragline MaSp 2, thus containing a polyalanine motif next to proline-containing pentapeptides, but in which the MaSp 2 like (GPGQQ GPGGY)₂ was replaced with one of two Flag native variants.³⁹ These two Flag/MaSp 2 chimeric proteins, named A1S8₂₀ and Y1S8₂₀ had basic repeats that juxtaposed respectively, the Flag-like (GPGGA GPGGA)₂ or (GPGGY GPGGS)₂ motif with the MaSp 2-like [linker-(A)₈] sequence. Despite the absence of native C- and N-terminal sequences, these chimeric Flag/MaSp 2 chimeric proteins provided interesting self-assembly abilities in aqueous solution, triggered by heat-treatment and/or shearing, that depended on the nature of their Flag structural motif. Both of these proteins could also form synthetic fibers in organic or aqueous conditions by extrusion or fiber-drawing from self assembled films.³⁹ In this study, we focused on improving the mechanical properties of both types of fibers generated by these two chimeric Flag/Masp 2 chimeric proteins that were formed under different conditions to investigate the function of these two different Flag structural motifs and fully analyze the potential of the fibers formed by these chimeric proteins. It is established that post-spinning modifications (*i.e.* fiber treatments with appropriate solvents coupled with specific fiber drawing ratios) improve the tensile strength and initial modulus of native, regenerated, and artificial silk fibers.^{30–34, 40–42} For each Flag/MaSp 2 chimeric fiber type,

adequate sequential post-spinning treatments, all involving fiber pre- or post-treatment with water, were applied. Mechanical (stress/strain) and morphological (SEM) analyses along with exhaustive structural analyses (NMR and XRD) at all stages of the processing provide a better understanding of the structure-function relationships in these different Flag/MaSp 2 synthetic fibers.

MATERIALS AND METHODS

Bacterial Chimeric Silk protein production

The pE(Y1S8)₂₀ and pE(A1S8)₂₀ plasmids previously engineered and cloned in the *E. coli* BL21 DE3 expression line³⁹ were used in this study. These plasmids contain two different 2,300 base pair highly repetitive spider silk-like sequence that combines a *Nephila clavipes* flagelliform silk-like putative elastic encoding motif (Y1) or (A1) with the major ampullate silk-like putative strength encoding motif (S8). The Y1, A1, and S8 motifs encode respectively the [GPGGYGPGGS]₂, [GPGGAGPGGA]₂, and [GGPSGPGS(A)₈]₈ sequences and the [Y1S8] or [A1S8] basic synthetic repeat units are iterated twenty times in the final (Y1S8)₂₀ and (A1S8)₂₀ construct sequence. The Y1S8₂₀ and A1S8₂₀ proteins have repetitive chimeric silk-like sequences of the form: [(GPGGY GPGGS)₂ GPSGPG(A)₈]₂₀ and [(GPGGA GPGGA)₂ GPSGPG(A)₈]₂₀.

The pE(Y1S8)₂₀ and pE(A1S8)₂₀ bacterial clones produce respectively a 62 kDa Y1S8₂₀ and 58 kDa A1S8₂₀ His-tagged recombinant chimeric spider silk-like proteins that were purified under the same conditions. Details on cloning, gene expression and protein purifications were described elsewhere.^{39,43}

Synthetic Silk Fiber Generation

Lyophilized A1S8₂₀ or Y1S8₂₀ proteins were resolubilized in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; TCI America, Portland OR) to prepare the 26–27% (w/v) spinning dopes. Fibers were extruded using the DACA Instruments SpinLine system (DACA Instruments; Santa Barbara, CA). The complete spinning line used comprised the spinneret followed by one set of godets and a winder to respectively extrude, collect, and wind the spun fibers at a controlled speed. The spinneret is composed of a 1 mL Hamilton Gastight[®] syringe (Hamilton company; Reno, NV) mounted with a 10 cm long PEEK tubing (inside diameter: 254 µm; Upchurch Scientific; Oak Harbor, WA).

Extrusion of the artificial silk fibers into a 90% isopropyl alcohol/10% water coagulation bath (90% v/v IPA) was performed at a plunger speed of 0.5 mm/minute. After coagulation and upon emergence of the synthetic as-spun fiber from the coagulation bath, the single as-spun fiber (step 1) was picked up carefully leaving enough slack before being fed through the godets, and collected on a winder both set at a speed lower than that of the extrusion (0.3 mm/min) to avoid premature stretching the fiber.

An alternative method of fiber formation was used to generate ‘pulled’ fibers or hand-drawn synthetic Y1S8₂₀ fibers.³⁹ This technique relies on the ability of the pure heat-treated Y1S8₂₀ synthetic silk-like protein to spontaneously form liquid surface films in aqueous solutions at low concentrations (0.5 mg/ml). For this experiment, these surface films were drawn manually into fibers (Y1S8₂₀-P as-pulled fibers). The film was lifted into a fiber using forceps at a very low and fairly constant speed to avoid stretching the fiber. Y1S8₂₀ as-pulled control fibers of different lengths and diameters were obtained depending on the local thickness and total area of the films.

Fiber post-spinning treatments

For the extruded fibers, only A1S8₂₀ as-spun fibers could be processed since the Y1S8₂₀ as-spun fibers were too brittle to be handled. The dry as-spun or step 1 fibers were cut in 10 cm pieces. These shorter fibers pieces were subjected to single-step (step 2) or double-step (steps 2 and 3) post-spinning modifications. For step 2, the as-spun fibers were stretched (draw ratio/DR= 2–2.5) while soaked in 90% v/v IPA. The step 2 or IPA-stretched fibers were then lifted up carefully and air-dried within seconds while physically constraining the fiber to maintain its length. For step 3, the dry step 2 or IPA-stretched fibers were soaked for 2 minutes in water while under physical constraint, removed carefully from the bath and air-dried.

Before individually processing the as-pulled Y1S8₂₀-P fibers, their initial lengths were measured. Each fiber was then completely immersed in water at room temperature for 2 minutes and held at a constrained length. The fiber was then lifted up above the bath and stretched twice its length into the air while wet (DR or draw ratio= 2).

Mechanical Testing

All fibers tested had an initial fiber length of $L_0 = 15$ mm. The average diameter of each fiber was measured according published methods prior to mechanical testing.³⁹ The synthetic silk fibers were tested at ambient conditions (21–22°C; relative humidity= 18–20%) using a 10 gram load cell (Transducer Techniques, Temecula CA) mounted on a MTS Synergie 100 system (MTS Systems Corporation, Eden Prairie MN). The mechanical data were recorded at a strain rate of 5 mm/min and a frequency of 250 MHz. The recorded load and elongation parameters were used to calculate engineered stresses ($\sigma = F/A$; F = Load and A = fiber cross section) and strains ($\epsilon = \Delta L/L_0$). Stress/strain profiles were plotted using MATLAB (Version 7.1) and fitted to calculate the energy to break or toughness (area under the stress/strain curve). Young's modulus (E_{initial} or initial stiffness), yield (first yield point), maximum stress (= tensile strength) and extensibility (= maximum strain $\times$ 100) were determined from the stress strain curves.

