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Review

Molecular mechanisms of spider silk

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Abstract. Spiders spin high-performance silks through the expression and assembly of tissue-restricted fibroin proteins. Spider silks are composite protein biopolymers that have complex microstructures. Retrieval of cDNAs and genomic DNAs encoding silk fibroins has revealed an association between the protein sequences and structure-property relationships. However, before spider silks can be subject to genetic engineering for commercial applications, the complete protein sequences and their functions, as well as the details of the spinning mechanism, will require additional progress and collaborative efforts in the areas of biochemistry, molecular biology and material science. Novel approaches to reveal additional molecular constituents embedded in the spider fibers, as well as cloning strategies to manipulate the genes for expression, will continue to be important aspects of spider biology research. Here we summarize the molecular characteristics of the different spider fibroins, the mechanical properties and assembly process of spidroins and the advances in protein expression systems used for recombinant silk production. We also highlight different technical approaches being used to elucidate the molecular constituents of silk fibers.

Keywords. *Latrodectus hesperus*, spider silk, fibroin, major ampullate gland, tubuliform, egg case silk.

Introduction

The order Araneae contains over 37,000 described species, all of which have the ability to spin multiple task-specific silks [1]. Spider silk is an intriguing biomaterial that is lightweight, extremely strong and elastic, and exhibits mechanical properties comparable to the best synthetic fibers produced by modern technology [2]. Silk fibers are protein-based biopolymer filaments or threads secreted by specialized abdominal glands connected to the spinnerets, ducts or spigots, and are used in different combinations to produce structures for prey capture, reproduction and locomotion [3–5]. Exceptionally complex spinning processes are used by the spider to transform soluble silk proteins into solid fibers with specific mechanical and functional properties. Spider silk is spun near ambient temperatures and pressures using water as the solvent, which gives rise to an environmentally safe, biodegradable material [6]. Based upon the desirable properties of spider silk, scientists have recognized the potential for large-scale production of silk for commercial applications. Because spiders are territorial carnivores, domestication of these organisms to create spider farms for silk production is impractical. Therefore, to obtain large quantities of spider silk for industrial applications, molecular biologists have been attempting to clone silk genes for overexpression in bacteria, yeast and mammalian cell culture systems. In addition to being able

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to produce large quantities of recombinant silk proteins, material scientists and engineers have been attempting to copy the spinning process in the laboratory, but with much faster reeling speeds to facilitate commercial viability. Advancements in both areas of research are essential constituents that will facilitate the development of synthetic fibers that resemble the mechanical properties and features of natural silk for commercial applications.

Spider silk types and glands

Orb web spiders are capable of producing up to six different types of silk, as well as a glue substance, that have various biological functions (Fig. 1a, b). The different silk types are protein-based polymers that are members of the spider silk protein superfamily and display restricted expression in seven morphologically distinct silk glands. These distinct abdominal glands are thought to have evolved from a single type of gland, and have subsequently diverged in their anatomy, luminal contents and morphology [4]. Based upon the differential amino acid compositions of the luminal contents, the silk proteins within each gland are proposed to be assembled to create specific fibers with particular functions. To date, most research has focused on the major ampullate gland, which manufactures dragline silk constituents. Dragline silk is well known for its combination of high tensile strength and elasticity, which leads to a fiber with extraordinary toughness [3]. Spiders use dragline silk to create web anchors, as well as safety-lines for survival (Fig. 1a, b). The minor ampullate gland, which shares morphological similarity to the major ampullate, synthesizes web radii filaments and temporary capture silk. Other abdominal glands produce silk types that participate in the direct capture of prey; these tissues include the flagelliform, aggregate and aciniform glands (Fig. 1a, b).

Capture silk, also known as viscid silk, is a composite silk that contains material derived from the flagelliform and aggregate glands. Flagelliform gland silk is extremely extensible and forms the capture spiral of an orb web; this elasticity has been proposed to facilitate prey capture, enabling webs to arrest the motion of flying organisms without breaking [7]. Aggregate glands have been postulated to manufacture spider glue proteins for silk fibers that coalesce to form sticky droplets, which interact with the capture spiral silk to influence the mechanical properties of the spiral filaments. The droplets have been shown to consist of a glycoprotein mixture, as well as a number of small molecules related to neurotransmitters [8, 9].

a

Spider silks and their natural functions

Silk	Location	Core Fiber Proteins
Major ampullate dragline	Web frame, safety line	MaSpl, MaSp2
Minor ampullate	Web reinforcement and temporary capture silk	MiSpl, MiSp2
Flagelliform	Capture spiral	Flag
Aciniform	Wrapping silk, small diameter egg case fiber	AcSpl
Tubuliform	Large diameter egg case silk fiber	TuSpl, ECP-1, ECP-2
Aggregate	Glue coating for capture spiral	Unknown
Pyriform	Attachment disk and joining fiber	Unknown

b

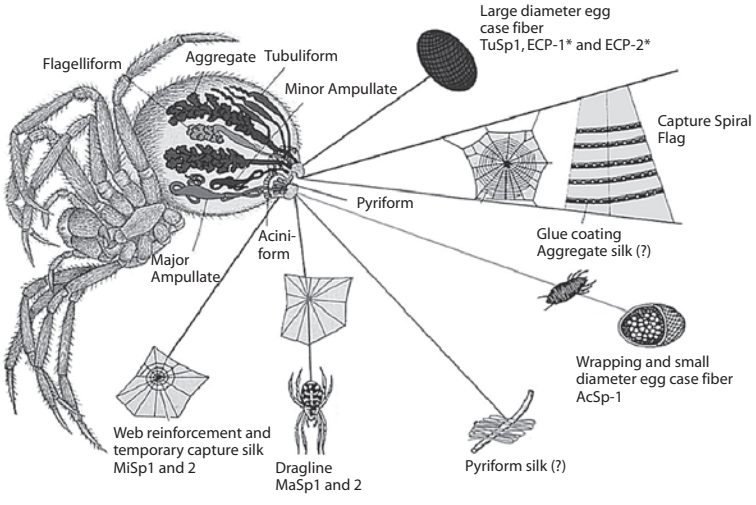


Figure 1. The molecular constituents of silks and their natural functions in orb weavers are diverse and specialized. (*a*) Spider silks and their natural functions. ECP-1 and ECP-2 have been shown to be part of the egg case core fiber of the cob-weaver spider, *Lactrodectus hesperus*. (*b*) The silk glands, fibers and molecular constituents found in the orb-weaver *Araneus diadematus* [adapted from ref. 84].

