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Spider Silk: From Soluble Protein to Extraordinary Fiber

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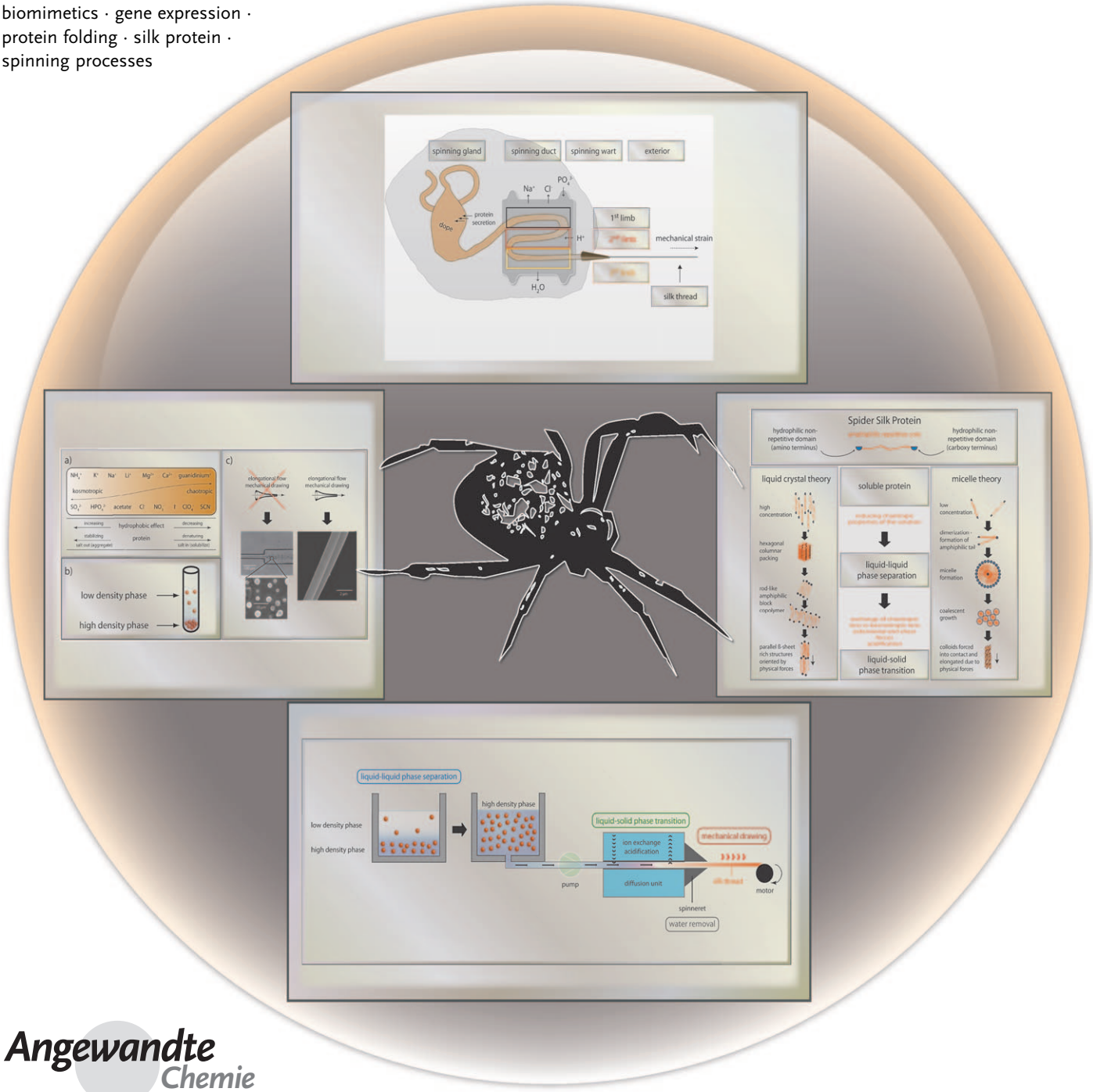

Spider silks outrival natural and many synthetic fibers in terms of their material characteristics. In nature, the formation of a solid fiber from soluble spider silk proteins is the result of complex biochemical and physical processes that take place within specialized spinning organs. Herein, we present natural and artificial silk production processes, from gene transcription to silk protein processing and finally fiber assembly. In-vivo and in-vitro findings in the field of spider silk research are the basis for the design of new proteins and processing strategies, which will enable applications of these fascinating protein-based materials in technical and medical sciences.

From the Contents

1. Introduction

Mankind has used spider silk as a material long before it appeared in the focus of research. In ancient Greece, natural cobwebs were used to seal bleeding wounds, and in Australasia, spider silk threads or whole spider webs were used for fishing. Later, spider silks were also utilized for military purposes, and in particular for the construction of crosshairs.[1] The variety of applications for spider silk is due in part to its extremely high mechanical stability, biocompatibility, smoothness, and thinness in comparison to other available materials.

Unlike other arthropods, spiders produce a variety of different silks with diverse properties. Female orb-weaving spiders (ecribellate spiders) utilize up to six different silks and a silk-like glue, each produced in a specialized gland, and each tailored to fulfill a certain task (Figure 1 and Table 1).[2,3]

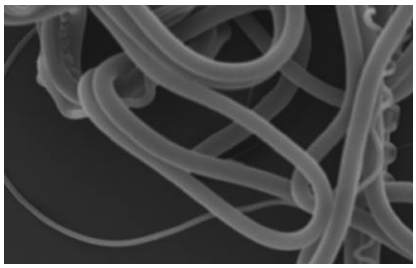

Figure 1. Scanning electron microscopy image of spider silk taken from a web of the garden spider *Araneus diadematus*.

