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Synthetic Spider Silk Proteins and Threads

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E. coli can be genetically engineered to mimic the complex natural process by which a spider makes the proteins that it uses to spin the fibers for its web. With additional processing, fibers with properties similar to those of natural spider silk can be obtained.

Silk is an amazing material produced naturally by various species, such as the silk moth and silk worm (Lepidoptera), bees, wasps, and ants (Hymenoptera), and spiders (arthropods). Each species' silk has its own unique set of properties.

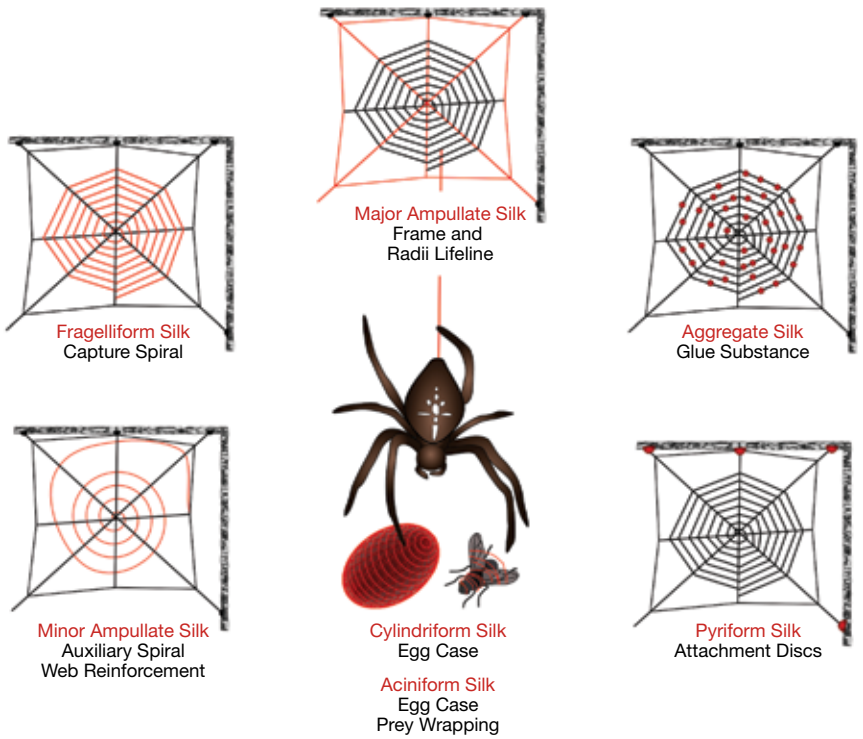
For example, silk from the silk moth *Bombyx mori* is ideally suited for fashion textiles due to its light weight, soft touch, and luxurious appearance. It has been produced commercially and traded in China for at least 4,000 years — an enduring commercial success that can be attributed to the high yields achieved via breeding the silk-producing larvae on large farms. Although silks from other species, especially spider silk, have even higher toughness and tensile strength, as well as better chemical resistance — properties that make them of great interest to industry — they have not been produced commercially to date.

Spiders such as the golden orb-weaver *Nephila clavipes* (Figure 1) can produce various kinds of silk — each perfectly adapted to the specific requirements demanded by nature. Female orb-web weavers can produce more than six different silks, each made from a different combination of silk proteins.

The most ancient silks produced by spiders, in an evolutionary sense, are those used for reproduction. The tough outer egg case is composed of a cylindrical silk, while the soft inner part of the casing is formed by aciniform silk, which is also used for the wrapping of prey.

Spider webs consist of various evolutionarily younger, and thus more developed or specialized, types of silk (Figure 2). These webs can withstand high deformations caused by wind or prey, because of their complex geometrical structure and because the different threads can adapt their mechanical response depending on the load *(1)*. Dragline, or major ampullate, silk forms the frame and radii of the web and serves as a lifeline for the spider during escape. Flagelliform silk, which is made of more-elastic proteins, makes up the capture spiral of the net. Piriform silk connects the dragline and flagelliform silks and attaches the web to a substrate (*e.g.,* a tree or a door frame). Minor ampullate silk is used in the early stages of web building and as auxiliary spiral or web reinforcement. Aggregate silk is sticky and is applied to the nonsticky flagelliform silk spiral to prevent prey from escaping.