An unpaired T-test was performed between the different samples and differences were found statistically significant when $p < 0.05$.

Scanning Electron Microscopy

To prevent charging, the fiber samples were gold coated (Denton Vacuum Sputter Coater Desk II) for 180 seconds to give an estimated 15 nm Au layer on the samples. The samples were then placed into the SEM specimen chamber (XL30 ESEM-FEG; FEI). The images were taken in Hi-Vac mode with a vacuum reading of less than 9×10^{-5} mbar. The beam (20 kV) had a beam diameter of 1.3 nm and a beam current of 148 pA. All images were recorded with a line time of 66.4 ms, with 484 lines per frame.

Solid State NMR

Nuclear Magnetic Resonance (NMR) experiments were conducted on a 400 MHz Bruker Avance-III spectrometer using a 4 mm cross-polarization magic angle spinning (CP-MAS) probe configured in ^1H - ^{13}C double resonance mode. A1S8₂₀ or Y1S8₂₀ synthetic fibers were packed in a 12 μl drilled-out 4 mm Zirconium rotor fitted with a Kel-F drive tip, while lyophilized protein powders were packed in a full 4 mm rotor. Typical experimental conditions for ^1H - ^{13}C CP-MAS experiments were a 3 μs $\pi/2$ pulse on the ^1H channel, a 1 ms ramped (~50%) CP spin-lock pulse on the ^1H channel with a 65 kHz rf field strength and square ^{13}C contact pulse, 8192 scan averages, 2048 complex points, a 50 kHz sweep width, and 5 s recycle delay. ^{13}C direct detection (DD) were collected using a ^{13}C 4 μs $\pi/2$ pulse, 10240 transients, 2048 complex points, a 50 kHz sweep width and a 1 s recycle delay. The

MAS frequency was 10 kHz and 80 kHz two-pulsed phase-modulated⁴⁴ (TPPM) proton decoupling was applied during acquisition in all experiments. All spectra were processed using 25 Hz exponential line broadening before Fourier transformation (FT). ¹H-¹³C CP-MAS data were collected on an 800 MHz Varian VNMRs spectrometer with a triple resonance 3.2 mm BioSolids CP-MAS probe for the Y1S8₂₀ film and lyophilized protein. Typical experimental conditions for these experiments were 20 kHz MAS, 100 kHz sweep width, 5 s recycle delay, and a 2 ms CP contact time. For CP, a ramped (~20%) ¹H spin lock pulse (rf field strength 70 kHz) and a square ¹³C contact pulse (rf field strength 50 kHz) were used with 100 kHz TPPM ¹H decoupling during acquisition. These spectra were processed using 150 Hz exponential line broadening prior to FT. All ¹³C chemical shifts were referenced indirectly to TMS by setting the downfield adamantane resonance to 38.56 ppm.

X-Ray Diffraction

X-ray fiber diffraction experiments were conducted at the BioCARS beamline (sector 14-BM-C) of the Advanced Photon Source (APS) at Argonne National Laboratory (Argonne, IL, USA).⁴⁵ The x-ray diffraction (XRD) data were recorded with a large area 9-chip ccd detector (ADSC Quantum-315) placed 300 mm behind the sample. The x-ray monochromatic beam was focused to 150 × 200 micron at an incident energy of 12.67 keV (0.978 Angstrom). The beamstop was mounted vertically. The fiber samples were also positioned vertically, thus perpendicular to the x-ray beam, in the identical geometry as the beamstop. The data (10 frames) were collected with a 50 mm distance between the sample and the beamstop and an x-ray exposure time of 60 seconds. The background (air scattering) was subtracted from all x-ray intensities shown.

RESULTS

Effects of fiber formation methods and post processing with water on mechanical performances

Synthetic A1S8₂₀ and Y1S8₂₀ single fibers were extruded from 26–27% w/v silk protein-HFIP dopes into a 90% IPA bath. To generate more malleable as-spun fibers that could be handled easily during further extensive processing, the inside diameter of the extrusion needle selected (254 μm) was twice the size of that used in previously published experiments.³⁹ Regardless of the spinning conditions, *i.e.* larger needle diameter and with or without prior adjustment of the dope with 15% v/v H₂O, the thicker Y1S8₂₀ as-spun fibers which extruded easily were extremely brittle once out of the coagulation bath and thus did not allow for further processing. This confirmed the observed ineptitude of this specific silk-like protein to form usable fibers in these organic spinning conditions. Only the A1S8₂₀ as-spun fibers, which had with diameters roughly twice the size (~70 μm) of those from the original A1S8₂₀ as-spun fibers,³⁹ were further processed (Table I). Both as-spun types were thick white water-soluble fibers.

The A1S8₂₀ fibers exhibited distinct mechanical properties at the different stages of the processing as shown in Figure 1 and Table I. The as-spun fibers were extremely brittle (0.3 MJ/m³ and 1.7%). Successive stretching in 90% IPA increased their average toughness (18.8 MJ/m³) due to drastic increases in all average mechanical performances. The stretched fibers were on average noticeably stiffer and stronger (3 and 3.5 fold; p=0.01 and p=0.0003) showing little variability for these two parameters. However, their recorded average extensibility, and thus average toughness, were still highly variable indicating that this process was only successful in refining tensile strength and initial modulus, but failed to optimize the overall mechanical properties of these fibers. These more uniform fibers showed record stress and initial modulus of 125.1 MPa and 5.5 GPa, respectively after

exhibiting pronounced strain-hardening before failing. The highest extensibility and toughness recorded at this stage were 58.6 % and 53.3 MJ/m³. After water treatment, the fibers became about 3 times tougher ($p = 0.0005$) than the stretched fibers due to slight increases in tensile strength and stiffness ($p = 0.04$), but mainly due to an impressive 2.8 fold average increase in extensibility ($p = 0.0005$) as shown in Figure 1B and in Table I. Almost all fully processed fibers exhibited a delayed and softer strain-hardening tendency. Overall, this step 3 group was more homogenous since it showed the least variability in the data (Table I). Record best tensile strength and stiffness (184.4 MPa and 6.8 GPa) were observed for fibers of smaller diameter than average (21.7 μm vs. 28.3 μm). Fibers in this group also showed record best values for extensibility and toughness (80.2 % and 93.5 MJ/m³). The processing sequence for these A1S8₂₀ fibers was successful to increase their mechanical properties but the final processing stage of these spun fibers with water was critical to optimize their properties and lower data variability.

