Spiders make their webs and perform a wide range of tasks with up to seven different types of silk fiber. These different fibers allow a comparison of structure with function, because each silk has distinct mechanical properties and is composed of peptide modules that confer those properties. By using genetic engineering to mix the modules in specific proportions, proteins with defined strength and elasticity can be designed, which have many potential medical and engineering uses.

**Synthetic spider silk: a modular fiber**

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For eons, nature has been testing biological polymers as structural and functional materials. Proteins, which are polymers with 20 different possible amino acid monomers, have proved to be extremely adaptable as biomaterials. The spider uses as many as seven different kinds of silk fiber for various functions. Table 1 provides a summary of silks, their uses and glands of origin for the golden orb-web-weaving spider *Nephila clavipes*. *N. clavipes*, a subtropical species, has been used primarily as a result of its availability and relatively large size, which makes its glands easy to dissect. Preliminary mechanical and chemical properties of its dragline silk were determined in the 1960s [Ref. 1].

Synthesis of silk protein(s) takes place in specialized columnar epithelial cells², with newly synthesized protein being secreted into the lumen of a storage gland. Ducts lead from each gland to one of three sets of spinnerets, from which silk is drawn. The protein in the gland is believed to be in a liquid-crystal form³ but the fiber is not formed until the protein passes down the duct leading to the exit spinneret. It has been shown that silk becomes increasingly bi-refrangent as it passes down the duct, orienting the protein’s structure during its passage. This appears to be caused by the mechanical and frictional forces aligning the secondary structure of the protein molecules. Iizuka has proposed a similar mechanism for silkworm (Bombyx mori) silk formation⁴.

Dragline silk has a tensile strength that is comparable to Kevlar⁵ (4 × 10⁹ N m⁻²) coupled with a reasonable elasticity (35%), and is therefore an extremely strong fiber. It is used as a strong yet flexible structural element in the web, providing a framework to which other silks are attached, and as a belaying line for spiders when they are climbing. Capture silk (flagelliform-gland silk), which forms the spirals of the web, is already under tension and can more-than-triple its length⁶. Gosline et al. have reviewed aspects of different silk properties and concluded that the spider silks and web architecture are optimally designed for each other⁷,⁸.

Minor ampullate silk is similar to major ampullate silk in tensile strength but has little elasticity. It is used for structural reinforcement of major ampullate silk in construction of the web. The major and minor ampullate and tubuliform silks from both *N. clavipes* and *Araneus gemmoides* have been tested using standard mechanical testing methods⁹. Table 2 lists mechanical properties of several spider silks from *N. clavipes* compared with some natural and manmade materials. Spider-silk fibers are nearly as strong as several of the current synthetic fibers and can outperform them in many applications in which total energy absorption is important.

**Modular nature of silk proteins**

The repetitive nature of silkworm silk and spider silks is apparent upon examination of the amino acid
sequences. Some of the first work on synthetic silk products involved the genetic engineering of peptides to create potentially different physical properties based on silkworm silk sequences and the production of fibers from those peptides. The modular nature of silk fibers was emphasized by the entirely different sequences found in spider silk, which presumably corresponded to the differing properties of silkworm silk and spider dragline silk.

An examination of the cDNAs and genes of the spider silks sequenced to date shows that all silks are chains of iterated peptide motifs. The consensus sequences for the repeating peptides of major and minor ampullate silks and flagelliform silk from N. clavipes are repeated multiple times throughout the length of each protein. Major ampullate silk is composed of two proteins, major ampullate spider silk 1 (MaSp1) and major ampullate spider silk 2 (MaSp2). Minor ampullate silk is also composed of two proteins, called MiSp1 and MiSp2.

The small peptide motifs can be grouped into four categories: (1) GPGXX/GPGQQ; (2) (GA)$_n$/A$_n$; (3) GGX; and (4) spacers. On the basis of physical studies, the peptide motifs in spider silks have been assigned structural roles. The (GA)$_n$/A$_n$ module has been found to be a β sheet. The GGX motif is probably a helix with three amino acids per turn, called a 3$_{10}$ helix. The GPGXX repeat unit (in many cases, GPGQQ is the major form) has been suggested to be involved in a β-turn spiral, based on structures in comparable proteins. The spacers contain charged groups and separate the iterated peptide motifs into clusters. Figure 2 shows the categorization of silk fibers by the types of modules that they contain, based on deduced protein sequences from N. clavipes and A. diadematus. N. clavipes proteins are denoted as mentioned previously, A. diadematus proteins are designated ADF1–4.