▲ **Figure 1.** A female *Nephila clavipes* uses several different types of silk to weave her web. Photo courtesy of S. Schmidt.



◀ **Figure 2.** Each type of spider silk has evolved with a unique set of properties needed for various applications.

Silks of the genera *Araneus* (European garden cross spider), *Nephila* (golden silk orb-weaver), *Argiope* (wasp spider, St. Andrew's Cross spider) and *Latrodectus* (widow spider) are the best-characterized ones. Fragments of coding DNA of almost all silk proteins can be found in public databases, although complete sequences are available for only a limited number of silks, such as the dragline silk proteins of the western black widow spider (*Latrodectus hesperus*) and the egg case silk protein of the wasp spider (*Argiope bruennichi*) *(2)*.

Silk proteins and properties

For decades, silk research has focused mainly on the dragline silk. The interplay of its high tensile strength and elasticity results in a very high toughness that is competitive with (and sometimes even better than) that of modern synthetic fibers like Kevlar, a polyaramid brand from DuPont. The thickness of spider dragline silk varies from 1 to 20 µm, depending on the spider species and its size.

Natural silk thread has been studied intensely, but some details remain obscure. The protein core is thought to be covered with a thin glycoprotein layer and a lipid outer coating. Two main protein components, each with a molecular size of more than 300 kDa, have been identified in the core: MaSp1 and MaSp2. Both amino acid sequences consist of highly repetitive motifs with an unusual amino acid composition, namely a significantly high alanine and glycine content; MaSp2 also has a high proline content. These short motifs consist of repeating units of 20 to 40 amino acid residues in which hydrophobic and hydrophilic regions alternate. The repetitive core region, formed by several of these repeating units, is flanked by nonrepetitive domains at the carboxyl and amino terminal ends. These terminal ends often contain cysteine residues to link two or more protein monomers covalently (Figure 3) *(2)*.

The silk protein's amino acid sequence determines the three-dimensional structure of the protein, which is directly associated with the mechanical properties of the thread. Alanine-rich motifs adopt β-sheet structure and assemble into crystalline substructures in the thread. They are thought to be responsible for toughness. The amorphous matrix around these crystallites consists of glycine blocks that form β-spiral structures, or random coils, that behave like springs and provide elasticity to the thread. Thus, the performance of the dragline fiber depends on the ratio of the two protein components, MaSp1 and MaSp2, and varies among spider species.

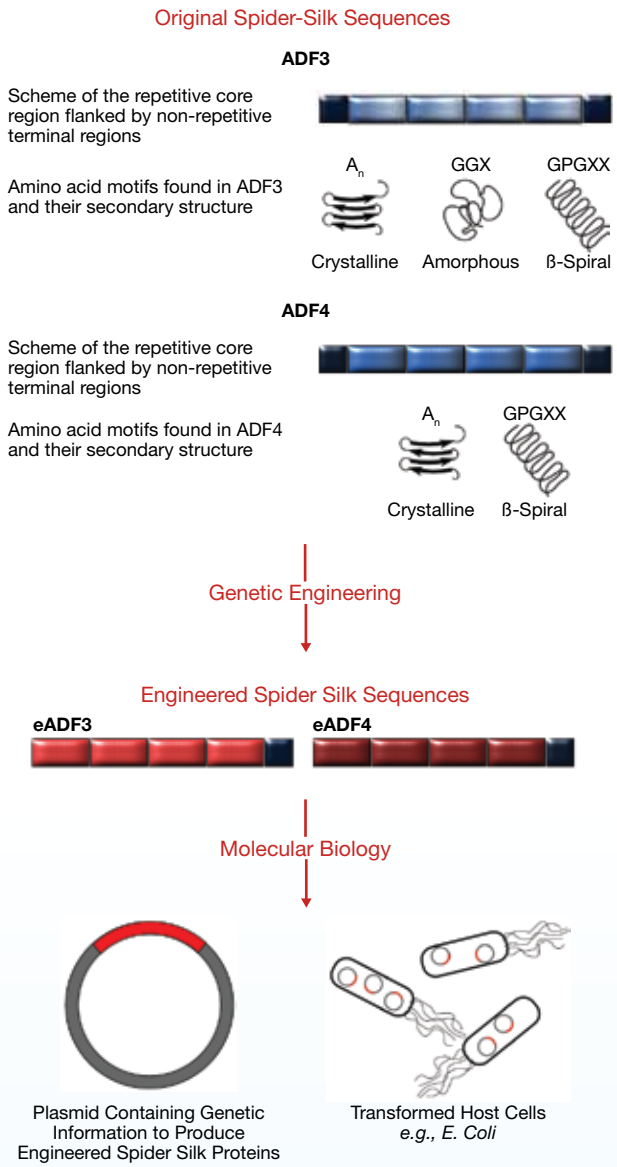
The dragline of *Nephila clavipes,* for instance, is composed of approximately 80% MaSp1 and approximately 20% MaSp2 and has a high tensile strength that exceeds 1,500 MPa, which is comparable to that of steel *(3)*. It was recently discovered that the Darwin's bark spider (*Caerostris darwini*) produces a silk thread with a toughness of more than 250 MJ/m^3 *(3)*, which is about five times higher than that of Kevlar, making it the toughest silk fiber measured to date.