The frame and radii of an orb web are constructed by the so-called dragline silk, the main constituents of which are typically two major ampullate spidroins (MAS). Among all types of silk, draglines have the greatest toughness, therefore providing shape and stability for the web and serving as the spider's lifeline. The capture spiral, which is designed for dissipating the kinetic energy of impacting prey, is built of a single flagelliform silk protein.[3] Because flagelliform silk itself is not sticky, the capture spiral of ecribellate spiders receives an additional adhesive coating secreted by the aggregate silk gland to tether the captured prey to the net.[3–5] When constructing a web, an orb-weaving spider first uses silk proteins produced in the minor ampullate gland (minor ampullate spidroins, MIS) to form an auxiliary spiral that serves as a scaffold for the emerging web and as a template for the capture spiral.[6] To interconnect the different silk types and to attach the web to the environment, spiders use "attachment cement", silk proteins originating from the piriform gland.[7] Other silks are used to protect the offspring; the silken egg case is built from two different types of silk. Silks from the tubulliform (cylindrical) gland form a tough shell that provides structure and stability to the egg case, protecting a spider's offspring from mechanical injury. Aciniform silk is used as a soft inner egg case layer, thus providing additional protection, or to wrap captured prey.[3,8]

In the 1950s, spider silk, and in particular dragline silk, entered the focus of material sciences owing to its outstanding mechanical properties, which outperform most other natural and man-made fibers.[9] Available data are summarized in Table 2 for *Araneus diadematus* dragline silk in comparison to other fibrous materials, and to steel and copper.

Dragline silk is five times tougher than steel by weight and even three times tougher than man-made synthetic fibers, such as Kevlar 49.[10–12] Apart from its classical mechanical properties, dragline silk has the ability to undergo supercontraction. When a native dragline thread comes in contact with water, or a relative humidity greater than 60%, the thread starts to swell radially, leading to an increase in diameter and a shrinking in length of about 50%.[13–15] In nature, this characteristic property allows reorientation of hydrogen bonds between the spider silk protein molecules

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Table 1: The seven different silks produced by the female orb-weaving spider *Araneus diadematus*.

Silk	Origin	Mechanical data	Sequence data
structural and dragline silk	major ampullate silk gland	strength: 1.1 GPa [3] extensibility: 27% [3] toughness: 180 MJ m^{-3} [3]	partial sequence data for *Araneus diadematus* and *Nephila clavipes*, complete sequence data for *Latrodectus hesperus*[22]
auxiliary spiral thread	minor ampullate silk gland	n/a	partial sequence data from *Nephila clavipes*
capture spiral (flagelliform) thread	flagelliform silk gland	extensibility: 300% [3] toughness: 150 MJ m^{-3} [3]	partial sequence data from *Nephila clavipes*
tough outer egg case	tubulliform and cylindrical silk gland	n/a	partial sequence data from *Latrodectus hesperus*
soft inner egg case layer and wrapping	aciniform silk gland	strength: ca. 0.7 GPa[a] extensibility: 86%[a] toughness: 250 MJ m^{-3} [a]	partial sequence data from *Araneus diadematus* and *Argiope trifasciata*
attachment cement sticky aqueous coating	piriform silk gland aggregate silk gland	n/a n/a	n/a composition of low-molecular-mass compounds from araneoid spiders,[5] isolation of two cDNAs for *Latrodectus hesperus*[100]

[a] Data is from *Argiope trifasciata*.

Table 2: Comparison of mechanical properties of *Araneus diadematus* dragline silk and other well-known natural and synthetic fibers.[a]

Material	Density [g cm^{-3}]	Strength [GPa]	Stiffness [GPa]	Extensibility [%]	Toughness [MJ m^{-3}]	Specific Properties
Araneus diadematus silk (dragline)	1.3	1.1	10	27	180	torsional shape memory without external stimulus,[20] reversible supercontraction (to 50% of original length)
Bombyx mori silk (cocoon)	1.3	0.6	7	18	70	availability (silkworm farming)
elastin	1.3	0.002	0.001	15	2	shape memory when poked or pinched
nylon 6.6	1.1	0.95	5	18	80	high resistance to heat and friction[102]
kevlar 49	1.4	3.6	130	2.7	50	high strength-to-weight ratio[103]
steel	7.8	1.5	200	0.8	6	versatility (alloying, tempering, swaging)
copper (soft)	8.9	0.2	120	40	–	exceptional electrical conductivity
wool (at 100% RH)[b]	1.3	0.2	0.5	5	60	circa 40% water uptake before wet to touch, high ignition temperature
carbon fiber	1.8	4	300	1.3	25	high strength-to-weight ratio

[a] If not otherwise mentioned, the data shown are taken from Ref. [101]. [b] RH: relative humidity.

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during the uptake of water,[3,15–17] thereby plasticizing the thread and changing its mechanical properties.[18] By this process, "worn-out" silk threads within a spider's net are renewed in the morning dew, and the web regains its rigidity.[1,17,19] Interestingly, supercontraction of spider silk takes place at ambient temperatures, whereas induction of the same process in man-made fibers generally requires elevated temperatures or harsh solvent conditions (e.g. hexafluoroisopropanol, or other alcohols).[18] Furthermore, spider silk also has a torsional shape memory, which allows the spider dragline thread, after being twisted, to oscillate only slightly, and by this means to totally recover its initial form.[20,21] This unique property allows spiders to rapidly descend using dragline silk as a lifeline in case of danger.

The intriguing characteristics of spider silk have attracted the interest of scientists to investigate the molecular building blocks of spider silk (mainly the proteins), the self-assembly properties of the spider silk proteins, and the fiber spinning process, all with the aim of employing spider silk for technological applications.

In this Review, we will shed light on the very complex processes involved in going from genetic information to a solid silk thread. In each section, in-vivo processes are compared to in-vitro findings, thus providing a basis for the production of artificial spider silk fibers for technical applications in the near future.