Wrapping silk, which is used to entangle prey and build sperm webs as well as some constituents of egg case silk, is manufactured by the aciniform gland and has different molecular properties relative to viscid silk (Fig. 1a, b).
Tubuliform silk, also known as egg case silk, is produced from female tubuliform glands and provides protection for the encased eggs (Fig. 1a, b). Egg cases must be tough enough to resist parasitic invasion and climate changes, and studies indicate that ancient female spiders constructed egg case sacs for protection of their developing offspring, whereas males used the egg case fibers to deposit sperm that could be transferred to the copulatory pedipalps. Perhaps the least studied gland, known as the pyriform gland, has been proposed to secrete materials that form attachment discs; these discs fasten threads of dragline silk to each other or to substrates [10].

Common modules found within silk proteins

To begin to manufacture synthetic silks that mimic the diverse mechanical properties observed in nature, a complete understanding of the protein modules that control the physical properties of the fibers must be elucidated. Scientists have therefore turned to cloning silk genes for sequence analysis in order to reveal the amino acid sequences of fibroins and better understand their structure-function relationship. DNA sequence analysis of different silk genes retrieved from diverse spider species has revealed common protein architectures in the silks [5, 11]. Analyses of the primary sequences of spider silks demonstrate regular, small peptide motifs that can be grouped into four categories: (i) $(Gly\text{-}Ala)_n/A_n$ (ii) Gly-Gly-X (iii) Gly-Pro-Gly-X-X and (iv) spacers. Based on physical studies, the peptide modules in spider silks have been assigned different structural roles [12–14]. Polyalanine (A_n) and $(GA)_n$ regions that typically contain six to nine residues have been shown to form β-sheet structures in the fiber [2, 12, 13]. These regions form crystallites that are responsible for the high tensile strength, whereas elasticity is governed by glycine-rich stretches that localize predominantly to the noncrystalline or amorphous segments. However, the precise structure adopted by the glycine-rich regions (Gly-Gly-X repeats) has been somewhat controversial. Glycine-rich regions have been described as an amorphous rubber from X-ray diffraction studies [15], as well as 3_{10}-helical structures by two-dimensional (2D) spin-diffusion nuclear magnetic resonance (NMR) spectra [14]. Gly-Pro-Gly-X-X motifs have been proposed to be involved in a β-turn spiral or helical structure [16]; these modules have been identified in flagelliform and major ampullate silks. The spacers, which contain charged residues, separate the iterated motifs into clusters.

Molecular constituents of spider silks

Dragline silks

All dragline silk core fibers have been shown to contain more than one protein, with molecular weights up to several hundred kilodaltons [17, 18]. Spidroin 1 (contraction of spider and fibroin) and spidroin 2 are two of the dragline silk subunits that are produced in the major ampullate glands [18]. Based upon their patterns of expression and primary sequence similarities, these proteins have been named major ampullate spidroin 1 and 2 (MaSp1 and MaSp2). MaSps have been shown to contain short peptide motifs that are repeated multiple times with various combinations to form repetitive structural modules, which range in size from 19 to 46 residues [2, 11, 19]. The repetitive regions of MaSp1 have been shown to be essentially proline free, whereas MaSp2 iterated sequences contain ~15% proline (Fig. 2). Partial cDNA clones that encode the two protein components have been isolated and characterized, largely from orb- and cob-weavers [20–22]. Western blot studies indicate MaSp1 protein levels are higher in *Nephila clavipes* dragline silk, relative to MaSp2 [18]. In addition, structural studies of this dragline fiber using immunostaining are consistent with this finding, demonstrating that MaSp1 constitutes the bulk of the threads [23]. Collectively, these reports support the calculated amino acid composition of the fiber based upon proline content, which suggests the fiber contains 81% MaSp1 and 19% MaSp2 [24]. The question arises whether these percentages are universal in other dragline silks from other spider species. Recent evidence suggests that different spider species have markedly different ratios of MaSp1 and MaSp2 assembled into dragline silk relative to *N. clavipes*. For example, the orb weaver *Argiope aurantia* produces a dragline silk fiber that contains 41% MaSp1 and 59% MaSp2 [24]. In part, these differences may reflect the diet of the particular spider species, as dietary effects have been shown to affect the amino acid composition profiles of dragline silk [25]. Based upon the well-documented interspecies and intraspecies variations in the mechanical properties of dragline silks, it would seem plausible that changes in these spidroin ratios, along with the spinning conditions, could dictate the performance of the fibers [26]. Therefore, in the end, the composition of MaSp1 and MaSp2 assembled into the fiber is likely balanced by the pool of amino acid resources available to the spider, as well as the particular habitat of the spider [27]. When resources and spinning conditions are ideal, a higher-performance fiber suited for the spider's needs is spun; however, when resources are limiting, the spider likely shifts the ratios of major ampullate spidroins to engineer a metabolically cheaper, lower-performance silk.
In addition to the presence of internal repetitive modules within the primary sequences of MaSp1 and MaSp2, they

both have been shown to contain nonrepetitive N and C termini [20, 28–30]. C terminus length, as well as amino acid sequence, has been shown to be conserved across spiders of diverse phylogenetic origin for the Flag, MaSps and MiSps silk paralogues [11, 31–33]. The strong conservation of the C-terminal region over several hundred million years implies an important structural role [34]. Initially, two functions were proposed for the C-terminal region, which included (i) a role as a signal peptide and (ii) as a participant necessary for controlling the solubility of the spidroins in the highly concentrated spinning dope [35–37]. Although these functions are not mutually exclusive, recent studies have revealed that the major ampullate silks retain their C termini after extrusion [28]. Because signal sequences are typically removed in the mature protein, their retention in the extruded fiber reduces the likelihood that they function as signal sequences. Evidence to support a role of the C termini in the conversion between the liquid to crystalline phase is beginning to gain scientific support. Localized regions of the nonrepetitive C termini have recently been demonstrated to modulate both the solubility and aggregation of the fibroins, with the conversion between these different physical states being highly dependent upon the environmental conditions [29, 36].