Table 1 compares the properties of common spiders' dragline silk.

Limitations on the production of spider silk

Considering its mechanical and physico-chemical properties, spider silk is perfectly suited for many industrial applications. Yet, no spider silk product is commercially available. Spiders are cannibals, so they cannot be housed together, and thus, cannot be bred on a large scale. In addition, the silk must be harvested manually by pulling each thread directly out of a spider's abdomen, which is prohibitively expensive.

Alternatively, spider silk can be produced as recombinant proteins using engineered host organisms. Many prokaryotic



▶ **Figure 3.** How to produce recombinant silk proteins: The modules of the highly repetitive original silk amino acid sequences (ADF3 and ADF4) contain motifs (A_n, GGX and GPGXX) with distinct secondary structures. Characteristic amino acid repeating units (modules consisting of motifs) are then selected and backtranslated *in silico* to get a gene sequence that is adapted to the host's codon usage. The synthesized gene sequences are combined to obtain whole engineered spider silk genes. The genetic information coding for silk proteins is then transplanted into the host organism (*e.g., E. coli*), which is then able to produce the engineered spider silk proteins (eADF3 and eADF4).

and eukaryotic hosts have been employed, each with its own advantages and disadvantages with regard to costs, handling, productivity, and impurities (*e.g.,* other proteins, degradation products, production remnants). The main benefit of this approach is that the process is independent of the spider.

Recombinant strategies, however, have been limited because few full gene sequences for spider silk proteins are available. Genetic instability, recombination due to repetitive sequences, and undesirable mRNA secondary structures lead to problems with rearrangements and truncated translation products — further complicating the recombinant approach.

Two strategies have been used to date to produce recombinant spider silk proteins. One is based on the complete or a fragmented original (*i.e.,* natural) gene sequence coding for spider silk proteins. The other uses a synthetic sequence that mimics or even enhances the original silk proteins.

Copying nature with original gene sequences

When producing spider silk proteins based on the authentic (or largely authentic) gene, the chosen sequences are transferred into a suitable production host. The expression of authentic, full-length gene sequences in *Escherichia coli* results in ineffective protein production, mainly because the particular codons used by spiders are different from those used by *E. coli.*

Codons are the genetic basis of every amino acid. A codon consists of a sequence of three nucleotides that specifies which amino acid will be added next during protein synthesis. The genetic code is degenerated, which means that multiple codons can code for the same amino acid. The use of these codons varies from species to species — as a result, *E.coli* is not able to process some of the spider-specific codons.