2. Silk Production: From Gene to Protein

2.1. Protein Secretion from Spider Glands

Spider silk proteins are encoded by a diverse set of genes, almost all of which belong to a single gene family.[22–24] Members of this gene superfamily have many similar molecular characteristics, such as a highly repetitive core sequence composed of tandemly arrayed consensus motifs flanked by two nonrepetitive terminal regions. However, the organization of the respective loci can differ markedly, as seen in the sequences of different spidroin types. Whereas the completely sequenced major ampullate spidroins 1 and 2 (MaSp1 and MaSp2) of black widow spider (*Latrodectus hesperus*) dragline silk are each encoded by single exons comprising 9390 and 11340 bp, respectively,[22] the genetic information of the flagelliform spider silk protein of the golden orb weaver *Nephila clavipes*, which is not completely but substantially sequenced, is estimated to split over 13 exons divided by highly conserved introns.[25]

Transcription of certain spider silk genes may lead to different versions of the same spidroin, called isoforms, as alternative start codons exist in the 5′ region.[26] Furthermore, for spider silk genes displaying an intron–exon structure, premature mRNA, which still contains the transcribed introns, has to be processed before translation. Translation of the genetic information into the amino acid sequence of spider silk proteins takes place within tall columnar endothelial cells lying in the uppermost part of a spider's silk gland in an elongated, convoluted diverging region.[27] These cells harbor an extensive endoplasmic reticulum (ER) and a large number of secretory vesicles.[28,29] In the case of dragline silk, the expression of the respective genes within the epithelial cells of the major ampullate gland is followed by the secretion of the major ampullate spidroins MaSp1 and MaSp2. These spider silk proteins generally have a highly repetitive core sequence consisting of iterated tandem repeats of certain consensus motifs. Alanine-rich stretches (A_n or $(GA)_n$; A alanine, G glycine), GPGXX (P proline, X often representing glutamine), and GGX (X represents alanine, leucine, glutamine, or tyrosine) are the consensus motifs of the core region of major ampullate silk proteins,[10] which have been highly conserved between the major ampullate spidroins (MAS) of different orb-weaving spiders for the last 125 million years.[30] Owing to the extensive repetition of relatively short consensus motifs, spider silk proteins contain an unusually high content of the five amino acids glycine, glutamine, alanine, proline, and serine relative to many other proteins (Figure 2a). The core region is flanked by non-repetitive carboxy-[31,32] and amino-terminal[26] sequences, which are also conserved. Molecular weights of dragline silk proteins are estimated to range from 250–350 kDa.[10,33] The strong conservation of the consensus motifs and, to a lesser degree, the termini, and the unusually high content of non-polar and of polar amino acids, coupled with a very low content of charged acidic and basic amino acids (Figure 2b), leads to the conclusion that the primary structure of the silk is extremely important for both the fiber assembly process and the characteristic features of the solid silk fiber. The extremely low content of charged amino acids and the extremely high abundance of glutamine differentiates spider silk proteins further from other extracellular and structural proteins, such as collagen.

The secondary structure of secreted MAS reflects that of natively unfolded proteins, mainly consisting of random-coil and polyproline-II helix-like structures.[34] The extended polyproline-II helix-like regions in particular are thought to maintain the solubility of MAS in the spinning dope (feedstock solution) with protein concentrations of up to 50% w/v by preventing the formation of intramolecular hydrogen bonds, favoring instead hydrogen bonding between side chains and the solvent.[35] Interestingly, apart from maintaining solubility, the polyproline-II helices in MAS can be readily

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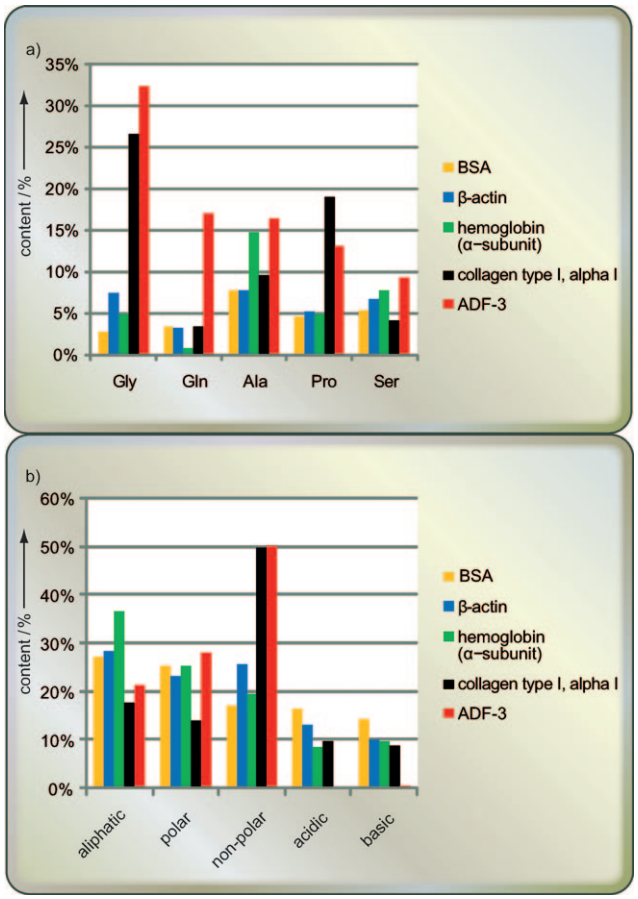

Figure 2. a) Content of the five most abundant amino acids glycine (Gly), glutamine (Gln), alanine (Ala), proline (Pro), and serine (Ser) in the known fragment of major ampullate spidroin 3 (ADF-3) of *Araneus diadematus* compared to the intracellular protein β-actin, the extracellular globular proteins bovine serum albumin (BSA) and the hemoglobin α subunit, and the fibrous extracellular protein collagen type I (α1 subunit). b) Comparison of these five proteins with respect to total amino acid composition, grouped by their chemical characteristics, based on published sequences: U47855 (ADF-3), NP 001092 (β-actin), NP 851335 (BSA), P69905 (hemoglobin α subunit), and NM 000088 (collagen type I, α1).

transformed thermodynamically into a β-sheet structure owing to their characteristic dihedral angles. This transformation is important during the spinning process discussed below.[34]

After secretion, MAS apparently form droplet-like structures made of tightly hexacolumnar-packed spider silk protein molecules in the glandular ampulla.[11,27] The highly concentrated and dissolved spider silk protein undergoes further rather complex processes, finally yielding a solid silk fiber.