The correlation of the modular structures with the physical properties of each silk is striking. Major-ampullate and flagelliform silks share the GPGXX motif, and are the only silks with elasticity >5–10%. Major ampullate silk has 35% elasticity, with an average of five β turns in a row, whereas flagelliform silk, with >200% elasticity, has this same module repeated ~50 times. The major and minor ampullate silks are both very strong, and at least one protein in each silk contains the (GA)$_n$/A$_n$ β-sheet module. Several proteins, but not MaSp2 or ADF-4 (a major ampullate–like silk from A. diadematus), share the postulated GGX helices. The effect of the spacer regions is currently underdetermined, although these might provide organizational areas within silks or surface regions for interactions when a mature fiber is formed. Owing to the inability to detect any protein secondary structure other than the β sheet, several groups have suggested that the other modules form random coils. When combined with the β-sheet regions as anchors, the random-coil segments would provide elasticity.

The unique modular nature of spider silks and the relationship of the modules to the properties of the silks has proved to be a driving element in the design of synthetic silk genes for the expression and production of silk proteins. Several laboratories have evolved strategies to generate long, iterated peptides that can be produced by hosts other than spiders, such as bacteria, yeast, insects and goats.

### Strategies for synthetic gene construction

The cDNAs and genes for several spider silks have already been cloned and sequenced. They are all GC (guanine and cytidine) rich and this causes several problems. First, translational pauses are common for these messages, as they are in silkworm silk. Second, the high alanine and glycine content of the proteins, and thus the prevalence of these codons in the mRNAs, appear to have caused the coevolution of tRNA pools specially designed to deal with Gly–Ala–rich messages. Third, the high GC nature of the DNA

| Table 1. Spider silks and their uses |
|---|---|---|
| **Silk** | **Use** | **Spinneret** |
| Major ampullate dragline | Web frame and radii | Anterior |
| Minor ampullate | Web reinforcement | Medial |
| Flagelliform | Core fibers of adhesive spiral | Posterior |
| Aggregate | Adhesive silk of spiral | Anterior and posterior |
| Cylindrical | Cocoon | Posterior |
| Aciniform | Swathing and inner egg sac | Anterior |
| Pyriform | Attachment disk and joining fibers | Anterior |

| Table 2. Comparisons of mechanical properties of spider silk |
|---|---|---|
| **Material** | **Strength (N m$^{-2}$)** | **Elasticity (%)** | **Energy to break (J kg$^{-1}$)** |
| Dragline silk | 4 × 10$^9$ | 35 | 1 × 10$^5$ |
| Flagelliform silk | 1 × 10$^9$ | >200 | 1 × 10$^5$ |
| Minor ampullate | 1 × 10$^9$ | 5 | 3 × 10$^4$ |
| Kevlar | 4 × 10$^9$ | 5 | 3 × 10$^4$ |
| Rubber | 1 × 10$^6$ | 600 | 8 × 10$^4$ |
| Tendon | 1 × 10$^9$ | 5 | 5 × 10$^3$ |
| Nylon, type 6 | 7 × 10$^7$ | 200 | 6 × 10$^4$ |

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**Flag** (GPGXX)$_{43–63}$(GGX)$_{112}$ = flag spacer  
MaSp2 (GPGGYGPGQQ$_2$GPGGYGPGQQ$_2$GPGGYGPGQQ$_2$) = major spacer  
MaSp1 (GGAGQGGYGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQGAGRGGLGGQ
causes secondary-structure problems that lead to recombination of cloned cDNAs and genes. Most laboratories in this field have attempted to avoid these problems by expressing spider silk proteins from synthetic genes. By carefully selecting codons, the problems of secondary structure have been avoided and the codon balance can then be accommodated by the host’s tRNA pools. Two strategies using optimized codons and biosynthetic approaches have emerged after early experiments proved the advantages of biosynthetic rather than chemical construction10,34,35. In current strategies, synthetic DNA coding for a monomer repeat unit is cloned and large amounts of monomer DNA are produced. The two strategies diverge after that and can be broadly labeled the ‘condensation’ strategy and the ‘iterative polymerization’ strategy.