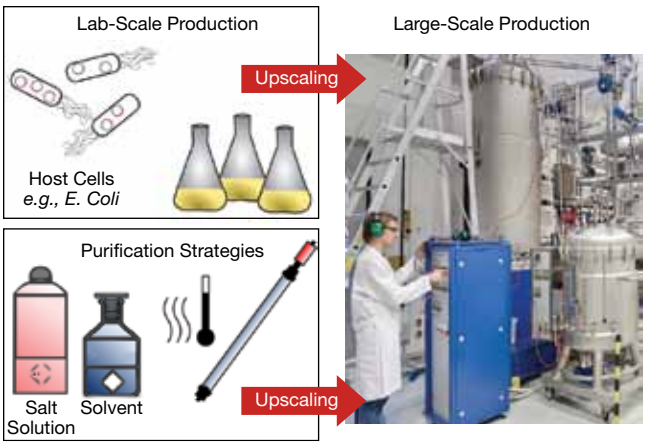
In addition, highly repetitive gene sequences are prone to homologue recombination, which results in truncated, and thus

Table 1. Properties of dragline silk collected from web radii of different types of spiders.

Genus and Species	Stiffness, GPa	Strength, MPa	Extensibility*, %	Toughness, MJ/m^3
Araneus diadematus	3.6	1,599	39	193
Nuctenea umbratica	7.4	1,778	35	196
Larinioides cornutus	7.7	1,760	31	197
Nephila clavipes	8.4	1,725	32	206
Verrucosa arenata	7.6	1,842	36	228
Gasteracantha cancriformis	5.2	1,929	49	254
Caerostris darwini	11.5	1,850	39	271

* with respect to maximum strain at the breaking point

Source: Adapted from *(3)*.



◀ **Figure 4.** The production of several spider silk proteins has been scaled up from laboratory to industrial scale.

unwanted, spider silk proteins.

The largest reported spider silk protein produced in a specially engineered *E. coli* was based on MaSp1 of *Nephila clavipes* and had a size of 285 kDa *(4)*.

Other processes use the insect cell lines of *Bombyx mori* and *Spodoptera frugiperda,* which are genetically more closely related to spiders than to *E. coli*. Spider silk protein fragments of 110 and 140 kDa have been obtained with these hosts. The yield generally decreases with increasing protein size, which can be attributed to inefficient transcription, low gene copy numbers, or limitations of the cellular system of the host.

Another approach to producing spider silk utilizes transgenic animals that carry a segment of silk DNA in the genome of their epithelial cells. These are found in the alveoli of the mammary glands of female animals and are involved in the production of milk proteins. The promoter and the regulatory regions of the genes that are specific for milk proteins are used to direct the silk gene expression in the mammary gland.

Mice and goats were transgenically modified to produce spider silk proteins, which are secreted into the milk. Milking goats was thought to be an easy and fast method to obtain silk protein. However, problems occurred with the separation of the silk and milk proteins. In addition, only females produce milk, and maintaining transgenic animals in large numbers is highly regulated and very expensive.

Transgenic silkworms can be used to express original sequences. A major advantage of this approach is that the harvested product is the finished fiber obtained from the cocoon, unlike silk protein, which must be purified and further processed into fiber *(2, 5, 6)*. Research on this promising approach is ongoing, but several problems must be solved before this system becomes commercially viable.

For instance, the inner core of the silk thread produced via transgenic silkworms is still mainly composed of the original *Bombyx mori*-fibroin with only a small amount of spider-silk-like protein (less than 6% w/w). This concentration is too low to obtain the desired mechanical properties of high-performance silk threads. *(7)*.

Also, spinning experiments have shown that post-drawing of the fiber, mimicking the way a spider pulls dope from the gland, is necessary to obtain good fiber performance. Once the fiber has dried, it cannot be drawn (silk worms are not capable of drawing), and therefore will likely remain inferior.

Enhancing nature using engineered gene sequences

The production of recombinant spider silk proteins based on engineered DNA sequences starts with the identification of the characteristic repeating motifs of the spider silk core sequence. The chosen amino acid sequence is then reverse-translated into a DNA oligomer. This oligomer is replicated using molecular biology methods until the desired gene size is obtained. Non-repetitive regions or any other sequence of interest can be added to the synthetic genes.