One biological event that likely controls the natural spinning process is the hydrogen ion concentration. Certain residues within the C termini have been proposed as targets for ionization state changes, which might play an important role in initiating assembly of spider silk proteins. In addition, support for a conserved, C-terminal cysteine residue participating in the formation of higher aggregate

Silk	Ensemble Repeats
MaSp1	GGAGQGGYGRGGAGQGGAGAAAAAAAA [22] poly(A) blocks, $(GA)_n$ and GGX
MaSp2	GGAGPGRQQGYGPGSSGAAAAAAA [22] GGX, GPGXX, $(GA)_n$ and poly(A) blocks
MiSp1	GAGAGAGAAAGAGAGAGGAGYGGQGGYGAGAGAGAAAAAGAGAGGAGGYGR [40] GGX, GA_n, poly(A) blocks and spacer
MiSp2	GAGVGAGAAAGFAAGAGGAGGYR [40] GGX, GA_n, poly(A) blocks and spacer
Flag	ISEELTIGGAGAGGVGPGGSGPGGVGPGGSGPGGVGPGGSGPGGVGSGGSGPGGVGPGGSGPGGVGSGGFGPGGIGPGGSG PGGVGPGGVGGPYGPGGSGPGGAGGAGGSYGPGGPYGPGGSGGPGGAGGPYGPGGAGGPYGPGGPYGPGGAGGPGGEGP GGAGGPYGPGGPGGAGPGGYGPGGAGPGGYGPGGAGPGGYGPGGAGSGGYGPGGAGPGGYGPGGPGPGGYGPGGAGPG GYGPGGTGPGGSAPGGAGPGGAGPGGYGPGGSGPGGYGPGGGPGGAGPGGAGPGGAGPGGAGPGGAGPGGAGP GGAGPGGAGPGGAGPGGVGTGGLGRGGAGRGGAGRGGAGRGGAGRGGAGRGGTGGVGGAGGAGGAGGVGGAGGSGG TTVIEDLDITIDGADGPIT [19] $GPGXX_n$, GGX and spacer
AcSp1	GSAGPQGGFGATGGASAGLISRVANALANTSTLRTVLRTGVSQQIASSVVQRAAQSLASTLGVDGNNLARFAVQAVSRLPA GSDTSAYAQAFSSALFNAGVLNASNIDTLGSRVLSALLNGVSSAAQGLGINVDSGSVQSDISSSSSFLSTSSSSASYSQASASS TSGTGYTGPSGPSTGPSGYPGPLGGGAPFGQSGFG [32] poly(S) blocks and GGX
TuSp1	RTVGVGASPFQYANAVSNAFGQLLGGQGILTQENAAGLASSVSSAISSAASSVAAQAASAAQSSAFAQSQAAAQAFSQAAS RSASQSAAQAGSSSTSTTTTTSQAASQAASQSASSSYSAASQSAFSQASSSALASSSSFSSAFSSASSASAVGQVGYQIGLNAA QTLGISNAPAFADSVSQAV [52] poly(S), GX, AAQAASAA, AAAQA, AASQAA, and SQ_n
ECP-1*	AGVGNNARFINGAGNNWSVSSMSGAGAFSGRRNSVYSGSSAGAAAGAHAASGGRAGAVAGAGAGASARAGAGARAAAG AGAGASAEAGAGARAAAGAGAGTGAGSGAGAAAGAGAAATSNAQAGAAVGSRGRASAGSRARAASFSEANTLAGAGAS SNARAASFSGANALAGAGSRAGAEAQAGARAGAGAASEASAAASAEARAGARAGAGAASEASAAASAEANAGARAVAG AGASAGAESNAGAKAVTRGRARAAAGAGATASSSASSLASSLSEAASSSSSSSSSSSSSSFGESLA SSAASAASALGA [47] Short poly(A) blocks and GA repeats
ECP-2*	GAGATAGAEAGAASGAAAGAGASSGAGAGAGASSGAGAGAGAGASSGAGARAGAGAGAGAGAGASSGADANAGAVAS SGAGANAGAGASSGAGANAGVGAGAGAGANAGAGAGAGANAGAGAGAGANAGAGAGAGAGASSGAGAGAGAAASA GAEA [56] Short poly(A) blocks and GA repeats

Figure 2. Comparison of the core repeats (also called ensemble repeats) identified in spider silk proteins. Spider silk proteins are modular in nature, containing ensemble repeat units with different molecular characteristics. Only one representative internal repeat unit for each fibroin type is illustrated from either orb- or cob-weavers. Underlined sequences in AcSp1 and TuSp1 represent SQ and/or poly(S) modules. For poly(S) there must be three or more consecutive residues. Areas colored red indicate polyalanine or GA repeats; blue letters denote GGX repeats; orange lettering represents GPGXX motifs; and pink-colored regions denote GX iterations. MaSp1 (DQ409057), MaSp2 (DQ409058), TuSp1 (AAZ15706), ECP-1 (AAX92677) and ECP-2 (ABC68105) are from *L. hesperus*. Flag silk is from *Nephila madagascariensis* (AAF36091); AcSp1 is from *Argiope trifasciata* (AAR83925); MiSp1 (AAC14589) and MiSp2 (AAC14591) are from *Nephila clavipes*. Asterisks after ECP-1 and ECP-2 indicate that these sequences are derived from their C termini and do not reflect ensemble repeat units; they are presented to show the presence of short modules commonly found in silks.

complexes through intermolecular disulfide bond linkages has been implied [28, 38]. In particular, recombinant MaSp C-terminal sequences have been shown to form dimers under nonreducing conditions, whereas similar structured MiSps lacking the conserved cysteine residues fail to form higher-aggregate complexes [28]. In addition to the C-terminal region playing an integral part of the liquid to crystalline phase transition, intrinsic chemical properties embedded within the primary sequence of silk fibroins have been demonstrated to display remarkable differences in their solubility and assembly characteristics. Comparative studies of the two major dragline silk proteins from *Araneus diadematus*, ADF-3 (MaSp2-like) and ADF-4 (MaSp1-like), demonstrate that ADF-3 is soluble at high concentrations [37], whereas ADF-4 is essentially insoluble and self-assembles into filamentous structures under certain experimental conditions [39]. A closer analysis of the hydropathicity demonstrates that ADF-4 is more hydrophobic in nature, which facilitates aggregation with other protein molecules. Interestingly, dragline silks have been found to contain pairs of proteins, which is a feature observed across a large number of spider species. Strikingly, different species display a common distinct distribution of hydrophobicity, with one protein being more hydrophilic (ADF-3/MaSp-2) and the other member more hydrophobic (ADF-4/MaSp1) [39].