2.2. Biotechnological Spider Silk Production

The ability to produce spider silk proteins in sufficient amounts and in a cost-effective way is essential for the application of spider silks as high-performance materials. As the farming of spiders is hampered by their territorial and cannibalistic behavior,[36] biotechnological production of spider silk proteins is a promising alternative. Therefore, scientists have put remarkable efforts into developing various cloning and production strategies. The main obstacle to a successful biotechnological production of spider silk proteins was a limitation of the polymerase chain reaction (PCR), which is unable to reliably amplify repetitive sequences as found in spider silk genes. The repetitive structure of silk genes is also a challenge to finding a suitable expression host. Therefore, modern biotechnology is needed to specifically design spider-silk-like genes and distinct host organisms for protein production.[37] A host must provide the genetic stability of the transgenic sequence, and its translational machinery must cope with repetitive mRNAs, which often tend to form large secondary structures. Furthermore, upon induction, tRNA and amino acid stocks are often rapidly depleted owing to the disproportionately high incorporation of the five amino acids glycine, glutamine, alanine, proline, and serine (Figure 2a). To overcome the mentioned hurdles, spider silk proteins have been produced in genetically modified bacteria,[38–42] yeasts,[40,43] plants,[44–46] insect[47] and mammalian cells,[48] and also in transgenic animals.[49] Each host offers certain advantages, but also presents certain obstacles (Table 3).

Several approaches have been employed regarding gene design.[10,38,40,46,48,50–52] Our group, among others, has developed a cloning system that allows the creation of artificial spider silk genes by seamlessly joining solid-phase synthesized oligonucleotides.[53] This method not only enables the mimicking of the modular arrangement of spider silk consensus motifs, but also allows the codon usage to be adjusted according to the needs of the designated expression host. Using this system, we were able to recombinantly produce a variety of spider-silk-like proteins that are based on sequences of dragline silk proteins of *Araneus diadematus* and of flagelliform silk proteins of *Nephila clavipes* in bacteria and in insect cells.[47,53]

3. Silk Protein Assembly: Conformational Changes and Phase Separation

Spider dragline silk proteins are stored in the ampulla of the major ampullate gland until they are processed into fibers. During the natural spinning process, the proteins move distally through the gland (Figure 3), where they encounter changes in their biochemical environment and elongational and shear forces.

The biochemical and physical changes are accompanied by a liquid–liquid phase separation followed by a liquid–solid phase transition that results in a preliminary silk fiber. The final structure of the fiber is reached after a drawdown process in the last limb of the duct and evaporation of some of the solvent water in air.

The assembly pathways of natural spider silk proteins have been explained by two different theories, which were obtained from in-vivo (Section 3.1) and in-vitro results (Section 3.2) that concern the molecular orientation during storage, phase separation process, and conformational changes of the proteins (Figure 4).

Table 3: Summary of organisms used for recombinant production of spider silk proteins.

Expression Host	Silk	Spider[a]	Advantages	Disadvantages
bacteria:				
Escherichia coli (B and K12 derivatives)[38–42,53]	various engineered spider silk proteins	*N.c.* *A.d.*	easy to handle expression system; easy to manipulate; rapid growth; easy to upscale; cost-efficient fermentation	nucleotide sequences must be adapted to prokaryotic codon usage; poor production of larger spidroins; genetic instability of repetitive nucleotide sequences (deletions, insertions); premature translation termination→product inhomogenity
yeast:				
Pichia pastoris[40,43]	engineered MAS	n.m.	easy to upscale; cost-efficient fermentation; production of larger silk proteins possible in eukaryotes; no premature translation termination; post-translational modifications possible; secreted production possible, enabling higher protein yields	multiple gene insertions may occur→product inhomogeneity; expression efficiency decreases with increasing gene size
plants:				
Arabidopsis thaliana[44] *Solanum tuberosum* (potato) [45,46] *Nicotiana* (tobacco)[45,46]	MAS and derived proteins	*N.c.*	only 10–50% of the cost of bacterial fermentation; easy to upscale; stable production of larger spidroins; post-translational modifications possible	genetic manipulation more complicated than for bacteria; longer generation intervals; large-scale field cultivation may raise legal issues
insect cells:				
Bombyx mori cells[51] *Spodoptera frugiperda* cells (sf9, sf21)[47]	flagelliform silk, MAS (originating from cDNA) and mutated fragments thereof	*N.c.* *A.d.*	among all used expression systems, insects are phylogenetically closest related to spiders; production of larger silk proteins possible in eukaryotes; availability of convenient commercial cell-culture systems; no translational pausing→higher product homogeneity; secreted production possible, enabling higher protein yields; post-translational modifications possible; fermentable cell cultures→large-scale biomass production	time-consuming owing to longer generation intervals compared to bacteria and to more complicated cloning procedures; cytosolic production of certain spider silk proteins resulted in protein aggregation→subsequent renaturation reduces protein yields
animal cells:				
baby hamster kidney (BHK) cells[48] bovine mammary epithelial alveolar (MAC) cells[48]	MAS cDNA sequences and variations thereof	*N.c.* *A.d.*	production of larger silk proteins possible in eukaryotes; secreted production possible, enabling higher protein yields	fast depletion of tRNA pools owing to the unique amino acid composition of spider silk proteins; translational pausing resulting in heterogenous protein expression; time-consuming owing to longer generation intervals compared to bacteria and to more complicated cloning procedures
transgenic animals:				
BELE goats[b] *Mus musculus*[52]	subunits of silk molecules engineered MAS	n.m.	production of larger silk proteins possible in eukaryotes; post-translational modifications possible; protein is secreted to milk or urine, enabling high protein yields; constitutive production of silk proteins; production and secretion last for duration of lactation (milk) or lifetime (urine) of the transgenic animals	creation of transgenic mammals is very time-consuming; separation of spider silk proteins and milk caseins during purification is challenging; creation of transgenic animals may raise ethical and/or legal issues; mice produce only low amounts of milk, milking may be challenging

[a] *N.c.*: *Nephila clavipes* (golden orb weaver); *A.d.*: *Araneus diadematus* (garden cross spider); n.m. not mentioned in the cited publication(s). [b] The method is patented for mammals in general.[49]

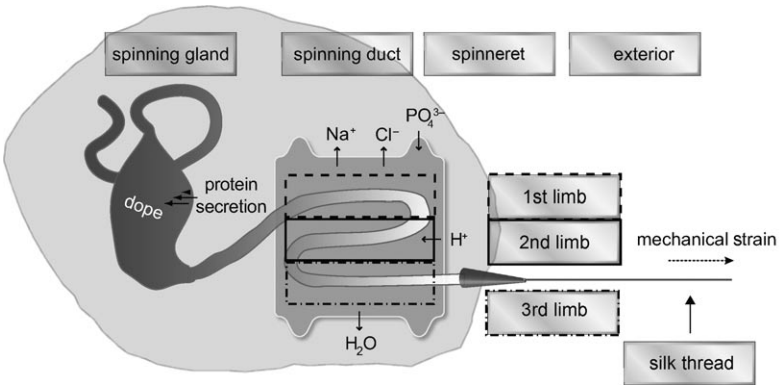

Figure 3. Spider silk processing. Major ampullate spidroins (dragline silk proteins) are secreted by epithelial cells lining the gland. The secreted protein is stored as highly concentrated spinning dope. Towards the spinneret, the silk proteins pass three limbs of the tapering spinning duct, accompanied by changes in their biochemical environment, extensional flow, and shear forces. The preliminary dragline silk fiber finally exits the gland through the spinneret and is finished by post-spin drawing and evaporation of some of the remaining solvent in air.