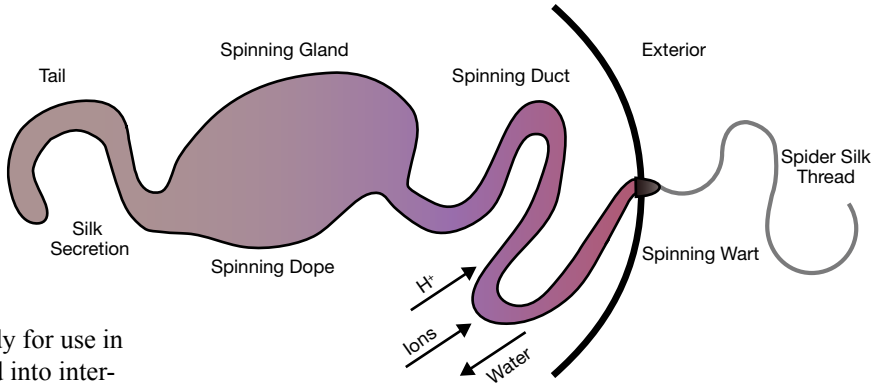
The synthetic spider silk genes are then transplanted into an established host organism to produce the target proteins. Synthetic spider silk modules have different hydrophobicity and charge properties, as well as different reactive groups. This allows for the production of customized spider silk proteins that are tailored for specific purposes.

The recombinant production of spider silk protein using engineered DNA sequences offers some solutions to the difficulties of working with the authentic genes. One of the major problems, the degenerated code, can be avoided by adapting the codons used in the synthetic gene to the host organism. Host organisms are manifold — *E. coli*, *Pichia pastoris*, plants (*e.g.*, tobacco, potato, *Arabidopsis thaliana*), and various eukaryotic cells have been successfully used so far.

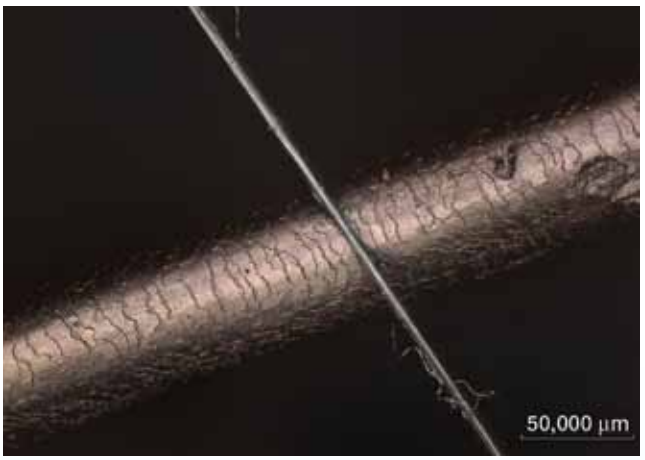
Engineered spider silk proteins derived from the dragline components ADF3 and ADF4 of the European garden cross spider (*Araneus diadematus*) have been designed by Scheibel and coworkers and produced in *E. coli (8)*. *E. coli* is a well-characterized, established, and cost-efficient production host, and high protein titers (concentrations) are possible. Many recombinant proteins, including insulin and, most recently, several spider silk proteins, have been successfully scaled up from laboratory to industrial scale (Figure 4). One disadvantage is that the molecular weight of proteins produced in *E. coli* is limited, but good yields can be achieved up to 150 kDa, which appears to be large enough for most applications.

The recombinant silk proteins eADF3 (engineered ADF3, molecular weight 48–106 kDa) and eADF4 (engineered ADF4, molecular weight 25–104 kDa) can be produced in *E. coli* with high protein yields on a large scale. The target proteins are separated from host cell proteins and other impurities based on the unique properties of spider silk (*e.g.,* thermal stability).

Before the spider silk proteins are ready for use in final end products, they must be processed into intermediate products, such as particles, films, nonwovens, hydrogels, and threads. The production of silk threads with mechanical properties similar to those of the original spider silk fiber is the most significant challenge and is discussed in the next section.



▲ **Figure 5.** Silk proteins are produced in the tail and stored in the ampulla. Fiber assembly occurs in the spinning duct, where ion exchange, pH adjustment, and protein dehydration take place.



▲ **Figure 6.** Spider silk has a diameter of a few microns — roughly one-thirtieth the thickness of a human hair.

Spiders show how it's done

Spiders are able to produce a high-performance polymer material (Table 1) under environmentally friendly conditions using aqueous solutions, at ambient temperature, and with low energy consumption *(9)*. However, the complex mechanisms behind the seemingly simple process of natural thread formation and web construction are not yet fully understood and therefore cannot be replicated synthetically.