Minor ampullate silks

Scanning electron microscopy demonstrates that minor ampullate silks form smaller-diameter fibers than do major ampullate silks. Two partial cDNAs that encode constituents of minor ampullate silk have been isolated from *N. clavipes*. These cDNAs encode two proteins named minor ampullate spidroin 1 and 2 (MiSp1 and MiSp2). Based upon mRNA lengths, the estimated protein size for MiSp1 is 320 kDa, whereas MiSp2 is 250 kDa. Southern blot analysis of genomic DNAs isolated from *N. clavipes* support the fact that these mRNAs are derived from two distinct genes [40]. Structurally, both proteins are organized into predominantly repetitive regions, with small non-repetitive C termini [40]. The highly repetitive regions consist mainly of alanine and glycine, with lower levels of tyrosine, glutamine and arginine being present. Gly-Gly-X and Gly-Ala repeats are significantly represented throughout the primary amino acid sequence (Fig. 2). Repetitive regions of both MiSp proteins are interrupted by ~137 amino acid serine-rich spacer regions; these spacer regions have similar serine composition relative to the amorphous region of *Bombyx mori*. Solution state conformational studies using Fourier transform infrared spectroscopy (FTIR) of minor ampullate silk collected from *N. edulis* demonstrate a dominant α-helical nature, with reduced β-sheet structure [41]. Solid-state NMR data suggest that the conformations of the alanine residues found in minor ampullate silk fibers are more heterogeneous in nature, with a large fraction present in a non-β sheet conformation [42]. The lower amount of β-sheet structure, yet high tensile strength of the fiber, suggests that minor ampullate silks may have different cross-linking mechanisms and matrix proteins, relative to major ampullate silks. From a mechanical perspective, minor ampullate silk is similar to major ampullate silk in tensile strength, but has lower elasticity [2, 3].

Capture silks

Orb-weavers manufacture capture silks, which form the major component of the capture spiral of the web. Capture silk (flagelliform silk) has been proposed to consist largely of a single protein produced from the flagelliform gland, called Flag silk [16]. Partial cDNA sequences that encode for Flag silk have been retrieved, as well as extensive genomic DNA information. The *flag* gene represents the most extensively studied spider gene at the genomic DNA level. Characterization of the *flag* gene has demonstrated the presence of 13 exons that span over 30 kb of genomic DNA sequence. Flag transcripts have been determined by Northern blot analysis to be ~15 kb, with an estimated protein size of about 500 kDa [16]. Translation of the cDNA predicts a protein with multiple iterations, largely with the dominant repeat of this protein representing a pentapeptide Gly-Pro-Gly-Gly-X sequence, which can appear up to 63 times in tandem arrays [19]. Concatenated repeats of the Gly-Pro-Gly-Gly-X sequence have been proposed to form a β-spiral structure that can function as molecular 'nanosprings', which provide fiber elasticity. A second motif commonly found within the predicted primary sequence represents the tripeptide Gly-Gly-X, which occurs approximately tenfold fewer times than Gly-Pro-Gly-Gly-X. The last repetitive flagelliform element, which is the longest and least abundant, represents a 28-amino-acid spacer region that is glycine poor. These motifs are woven together to form ensemble repeats that range from 368 to 411 residues (Fig. 2). Comparison of the mechanical properties of the different silk types in orb web spiders demonstrates that Flag silks are the most extensible, but have lower tensile strength relative to dragline silk [7]. Within each monomeric protein, more than 14 ensemble repeats may be present. Both N- and C-terminal sequence data demonstrate a nonrepetitive nature for Flag silk [19]. The Flag C terminus differs substantially from the primary sequences of the MaSp and MiSp silks, even though, presumably, these regions may perform similar functions.

Although particular silk types in the same species have been found in pairs (e.g. MaSp1 and MaSp2), only one protein has been reported to assemble into flagelliform silks. Spidroins with similar functional properties likely exist in cobweb weavers. Although cob-weavers have

been reported to lack capture silk, they have threads that are coated with glue-like substances [43]. Instead of capture spirals, black widow spiders manufacture gumfooted lines that extend downward to the substrate from the supporting scaffolding of the cobweb, with only the bottom 5–15 mm being coated with aqueous glue [44]. Gumfooted lines may use structural related fibroins, but their molecular constituents remain to be determined.

Aciniform silks

Araneoid spiders use aciniform silks to wrap and immobilize prey, build sperm webs and web decorations [45]. Relative to dragline silk amino acid compositions, which are rich in glycine and alanine, analyses of the luminal contents from aciniform glands have revealed low percentages of glycine and alanine [46]. Evidence for the expression of a single silk fibroin gene that encodes an inferred protein closely matching the amino acid contents of the aciniform glands has been reported [32]. The predicted protein, aciniform spidroin 1 (AcSp1), displays a different structural organization relative to Flag, major ampullate and minor ampullate silks. AcSp1 contains >14 highly homogenized repeats, with each ensemble repeat consisting of 200 amino acid blocks; the repetitive sequences predicted from the AcSp1 cDNA do not resemble iterations found within other araneoid fibroins (Fig. 2). Of the silk types studied in orb weavers, aciniform silks have lower tensile strength relative to dragline silks but higher extensibility and toughness [32]. The high degree of extensibility of aciniform silks is second only to flagelliform silks [7]. Phylogenetic analyses of the C-terminal aciniform sequence demonstrate that this fibroin groups weakly with major ampullate, minor ampullate and flagelliform silks, suggesting aciniform silks represent divergent members of the silk gene family.

Tubuliform silks

Significant progress to unravel the structural and molecular properties of egg case (also known as tubuliform) silk has recently been reported. Aside from dragline silk, these threads represent the second most characterized spider silk at the molecular level. Egg case silk is produced by female spiders during the reproductive season. Scanning electron microscopy studies have revealed that spider egg cases contain two different diameter fibers in the cob-weaver, *Latrodectus hesperus* [47], as well as the orb-weaver, *A. aurantia* [48]. It has been suggested that the larger-diameter fibers are derived from the tubuliform gland, whereas the smaller-diameter fibers originate from the aciniform gland [48]. Morphological studies indicate that the tubuliform glands undergo structural changes prior to egg laying and egg case formation [49, 50]. Solid-state NMR and FTIR studies of egg case silks from *L. hesperus*, *A. diadematus* and *Achaearanea tepidariorum* demonstrate profound differences in their secondary structures. *A. tepidariorum* silk largely consists of α-helices and parallel β-sheets, whereas *A. diadematus* silk shows a more complex structure that also contains antiparallel β-strands and β-turns [51]. For *L. hesperus*, alanine residues are primarily found in a β-sheet environment [52]. The differences in these structures have been hypothesized to correlate with the different habitats of the spiders.

Some of the molecular constituents of egg case silk have recently begun to be elucidated. Denaturing polyacrylamide gel electrophoresis of egg case silk proteins from *N. clavata* have detected two proteins with sizes of 342 and 303 kDa [53], whereas a complex protein profile has been observed for *L. hesperus* [47, 54]. One of the major components of the larger-diameter fiber has been determined at the molecular level; this protein represents tubuliform spidroin 1 (TuSp1). Analyses of the primary sequence of TuSp1 reveal large ensemble repeats that are ~184 amino acids in length, rich in serine, but low in glycine [33, 52, 53, 55] (Fig. 2). TuSp1 orthologs have been isolated from a variety of different species (includes orb- and cob-weavers) and their primary amino acid sequences are highly conserved across species [33]. New amino acid motifs which include S_n, $(SA)_n$, $(SQ)_n$ and GX (X represents Q, L, Y, I, V and A) have been identified. Analysis of the primary sequence of *L. hesperus* TuSp1 reveals motifs that are potentially involved in β-sheet structure, which include the modules AAQAASAA, AAAQA and AASQAA (Fig. 2). Relative to the polyalanine blocks found in major ampullate silks, these motifs contain larger side chain groups that are more hydrophilic in nature (Q and S). The bulkier side chains of serine and glutamine are consistent with the larger side chain spacing detected in the β-sheet regions by X-ray diffraction studies of egg case silks from *N. clavipes* [12].

