Artificial Spinning of Spider Silk

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1. Introduction. Silks have attracted the interest of scientists of various disciplines for a long time.[1,2] Initially, this interest was inspired by the importance of silkworm silk in the manufacture of high-quality textiles. Attempts to make synthetic fibers mimicking the basic structural motifs and properties of native silk resulted in the invention of high-performance polyamide materials such as Nylon and Kevlar, which have had an important impact extending beyond the textile industry. Although many insects and spiders are known to produce silks, until today the cocoon silk of the silkworm, *Bombyx mori*, remains the only native silk fiber that can be cultivated and commercialized. Since advances in biotechnology now have opened new pathways for the controlled large-scale production of proteins with predetermined amino acid sequences, materials scientists, polymer chemists, and bioengineers have become increasingly interested also in other kinds of silks and silk-producing organisms that so far have escaped cultivation. In contrast to silkworms, spiders are remarkable in their ability to spin a variety of different silks, each of which has been optimized with respect to its specific biological function by nature. Thus spiders can teach us important lessons about structure−function relationships of biomaterials based on proteins.

Due to its excellent mechanical properties, the dragline silk of the golden-orb weaver, *Nephila clavipes*, has been the subject of the most intense studies. A balanced combination of high tensile strength, stiffness, and elasticity makes this fiber one of the toughest, if not the toughest material of its kind known so far.[2] The amino acid sequences of two different proteins building up the silk fibers have been reported.[3,4] Basically, both proteins consist of repeats of polyalanine runs that exist in antiparallel β-sheet crystals[5,6] and glycine-rich sections containing the bulkier amino acid residues. The latter portion probably does not adopt any well-defined secondary structure and is considered to form an amorphous or an oriented-amorphous polymer matrix. While it is the oriented β-sheet crystals that are believed to be responsible for the high strength and stiffness, it is the amorphous material portion causing the elasticity of the fiber and mediating stress transfer between the crystalline domains.[7]

Recently, synthetic[8,9] and native[10] genes coding for amino acid sequences based on the consensus repeats in the dragline silk proteins of *N. clavipes* have been successfully expressed in *E. coli* bacteria. Consequently, laboratory-scale amounts of silklike protein powders are readily available. The final hurdle on the way to the production of manmade silks now lies in the development of an appropriate spinning technology capable of converting these powders into high-performance fibers. In this respect, it is of special importance to work out a comprehensive understanding of the molecular processes occurring during the spinning of protein fibers and to learn how the spinning conditions can influence the properties of the final material. To date, there have been reported limited attempts to artificially spin protein fibers.[1,8,11−14] Most of what is known in the art is deduced from the wet-spinning of *B. mori* silk fibers.[11−13] There has also been a preliminary report of electrospun spider silk nanofibers.[14] Before plunging into trials to spin genetically engineered materials, it is advantageous to focus on the regeneration of biological silks because in this case the native fiber can be used as a benchmark for evaluating the success of the spinning process. The present paper reports on the first controlled wet-spinning of dragline silk of *N. clavipes*.

2. Experimental Section. 2.1. Spinning Procedure. The dragline silk was harvested from female *N. clavipes* spiders (Central Florida) by forced silking according to a technique established by Work and Emerson.[15] The spiders were silked every second day for a period of 40 min; the silking rate was 2 cm/s. This procedure yielded an average of about 0.8 mg of dragline silk per spider and silking day. Following two patents held by du Pont de Nemours & Company,[8,11] hexafluoro-2-propanol (HFIP, 99+ %, Aldrich, Milwaukee, WI) was chosen as the solubilizing agent and was applied to the dragline silk in such an amount as to obtain a fibroin solution with a concentration of 1% (w/w). Portions of the fibers that did not dissolve overnight were separated by filtering the solution through a glass fiber syringe filter with a pore size of 1 μm (Gelman Sciences, Ann Arbor, MI). After allowing evaporation of most of the HFIP at ambient temperature over a 24 h time period and further annealing in a vacuum at 60 °C for 16 h, a fibroin film remained. Comparison of the masses of this film and of the native fibers that had been dissolved showed that even after prolonged heating in vaccum approximately 10% (w/w) of HFIP could not be removed from the fibroin. This interesting result, suggesting strong bonding of some HFIP molecules to the fibroin protein, was supported by element-specific X-ray fluorescence spectroscopy, which also gives evidence for the presence of fluorine in the film. The concentration of fluorine determined by this analytical technique corresponds very well to the HFIP content obtained from the mass measurements. A 2.5% (w/w) solution of the film in HFIP was prepared and used for spinning without further treatment.