Since the Y1S8₂₀ as-spun fibers could not be processed, we resorted to the alternative existing method of fiber generation for this protein to investigate the full potential of this type of fiber after processing. As-pulled Y1S8₂₀ fibers, which were generated in aqueous environment by fiber-drawing from a spontaneously formed film, were further treated with water and then drawn into the air (2 $\times$ the fiber length). This specific treatment of the Y1S8₂₀ pulled fibers (Y1S8₂₀-P) substantially improved their mechanical properties compared to the average performances of all as-pulled fibers. This is best illustrated by the overall increase in average toughness (22.6 vs. 5.6 MJ/m³; $p = 0.007$) of these fibers (Table I). Despite efforts to control the speed of fiber generation, due to the non-automated fiber formation technique and potential differences in local thickness of the spontaneously assembled Y1S8₂₀ surface film, which may depend on local protein concentration, the diameters of the as-pulled fibers were highly variable. Consequently, those of the processed fibers were also highly variable. The diameter variability of the processed fibers was also worsened by the lack of complete control over the speed of the successive manual stretching despite efforts to keep this parameter as constant as possible. The average diameters of the Y1S8₂₀-P processed fibers were overall thinner than those of the control fibers since fiber-drawing inevitably reduces fiber diameter (Table I). While the majority of the data for the control fibers were collected on thicker fibers (20–40 μm), the one obtained for the processed fibers was collected on fibers with thinner diameters (6–15 μm). Though overall, the average extensibility of the Y1S8₂₀ water-treated fibers decreased slightly compared to that of the control fibers, their toughness, ultimate tensile strength, elastic modulus, yield and breaking stresses were greatly enhanced compared to those of the control fibers ($p < 0.01$). The high variability in the stress/strain data for both control and processed Y1S8₂₀-P fibers was reduced when grouping the fibers by diameters within one treatment. This grouping brought up significant differences in tensile strength between the control fibers subgroups ($p = 0.02$), and in both tensile strength and stiffness between the processed fibers subgroups ($p < 0.0001$). For the control fibers, the thicker fiber group (20–40 μm) exhibited a more elastic deformation after yield, and yielded at lower stresses than the thinner fiber group (9–18 μm). On average, these fibers achieved lower maximum stresses (13.3 MPa; $p = 0.02$) and were almost twice more extensible than the thinner diameter fibers (57.3 vs. 30 %). However, on average, the thinner control fibers exhibited a higher yielding point (23.9 vs. 5.5 MPa), a pronounced strain hardening behavior, and were twice tougher (8.0 MJ/m³) than the thicker fibers as presented in Table I. The two groups of processed Y1S8₂₀-P fibers with smaller and intermediate diameters (6–9 μm and 10–15 μm) achieved higher tensile strengths and were stiffer than the thicker fibers (16–35 μm ; $p = 0.00013$ and $p = 0.02$). The Y1S8₂₀-P processed fibers with the smallest diameters (under 10 μm) were the stiffest (5.5 GPa; $p = 0.02$ and $p = 0.03$) and strongest (123 MPa; $p = 0.01$ and $p = 0.000013$), had a pronounced strain-hardening, but had highly variable extensibility (Figure 2). Fibers with intermediate diameters (10–15 μm) underwent a constant strain-hardening, achieved higher average

extensibilities (43.8%) while breaking at slightly lower stresses (92.0 MPa; $p=0.01$) than the thinner diameter group. Finally, the thickest fibers (16–35 μm) also had improved tensile strength and stiffness compared to all control fibers but had overall reduced extensibilities and ultimate breaking stress compared to the 20–40 μm controls and the 6–9/10–15 μm processed fiber groups, respectively. On average, the fibers belonging to the intermediate diameter group were the toughest (31.4 MJ/m³) and showed the highest extensibility and toughness (80.3% and 61.6 MJ/m³). However, the maxima for tensile strength and stiffness were observed among the thinnest fiber group (143.1 MPa and 8.3 GPa). Overall, the 10–15 μm Y1S8₂₀-P processed fiber group best compared mechanically to the fully processed A1S8₂₀ (step 3) but remained 1.7 fold weaker (Table I and Figures 1 & 2).

Morphological changes associated with fiber formation and post processing with water

SEM images show the overall morphology of the A1S8₂₀ fibers at all 3 stages with details of fracture points versus that of the Y1S8₂₀ as-spun fiber (Figure 3A–E & G–H). The Y1S8₂₀ fiber was covered with salt crystals. These salts were probably trapped inside the self-assembled film material during purification. Despite extensive dialysis, with conditions identical to those used for the A1S8₂₀ protein, all the salts were not removed from the dialyzed Y1S8₂₀ protein sample, thus ended up mixed with the lyophilized protein, and were incorporated into the as-spun fiber. The Y1S8₂₀ fibers appeared extremely spongy and porous, with lots of imperfections and cracks unlike the smoother more compact A1S8₂₀ fibers (Figure 3A & G). The lower density and porous nature of Y1S8₂₀ explains its much higher brittleness. After stretching, the A1S8₂₀ fibers were half as thick (Table I), had a visible greater sheen, and had become insoluble in water. These fibers were more uniform and had a smooth surface (Figure 3B). The successive water-treatment did not change the diameter of the fibers (Table I) but drastically affected their overall morphology revealing a defined, but still imperfect, microfibrillar structure with visible bundles (Figure 3C). When looking at accidental fracture points, the brittle A1S8₂₀ as-spun fibers showed a poorer internal structural organization than the much denser stretched fibers, since they experienced sharper or cleaner transversal breaks (Figure 3D & E). The water-treated fibers were denser but still contained smaller cavities along the fiber axis or hollow grooves, showing a loose and flexible network. Instead of breaking ragged, these fibers would tear off exposing different bundles of microfibrils looking like twisted cable ropes, thus showing the highest level of fiber network organization of all three groups (Figure 3C & F).

After water-treatment and stretching, the Y1S8₂₀-P fibers appeared more uniform and had a visible greater sheen than the control fibers, just like that observed for both the stretched and water-treated A1S8₂₀ fibers. These fibers also showed a loose microfibrillar and bundle-type structure with apparent longitudinal grooves in the fiber like that described for the water-treated A1S8₂₀ fibers (Figure 3I). Additionally, along this fiber, veil-looking pieces corresponding to remaining un-stretched film material surrounded the fiber.

Structural analyses of the synthetic fibers

NMR and XRD data were collected on the A1S8₂₀ lyophilized protein and fibers at all stages of the processing. ¹H-¹³C CP-MAS NMR data on the A1S8₂₀ lyophilized protein and fibers are presented in Figure 4. The secondary structure of the protein and subsequent fibers can be determined from the conformation-dependence of the ¹³C isotopic chemical shift. This approach has been used extensively to characterize secondary structure in biopolymers and native spider silks.^{8–9, 46–56} The Ala C β resonance displays two components (17.8 and 21.2 ppm) in the ¹H-¹³C CP-MAS spectra of the lyophilized protein and processed fibers that can be ascribed to a random coil and β -sheet conformation, respectively. In the as-spun fibers, the β -sheet fraction increases considerably compared to the lyophilized protein showing that the spinning process drives more β -sheet formation. However, the as-spun

fibers still exhibit some mobile components as evidenced by the sharp Ser C α and Ala C α resonances observed. These mobile components are due to the presence of solvent following the spinning process that interacts with the protein backbone. After the fiber is post-spin-stretched and hydrated with water, the β -sheet content is increased further and the solvent is expelled (note lack of sharp Ala C α and Ser C α resonances).