Comparison of the C terminus of TuSp1 with other silk family members demonstrates a highly divergent silk fibroin family member. Based upon this lack of similarity, TuSp1 has been proposed to represent an evolutionary ancient silk. Real-time PCR analysis has confirmed the tubuliform gland-restricted mRNA expression pattern for TuSp1, whereas matrix-assisted laser desorption ionization (MALDI) tandem time-of-flight (TOF) mass spectrometry of peptides generated from tryptic digestion of solubilized egg case material provides direct evidence for the assembly of TuSp1 into the fiber [52]. Digestion of the egg case core fiber with trypsin, followed by the analysis of the peptides using MALDI tandem TOF MS, has lead to the discovery of two other proteins that are assembled into the core fiber of *L. hesperus*, egg case protein 1 and 2 (ECP-1 and ECP-2) [47, 56]. Although the core fiber of dragline silk is presumably dimeric in nature, the core fiber of egg case silk has been demonstrated to represent a

trimeric complex, consisting of TuSp1, ECP-1 and ECP-2 [56]. Similar to the mRNA expression profile for TuSp1, the ECPs also display tubuliform-restricted patterns of expression. Real-time PCR analysis indicates that TuSp1 mRNA levels are approximately 20-fold higher relative to the ECPs [56]. This suggests that the ECPs represent lower-abundancy species in the egg case fiber. Analyses of the ECP primary sequences indicate that they share similarity to fibroins from spiders and silkworms, with short polyalanine and poly(Gly-Ala) modules (Fig. 2). The ECPs show extreme codon biasness at the wobble position (A or U), similar to other fibroins, as well as constituting a pair of proteins, which is a common feature in the spidroin family (e.g. MaSp1 and MaSp2) [47]. Similar to TuSp1 and AcSp1, the ECPs lack the conserved, nonrepetitive C termini, which is characteristic for some fibroin family members. Perhaps more intriguing is the primary sequence of their N termini, which contain 16 conserved cysteine residues (Fig. 4a). Evidence that these residues mediate higher-aggregate complex formation, presumably by intermolecular disulfide bond linkages, is supported by their increased monomeric accumulation after prolonged treatment of solubilized egg case material with reducing agents [47]. Collectively, these data suggest the ECPs may function as intermolecular cross-linkers, with potential structural roles in the egg case fiber. It will be interesting to determine whether ECP-like molecules are present in orb-weavers or more divergent spider species.

Glue and pyriform silks

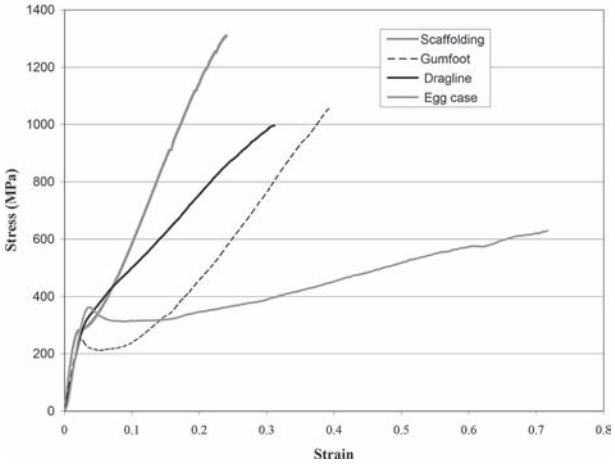
No cDNAs encoding proteins that represent glue or pyriform silks have been reported. Comparatively little is known about these silks types. Amino acid compositions have been determined for both silks using luminal gland materials from orb-weavers. Pyriform and aggregate silks contain very polar amino acids, which include Ser, Asp, Glu, Thr, Lys and Arg [57]. Although the precise molecular identities of the proteins involved in both silks/glues remain to be determined, the aggregate glands have been shown to produce a glycoprotein coating that likely provides stickiness to the capture silk fiber [58].

Mechanics of spider silks

Because spiders produce a diverse family of high-performance structural fibers, they provide an ideal model system for studying the functional design of protein-based materials. The mechanical properties of single fibers can be described by stress-strain curve profiles, which are generated by stretching individual fibers. Stress-strain curves have been shown to display variability for the same silk type from different spider species [26]. In addition, the curves vary between different silks from the same spider species [59]. Stress-strain curve variance can be attributed to differences in the spinning environment and conditions, as well as the composition of the spinning dope. Recently, environmental parameters that include diet, time of the day for web construction, as well as reeling speed, have been shown to influence fiber performance [25, 27, 60]. This underscores the importance of the spinning conditions and their influence on fiber performance. Spider silk is lightweight and strong, having a strength of 1.1 GPa. High-tensile steel and nylon have strengths of 1.5 and 0.95 GPa, respectively [7]. When equivalent weights of spider silk are compared with high-tensile steel, spider silk is about five times stronger.

The mechanical properties of silk are determined by stretching a fiber at a particular strain rate, $d(l/l_o)/dt$, and measuring the force required to extend the fiber a certain length, defined as dl. The stress (σ) is defined as the force (F) divided by the cross-sectional area of the fiber ($\sigma = F/A$). Upon extension, the cross-sectional area is considered to be constant, even though A will decrease somewhat during extension. The strain (ε) represents the normalized deformation, which is defined as the ratio of change in length (dl) to the initial length (l_0) or $d(l/l_0)$. Young's modulus (E), which can be obtained from the slope of the stress-strain curve, is a measure of the stiffness of the fiber. A steeper slope indicates a stiffer material. If the fiber breaks during extension, the area under the curve is a measure of the toughness of the fiber, a parameter that indicates the amount of energy absorbed by the fiber. During extension, stress-strain profiles can often display sudden slope changes, indicative of major structural transitions in the material; these slope changes are known as 'yield points'. Here we briefly compare the mechanical properties of silk types from *L. hesperus* with other silks collected from orb-weaving spiders. Other studies that emphasize additional details regarding the mechanical properties of silks from orb-weavers have been extensively covered in other articles [2, 3, 7]. Figure 3a demonstrates the typical experimental data for stress-strain profiles of *L. hesperus* dragline, egg case, scaffolding and gumfooted silks. The stress-strain curves reveal that dragline, egg case, scaffold and gumfooted silks are similar in their initial stiffness; however, after their yield points, each material displays markedly different mechanical properties. *L. hesperus* dragline silks have a higher tensile strength relative to the other black widow spider fibers, whereas egg case silks display higher extensibility and toughness (Fig. 3a, b). Scaffolding silks, which are found in the web of black widows and have little molecular characterization, exhibit the lowest extensibility and toughness for black widow silk types (Fig. 3a, b). Comparison of *L. hesperus* dragline silks with major ampullate silks collected from two different orb weavers, *Argiope trifasciata* and *A. diadematus*, display similar strengths and extensibilities (Fig. 3b). Flagelliform silks