The fibroin solution was wet-spun using a spinneret designed for handling minimum amounts of material. The experimental setup as well as the spinning procedure have been described in detail elsewhere.[13] Basically, the spinneret is composed of a silicon wafer with a square aperture (180 μm × 180 μm) held in place by a stainless steel jig to which a disposable syringe with the spinnable solution can be fitted. Water, methanol, 2-propanol, and acetone were tested as potential coagulants. Though under similar conditions methanol and 2-propanol have been reported to be appropriate coagulants for spinning of solubilized *B. mori* silk[11−13] and genetically engineered analogues of *N. clavipes* dragline silk,[8] respectively, in our tests only extrusion of the

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fibroin solution into acetone was observed to lead to any appreciable fiber formation. Successful spinning of the fiber, which was characterized by SEM and solid-state NMR (see Results and Discussion), was performed by forcing with a syringe pump 6 μL/s of the fibroin solution through the spinneret into a 30 cm column filled with acetone (99.7%, Mallinckrodt, KT). This procedure resulted in fiber formation at an approximate rate of 10 m/min. Spinning of 0.4 cm^3 of fibroin solution, containing 16.0 mg of protein, yielded approximately 7 mg of coagulated solid, from which 4.0 mg of regenerated fibers could be manually spooled. The fibers were dried for 1 h in a vacuum at 40 °C and afterward equilibrated under ambient conditions.

2.2. Fiber Characterization. Scanning electron microscopy (SEM) and ^{13}C cross-polarization magic angle spinning nuclear magnetic resonance (CP-MAS NMR) spectroscopy have been chosen to examine the macroscopic morphology/surface texture and the microscopic structure, respectively, of both regenerated fibers and native dragline silk.

For SEM investigations, the specimens were fixed on aluminum sample holders with a double-sided electrically conductive carbon tape (SPI Supplies, West Chester, PA) and covered with a layer of gold−iridium. SEM micrographs were taken with a Stereoscan 440 (Leica, Cambridge, England).

^{13}C CP-MAS NMR spectra were acquired on a home-built spectrometer using a Doty Scientific (Columbia, SC) magic angle spinning probe. The system operates at a ^{13}C Larmor frequency of $\omega/2\pi = 90.556$ MHz. Randomly coiled fiber samples were either directly filled into 5 mm o.d. ZrO_2 rotors (native silk) or, in the case of the small weight samples (regenerated fibers), centered in the middle of such rotors by embedding them in ICN Silica 32-63 (60 A, ICN Bimedicals, Eschwege, Germany). Cross-polarization was performed at 60 kHz with a $^{13}C-^{1}H$ contact time of 2.5 ms. During acquisition protons were decoupled at 90 kHz. Samples were spun at the magic angle with a frequency of 5 kHz. FIDs were accumulated with a recycle delay time of 2.5 s. Chemical shifts are reported relative to TMS as the reference.

3. Results and Discussion. 3.1. SEM Micrographs. Figure 1a,b shows SEM micrographs of native dragline silk and of the regenerated fiber, respectively. The native silk fiber exhibits a slightly rough surface and a diameter of 2.7 μm. Micrographs taken from a bundle of native fibers at lower magnification (not shown) revealed that the diameters of the individual fibers vary between 2.5 and 4.0 μm, whereas the variation of the diameter of each of the fibers along its axis is only ± 0.3 μm. These results are in reasonable agreement with data published in the literature.[1] The regenerated fiber at the displayed magnification exhibits a smooth surface. However, at the magnification used in Figure 1a, a surface roughness similar to that one of the native fiber was observed (micrograph not shown). The part of the fiber shown in the micrograph exhibits a diameter of about 40 μm. This value represents an average size; lower magnification pictures of different parts of the regenerated fiber reveal variations in diameter between 20 and 80 μm. These large variations may be due to the manual reeling of the fresh, still plastic fiber, which unpreventably results in some nonconstant draw.