In order to test if the fully processed A1S8₂₀ will get plasticized by water a ^1H - ^{13}C CP-MAS spectrum was collected on hydrated fibers. The ^1H - ^{13}C CP-MAS spectrum on the hydrated fibers (Figure 4d) shows a decrease in intensity for the Gly, Pro, Ser and a fraction of the Ala resonances that represent random coil structures while, the signal from the Ala β -sheet resonances remain strong. This result indicates that the water plasticizes the non- β -sheet regions of the fiber increasing mobility while the Ala β -sheet fraction remains rigid. The increase in mobility for the non- β -sheet forming region was further confirmed by collecting a ^{13}C DD-MAS spectrum that will enhance the mobile amino acids that interact with water (see Figure 5). Gly, Pro, Ser and the random coil fraction of Ala increase in intensity and display sharp resonances illustrating that these components of the silk fiber are readily accessible to water and are plasticized. It should be noted, that although a large fraction of Ala is present in a rigid β -sheet conformation that is not plasticized by water, there is a minor fraction of Ala that is not converted to a β -sheet and can be plasticized by water following fiber processing. This indicates that the fiber processing conditions can still be perfected to convert all the Ala in the polyalanine regions to a β -sheet structure. This would likely further improve the mechanical properties of these synthetic spider silks.

The ^1H - ^{13}C CP-MAS NMR results for the Y1S8₂₀ lyophilized protein and as-spun fibers are shown in Figure 6. The lyophilized powder displays a very low population of Ala in a β -sheet compared to the A1S8₂₀ protein. In addition, the appearance of sharp resonances in the spectrum of the lyophilized powder illustrate that this protein is highly hygroscopic and has bound water. The presence of bound water was confirmed by conducting ^1H fast MAS NMR spectroscopy where a strong resonance appears at 4.5 ppm that can be removed by vac drying (data not shown). The presence of bound water in the lyophilized protein increases the mobility of the Ala C α , Ser C α , Pro C α and some of the CO resonances. For the as-spun fiber, sharp resonances are still observed in the ^1H - ^{13}C CP-MAS spectrum due the presence of spinning solvent and the Ala is not converted to a β -sheet structure (see Ala C β resonance). When the fiber is water-hydrated following the spinning process, the Ala C β shows almost a complete conversion to a β -sheet structure. This shows that treating these synthetic spider silks with water is required to drive β -sheet formation. This protein readily forms a β -sheet structure when in contact with water. In fact, when conducting protein purification, a film form on top on of the aqueous buffered solution that is aggregated in a β -sheet structure (see Figure 7 for ^1H - ^{13}C CP-MAS on the film).

X-ray diffraction (XRD) of as-spun A1S8₂₀ fibers shows a single diffuse ring centered at 4.4 Angstrom (Figure 8A). This is consistent with a disordered amorphous structure and poorly stacked β -sheet structures. The XRD of stretched then water-treated (step 3) A1S8₂₀ fibers is shown in Figure 8B. There is a clear increase in Bragg reflections, both isotropic and anisotropic components. The equatorial Bragg reflections (5.2 and 4.4 Angstrom) are similar to what is observed in other spider and silkworm silk fibers, and results from β -sheet nanostructures oriented along the fiber axis.^{13, 57} These results support the suggestion by Seidel et al³¹ who have argued in the case of regenerated silks, that the presence of water increases the local mobility in the protein which aids in the reorganizational process. The XRD 2D image shown in Figure 8B is typical for semi-nanocrystalline morphology, which is characterized by Bragg reflections and diffuse diffraction from amorphous components. XRD data confirm the NMR interpretation of the processed fibers to increase in β -sheet content. Furthermore, the azimuthal width of the equatorial reflections in the (step 3)

processed A1S8₂₀ fibers indicates anisotropic β -sheet nanocrystalline domains along the fiber axis.

DISCUSSION

The basic repeats of the two small chimeric Flag/MaSp 2 proteins used in this study were based on the consensus repeat published for *Nephila clavipes*' dragline MaSp 2 protein,⁴ thus combined the same number of proline-containing pentapeptides with a linker-polyalanine segment. However, instead of the MaSp 2 like (GPGXX)₄, the presumed elastic motifs under study were two smaller variants of the [GPGGX]_n found in the Flag protein (n= 4 instead of n>43) making up the highly extensible capture spiral.⁴

To investigate the functional role of each of these two shorter Flag-like motifs, synthetic fibers were formed in optimum, but drastically different conditions adapted to each chimeric protein. The A1S8₂₀ protein was spun into fibers using organic solvents during an automated extrusion process while the Y1S8₂₀ protein spontaneously assembled into a surface film upon elution in aqueous buffers, and this film was used to hand draw fibers. The length and thickness of these Y1S8₂₀ fibers could not be controlled as they depended on the area and local thickness of the formed film.

Both types of Flag/MaSp 2 fibers were subjected to sequential post-processing techniques that combined pre- or post-treatment with water and single fiber stretching at similar draw ratios (2–2.5x). As a result, both chimeric fiber types had greatly enhanced and more refined mechanical properties. Our data suggest that despite their clearly different modes of fiber formation and sequences of processing, both fully processed Flag/MaSp 2 protein fibers exhibited the same range of mechanical properties, thus emphasizing the prevalent role of the overall structure of their basic consensus repeat and their molecular weight in dictating fiber mechanical performance. Similar observations about the effect of polymer size on the mechanical properties were also made for two different types of synthetic MaSp 1 like fibers.^{30, 33} The functional role of these two Flag-like structural motif variants in the context of the MaSp 2-like basic repeat structure is best exemplified by the mechanical performances achieved by the toughest synthetic fibers produced from the two chimeric proteins after undergoing similar drawing ratios (2–2.5x), followed or preceded by fiber water treatment. These best performances for each fiber category also hinted to the optimum potential of these particular chimeric silk protein fibers under these specific spinning and processing conditions (Table II). Despite the slight but consequential differences of Flag-like (GPGGX)₄ structural motifs used in these proteins, the best fibers for both categories achieved the same extensibility (80%), while showing similar high increases in ultimate tensile strength and initial modulus. In the most controlled experimental settings for fiber generation like those described for the spun and processed A1S8₂₀, the toughness of the fully processed fiber reached 93.5 MJ/m³, thus was 1.6–1.7 fold lower than those of native dragline and flagelliform silks. The highest level of extensibility observed for the fully processed Flag-MaSp 2 fibers compares well to the data obtained for processed fibers spun from a recombinant ADF-3 protein encoded by a native partial MaSp 2 sequence despite the use of very different spinning and processing conditions, *i.e.* the use of methanol for coagulation and fiber double drawing with methanol and water.³⁴ Additionally, besides showing the compatibility of these Flag-like elastic motifs with the MaSp 2 like crystalline domains in the hybrid chimeric protein, the roles of the short Flag-like motifs in providing extensibility to the fiber were preserved despite the rigidity of the basic repeat, and the lesser number of elastic-like motifs present compared to that present in the native-like ADF3 sequence⁶¹ ((GPGGA)₄ vs. (GPGXX)_{3–9}).