The condensation strategies

The condensation strategies rely on the use of biosynthesis to produce a large amount of the monomer DNA. The monomers are then ligated to form multimers and the mixture is cloned. Post-transformational screening separates clones of different lengths. A good example of the use of the condensation strategy with silkworm silk is the design of a monomer with a nonpalindromic restriction site that caused the resultant monomers to be ligated only in a head-to-tail fashion36. A family of products was obtained that varied by length. A further study used a similar scheme for MaSp1-like monomers37. In another synthetic silkworm silk, the monomer had 5 protruding nonpalindromic extensions six bases long34. These monomers were ligated, forming a family of products. The degree of polymerization of the monomer varied from ~20 (Ref. 32) to 80 (Ref. 36), as demonstrated by gel electrophoresis, but clones of six repeats34 (~108 amino acids (aa)) and 13 repeats37 (221 aa) were the largest clones used for expression.

Figure 2

Structural modules found in spider silk proteins. Flag is the flagelliform silk protein. MaSp1, MaSp2, ADF-3 and ADF-4 are the proteins composing major ampullate (and major-ampullate-like) silk. MiSp1, MiSp2, ADF-1 and ADF-2 are the proteins composing minor ampullate (and minor-ampullate-like) silk. ADF proteins are from Guerette15. To determine which silk contains which modules, read the dotted line that connects the boxes from left to right, starting with the silk title. The boxes represent modules (and, in some cases, subsets of modules) that are found in that silk, without regard to their actual positions within the silk molecule. For example, MaSp2 contains both GPGGX and GPGQQ, so both boxes are included, and it contains only A modules, not (GA)n. Flag has GPGGX, GGX and spacer modules, but neither the GPGQQ nor the (GA)n/A module. (Figure reproduced, with permission, from Ref. 23)
constructing the original series of clones, a monomer or multimer was double digested from one clone and ligated into a second clone, which had been linearized with only one enzyme, generating larger multimers.

This second scheme was also used to introduce structurally dissimilar peptide elements into the repeating multimers, to change the protein’s properties. The condensation portion of the strategy was responsible for fewer of the clones than the use of compatible, non-regenerable enzymes. The largest clones produced by this mixed strategy encoded 684 amino acids, although one encoding 494 amino acids was chosen as the largest to express for other studies. When the insert contained multimers with six or more DNA repeats, a slight laddering effect was noticed (the DNA from a putative single clone shows a family of products separated by regular, insert-sized intervals), suggesting that the insert was subject to internal deletions.

Exclusively iterative strategies

An iterative polymerization strategy that uses only compatible, nonregenerable restriction enzymes has been described\(^2\), based on original work by Kempe\(^3\). After introducing the monomer into a plasmid, the synthetic gene was constructed using successive restriction digests, purifying fragments and ligating them. This strategy has several advantages, notably the controlled construction of the insert and the larger possible insert size. When considering the modular nature of spider silks, this strategy has the additional advantage of being able to mix different modules easily in defined ratios in order to tailor the expressed protein’s properties. A double-stranded monomer of DNA was constructed that coded for the basic repetitive unit of MaSp2 (35 aa):

\[
\text{PGGYPGQGPGPGYGPGQQGPS- GPGSAAAAAAAAG}
\]

The DNA monomer was directionally cloned and double-digested by manipulating the restriction sites embedded at the ends of the monomer sequence as well as a unique Sal site in the ampicillin-resistance gene of the plasmid; the resultant multimers were transferred to an expression vector\(^2\). This demonstrated the construction of multimers as large as 32 repeats, representing 1120 aa of repeating protein. The copy number of the cloning plasmid appeared to decrease as the insert size increased. No laddering effect was reported during digestion of the DNA from any individual clone.

One ambitious study used iterative polymerization to make large inserts\(^4\). The monomers were made of overlapping oligomers that encoded at least four different native deletion patterns of the repeat sequence. Three variations of MaSp1 were constructed, to study the difference between native and optimized codons. One monomer of MaSp2 was constructed, containing mostly codons optimized for Escherichia coli. Iterative polymerization was used to multiply the insert size to eight or 16 repeats. Although the inserts obtained were significantly larger than in other studies (>4.8 kb (1616 aa) to 5.6 kb (1904 aa)), the inserts were subject to internal deletions and duplications, forming a ladder that showed integral multiples of the monomer\(^4\).