a



b

Spider	Material	Tensile breaking strength (MPa)	Extensibility
L. hesperus	Dragline silk	996	0.311
L. hesperus	Egg case silk	629	0.717
L. hesperus	Scaffolding silk	1309	0.240
L. hesperus	Gumfoot silk	1057	0.393
*A. trifasciata**	Major ampullate silk	1290	0.220
A. trifasciata	Minor ampullate silk	342	0.540
*A. trifasciata**	Aciniform silk	687	0.860
A. diadematus[#]	Major ampullate	1100	0.270
A. diadematus[#]	Flagelliform silk	500	2.700

Figure 3. Different silk fibers from spiders display different mechanical properties. (*a*) Stress-strain curve of *L. hesperus* dragline, egg case, scaffolding and gumfooted silks. The curves represent data collected for threads from eight to ten different spiders. (*b*) Comparison of the breaking strain and stress (MPa) for *L. hesperus* silk fibers to other spider fibers reported from orb-weavers. *A. trifasciata** = *Argiope trifasciata*, data from Hayashi et al. [32]. *A. diadematus*[#] = *Araneus diadematus*, data from Gosline et al. [7].

display markedly different mechanical properties relative to black widow gumfooted silks, with flagelliform silks exhibiting more elasticity and less strength (Fig. 3b). Aciniform silk, which has only been characterized at the molecular level from orb-weavers, has less strength relative to major ampullate silks, but more elasticity (Fig. 3b).

Biophysical studies to establish the relationships between the mechanical properties of the fibers and the modular features of the fibroins have been most extensive for dragline silk. Dragline silk consists of alanine-rich domains that form crystalline regions. These crystalline regions are proposed to come largely from polyalanine stretches and GA repeats found within the primary sequence of MaSp1, providing high-tensile strength. The MaSp2 fibroins, which exclusively contain the GPGXX motifs, provide the fiber elasticity and represent the major constituent of the amorphous region [23]. Other models continue to be developed to predict the relationships between the primary sequences and the material properties of silk types [61]. In the end, the ability to predict the material properties of natural as well as engineered fibers will be an essential aspect of silk biology, enabling the field to move one step closer to large-scale production of synthetic silks for commercialization.

Production of silk proteins for assembly into fibers

In order to successfully produce synthetic silk fibers on a large scale, the intricacies of the assembly process for silk proteins must also be fully understood. Because the assembly process is a complex event, scientists have turned their attention to studying the process *in vivo*. It has been established that dragline silk proteins are stored in a concentrated liquid form (up to 50% weight/volume) in their particular silk-producing glands [62]. This highly concentrated liquid, often referred to as the spinning dope, displays liquid crystal properties [63]. Within the gland, the evidence indicates that the polyalanine motifs form α-helical structures, while the glycine-rich regions adopt either β-turns or random-coil conformation [22, 62, 64]. However, during assembly of the fiber, the proteins are known to undergo dramatic changes in their secondary and tertiary structure. During the passage of the liquid silk through the spinning duct, sodium, chlorine and water are removed [65]. It has also been shown that potassium ions and hydrogen ions are secreted into the lumen of the duct [63, 65]. As the silk dope passes through the spinning duct, the pH changes from 6.9 to 6.3. The alteration of hydrogen ion concentration is likely controlled by a proton pump. A similar mechanism has been observed in the spinning duct of the silkworm silk [66]. The progressive pH change has been proposed to facilitate the denaturation of localized protein regions by disrupting their water shell, presumably by changing the ionization state of charged groups on amino acid side chains as they move through the duct [63]. Special sequence parts of the conserved C termini have been suggested to be targets for ionization state changes, playing a role in the maintenance of the solubility and aggregation of the fibroins [29, 36]. As the pH decreases in the spinning duct, acid side chain groups positioned within the C termini have been postulated to become uncharged via protonation, resulting in the attenuation of expulsion forces and the formation of strong hydrophobic interactions that facilitate parallel alignments of the spidroins [29]. Both the pH change and the geometry of the duct likely contribute to the denaturation and the proper alignment of protein molecules as they are subject to a rapid extensional flow field from passing through a draw-down taper positioned

a

Silk	Non-repetitive N-termini of Silk Proteins
MaSp1	**MYSLSIQSDFPTTTMTWSTRLALSFFAVICTQSIYA**LGQGNTPWSTKANADNFMNGFLSACAQSGVFSADQVDDMTTIGK TLMIAMDKMGGKISSSKLQALDMAFASSVAEIATAEGGANINDITDTIRYALQNAFYQTTGAVNSKFINEISNLIYMFAQTNI NDVN
MaSp2	**MTTMNWSTRLVLSILVVLCTQSLCALG**QANTPWSSKENADAFIGAFMNAASQSGAFSSDQIDDMSVISNTLMAAMDNM GGRITQSKLQALDMAFASSVAEIAVADGQNVGAATNAISDALRSAFYQTTGVVNNQFITGISSLIGMFAQISGNEVSYSSA
MiSp1	Unknown
MiSp2	Unknown
Flag	**MGKGRHDTKAKAKAMQVALASSIAELVIA**ESSGGDVQRKTNVISNALRNALMSTTGSPNEEFVHEVQDLIQMLSQEQINE VDTS
AcSp1	Unknown
TuSp1	**MVWLTSTVLLASLLGTLG**LPANSLSGVSASVNIFNSPNAATSFLNCLRSNIESSPAFPFQEQADLDSIAEVILSDVSSVNTASS ATSLALSTALASSLAELLVTESAEEDIDNQVVALSTILSQCFVETTGSPNPAFVASVKSLLGVLSQSASNYEFVETADESIAAN VPGITTTGNFTPLNAIEQNFV [56]
ECP-1	**MFTFLGLISLLGVQIGIALG**QGDVCFNKCLSRISGGCQSLIYTQVNPCAFQCTCDGVVTYHVEETFTKCGSRKLCYQGECL TEVPNRCERRYGYGYIGLLNPYNPCVFYCHNADVPCESFEENFVDGTTCYSSNSVIGQCLLGRCAEGGL [47]
ECP-2	**MFTLVGLLSLLGVQIGIALG**DDVCFNKCLSKISGECQSLVYTQINPCSFECNCDGVYSYHVEETFTRCGSHKLCYQGECLT EVPRQCQRRYGYGYIGLLNAYNPCAFSCYNADVPCELYEENLVDGTACTTSNSVIGQCLLGRCTKVRIP [56]
Aggregate	Unknown
Pyriform	Unknown