The original combination of the Flag motifs with the MaSp 2 dragline silk crystalline motif in the case of the Y1S8₂₀ protein also provided a unique self-assembly ability in aqueous solutions similar to that observed for other synthetic MaSp 1 and 2 recombinant proteins containing native C- and/or N-termini sequences.^{23–25} This result shows that silk protein re-design, or rather the reengineering of non β -sheet crystalline forming motifs in the synthetic consensus repeat, can also be used to control protein self-aggregation. NMR data on the lyophilized Y1S8₂₀ film showed formation of β -sheet structures in the polyalanine regions in aqueous environments while the lyophilized Y1S8₂₀ protein, which is highly hygroscopic, is primarily disordered and in a random coil geometry. Water seemed to have promoted the crystallization of the polyalanine motifs which drove the spontaneous assembly mechanism seen for this specific Y1S8₂₀ chimeric protein in aqueous conditions. Furthermore, the mechanical data between the as-pulled versus processed Y1S8₂₀-P fibers, though highly variable, due to the less standardized mode of fiber generation, showed that the successive 2x drawing into the air of these water-saturated fibers was responsible for increases in fiber stiffness and ultimate strength, probably due to an increase in crystallite orientation though no XRD data could be collected due to the high salt content of this fiber. As a result of this treatment, the processed fibers were significantly tougher. Mechanically, the processed Y1S8₂₀-P fibers had a similar stress/strain response as that seen for native dragline silk^{1–3}. For these fibers formed in aqueous environments, it is difficult to separate the role of water versus that of stretching in improving mechanical properties. However, due to the inability to process the Y1S8 as-spun fibers, these processed Y1S8₂₀-P fibers were valuable to compare to the A1S8₂₀ chimeric protein fibers since SEM data showed that they seemed to have attained similar levels of fiber organization.

The mechanical performances of these processed Y1S8₂₀-P fibers depended on fiber diameter, and seemed best and more reproducible for fibers with diameters ranging between 10–15 μ m. This optimum fiber diameter range is 2–3 times larger than those of native silk fibers while the size of the recombinant protein is 5-fold smaller. It is important to note that chemical polymer-based fibers,^{62–63} and natural protein-based fibers such as spider and silkworm silks, as well as wools^{64–65} also have variable diameters leading to variation in their mechanical properties though these are much less pronounced than what is seen for synthetic fibers. As a general rule, tensile strength and moduli increase when the diameter of the fiber decreases. This tendency was also observed for fibers produced by extrusion of spinning dopes made of resolubilized spider or silkworm silks,^{31, 66} bovine Achilles tendons (collagen),⁶⁷ or for fibers generated from self-assembled non fibrous protein films.⁶⁸ Additionally, in combination with the effect of fiber diameter, the presence of imperfections or flaws, a still hollow core, and the solidification of trapped salts in the dried Y1S8₂₀-P fibers, most likely contributed to the more random breakage of the fibers. While the Y1S8₂₀ control fibers had already an initial extremely high extensibility, probably due to their saturated water content, it is evident that the major increase in extensibility seen throughout the processing of the A1S8₂₀ spun fibers generated in a more controlled experimental setting, only reached its maximum in the last processing step which consisted in full water-treatment while physically constraining the pre-stretched fiber. This further confirms the similarity in behavior of both chimeric proteins and native spider silk proteins for which water is a well-known plasticizer that affects both amorphous and crystalline regions^{41, 52} allowing molecular rearrangements in the fiber during post-spinning treatments.

In the case of the A1S8₂₀ fibers, we provided a complete morphological (SEM) and structural analysis (NMR and XRD) from the lyophilized protein stage throughout the spinning and different processing treatments to understand the nature and origin of the molecular transformations and internal network reorganization that are associated with the observed evolving mechanical properties of the fibers. NMR data showed that the spinning process drove β -sheet formation in the polyalanine regions for the A1S8₂₀ protein since the

as-spun fibers had a higher β -sheet content than that seen in the lyophilized protein, but it failed to give the same result for the Y1S8₂₀ protein. SEM data showed that the imperfect thick A1S8₂₀ as-spun fibers experienced very clean or sharp transversal breaks that are characteristic of an extremely poor fiber internal network organization. However, these A1S8₂₀ as-spun fibers were not obviously spongy or porous like the Y1S8₂₀ ones which shattered when dry.

The fact that once wetted, the Y1S8₂₀ as-spun fibers did show the presence of formed β -sheet structures reinforces the argument that this protein is more suited for fiber formation in aqueous environments. This clearly shows the unique capacity of the (GPGGA GPGGA)₂ or A1 motif to better accommodate the structural transition of the polyalanine segment into β -sheet structures in this chimeric protein as a result of mechanical shearing and dehydration due, respectively, to extrusion and coagulation in organic conditions. Thus again, these findings show the impact of the primary structure of the silk-like protein on its spinnability under given fiber formation conditions.

XRD data revealed a disordered and overall amorphous structure for the A1S8₂₀ as-spun fibers. This isotropic characteristic indicates the presence of poorly stacked β -sheet structures that have no preferential orientation, thus further explained the brittleness of the fiber. In both case, the Flag-like elastic motifs, may also have been negatively affected in the drying fiber, contributing to further fiber brittleness. This is particularly likely since native Flag proteins are naturally hydrated and the fiber shows major decreases in mechanical properties when dried.⁵⁹ After stretching the A1S8₂₀ as-spun fibers in 90% IPA at the maximum draw ratio possible (2–2.5x), the A1S8₂₀ fibers became insoluble in water. The stretched fibers also displayed impressive increases in ultimate strength and initial modulus. SEM data showed that morphologically, the fibers were more uniform and denser with more rugged breaking points. NMR data support that these morphological and mechanical changes are the results from an increase in β -sheet structures while XRD data (data not shown) indicated that it was concomitant with a slight alignment of the nanocrystals parallel to the fiber axis although the vast majority of the structure remained disordered. Such correlation between increased tensile strength and initial modulus as a result of crystal orientation during post spin draw is in agreement with previous findings for dragline silk⁴⁰ and regenerated dragline silk fibers.³¹ However, although these A1S8₂₀ stretched fibers were still characterized by both higher extensibilities and toughness, these were extremely variable indicating the need for further processing to refine their core structure. The last step of the treatment consisted in wetting the fiber under tension in water. As revealed by NMR and XRD data, not only the A1S8₂₀ proteins in the water-treated fibers experienced an increase in β -sheet structures in their polyalanine regions but also displayed a more pronounced anisotropy indicating the packing and 3-dimensional growth of these β -sheet nanocrystals in a similar motif to that found in spider silk fibers.^{13, 57} Water essentially appears to help orientate the β -sheet crystals together, probably through hydrogen bonding, thus allowing them to find a stable ordered configuration. These results are in line with findings for regenerated spider silks.³¹ These structural changes translated mechanically in much tougher fibers that achieved maximal performances in ultimate strength, initial modulus with much higher and less variable extensibilities. Water acted as a lubricant of the polymer chains for the entire fiber network thus allowing drastic, though incomplete, fiber network reorganization. SEM data showed that these A1S8₂₀ fibers were clearly much denser, and had the highest level of fiber internal organization with both visible longitudinal microfibrils and bundle-type structures running parallel to the fiber axis. However, hollow cavities were still visible along the fiber axis, thus showing a very loosely organized and poorly interwoven fiber network. This observed fiber internal organization was reminiscent of that observed for the more flexible inner core structure seen in native dragline silk, which is uniquely composed of MaSp 2 proteins²⁶ and also characterized by the numerous

presence of *canniculis* or longitudinal interspaces. It is possible, as suggested for the role of the flexible native MaSp 2 fiber inner core, that the large longitudinal interspaces in the synthetic fibers could have accounted for their ability to absorb and retain water. Thus, the remaining variability seen for the extensibility of these synthetic fibers could stem from slight differences in fiber network organization and composition between fibers. Indeed, from our study, we can hypothesize that crystallinity and/or crystal orientation levels could potentially affect the water absorption and retention levels by the fiber and thus, result in differences in fiber network plasticization that would come in play in their extensibility.