3.1. The Silk Assembly Process within a Spider's Spinning Duct

As determined from research by Vollrath and Knight, the freshly synthesized, rod-shaped spider silk proteins first adopt a nematic liquid-crystalline phase within the dope, with the long axis of the molecules oriented parallel to each other and perpendicular to the secreting epithelium.[11] Upon movement through the ampulla, the orientation of the long axes turns until they lie parallel to the epithelial walls. The spider silk proteins retain this nematic orientation until they enter the second limb (Figure 3) of the spinning duct, where they are organized in bilayered disks with their long axes perpendicularly arranged to the plane of the disk. This arrangement is commonly known as cellular optical texture,[54] and is achieved under relatively low stress forces. Accelerating elongational flow and shear forces in the third ductal limb act on the preorientation of the spider silk protein, leading to an elongation and alignment of the disk-like structures (Figure 4).[27] In this step, the conformational transition of the silk proteins from random-coil and polyproline-II helix-like conformations to mainly β-sheet-rich structures is promoted.[11]

The conformational change is further supported by a slight acidification of the spinning dope.[27,55–58] Acidification of silk proteins results in neutralization of glutamate residues, which are typically negatively charged under physiological conditions, thereby promoting hydrophobic interactions. As a consequence, the spinning dope undergoes gelation in the distal part of the duct, resulting in an increased viscosity, which in combination with rapid extensional flow supports the internal drawdown process.[56,57,59]

Finally, in the third limb, epithelial cells with apical microvilli provide a large surface area for resorbing water, which is additionally facilitated by the thin cuticle lining the duct in this region.[11,58,60] Assuming that the convective removal of water by the epithelial lining is fast, the process fits well to a numerical model proposing that further water removal is solely governed by internal diffusion. Diffusion of residual water, which is dependent on its diffusion coefficient, out of the silk assembly is the rate-limiting step.[61] Slow diffusion of water leads to increased fiber plasticity, as intra- and intermolecular hydrogen bonds have more time to reorient. Shortly before the fiber exits the spider's abdomen, the lips of the spigot, which fit tightly around the silk fiber as it forms, remove most of the remaining residual water.[11]

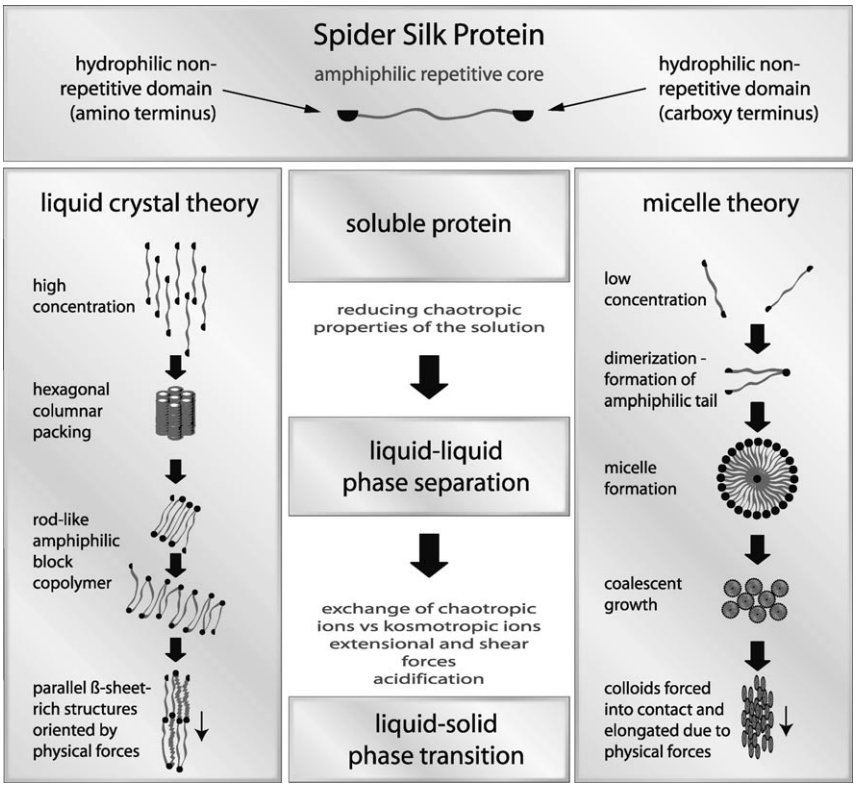

Figure 4. Two established models that describe spider silk thread formation.[11,62]

3.2. Analyzing Silk Assembly In Vitro

In general, two different approaches have been employed to investigate silk assembly in vitro: 1) dissolving native silk fibers in harsh solvents (e.g. highly concentrated LiBr or LiSCN solutions, hexafluoroisopropanol, hexafluoroacetone hydrate) to obtain reconstituted/regenerated silk dope solutions; and 2) producing recombinant silk proteins based on sequences derived from the native sequence (see Section 2.2), which are then dissolved in aqueous solutions (native-like conditions).