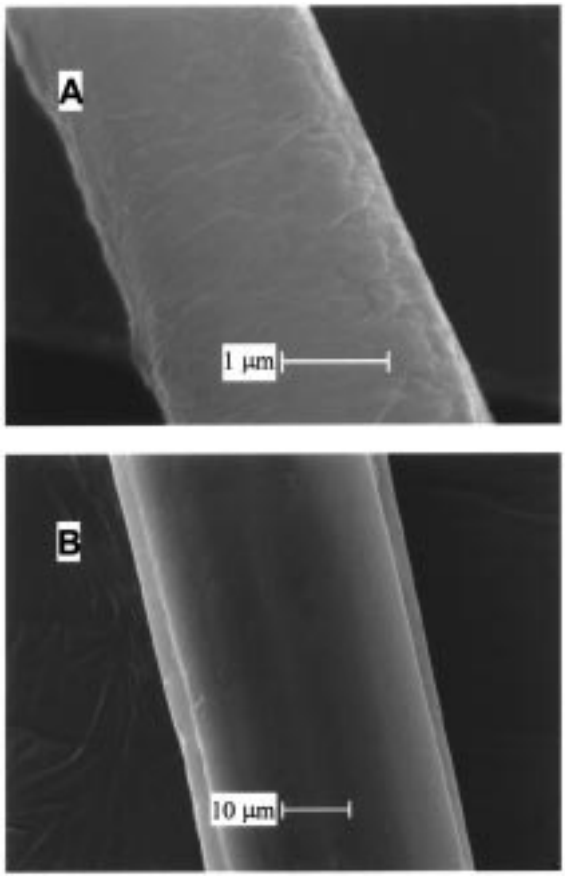

Figure 1. SEM micrographs of a native (A) and artificially spun (B) silk fiber from the spider *N. clavipes*.

3.2. ^{13}C CP-MAS NMR. The isotropic ^{13}C NMR chemical shifts of carbon atoms in proteins are sensitive to the secondary structure in which the amino acids are involved. In most cases, they are not as strongly influenced by the local sequence of the amino acids.[16−18] For alanine, which is a major constituent of the silk protein, relations between local conformation and ^{13}C chemical shift have been well established.[16,17] The variations of chemical shifts of the C_α and C_β carbons of alanine in different structural elements are so pronounced that ^{13}C NMR has repeatedly provided valuable information on the molecular structure of silk fibroin materials.[5,13,17,18] Particularly, alanines in the antiparallel β-sheet conformation can be well distinguished from all other alanine residues by their typical downfield shift of the C_β carbons.[16,17]

Figure 2a−c shows the ^{13}C CP-MAS NMR spectra of the native fiber, the fibroin film, and of the regenerated fiber, respectively. The shaded areas highlight the ranges of isotropic chemical shifts reported in the literature for the alanine C_α (48−53 ppm) and C_β (15−22 ppm) carbons in proteins.[16,17] The regions between the dotted lines show the chemical shift ranges typical of alanine in the antiparallel β-sheet conformation. The sequence of NMR spectra shows that the protein molecules undergo significant conformational changes during the various steps of fiber processing. Whereas the features of Figure 2a recall that the majority of the alanine residues in the native spider silk adopt the β-sheet conformation,[5,6] in the film obtained from the fibroin solution in HFIP (Figure 2b) this structural element is almost completely missing. Here, the NMR signal of the C_α carbons of alanine is displaced consider-