For these specific types of chimeric fibers, longer water-treatments, and/or additional fiber stretching after water-treatment may provide the additional structural changes needed to increase fiber density and refine fiber mechanical properties.

CONCLUSION

We have provided a very detailed study on synthetic fibers formed from two, slightly different, Flag/MaSp 2 chimeric proteins, in very different conditions. The morphological and structural transformations responsible for the changes in mechanical properties of these synthetic fibers were tracked using solid state NMR and XRD, and SEM, respectively. These combined studies were critical to assess the quality of the processed fibers, and thus help evaluate the nature of the additional post-spinning modifications required to optimize their mechanical properties.

Both Flag/MaSp 2 chimeric spider silk proteins were 1/5 the length of the native silk proteins^{22, 60} with much simplified basic repeat sequences, yet both proteins in the processed fibers retained structural and functional characteristics of both native dragline and flagelliform silk proteins when in presence of water (hydration-dependent crystallization and network flexibility). Furthermore, this study showed that the overall structure of the basic repeat of these chimeric silk proteins and their molecular weight restricted the range of the mechanical performances of the fibers that were produced in drastically different conditions, but processed in similar ways using water-treatment. Additionally, both Flag-like motifs offer advantageous rapid mechanisms to promote self-assembly through β -sheet driven mechanisms in these chimeric Flag/MaSp 2 proteins. Depending on the spinning conditions, these MaSp 2-like chimeric protein variants may be useful alternatives to control premature aggregation during spinning (switches triggered by heat or shearing).

For artificial silk-like fiber production, because of the more simplistic and more closed spinning mechanisms (single step, no control over pH or protein concentration), the engineering of the silk protein sequence appears to be more critical than the process of fiber formation, as long as appropriate post-spinning modifications can tailor the ultimate mechanical properties by refining the organization of the internal fiber network. The standards for such shorter silk-like fibrous protein sequences are thus bound to differ slightly from those required for *in vivo* silk spinning. The challenge for fibrous protein engineers is to establish the simplest, yet optimum, basic repeat sequences and sequence combinations of silk-like structural amino acid motifs that ensure fiber formation and mechanical performances. Working with such smaller and simplified silk protein versions, protein behavior and secondary structure are more predictable, thus overall more controllable. Implementing the sequence design of a more predictable spinnable silk-like protein is always an alternative means to optimize the mechanical properties of the resulting fiber when post-spinning treatments are failing to do so.

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References

1. Denny M. J Exp Biol. 1976; 65:483–505.
2. Stauffer S, Coguil S, Lewis R. J Arachnol. 1994; 22:5–11.
3. Gosline JM, Guerette PA, Ortlepp CS, Savage KN. J Exp Biol. 1999; 202:3295–3303. [PubMed: 10562512]
4. Hayashi CY, Shipley NH, Lewis RV. Int J Biol Macromolecules. 1999; 24:271–275.
5. Xu M, Lewis RV. PNAS. 1990; 87:7120–7124. [PubMed: 2402494]
6. Hinman MB, Lewis RV. J Biol Chem. 1992; 267:19320–19324. [PubMed: 1527052]
7. Hayashi CY, Lewis RV. J Mol Biol. 1998; 275:773–784. [PubMed: 9480768]
8. Hayashi CY, Lewis RV. Science. 2000; 287:1477–1479. [PubMed: 10688794]
9. Jenkins JE, Creager MS, Butler EB, Lewis RV, Yarger JL, Holland GP. Chem Comm. 2010; 46:6714–6716. [PubMed: 20733981]
10. Becker N, Oroujev E, Mutz S, Cleveland J, Hansma P, Hayashi C, Makarov D, Hansma H. Nat Mater. 2003; 2:278–283. [PubMed: 12690403]
11. Holland GP, Creager MS, Jenkins JE, Lewis RV, Yarger JL. J Am Chem Soc. 2004; 130:9871–9877. [PubMed: 18593157]
12. Jelinski LW, Blye A, Liivak O, Michal C, La Verde G, Seidel A, Shah N, Yang Z. Int J Biol Macromolecules. 1999; 24:179–186.
13. Riekel C, Bränden C, Craig C, Ferrero C, Heidelbach F, Muller M. Int J Biol Macromolecules. 1999; 24:79–186.
14. Simmons A, Michal CA, Jelinski LW. Science. 1996; 271:84–87. [PubMed: 8539605]
15. Lefèvre T, Rousseau ME, Pézolet M. Biophys J. 2007; 92:2885–2895. [PubMed: 17277183]
16. Hirijida DH, Do KG, Michal C, Wong S, Zax D, Jelinski LW. Biophys J. 1996; 71:3442–3447. [PubMed: 8968613]
17. Vollrath F, Knight D. Int J Biol Macromolecules. 1999; 24:243–249.
18. Knight DP, Vollrath F. Proc R Soc Lond B. 1999; 266:519–523.
19. Knight DP, Vollrath F. Naturwissenschaften. 2001; 88:179–182.
20. Vollrath F, Knight DP. Nature. 2001; 410:541–548. [PubMed: 11279484]
21. Challis RJ, Goodacre SL, Hewitt GM. Ins Mol Biol. 2006; 15:45–56.
22. Sponner A, Schlott B, Vollrath F, Unger E, Grosse F, Weisshart K. Biochemistry. 2005; 44:4727–4736. [PubMed: 15779899]
23. Sponner A, Vater W, Rommerskirch W, Vollrath F, Unger E, Grosse F, Weisshart K. Biochem Biophys Res Comm. 2005; 338:897–902. [PubMed: 16253207]
24. Stark M, Grip S, Rising A, Hedhammar M, Engstrom W, Hjalm G, Johansson J. Biomacromolecules. 2007; 8:1695–1701. [PubMed: 17402782]
25. Hedhammar M, Rising A, Grip S, Saenz Martinez A, Norling K, Casals C, Stark M, Johansson J. Biochemistry. 2008; 47:3407–3417. [PubMed: 18293938]
26. Sponner A, Vater W, Monajembashi S, Unger E, Grosse F, Weisshart K. PLoS. 2007; 10:e998.
27. Rammensee S, Slotta U, Scheibel T, Bausch AR. PNAS. 2008; 105:6590–6595. [PubMed: 18445655]