Using regenerated *B. mori* fibroins, Kaplan and Jin attempted to clarify the process of silk self-assembly during natural spinning.[62] In contrast to the theory of Vollrath (Section 3.1), fracture surfaces of native silks often show

globular structures in their internal core region. Furthermore, elongated fibrillar structures have been found in coat regions of dragline silks.[63] *B. mori* fibroins and spider silk proteins usually show an amphiphilic sequence, implying short alternating hydrophilic and hydrophobic amino acid stretches flanked by larger hydrophilic terminal regions, which renders these molecules surfactant-like with the ability to form micelles.[3,10,53,64] In a protein concentration-driven process, it could be shown that these micelles coalesce to form larger globular structures. The forcefield created by elongational flow and ductal wall boundaries elongates the globular structures, shaping them into fibrillar morphologies. These fibrillar structures are thought to be the precursors of the subsequent spider silk fiber.[62,63]

Although the same aspects of silk protein preorientation have been highlighted in both in-vitro and in-vivo studies, several important effects influencing secondary, tertiary, and quaternary structures of the proteins have not been considered. Upon passage through the spinning duct, the proteins encounter remarkable changes in their solvent environment, leading to salting-out effects accompanied by structure formation. The changes include an increase in potassium and phosphate concentration, a decrease in sodium and chloride concentration, removal of water, and slight acidification.[27,55,58,65] The stability of proteins in aqueous solution in general is affected by its surrounding ions: according to studies by Hofmeister in the early 20th century, chaotropic ("salting-in") ions stabilize soluble proteins, whereas kosmotropic ("salting-out") ions promote structure formation and protein aggregation (Figure 5a).[66,67] To unravel Hofmeister effects on silk proteins, we determined the solubility of recombinant, engineered silk proteins based on sequences of MAS from *Araneus diadematus*. The solubility is determined by the hydrophobic/hydrophilic properties of the repetitive sequences in the individual protein: the more hydrophilic eADF3 protein is water soluble up to 30% w/v, whereas the more hydrophobic eADF4 protein gelates at concentrations of around 10% w/v.[53,68] These findings are consistent with those from other groups, who achieved solubility of recombinant silk proteins (MA spidroin 1 and 2 analogues) from *Nephila clavipes* in the range of 20% w/v in aqueous solution.[69] We observed that in the absence of chaotropic ions (for example, using deionized water) and at subcritical protein concentrations (the proteins are completely in solution), a liquid–liquid phase separation takes place, resulting in an increased protein concentration in a high density phase; that is, having large colloidal assemblies without detectable secondary structure (Figure 5b).[64]

In contrast, the presence of chaotropic salts, such as sodium chloride, as found in the spider's storage dope, inhibited aggregation and assembly of the silk proteins and even prevented liquid–liquid phase separation. Moreover, as soon as sodium chloride was exchanged with "salting-out" ions, structure formation began.[64] The "salting-out" effect depended not only on the ions employed, but also on the sequence of the repetitive core and on the flanking nonrepetitive (NR) domains, which amplify the response of the repetitive (rep) domain to factors promoting "salting-out".[53,68]

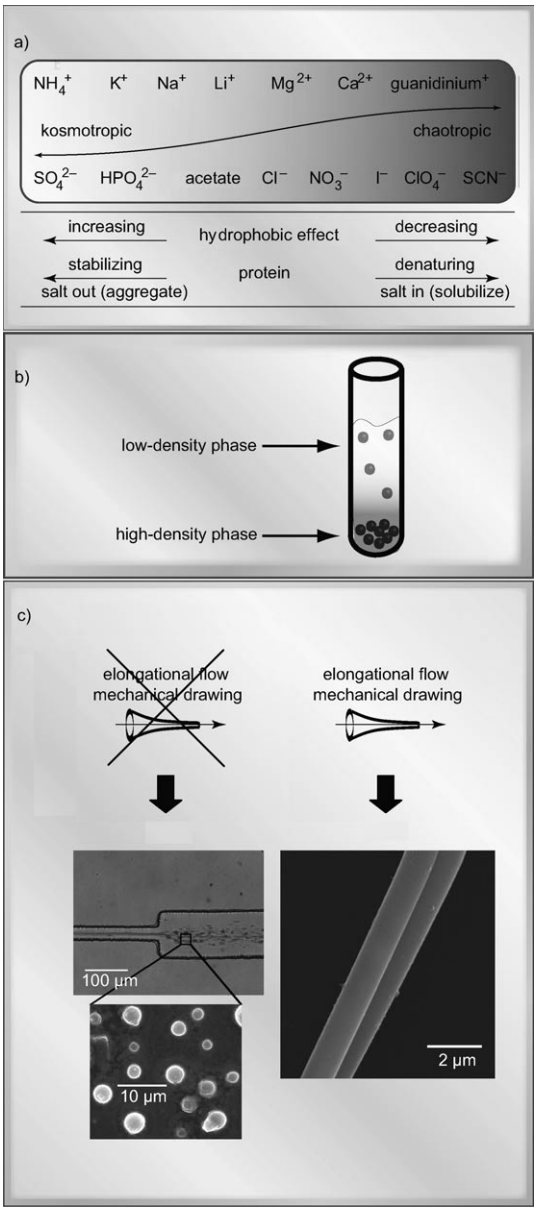

Figure 5. Prerequisites for in-vitro silk fiber assembly. a) Effect of salts on proteins (Hofmeister series);[66] b) liquid–liquid phase separation; c) importance of elongational flow and mechanical drawing on fiber formation. Without elongational flow or mechanical drawing, only spherical aggregates are formed (left).

Similar to conditions found in-vivo, in-vitro processing and assembly of silk proteins was influenced by the pH value and by physical stress.[27,65,70–72] At pH > 8.5, phase separation is inhibited owing to deprotonation of tyrosine residues. Anionic tyrosylates within the hydrophobic sequences of the proteins increase the hydrophilicity and thus reduce interchain hydrophobic interactions.[64] To more closely analyze the influence of acidification during silk assembly, a microfluidic device was employed in which the ion concentrations and pH value could be controlled, and simultaneously, physical stress could be applied by channel design.[65]