b

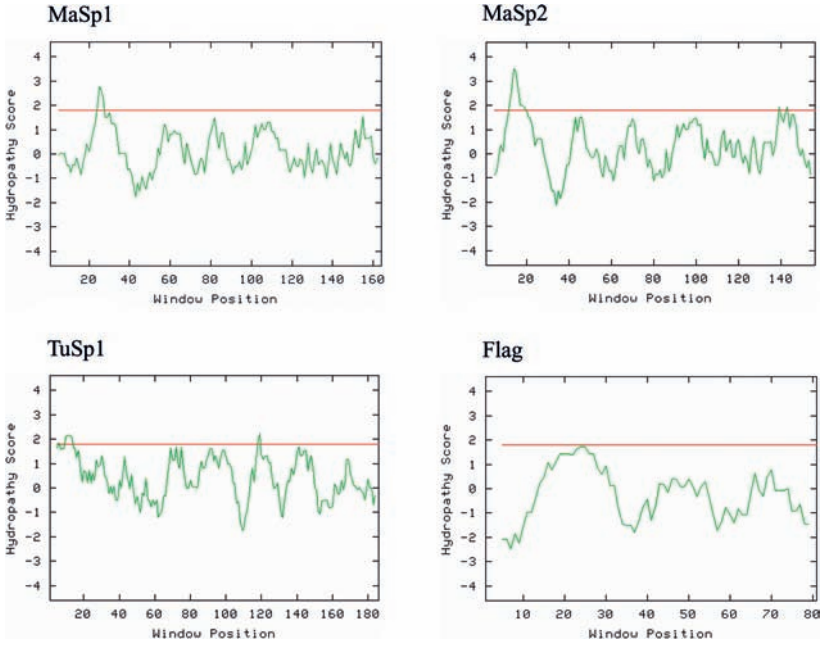


Figure 4. Spider fibroin N termini are nonrepetitive and contain alternating hydrophobic-hydrophilic block periodicity. (*a*) N termini of silk fibroins that are representative of the different silk types. N termini are from *L. hesperus* MaSp1 (DQ379381), MaSp2 (DQ379382), TuSp1 (DQ379383), ECP-1 (AAX92677) and ECP-2 (ABC68105). Flagelliform silk is from *N. madagascariensis* (AF218623) [19]. Residues in bold represent predicted signal secretion sequences identified by the SignalP 3.0 algorithm [85]. In the case of TuSp1, mass spectrometry has validated the cleavage position of the signal peptidase [56]. Cysteine residues that are potentially involved in disulfide bond linkages are indicated by underlines. (*b*) Hydropathy plots of the nonrepetitive N termini of the fibroin family members MaSp1, MaSp2, TuSp1 and Flag silk. Depicted is a Kyte and Doolittle scale mean hydrophobicity profile (scan window size of 9) [86]. Scores above 0 indicate hydrophobicity, whereas scores below 0 show regions of hydrophilicity. Window position indicates the location of the amino acids in the primary sequence. The red lines demonstrate regions (positioned at a hydrophobicity of 2) that are predicted to be either transmembrane or signal sequence segments.

in the distal duct [67]. The rapid extension is postulated to facilitate unfolding of the fibroins as well as to promote structural transitions of poly(A) and poly(GA) stretches from an α-helical to anti-parallel β-pleated structure, leading to the formation of crystallites [3, 68]. During the formation of the β-sheet structures and thread assembly, the process is considered a liquid-crystalline phase transition that involves the separation into a solvent-rich and polymer-rich phase [68].

Recombinant spider silk production

Recapitulation of the *in vivo* spinning process in the laboratory is the ultimate goal for the material scientists and engineers. Large-scale commercial production of synthetic spider silk would likely create a new generation of biomaterials with broad applications, ranging from medical sutures, bullet proof vests, fishing lines and ropes, sporting goods and materials for airplane construction [69]. To date, production of synthetic silks has been problematic for a number of different reasons: one of the barriers for silk production has revolved around the amplification of silk cDNAs. Because of their rich guanine-cytosine content (reflective of their high levels of glycine and alanine codons), their repetitive sequence nature, and their physical lengths, which can exceed >15 kb [16], manipulation of silk genes has been difficult when utilizing basic recombinant DNA methodologies. In addition, no natural full-length cDNAs encoding spider dragline silk components have been reported. Partial cDNA sequences (mainly the 3′ end of the cDNA) representing dragline silk genes have been reported from a variety of diverse spider species [5, 11, 20, 31, 70], and these nucleic acids have been used in expression studies. Difficulties in expression of silk protein is further compounded because of their high levels of glycine and alanine (combined over 50% of the entire amino acid composition of the protein), as well as their mRNAs displaying extreme codon bias at the wobble position [20, 32]. Mammalian cell lines (baby hamster kidney cells and bovine mammary epithelial alveolar cells immortalized with large T antigen) transfected with eukaryotic expression vectors carrying dragline silk cDNAs have led to the production of silks with molecular masses of 60 to 140 kDa; however, these filaments have lower tenacity relative to natural silks [37]. Dragline silk production has also been attempted in other host systems, which include bacteria [71], plants [72], goats [73] and the insect cell line Sf9 (generated from the fall armyworm *Spodoptera frugiperda*) [39], with the baculovirus expression system and Sf9 cells proving to be highly efficient in production of dragline silk. Synthetic spider silk sequences that resemble dragline silk genes have also been designed and expressed in plants [74–76], yeast [77] and bacteria [78–80]. Molecular masses of these recombinant dragline silk products have approached 163 kDa; however, the majority of these studies have led to size heterogeneity of their expressed products as a result of abortive transcription or translation. New approaches that combine synthetic repetitive silk sequences with authentic silk domains are also emerging as alternatives to protein expression systems [81]. Collectively, large-scale production of silk fibroins, including their assembly process, will need further progress.