28. Lefèvre T, Boudreault S, Coultier C, Pézolet M. *J Mol Biol.* 2011;238–253.
29. Heim M, Ackerschott CB, Scheibel T. *J Struct Biol.* 2010; 170:420–425. [PubMed: 20045468]
30. Xia XX, Qian ZG, Ki CS, Park YH, Kaplan DL, Lee SY. *PNAS.* 2010; 107:14059–14063. [PubMed: 20660779]
31. Seidel A, Liivak O, Calve S, Adaska J, Ji G, Yang Z, Grubb D, Zax DB, Jelinski LW. *Macromolecules.* 2000; 33:775–780.
32. Elices M, Guinea GV, Plaza GR, Karatzas C, Riekel C, Agulló-Rueda F, Daza R, Pérez-Rigueiro J. *Macromolecules.* 2011; 44:1166–1176.
33. Ann B, Hinman M, Holland GP, Yarger JL, Lewis RV. *Biomacromolecules.* 2011; 12:2375–2381. [PubMed: 21574576]
34. Lazaris A, Arcidiacono S, Huang Y, Zhou JF, Duguay F, Chrétien N, Welsh EA, Soares JW, Karatzas CN. *Science.* 2002; 295:472–476. [PubMed: 11799236]
35. Work RW. *J Arachnol.* 1981; 9:299–308.
36. Shao Z, Vollrath F. *Polymers.* 1999; 40:1799–1806.
37. Shao Z, Young RJ, Vollrath F. *Int J Biol Macromolecules.* 1999; 24:295–300.
38. Blackledge TA, Summers AP, Hayashi CY. *Zoology.* 2005; 108:41–46. [PubMed: 16351953]
39. Teulé F, Furin WA, Cooper AR, Duncan JR, Lewis RV. *J Mater Sci.* 2007; 42:8974–8985.
40. Carmichael S, Barghout JY, Viney C. *Int J Biol Macromolecules.* 1999:295–300.
41. Vollrath F. *Rev Mol Biotechnol.* 2000; 74:67–83.
42. Trabbic KA, Yagger P. *Macromolecules.* 1998; 31:462–471.
43. Teulé F, Cooper AR, Furin WA, Bittencourt D, Rech EL, Brooks AE, Lewis RV. *Nat Protocols.* 2009; 4:341–355.
44. Bennett AE, Rienstra CM, Auger M, Lakshmi KV, Griffin RG. *J Chem Phys.* 1995; 103:6951–6958.
45. Graber T, Anderson S, Brewer H, Chen Y-S, Cho HS, Dashdorj N, Henning RW, Kosheleva I, Macha G, Meron M, Pahl R, Ren Z, Ruan S, Schotte F, Srajer V, Viccaro PJ, Westferro F, Anfinrud P, Moffat K. *J Synchrotron Rad.* 2011; 18:658–670.
46. Saitô H. *Magn Reson Chem.* 1986; 24:835–852.
47. Saitô H, Tabeta R, Asakura T, Iwanaga Y, Shoji A, Ozaki T, Ando I. *Macromolecules.* 1984; 17:1405–1412.
48. Shoji A, Ozaki T, Saitô H, Tabeta R, Ando I. *Macromolecules.* 1984; 17:1472–1479.
49. Simmons A, Ray E, Jelinski LW. *Macromolecules.* 1994; 27:5235–5237.
50. Creager MS, Jenkins JE, Thagard-Yeaman LA, Brooks AE, Jones JA, Lewis RV, Holland GP, Yarger JL. *Biomacromolecules.* 2010; 11:2039–2043. [PubMed: 20593757]
51. Holland C, Terry AE, Porter D, Vollrath F. *Nat Mat.* 2006; 5:870–874.
52. Holland GP, Jenkins J, Creager M, Lewis RV, Yarger J. *Biomacromolecules.* 2008; 9:651–657. [PubMed: 18171016]
53. Holland GP, Jenkins JE, Creager MS, Lewis RV, Yarger JL. *Chem Comm.* 2008; 43:5568–5570. [PubMed: 18997954]
54. Holland GP, Lewis RV, Yarger JL. *J Am Chem Soc.* 2004; 126:5867–5872. [PubMed: 15125679]
55. Izdebski T, Akhenblit P, Jenkins JE, Yarger JL, Holland GP. *Biomacromolecules.* 2010; 11:168–174. [PubMed: 19894709]
56. Jenkins JE, Creager MS, Lewis RV, Holland GP, Yarger JL. *Biomacromolecules.* 2010; 11:192–200. [PubMed: 20000730]
57. Warwicker JO. *J Mol Biol.* 1960; 2:350–362. [PubMed: 13783274]
58. Kohler T, Vollrath F. *J Exp Zool.* 1995; 271:1–17.
59. Vollrath F, Edmond D. *Nature.* 1989; 340:305–307.
60. Ayoub NA, Garb JE, Tinghitella RM, Collin MA, Hayashi CY. *PLoS ONE.* 2007; 2(6):e514. [PubMed: 17565367]
61. Guerette A, Ginzinger DG, Weber BHF, Gosline JM. *Science.* 1996:112–115. [PubMed: 8600519]
62. Wong SC, Baji A, Leng S. *Polymer.* 2008; 49:4713–4722.

63. Abraham TN, George KE. *J Thermoplastic Composite Materials*. 2009; 22:5–20.
64. Rengasamy RS, Jassal M, Rameshkumar C. *Autex Res J*. 2005; 5:30–39.
65. Zhao HP, Feng XQ, Cui WZ, Zou FZ. *Engineering Fracture Mechanics*. 2007; 74:1953–1962.
66. Liivak O, Blye A, Shah N, Jekinski LW. *Macromolecules*. 1998; 31:2947–2951.
67. Gentleman E, Lay AN, Dickerson DA, Nauman EA, Livesay GA, Dee KC. *Biomaterials*. 2003; 24:3805–3815. [PubMed: 12818553]
68. Huang Z, Lu Y, Majithia R, Shah J, Meissner K, Matthews KS, Bondos S, Lou J. *Biomacromolecules*. 2010; 10:3644–3651. [PubMed: 21047055]

FIGURE 1.

Stress/strain analysis of the processed synthetic A1S8₂₀ extruded fibers.

Mechanical performances of the fibers after (A) post spin drawing in 90% IPA (step 2; diameter= 33.5 μm), and (B) water-treatment (step 3; diameter= 28.3 μm). In each graph, the maximum stress and strain observed for the as-spun fibers (step 1) is indicated in orange on the X and Y axes. Maximum values for stress (σ_{max}) and strain (ϵ_{max}), Young's modulus (E_{initial}) and toughness were recorded for fibers at each processing stage. These were: (A) σ_{max} = 125.1 MPa, ϵ_{max} = 58.6 %, E_{initial} = 5.5 GPa, and toughness= 53.3 MJ/m³, and (B) σ_{max} = 184.4 MPa, ϵ_{max} = 80.2 %, E_{initial} = 6.8 GPa, and toughness= 93.5 MJ/m³.

FIGURE 2.

Stress/strain analysis of the processed synthetic Y1S8₂₀-P fibers.