The silk-spinning compartment is localized in the abdomen of the spider and consists of three parts: a tail, where the silk proteins are produced and secreted; the ampulla, or spinning gland, where the silk proteins are stored in high concentrations (up to 50% w/v); and the S-shaped spinning duct, where fiber assembly takes place. Several biochemical processes take place within this compartment and eventually result in fiber formation, including ion exchange (H^+ and K^+), pH decrease (from 6.9 to 6.3), and dehydration of the silk proteins. The thread undergoes final structural changes as the spider's hind legs pull it out of the abdomen (Figure 5). The final product is a few microns in diameter (Figure 6).

The large-scale production of silk threads matching the performance of those made by spiders has not been shown to date, because many crucial parameters, such as the internal pressure of the abdomen and the stability of the highly concentrated viscous spinning dope (*i.e.,* the initial highly concentrated silk protein solution stored in the gland), are not yet fully understood or cannot be realized technically. Many attempts have been made to mimic the spinning process at the laboratory scale and significant progress has been made, but the mechanical properties of the natural dragline fiber are still unmatched.

At the molecular level, the secondary and tertiary structures of the amino acid chains are responsible for the overall self-assembly behavior of the fibers. The role of water in the mechanical performance of the fibers is still under investigation. Spider silks exhibit a unique behavior called supercontraction. When placed into water or even exposed to a high-humidity environment, the fibers shrink to half of their original length. In water, the fibers behave like an elastomer with a very low elastic modulus and increased stiffness. The water disrupts the hydrogen bonds and allows the elastic domain to return to its equilibrium by reducing the initial stress, which shortens the fiber. The fiber's elastic behavior is due to the absence of a rigid hydrogen bond network. This suggests that an aqueous environment and stretching are essential features of the silk fiber spinning process *(10)*.

How scientists try to produce silk threads

To produce a commercial fiber, either the natural process of silk spinning must be mimicked, or a completely new spinning process must be developed. To be commercially viable, any process must be cost-efficient and environmentally friendly.

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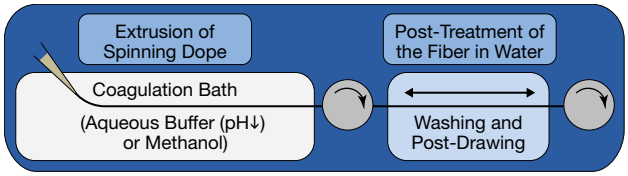

▲ **Figure 7.** Wet-spinning involves extruding a highly viscous polymer solution into a coagulation bath and post-drawing treatment of the fiber.

Wet-spinning using a coagulation bath is one process that has been used to produce silk fibers. Wet-spinning (Figure 7) involves extruding a highly viscous polymer solution into a coagulation bath. Neither the quantity nor the quality of the available recombinant spider silk stocks have been sufficient in the past, so the first studies used regenerated silkworm silk proteins obtained by separating the *Bombyx mori* cocoon silk proteins from the other components of the thread.

DuPont was the first company to design a spinning process for industrial silk fibers *(9)*. After degumming original silk fibroin from silkworms, the protein was dissolved in hexafluoroisopropanol (HFIP) at concentrations ranging from 5% to 25% and extruded into a coagulating methanol bath. However, the fiber did not have the desired mechanical properties. Consequently, different solvents have been tested to dissolve regenerated proteins at higher concentrations, but most of the solvents tested degraded the protein and thus resulted in weak fibers *(9)*.

Recently, Zhou *et al. (11)* dissolved regenerated *Bombyx mori* fibroin in a solution containing 9.3-M lithium bromide to final protein concentrations of 13–18% w/v. This solution was extruded into a coagulation bath containing 30% w/v ammonium sulfate at 60°C. After exiting the bath, the fibers were treated with steam and then stretched to 1.5 times their initial length by hand. The increased length improved the fibers' properties. Although the resulting fibers were relatively strong and extensible, they exhibited huge variation as a result of their manual processing.