A variation of this strategy has also been used to construct a gene encoding MaSp1 using codons optimized for expression in yeast\(^5\). Eight- and 16-unit multimers were constructed (808 and 1616 aa), and the multimers were then inserted into an alcohol oxidase (AOX1) gene under the control of the methanol-inducible AOX1 promoter. This construct was used to transform a Pichia pastoris yeast strain. Several of the transformants displayed the ladder effect owing to internal deletion or duplication events. However, all the clones were stable, producing the same patterns after more than 100 doublings of the host.

Finally, monomers for MaSp1 were designed with aa incorporations that let chemical-trigger sites either prevent or allow β-sheet formation between proteins\(^6\). The monomers were cloned using compatible, non-regenerable sites into two different plasmids, each with a different antibiotic gene. Multimers were constructed by digesting the monomer cassette from one clone and ligating it into the second plasmid linearized with only one enzyme. Transformant plasmids were screened for size, then dimers were double digested to confirm head-to-tail orientation. Iterative polymerization was pursued until clones of the desired size were achieved. The stability of the constructed genes was excellent, although the insert sizes were only around eight repeats of the monomer (~256 aa of repeating protein).

Expression of spider silk proteins

Several organisms have been used or proposed for use in expressing spider silk proteins after the construction of the synthetic silk genes. Microorganisms such as bacteria and yeast are common, although a few insect and mammalian systems have also been proposed. In bacterial systems, the most common strategy is to put the target gene under the control of a viral promoter. Alac operator is placed between the promoter and the gene, rendering the promoter subject to regulation by the Lac repressor. To begin stimulating expression, isopropylthiogalactoside (IPTG, a synthetic analog of lactose) is added to derepress the lac operator and allow transcription to proceed using a viral RNA synthetase that is much more efficient than the bacterial RNA synthetase. To simplify purification, a repeated segment of 6–10 histidines is appended to either the N or the C terminus of the construct, allowing the use of immobilized-metal affinity chromatography for purification.

Several of the studies cited use this strategy with a bacteriophage T5 (Ref. 38) or T7 (Refs 22,36,41,43) viral promoter in their expression vector. These extremely active viral promoters should force the bacteria to produce 50% of their total protein from the target gene. However, even codon-optimized spider silk proteins have low yields [1–20 mg purified protein l\(^{-1}\)] (Refs 22,37,38). These studies also reported that the level of expression is to some extent inversely proportional to the length of the insert. Several groups have transferred the kanamycin-resistance gene to their vectors to enhance expression\(^2\),\(^6\),\(^8\),\(^1\), because ampicillin resistance is conferred by a secreted enzyme that degrades ampicillin in the fermentation media. We found that this resulted in around a tenfold increase in protein production (R.V. Lewis et al., unpublished).

Excellent yields of about 300 mg l\(^{-1}\) (15% of total protein) have been reported\(^6\). These workers investigated several mechanisms for the appearance of ladders of
products during expression. Internal genetic deletions are at least partially responsible for shortened mRNAs, but truncated proteins are partly a problem of ribosomal fall-off. In inserts with rare or natural codons, the fall-off rates per codon were about twice as high as those for inserts with codons optimized for bacteria. Longer multimers had a higher fall-off rate. The same group constructed a synthetic gene with codons optimized for yeast, with the target gene under control of the alcohol-oxidase promoter. Upon integration into the yeast genome, longer genes were expressed less efficiently than shorter ones, but the effect did not seem to be due to truncated transcripts, as with \textit{E. coli}.

The cDNA for one of the major ampullate silk proteins has been cloned into an expression vector and expressed \textit{in vitro} and in \textit{E. coli} systems\textsuperscript{44}. Synthesis \textit{in vitro} produced relatively small amounts of protein with the cDNA in a vector that produced transcripts for translation, but the expression in fermentation studies with a different vector was comparable to that of synthetic genes ~4 (mg purified protein) \textit{l}\textsuperscript{−1}. A small proportion of the purified protein was expressed as a ladder of less-than-full-length proteins, as observed by western blotting. As the purification method (chelated-nickel-affinity chromatography) suggests that 3′ truncation does not occur, the ladder was presumably produced by a low level of recombination.

\textbf{Alternative hosts for expression}

Synthetic spider silk expression has been successful in bacterial and yeast hosts. In both organisms, the main problems are (1) the internal deletion arising from the repetitive and GC-rich nature of the messages, and (2) the inefficiency of translation of larger genes, which is probably due to mRNA secondary structure and ribosome fall-off.