The Y1S8-P fibers were soaked in water and stretched into the air. Mechanically the processed fibers segregate into 3 subgroups depending on their diameters (gray: 6–9 μm ; blue: 10–15 μm ; red: 16–35 μm). Maximum values of stress ($\sigma_{\text{max}} = 143.1 \text{ MPa}$) and Young's modulus ($E_{\text{initial}} = 8.3 \text{ GPa}$) were achieved by fibers in the 6–9 μm diameter group. Maximum values for strain ($\epsilon_{\text{max}} = 80.3 \%$) and toughness (61.6 MJ/m^3) were achieved by fibers in the 10–15 μm diameter group.

FIGURE 3.

SEM images of the different synthetic Flag/MaSp 2 fibers.

Images of (A–E) A1S8₂₀ extruded and processed fibers with (A) as-spun fibers (step 1), (B) IPA-stretched fibers (step 2), and (C) water-treated fibers (step 3). (D–F) fracture points of fibers presented in (A–C). (G–H) Y1S8₂₀ as-spun fiber (I) processed Y1S8₂₀-P fibers generated from a self-assembled film.

FIGURE 4.

^1H - ^{13}C Solid-state NMR analyses of the A1S8₂₀ protein and fibers at all processing stages. ^1H - ^{13}C CP-MAS of A1S8₂₀ lyophilized protein (a), as-spun fibers (b), post-spin-stretched fibers (c), and water-treated fibers (d). Alanine C β in β -sheet and helical/random coil conformations are assigned at 21.3 and 17.8 ppm respectively. The sharp resonances that disappear near 52 and 58 ppm after the post-spin-stretching procedure are assigned to mobile alanine and serine C α sites, respectively.

FIGURE 5.

^1H - ^{13}C Solid-state NMR of fully processed A1S8₂₀ synthetic fibers.

^1H - ^{13}C CP-MAS (a) spectrum is compared to ^{13}C direct observation using fast (1 second) recycle delay (b) after rehydrating the fully processed fibers with water.

FIGURE 6.

^1H - ^{13}C Solid-state NMR of the Y1S8₂₀ protein and extruded fibers.

^1H - ^{13}C CP-MAS of Y1S8₂₀ lyophilized protein (a), as-spun fibers (b), and water-hydrated as-spun fibers (c). The three broad resonances between 120 and 160 ppm are aromatic Tyrosine carbons, and the peak at 70 ppm is the spinning side band from the carbonyl.

FIGURE 7.

^1H - ^{13}C Solid-state NMR of the Y1S8₂₀ protein and film.

^1H - ^{13}C CP MAS of Y1S8₂₀ film formed in aqueous conditions (a) and the lyophilized Y1S8₂₀ protein (b).

FIGURE 8.

X-ray diffraction analyses of the A1S8₂₀ synthetic fibers.

(A) As-spun (step 1) and (B) stretched then water-treated (step 3) A1S8₂₀ synthetic fibers.

The air scattering background was removed from the data. The double arrow indicates the direction of both fiber axis and beam stop. The data were collected with a detector distance of 300 mm.

TABLE I

Average mechanical properties of the Flag/MaSp 2 synthetic fibers.

Fiber	Stage	Diameters μm (number of fibers)	E_{initial} GPa	σ_{max} MPa	ϵ_{max} %	Toughness MJ/m ³
A1S8 ₂₀	Step 1	(5) 68.3 $\pm$ 25.4	1.1 $\pm$ 1.3	28.4 $\pm$ 25.4	1.7 $\pm$ 0.6	0.3 $\pm$ 0.4
	Step 2	(14) 33.5 $\pm$ 5.6	3.3 $\pm$ 1.4	101.7 $\pm$ 32.8	18.7 $\pm$ 17.9	18.8 $\pm$ 20.7
	Step 3	(11) 28.3 $\pm$ 3.4	4.4 $\pm$ 1.0	127.5 $\pm$ 23.0	52.3 $\pm$ 23.6	54.6 $\pm$ 23.6
Y1S8 ₂₀ -P	As-pulled	All Fibers				
		(10) 22.9 $\pm$ 10.1	0.5 $\pm$ 0.8	22.9 $\pm$ 15.8	46.9 $\pm$ 23.0	5.6 $\pm$ 5.1
		Subgroups				
		9–18 (4) 12.9 $\pm$ 4.5	1.1 $\pm$ 1.1	38.9 $\pm$ 15.8	30.0 $\pm$ 23.7	8.0 $\pm$ 0.8
		20–40 (6) 28.8 $\pm$ 7.0	0.08 $\pm$ 0.07	13.3 $\pm$ 7.0	57.3 $\pm$ 17.0	4.3 $\pm$ 2.9
Processed	All Fibers					
		(18) 14.0 $\pm$ 8.7	3.8 $\pm$ 2.1	96.2 $\pm$ 28.8	29.6 $\pm$ 20.5	22.6 $\pm$ 15.7
		Subgroups				
		6–9 (7) 7.6 $\pm$ 0.7	5.5 $\pm$ 1.8	123.0 $\pm$ 15.0	17.2 $\pm$ 12.7	20.0 $\pm$ 12.9
		10–15 (6) 12.1 $\pm$ 1.5	3.1 $\pm$ 1.4	92.0 $\pm$ 21.0	43.8 $\pm$ 26.9	31.4 $\pm$ 22.0
		16–35 (5) 25.3 $\pm$ 8.8	2.1 $\pm$ 0.8	64.0 $\pm$ 8.2	29.9 $\pm$ 7.6	15.8 $\pm$ 4.0

At each stage of the fiber processing, the average values for the following parameters are presented with their respective standard deviation ($\pm$): diameter, maximum stress (σ_{max}) and strain (ϵ_{max}), Young's modulus (E_{initial}) and toughness. For the A1S8₂₀ synthetic fibers, the steps 1–3 indicate that the fibers were as-spun (step 1), stretched in 90% IPA (step 2), and water-treated (step 3). For the Y1S8₂₀ pulled fibers (Y1S8₂₀-P), the data are presented for the entire sample or after splitting the sample into subgroups based on the diameter range of the fibers. The number of fibers tested is indicated between brackets.

TABLE II

Mechanical properties of the toughest synthetic Flag/MaSp 2 fibers.
The mechanical performances for the native silk fibers were compiled from [1–3, 58–59]. The estimated sizes of native silk proteins were obtained from [4, 7] for Flag, and from [22, 60] for MaSp 1 and MaSp 2.

Spider Silk Fiber (<i>diameter</i>)	Protein (<i>molecular weight</i>)	E_{initial} GPa	σ_{max} GPa	ϵ_{max} %	Toughness MJ/m ³
Flag/MaSp 2 synthetic					
A1S8 ₂₀ (26.6 μm)	A1S8 ₂₀ (58 kDa)	4.6	0.136	80.2	93.5
Y1S8 ₂₀ -P (12.2 μm)	Y1S8 ₂₀ (62 kDa)	2.5	0.096	80.3	61.6
Native					
Dragline (4–6 μm)	MaSp 1 & MaSp 2 (250–350 kDa)	10	1–4	27–35	160
Flagelliform	Flag (360 kDa)	0.003	0.5	200–270	150