In the natural process, the linear velocity of the spinning dope during passage through the duct increases exponentially

before the drawdown taper, suggesting that wall shear may play a role in the transition from liquid dope to solid silk; moreover, controlled flow elongation and water removal provide an increase in β-sheet structures.[57,73,74] In the absence of salting-out conditions and acidification, elongational flow did not affect the structural state of the employed silk proteins, whereas salting-out in the absence of elongational flow led to the formation of spherical aggregates. However, in the microfluidic device, silk fibers formed only after addition of phosphate, application of a simultaneous elongational flow, and a pH change from pH 8 to pH 6.[65] It could be shown that fibers resulted from preformed spherical aggregates that were forced into contact by the elongational flow in the microfluidic channel.[65] The resulting fibers were highly flexible, having structurally highly-ordered regions (mainly β-sheet-rich) along the thread. The surface of the fibers obtained grainy structures, leading to the assumption that the resulting fibers are still not mature, and in fact most likely represented an early or intermediate stage of fiber formation.[65]

3.3. A Combined Model for Spider Silk Assembly

The two models shown in Figure 4 are not mutually exclusive. The characteristics of the spinning dope as depicted by these models reflect the physics of liquid crystals, implying that the micelle formation observed by Jin and Kaplan does not exclude liquid crystallinity. Lyotropic liquid crystals (i.e., liquid crystals that are able to undergo phase transitions dependent on the concentration of its main component) with amphiphilic character show concentration-dependent self-assembly behavior in solution: at low concentrations, they spontaneously assemble into micelles, whereas at higher concentrations, they are ordered into hexagonal columns.[75] We propose that this might explain why in-vivo investigations usually lead to the conclusion that the spinning dope displays liquid-crystalline behavior, whereas in-vitro studies (using either reconstituted or recombinant silks) give rise to a micellar-like preoriented spinning dope. It should be noted that native and reconstituted silk dope differ significantly in their rheological characteristics: native silk dope behaves like a molten polymer, whereas reconstituted silk dope does not.[76] Importantly, a higher protein concentration will lead to dramatically increased viscosities, enabling fiber formation at much lower elongational flow rates.[65] These findings indicate that liquid crystalline behavior of the spinning dope could be beneficial, but it is definitely not necessary for fiber assembly.

4. Fiber Formation: Liquid–Solid Phase Transition

4.1. Phase Transition in the Distal Part of the Spinning Duct

The final step of the spinning process is the transition from a high-density liquid to a solid phase that starts in the distal part of the duct.[11,58,59,74] As the spinning dope flows through the spinning duct, a liquid–solid phase transition is initiated by water removal in a rapid convective process, as described above,[11,61] which is contrary to the previous postulation that solidification occurs solely upon contact with air.[11,27,77–79] A semi-solid intermediate or premature fiber is moved through the duct by a pumping mechanism involving the cooperative work of two muscles, and finally exits through a spigot (often referred to as a valve).[27]

Mechanistic details of the process of moving a semi-solid spinning dope through a convergent die-like spigot could be explained by carrying out rheological studies.[80] It was shown that the force required to push the silk dope through the spigot is 500 times lower than that associated with corresponding viscous Newtonian fluids, owing to the non-Newtonian fluid behavior of the silk dope.[56,81] Viscous non-Newtonian fluids usually show shear-thinning behavior; that is, with increasing shear force, the viscosity (i.e., the resistance of the fluid to shear forces) of the fluid decreases.

Moreover, the silk dope displays increasing resistance to stretching with time and strain imposed during elongational flow, leading to a viscoelastic fluid filament,[80,82] which is not contradictory to the shear-thinning behavior. The thinning of the viscoelastic fluid filament (often referred to as "necking") driven by capillary pressure and resisted by the viscoelastic stress in the elongating filament, can be best described with the time-evolutionary necking model.[80,83] In this model, the thinning/drying process of a viscoelastic filament is based on the ratio of capillary thinning of the filament and the internal diffusion of water over time. The resulting necking rate can be further modulated by accessory evaporation of solvent from the thread, as the evaporation rate increases with time owing to the increasing ratio of surface area to volume. Thus, further loss of water leads to an increase in fluid viscosity and an additional slowdown in the necking rate.[83] The resistance of a fluid filament to further stretching is characterized by its extensional viscosity properties, which increase a hundred fold during capillary thinning. At large strains, the filament undergoes strain hardening, which inhibits capillary breakup and finally stabilizes the filament owing to the combined action of molecular elongation and solvent evaporation. Ultimately, a solid, uniform fiber is formed with constant diameter.[80]

4.2. Final Fiber Formation and Control over Mechanical Properties

The liquid–solid transition is initiated by environmental conditions, such as partial water removal, elongational flow, and shear forces (see Section 3.1). The liquid–solid transition is completed after exiting the spigot, and is caused by the combination of drawing and loss of water arising from evaporation in air. Factors influencing the evaporation process include the fiber radius, time of exposure to air, atmospheric humidity and temperature, and the speed of air flow.[84] However, solvent evaporation is not essential for fiber formation, as some natural silks are successfully spun in aqueous environments.[11,27,77,78] The drawing and/or stretching of the fiber from the spigot by the spider leads to a reduction in its diameter (supported by the fact that silks tend to display a moderate, positive Poisson ratio, with a linear relationship between diameter and extension) resulting in improved mechanical properties of the fiber.[85]

The freshly drawn dragline silk fiber, which is usually a two filament fiber known as a bave, is in most cases fixed to a substrate using a silk from the spider's piriform gland prior to further drawing by moving or descending using the spider's body weight and/or force of gravity. Alternatively, dragline silks are drawn out by a spider with its hind legs.[3,10,11,62,86,87]

These three different methods that are actively applied by a spider give rise to the broad range and variability of a dragline fiber's mechanical properties: 1) the vertical descent method, in which a spider exerts friction forces up to more than twice its body weight, results in strong fibers;[87] 2) a spider in free fall spins silk at low forces of approximately 10% of its body weight without applying any additional frictional force;[86,87] 3) fibers spun during undisturbed climbing of a spider represent the lowest limit in stress–strain curves.[15,87]

The effects of drawing speed on mechanical properties show a strong linear relationship, indicating that protein folding and molecular interactions between individual proteins are strongly affected by this process. The rate of drawing affects the time required for protein alignment, with higher draw rates reducing this time because of increased shear forces and forces of elongational flow.[85]

Apart from the active controls employed by a spider, silk fiber properties are affected by environmental influences, such as diet, temperature, and humidity, and body weight.[3,74,79,85,88–91] The resulting variability allows the production of a tailored material ideally suited for either an inhabited environment or immediate needs.[92]