Several alternative organisms have been suggested as hosts for the expression of synthetic or natural genes and cDNAs. Attempts to insert cDNAs and synthetic silk genes into the lactalbumin gene and express the proteins in goat’s milk are presently under way (C.N. Karatzas, pers. commun.). Another intriguing possibility, especially for commercial expression, is to replace the fibroin genes of silkworm with the natural or synthetic spider silk genes by gene targeting with baculovirus\textsuperscript{45}. This has already been done with a chimeric protein, light-chain fibroin fused to green fluorescent protein. The chimeric gene was expressed in the silk gland and spun into the cocoon. This is an interesting alternative, because the gene would be expressed as a fiber and there is already a commercial infrastructure to handle the fibrous product.

\textbf{Material studies of expressed proteins}

Several of the studies discussed used the expressed proteins to investigate secondary structures that might confer the physical properties exhibited by spider silks. Circular dichroism and Fourier-transform–infrared spectroscopy studies on soluble silk proteins support β-sheet and amorphous structures in expressed silks but differ on the relative percentages\textsuperscript{37,38,41} X-ray and electron diffraction support a β-sheet role for the polyalanine module\textsuperscript{43}, and transmission electron micrography suggests that the amorphous region might actually have structure.

Based on early work with silk fibroins\textsuperscript{36,47}, several films (R.V. Lewis \textit{et al.}, unpublished) have been made from native \textit{N. clavipes} major and minor ampullate silks, and from \textit{A. gemmoides} cylindrical-gland silk, using hexafluoroisopropanol (HFIP) as a volatile solvent. Although native silk fibers have tensile strengths on the order of 10\textsuperscript{5} N m\textsuperscript{−2}, major and minor ampullate silk films have strengths of 10\textsuperscript{6} N m\textsuperscript{−2}, and the \textit{A. gemmoides} silk film has a tensile strength of 10\textsuperscript{7} N m\textsuperscript{−2}. Their relative elongations (the ability to stretch before breaking) are 5.6%, 4.38% and 8.3%, respectively. The synthetic MaSp2-like protein has also been made into a film and has a tensile strength of 10\textsuperscript{6} N m\textsuperscript{−2} and an elongation of 18.2%, a tenfold increase in strength and threefold increase in elongation over a redissolved major ampullate silk protein film of similar dimensions. Triggers that disrupt the structure of MaSp1-like synthetic spider silk proteins were also used to make films\textsuperscript{49} and showed that, when the structural trigger is activated, the amount of β sheet in the protein decreases.

Although some effort has been expended on trying to make fibers of silkworm silks, both native and synthetic\textsuperscript{36,48}, studies have just started on spider silks. A 1994 patent application reported the formation of a fiber by dissolving the synthetic proteins\textsuperscript{42} in HFIP and extruding the material through a stainless-steel spinneret into isoamyl alcohol\textsuperscript{49}. The resultant fiber had characteristics that were similar to commercial textile fibers, such as 16.7 denier, elongation of 103.3% and initial moduli of 40.1 gram denier-1. One intriguing study involved the nanofabrication of an artificial spinneret, which was first tested on degummed silkworm silk\textsuperscript{50} and then on redissolved dragline silk\textsuperscript{51}. By assessing the mechanical properties of the fabricated fibers and performing \textsuperscript{13}C NMR, the mechanical properties of these fibers were found to approach those of native fibers as the diameter of the fabricated fiber decreased. An overall increase in orientation contributes to this effect because decreasing the aperture size and increasing the draw ratio (i.e. elongation of the fiber after formation) increased the proportion of β-sheet structure and therefore the magnitude of the maximum stress that the fiber can withstand.

\textbf{Modular fiber design}

The study of the modular nature of spider silks and the correlation of protein sequence and structure of these modules to the properties of the individual silks will allow the design of fibers with a diverse range of properties. As more protein modules conferring specific properties are discovered, mixing the modules in a defined manner will become easier to accomplish by following the prevailing trend towards constructing large multimers using the iterative polymerization of the DNA monomer to construct a multimer of specific design.

The use of compatible, nonregenerable restriction sites at the ends of the modules in an iterative strategy will ease construction by allowing: (1) the precise, sequential addition of individual modules; (2) the use of a mixture containing modules in defined ratios; and (3) the construction of larger multimers that more accurately reflect the length of natural spider silk proteins. With refinements in expression systems to produce larger amounts of the silk proteins and the
promising studies in spinning proteins into fibers, the possibility of producing protein fibers with tailored properties becomes an achievable goal.

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