Recombinant silk protein was developed in parallel by different laboratories and finally became available in small quantities sufficient for initial laboratory-scale wet-spinning tests. Harsh solvents, such as HFIP and formic acid, were used to reach the necessary high protein concentrations (above 20% w/v) more easily, which generally is difficult with hydrophobic high-molecular-weight proteins.

Many experiments using recombinant proteins produced mechanically poor silk fibers *(2)*. After the first hand-spinning successes, researchers genetically manipulated the protein sequences and mixed them with chimeric proteins that consist of partial sequence motifs of different silks (*e.g.,* motifs of the flagelliform silk combined with motifs of MaSp2) to study their respective roles in the mechanical properties, as well as to improve the spinning apparatus *(4, 7, 10)*.

For example, Elices *et al. (12)* tried to mimic the dragline spinning solution by producing dopes with varying ratios of recombinant silk proteins derived from MaSp1 and MaSp2 of dragline spider silk (100:0; 70:30; 30:70; 0:100). Once dissolved in HFIP, the dopes were spun in an isopropanol bath under the same wet-spinning process conditions. The fibers then underwent a post-treatment of further drawing under hot steam. The resulting tensile strength was between 280 MPa and 350 MPa (lower than that of natural spider silk) and the elongation break was between 35% and 50% extension.

Despite initial success in producing recombinant silk fibers with good mechanical properties, the use of HFIP and other organic solvents did not yield a fiber equal in performance to the natural silk fibers produced by the spider, which relies completely on a nontoxic and environmentally friendly aqueous system.

Arcidiano *et al. (13)* spun the first recombinant spider

Table 2. Mechanical properties of fibers consisting of recombinant proteins.

Recombinant Protein	Species*	Diameter, μm	Stiffness, GPa (Elastic Modulus, *E*)	Strength, MPa (Yield Stress, σ_{max})	Extensibility, % (Strain at Break, ε_{max})	Reference
(MaSp1) 96-mer	*N. clavipes*	60	21	508	15	4
$(MaSp1)_{24}$ as spun	*N. clavipes*	40.90	2.78	35.65	3.13	10
$(MaSp1)_{24}$ stretched	*N. clavipes*	17.44	5.70	132.53	22.78	10
MA rcSp1	*N. inaurata*	44	7.7	320	30	12
MA rcSp2	*N. inaurata*	36	7.4	330	35	12
MA rcSp1/Sp2 70:30	*N. inaurata*	46	3.6	350	51	12
$A1S8_{20}$ as spun	*N. clavipes*	68	1.1	28.4	1.7	7
$A1S8_{20}$ double stretched	*N. clavipes*	28.3	4.4	127.5	52.3	7

* Genus *Nephila*

silk fibers in an aqueous medium at concentrations up to 25% w/v. Fibers were spun into a coagulation bath containing a mixture of methanol and water to produce threads 10–60 µm in diameter. The fibers had an anisotropic structure (molecular orientation) along the fiber axis and were water-insoluble, but they were also so brittle that mechanical data could not be obtained.

Lazaris *et al. (14)* produced fibers with acceptable mechanical properties using the recombinant dragline silk proteins ADF3 precipitated in a mixture of water and methanol. However, those fibers had a tenacity (ultimate breaking strength) well below that of natural spider silk.

A biomimetic process, closely matched to the complex natural process, has been investigated. It entails using an aqueous protein solution and precipitating it into another aqueous buffer. Microfluidic devices with controls for the salting out, pH drop, and elongational flow were used to mimic the complex natural process observed in the silk glands of arthropods. The silk protein aggregated in spherical colloidal particles and formed a fiber under elongational flow and the correct salting-out conditions. No mechanical data for these fibers have been published to date.

Few data on the mechanical properties of synthetic silk fibers can be found in the literature. Most of the spinning processes create fibers that are so brittle that their mechanical properties cannot be properly measured. The mechanical data that do exist (Table 2) cannot be compared to each other because the measurement conditions and the devices used vary and have not been standardized. Moreover, compared with spider fibers, the resulting fibers have rather large diameters, and the diameters typically vary over the length of the fibers — making an accurate calculation of mechanical properties impossible.