4.3. In-Vitro Silk Spinning

Several studies have investigated artificial spinning of spider silk, but so far no process has resulted in silk fibers that perfectly mimic the mechanical properties of natural silks. Most of the techniques that have been applied to form fibers from a silk solution have been based on solvent extrusion, wet-spinning through a coagulation bath, electrospinning, and microfluidic approaches, sometimes using organic solvents.[10,93]

The natural spinning process is a complex combination of an extrusion and drawing process.[27,58] Such a combination distinguishes the natural spinning process from any known method of producing synthetic polymer fibers, and makes the task of mimicking the natural spinning process very challenging. In contrast to a technical spinning procedure, in which physical transformation, spinning, and drawing are sequential, the process in a spider is rapid and concerted.[11,48,73,94] Microfluidic devices are a promising tool to further investigate and thus understand the sequences and kinetics of silk assembly. However, the goal should be to integrate such findings into the development of a biomimetic spinning process, which is currently under investigation by several independent research groups. One example is given in Figure 6.[65,95,96]

As several factors, such as the internal water removal process, spin-dope fluid behavior, and environmental influences affect drawing of the silk thread and thereby its properties, all have to be considered when mimicking the natural spinning process. Moreover, it might be necessary to apply additional post-spinning procedures adapted from common technical spinning processes to yield high-performance silk fibers.

Post-spin drawing can influence the mechanical properties of silk fibers in a similar manner as the drawing rate in nature (see Section 4.2). Post-spin drawing leads to longer and thinner fibers as a consequence of a constant volume, resulting in improved mechanical properties. ^{13}C NMR spectroscopy studies indicate a linear increase in the fraction of alanine residues in the β-sheet conformation (otherwise present as random coil or helical structures) with the draw ratio (the ratio of drawn fiber length to original fiber length).[97]

As a spider web maintains its flexibility in nature by rehydration, giving rise to supercontraction, it may prove

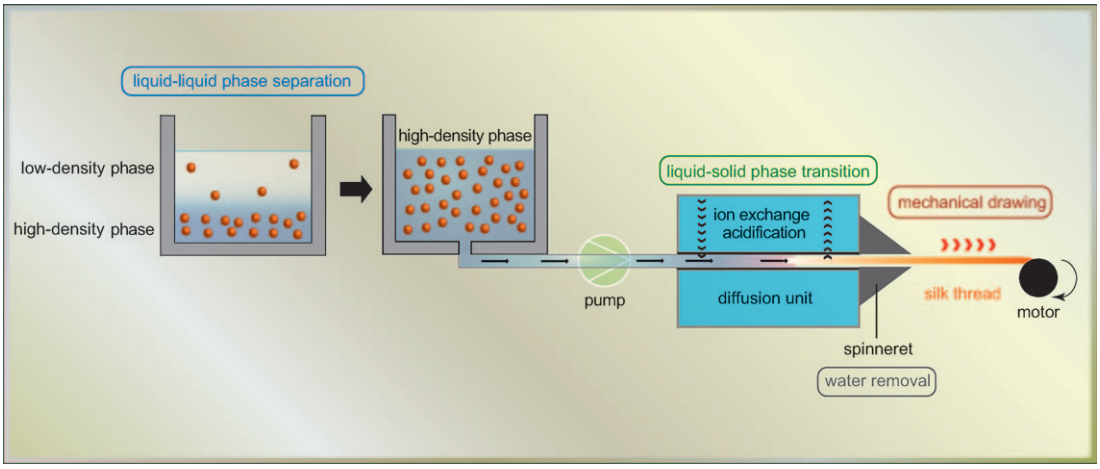

Figure 6. The biomimetic spinning process. Liquid–liquid phase separation results in the formation of a high-density phase, which is separated from the low-density phase for further processing. The high-density phase is pumped through a diffusion unit in which ion exchange and acidification lead to a liquid–solid phase transition. The semisolid fiber is drawn out at constant speed from the spinneret, in which the remaining residual water is removed, resulting in a solid silk fiber.

possible to use environmental conditions, namely humidity, to control the properties of technically spun fibers. Post-spin drawing of an artificially produced fiber through an aqueous bath results in stronger (manifested as an increase of yield stress, breaking stress, and initial modulus) but less extensible fibers (shown by the decrease of breaking strain, although the strain energy itself increased).[79] This improvement of the fiber's mechanical properties is related to the fact that immersing the silk fiber in water in combination with simultaneous drawing gives rise to improved orientation and thus improved mechanical properties.

Important aspects in mimicking the natural spinning process are the design of the artificial spinning duct and the post-spin processing. Moreover, it is known that the artificial duct geometry influences the elongational flow characteristics: 1) the slow decrease in diameter prevents premature crystallization owing to slow elongational flow rates; and 2) the hyperbolic geometry of the spinning duct accounts for constant elongational flow, reducing disorientation.[59]

5. Summary and Outlook

There is a great deal of knowledge about how certain factors, such as protein composition, biochemical environment, elongational flow, and shear forces, influence distinct processes during silk spinning. However, the control, the sequence, and complex interplay of these processes are still poorly understood. The combination of an extrusion and drawing process and numerous modification opportunities, such as drawing speed during postspinning, is one of the biggest challenges for today's research in the field of silk biomimetics.

Four prerequisites appear to be crucial to successfully mimicking spider silks:

1) gene design for efficient and accurate recombinant protein production and protein structure formation, including control of size and amount of β-crystals, which are essential for fiber strength, and the liquid crystal orientation, which affects the flow properties[57,59,98,99]
2) optimal chemical and physical conditions during silk protein processing following a distinct order of events without premature protein aggregation[57,65]
3) "water management" during the spinning process
4) controlled external parameters such as drawing speed and wetting

Although progress has been made in protein design and protein production and in understanding certain biochemical parameters, additional efforts are necessary to optimize the liquid–liquid phase separation behavior of the proteins involved. Moreover, the important point of water management has been neglected in in-vitro studies to date, although water removal plays a crucial role in the natural spinning process.

Once a biomimetic spinning process is established, producing engineered silk fibers will allow multiple technical applications. One day, biomimetic silk fibers can be envisioned as a substitute for many natural and man-made fibers in materials and medical sciences.

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