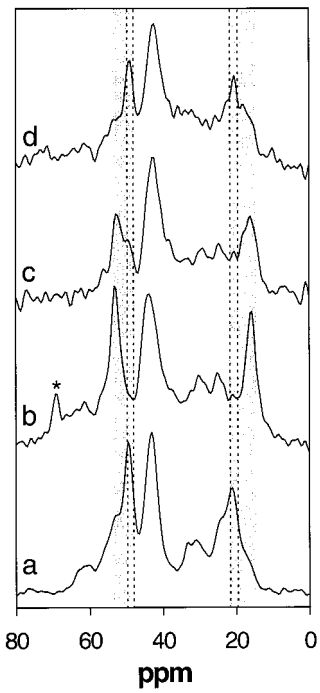

Figure 2. ^{13}C CP-MAS NMR spectra of the (a) native *N. clavipes* silk (55 mg, 6000 scans), (b) the fibroin film (16 mg, 28 000 scans), and the (c) fresh (4 mg, 26 000 scans) and (d) water-treated (3 mg, 38 000 scans), regenerated silk. Shaded areas highlight chemical shift ranges of C_α and C_β atoms of alanine; regions between dotted lines are typical of alanines in the β-sheet conformation. The asterisk denotes an NMR signal due to HFIP.

ably downfield. Its chemical shift of 53.0 ppm is characteristic of alanine in right-handed α-helices.[16,17] The observation that the NMR signal position of the alanine C_β carbons changes into the opposite direction to a value of 15.7 ppm is in line with the conclusion that these amino acids adopt the α-helix conformation in the film.[16,17] This finding has precedent, as α-helices have also been reported to be the major structural element in films cast from solutions of poly(L-alanine) in HFIP.[19] In solution, poly(L-alanine) in HFIP has been suggested to adopt a "double bonded" helical conformation in which the "normal" α-helix is slightly distorted by additional hydrogen bonds formed between the peptide carbonyl oxygens and the protons of the HFIP hydroxyl group.[19] This structural model is supported by our present finding that an appreciable amount of HFIP molecules is so strongly attached to the fibroin that it cannot be removed by vacuum treatment at 60 °C (see Experimental Section as well as the marked NMR signal at 69.0 ppm in Figure 2b, which arises from the CF_3 carbons of HFIP).

The NMR spectrum of the regenerated fiber displayed in Figure 2c shows that a small number of the alanines becomes involved in β-sheet structures during spinning. However, the portion of the alanine residues adopting this conformation in the regenerated silk is much lower than it is in the native material (Figure 2a). Interestingly, the regenerated fiber turned out to be very brittle. Because correlations between the β-sheet content and the tensile strength of silk fibers recently have been reported,[13] it seems reasonable to assume that at least one necessary prerequisite for improving the mechanical properties of the regenerated fiber is to force a higher percentage of the alanines to crystallize in the β-sheet conformation.

One well-known possibility to induce a conformational transition in proteins is to immerse them in an appropriate solvent. *B. mori* fibroin, for example, has been reported to undergo a transition from the α-helical silk I to the β-sheet silk II form during contact with polar solvents, especially when they are mixed with water.[18] This may occur because the partial replacement of intrahelical hydrogen bonds by intermolecular protein−solvent interactions plasticizes the molecular chains. New, kinetically favorable pathways for conformational transitions and/or recrystallization—energetically driven by hydrophobic effects—thus would be opened. Similar mechanisms might account for the well-known effect of supercontraction shown by spider dragline silk when immersed in water.[2] These considerations led us to investigate the effect of a water treatment on the structure of the artificially spun spider silk. The randomly coiled regenerated fiber used for recording the NMR spectrum displayed in Figure 2c was thoroughly soaked for 2 h in a large amount of water (ca. 2 mL of H_2O/1 mg of silk) at room temperature. After removal from the water, the fiber was dried at ambient temperature overnight and annealed for 1h in a vacuum at 40 °C. After equilibration in the laboratory atmosphere, an NMR spectrum of this water-treated material was recorded under the same conditions as applied before. The conclusion that can be drawn from the obtained spectrum (Figure 2d) is that the water treatment indeed produces the desired increase of the fraction of alanine residues adopting the β-sheet conformation. Closer inspection of the NMR line shapes shows that the β-sheet content in the water-treated fiber is approximately 3 times as large as it was in the freshly spun material. Though so far we have not been able to obtain any quantitative information on the tensile properties of the water-treated, artificially spun fiber, the fact that the main features of its NMR spectrum (Figure 2d) are very similar to those of the native silk (Figure 2a) suggests a strong structural similarity of these two materials. This finding is very encouraging for our continuing investigations.