However, these studies do provide some useful hints about the keys to spinning silk protein. A higher-molecular-weight protein produces a more-stable fiber *(4)*, but it is difficult to retain the protein at high concentrations, especially in an aqueous system. Studies have also found that fibers with a more orderly structure tend to be more elastic. And, the uniaxial drawing post-treatment under steam or in a water bath significantly improves fiber toughness.

The right combination of these and other factors can greatly improve the mechanical properties of the spun fibers. However, despite these promising approaches, the mechanical properties of natural dragline fibers have not yet been reproduced.

The future of spider silk

Spider silk — a high-performance, lightweight fiber capable of efficiently absorbing kinetic energy — has many applications, from ballistic vests and sporting goods to sutures. It has also been tested as a substrate for skin and neuron regeneration and wound healing, a coating for medical implants, and many other uses. Recent progress in scaling up the process for producing raw spider silk protein will accelerate application development. While a first commercial fiber will require further development, improvements in processing have paved the way for industrial fibers with properties similar to those of natural spider silk.

Attempts to reproduce in the laboratory what nature has perfected over millions of years will involve scientists and engineers from many fields, not only silk biochemists. As we begin to catch up with evolution in biochemical and material sciences, products will become better, as well as more in sync with nature. CEP

Literature Cited

1. **Cranford, S. W., *et al.*,** "Nonlinear Material Behavior of Spider Silk Yields Robust Webs," *Nature,* **482,** pp. 72–76 (2012).
2. **Rising, A., *et al.*,** "Spider Silk Proteins: Recent Advances in Recombinant Production, Structure-Function Relationships and Biomedical Applications," *Cellular and Molecular Life Sciences,* **68** (2), pp. 169–184 (2011).
3. **Agnarsson, I., *et al.*,** "Bioprospecting Finds the Toughest Biological Material: Extraordinary Silk from a Giant Riverine Orb Spider," *PLoS One,* **5** (9), e11234 (2010).
4. **Xia, X., *et al.*,** "Native-Sized Recombinant Spider Silk Protein Produced in Metabolically Engineered *Escherichia coli* Results in a Strong Fiber," *Proceedings of the National Academy of Sciences,* **107** (32), pp. 14,059–14,063 (2010).
5. **Humenik, M., *et al.*,** "Recombinant Spider Silks — Biopolymers with Potential for Future Applications," *Polymers,* **3** (1), pp. 640–661 (2011).
6. **Tomita, M., *et al.*,** "Transgenic Silkworms that Weave Recombinant Proteins into Silk Cocoons," *Biotechnology Letters,* **33** (4), pp. 645–654 (2011).
7. **Teulé, F., *et al.*,** "Combining Flagelliform and Dragline Spider Silk Motifs to Produce Tunable Synthetic Biopolymer Fibers," *Biopolymers,* **97** (6), pp. 418–431 (2011).
8. **Scheibel, T.,** "Spider Silks: Recombinant Synthesis, Assembly, Spinning, and Engineering of Synthetic Proteins," *Microbial Cell Factories,* **3** (14) (2004).
9. **Hardy, J. G., *et al.*,** "Polymeric Materials Based on Silk Proteins," *Polymer,* **49** (20), pp. 4309-4327 (2008).
10. **An, B., *et al.*,** "Inducing β-Sheets Formation in Synthetic Spider Silk Fibers by Aqueous Post-spin Stretching," *Biomacromolecules,* **12** (6), pp. 2375–2381 (2011).
11. **Zhou, G., *et al.*,** "Silk Fibers Extruded Artificially from Aqueous Solutions of Regenerated *Bombyx mori* Silk Fibroin are Tougher than their Natural Counterparts," *Advanced Materials,* **21** (3), pp. 366–370 (2009).
12. **Elices, M., *et al.*,** "Bioinspired Fibers Follow the Track of Natural Spider Silk," *Macromolecules,* **44** (5), pp. 1166–1176 (2011).
13. **Arcidiacono, S.,** *et al.,* "Aqueous Processing and Fiber Spinning of Recombinant Spider Silks," *Macromolecules,* **35,** pp. 1262–1266 (2002).
14. **Lazaris, A.,** *et al.,* "Spider Silk Fibers Spun from Soluble Recombinant Silk Produced in Mammalian Cells," *Science,* **295,** pp. 472–476 (2002).