Techniques used to identify silk constituents

Despite the large number of silk cDNA sequences that have been reported, the isolation of additional nucleic acid sequences that encode diverse fiber types must continue. To date, a variety of different molecular and biochemical approaches has been used to identify spider silk fibroins. The first spider fibroin cDNAs were isolated using a combination of biochemistry and reverse genetics. Dragline silks were dissolved and subject to acid hydrolysis to generate peptides for sequence analysis, followed by the design of oligonucleotide probes for screening silk-gland-specific cDNA libraries by nucleic-nucleic acid hybridization [20]. Translation of the retrieved dragline silk cDNA sequences revealed common architectures, with highly homogenized internal repetitive repeats and nonrepetitive C termini. Subsequently, other silk cDNAs have been isolated by either screening libraries with degenerate oligonucleotide probes corresponding to the internal repetitive repeat regions or the nonrepetitive C termini of dragline silk constituents [5, 11, 22]. Recently, more divergent silk family members have been isolated by randomly picking bacterial colonies carrying silk-gland-restricted recombinant plasmid cDNA libraries, followed by restriction digestion of the plasmids, to identify cDNAs that contain repetitive ensemble repeats [32, 55, 82]. The success of this strategy has relied on the premise that fibroin mRNAs are highly expressed within silk glands. Alternative approaches to identify new silk proteins embedded in the fiber are emerging, which combine biochemistry, molecular biology and genetics. One technique involves combining reverse genetics with MALDI tandem TOF mass spectrometric analysis of tryptic fragments generated from solubilized silk materials [47]. MALDI tandem TOF analyses of tryptic fragments generated from either in-solution or in-gel digestion of silk materials have helped elucidate the trimeric nature of the core fibroin from *L. hesperus* egg case silk [56]. Future applications that integrate this methodology should facilitate the identification of new silk molecules in spider silk fibers, which include skin coat proteins, glue and pyriform silks. This technology will also allow for the identification of lower-abundant species that potentially have important structural roles.

Although new techniques to identify novel silk proteins are surfacing, methods to retrieve N-terminal fibroin se-

quence information have been severely limited. Despite efforts to retrieve full-length cDNA sequences, the majority of nucleic acid sequences reported in the database for traditional spider silk fibroins represent partial cDNA sequences that correspond to the 3′ end of the gene, with the exception of Flag silk [19]. Full-length spider fibroin cDNAs have been difficult to retrieve because silk mRNAs are long and contain highly repetitive GC-rich modules (these modules are characteristic for alanine codons, which exhibit bias at the wobble position). Long and intrinsically repetitive mRNAs are technically more challenging to reverse transcribe into first-strand cDNAs, especially when oligo(dT) primers are used to initiate their synthesis. Northern blot analyses demonstrate that silk fibroin mRNAs have sizes that can approach ~15 kb [11, 16, 83]. Because long, full-length cDNAs can be difficult to find in cDNA libraries, scientists often rely on amplification methodologies, e.g. 5′ rapid amplification of cDNA ends (5′ RACE) to acquire rare full-length mRNAs. To date, however, no full-length cDNAs encoding traditional spider fibroins have been successfully amplified by PCR or retrieved by conventional cDNA library screens using nucleic acid probes. Attempts to sequence the N termini of fibroins by Edman degradation have been inefficient in elucidating N-terminal sequences from fibroins [18]. Other approaches to obtain N termini from silk fibroins have proved to be labor-intensive processes, requiring the conventional screening of genomic DNA libraries and sequencing of portions of large genomic DNA fragments to identify the exon(s) containing the coding information [30]. Recently, a method that employs a PCR-based promoter walking strategy, in which the 5′ ends of silk genes are retrieved using primary and secondary (nested) PCR, has proven successful for the rapid isolation of the N terminus for *L. hesperus* TuSp1 [56]. Application of this strategy has many advantages over conventional nucleic acid-nucleic acid screening, which include reduced labor, lower costs and a higher degree of specificity. In addition, this methodology has been successfully applied to retrieve the N-terminal sequences from two other silk fibroins, which include *L. hesperus* MaSp1 and MaSp2 (Fig. 4a). MaSp1, MaSp2, TuSp1 and Flag silk all display nonredundant N termini, comprising 167, 159, 189 and 84 amino acids, respectively (Fig. 4a). Both *L. hesperus* MaSp1 and TuSp1 were found to contain cysteine residues within their N termini, whereas both *L. hesperus* MaSp2 and *N. madagascariensis* Flag N termini lacked cysteine residues (Fig. 4a). Because the N-terminal region of *L. geometricus* MaSp1 has recently been shown to lack cysteine residues, it is presently unclear why certain orthologous members contain the N-terminal cysteines whereas others lack these residues [30].

Predicted signal sequences were identified for the *L. hesperus* fibroins using the SignalP 3.0 algorithm. The predicted cleavage site for TuSp1 has been confirmed by mass spectrometry studies [56]. Similar to the periodicity in the hydropathy pattern found within the internal repetitive parts of the MaSp1 and MaSp2, the N termini also maintain this periodicity (Fig. 4b). Previous experiments have demonstrated that the primary sequence for the fibroin heavy chain of *B. mori* contains a pattern of hydrophobic and hydrophilic blocks; these alternating hydrophilic-hydrophobic parts contribute to the formation of micelles, which under increased protein concentrations coalesce into larger globules and then gel states [35]. In a similar manner, the primary sequences of the spider fibroins, including their N termini, follow this same pattern. Therefore, this physical property supports the assertion that the N termini of the fibroins perform a dual function: secretion and fiber assembly. In summary, as technical advances allow for the incorporation of these modules into engineered silk proteins, scientists hope that designed silks will have mechanical properties that approach spider fibers found in nature.

Perspectives

The future objective will be to produce different silk fibroins that have a wide range of different mechanical properties for commercial applications. New insights revealing the molecular mechanisms used by spiders to assemble natural silks will help pave the way for the production of synthetic silks in the laboratory. Since silks have been shown to be modular in nature, key structural and functional features could be incorporated from different silk genes to create entirely new fibroins with enhanced mechanical properties. However, before this can be successfully achieved, collaborative efforts to advance silk gene manipulation, protein expression and spinning, will need to circumvent the current research barriers. Bioengineers will need to design machinery to manipulate large volumes of liquid that can be spun into synthetic fibers, a feat that will be technically challenging because these devices will need to mimic closely the natural spider spinning process. Protein chemists will need to find more efficient methods for increasing yield recoveries for expressed silk proteins that carry all the important silk domains required for proper assembly, which include the N and C termini of the fibroins. Minor constituents that have important structural roles will also need to be expressed and incorporated in the correct ratios into the fibers. Collectively, these represent challenging tasks for scientists across different fields – but the rewards of such accomplishments could have profound influences in the future on the materials used in our world.

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