4. Conclusions and Prospects. Fibers have been regenerated from the solubilized dragline silk of the golden-orb weaver, *N. clavipes*, by a conventional wet-spinning technique. This is the first time that spider silk is reported to be wet-spun in a controlled fashion outside the living organism. It is interesting to note that the conditions reported for the spinning of a genetically engineered spider silk analogue protein[8] have not resulted in successful fiber formation with the native fibroin. This finding seems to indicate that small changes in the protein's amino acid sequence can have a considerable impact on its spinnability.

At the moment we are improving our spinning equipment with the intention of further reducing the dead volume as well as allowing automatic reeling and drawing of the nascent fiber. This should lead to a decrease and a higher uniformity of the fibers' diameter as well as to an increase of the molecular chain orientation. We hope that with this advanced technology fibers can be produced that mimic the native material with regard to diameter and mechanical properties. In any case, however, such fibers will allow both NMR measurements and tensile tests and will thus lead to an understanding of the molecular processes occurring during the spinning of the protein material.

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References and Notes

(1) Kaplan, D., Adams, W. W., Farmer, B.; Viney, C., Eds. *Silk Polymers: Materials Science and Biotechnology*; ACS Symposium Series 544; American Chemical Society: Washington, DC, 1994.
(2) Kaplan, D. L.; Lombardi, S. J.; Muller, W. S.; Fossey, S. A. Silk. In *Biomaterials: Novel Materials from Biological Sources*; Byrom, D., Ed.; Stockton Press: New York, 1991.
(3) Xu, M.; Lewis, R. V. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 7120−7124.
(4) Hinman, M. B.; Lewis, R. V. *J. Biol. Chem.* **1992**, *267*, 19320−19324.
(5) Simmons, A.; Ray, E.; Jelinski, L. W. *Macromolecules* **1994**, *27*, 5235−5237.
(6) Simmons, A. H.; Michal, C. A.; Jelinski, L. W. *Science* **1996**, *271*, 84−87.
(7) Termonia, Y. *Macromolecules* **1994**, *27*, 7378−7381.
(8) Fahnestock, S. R. Novel, recombinantly produced spider silk analogs. Int. Application # PCT/US94/06689, Int. Publication # WO 94/29450, 1994.
(9) Fahnestock, S. R.; Irwin, S. L. *Appl. Microbiol. Biotechnol.* **1997**, *47*, 23−32.
(10) Arcidiacono, S.; Mello, C.; Kaplan, D.; Cheley, S.; Bayley, H. *Appl. Microbiol. Biotechnol.* **1998**, *49*, 31−38.
(11) Lock, R. L. Process for making silk fibroin fibers. U.S. Patent 5,252,285, 1993.
(12) Trabbic, K. A.; Yager, P. *Macromolecules* **1998**, *31*, 462−471.
(13) Liivak, O.; Blye, A.; Shah, N.; Jelinski, L. W. *Macromolecules* **1998**, *31*, 2947−2951.
(14) Zarkoob, S.; Reneker, D. H.; Eby, R. K.; Hudson, S. D.; Ertley, D.; Adams, W. W. *Polym. Prepr. (Am. Chem. Soc., Div. Polym. Chem.)* **1998**, *39*, 244−245.
(15) Work, R. W.; Emerson, P. D. *J. Arachnol.* **1982**, *10*, 1−10.
(16) Saitô, H.; Tabeta, R.; Shoji, A.; Ozaki, T.; Ando, I. *Macromolecules* **1983**, *16*, 1050−1057.
(17) Saitô, H.; Tabeta, R.; Asakura, T.; Iwanaga, Y.; Shoji, A.; Ozaki, T.; Ando, I. *Macromolecules* **1984**, *17*, 1405−1412.
(18) Ishida, M.; Asakura, T.; Yokoi, M.; Saitô, H. *Macromolecules* **1990**, *23*, 88−94.
(19) Parrish, J. R., Jr.; Blout, E. R. *Biopolymers* **1972**, *11*, 1001−1020.

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