The possible role of liquid-liquid phase separation as an intermediate step in spider silk assembly

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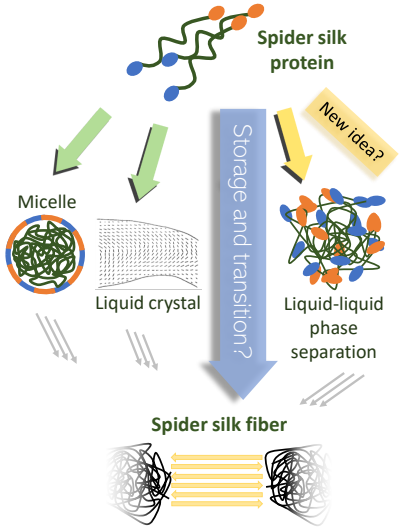
Spider silk exhibits exceptional properties that could bring great technological advances. Its high tensile strength, breaking strain and toughness can make it even stronger than Kevlar. Understanding the natural spinning process would allow to produce strong, highly engineered protein fibers but this has been facing challenges. Large efforts have resulted in an understanding of silk proteins and their responses to chemical and mechanical stimuli, as well as how the natural spider spinning gland produces these stimuli. However spinning is still hard to replicate due to an incomplete picture of the microscopic processes occurring to allow rapid fiber formation from a concentrated solution. Recently a new suggestion has emerged that spider silk proteins might phase separate into liquid condensates and this could help to bridge a gap in understanding. At first glance this idea seems to contradict long-standing theories involving micelles or liquid crystals for the storage of silk proteins and the transition to fibers. This review will consider the novel observations and try to relate them to the current views. Then it will be examined where the ideas meet or discrepancies can come from. It is concluded that the theory of liquid crystals and liquid-liquid phase separation do not mutually exclude each other. The distinction between the origin of micelles or coacervates is less clear but the assembly seems to depend on the structure of the N- and C- terminal domains and the amino acid sequence and length of the repetitive domain. This work is not able to present a definite theory to reconcile all ideas and neither shows a comprehensive overview of where differences arise, but it can be used as an outset to more discussion and research on the microscopic nature of spider silk.



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Introduction

Silks are natural materials with a range of interesting properties. Silk fibroin can be used to construct biodegradable materials such as films, gels and sponges that can be applied for tissue regeneration, drug delivery, optics and electronics.[1–4] The most prominent are its mechanical properties, showing high tensile strength, breaking strain and toughness.[5] Silkworm and spider silk can even outperform Kevlar or steel while still showing large elongation.[6] It has been a quest to replicate biological silk production to use it for widespread application.

The use of proteins in materials science is attractive for their high degree of tunability in primary, secondary and tertiary structure, the ease of fabricating amino acid sequences and

the fact that there are a plethora of examples from nature. In case of spider silk production large efforts have been put into understanding the remarkable characteristics of spider glands and the properties of spider silk proteins, called spidroins, for decades. This concerns the physical and chemical characterisation of the spider spinning gland such as shear forces, pH and ion content and exchange but also the response of spidroins or their separate domains on these triggers. That being said, questions arise on the sub-microscopic events taking place allowing rapid spinning of fiber. While much is known, still difficulties arise when fabricating spider silk. Rising and Johansson reviewed the efforts towards artificial silk in 2015 and concluded that no silks have accomplished comparable properties to those obtained from spiders.[7] This is partly due to a lack of understanding of the exact mechanisms that come into play at the nano- and micrometer scale when transferring from a liquid to solid fibers in a short time scale, and in the difficulty to translate all knowledge into biomimetic devices. Recently novel insights have been published on the microscopic behaviour of spidroins and spider silk like proteins showing that spidroins might undergo liquid-liquid phase separation that could shed light on the assembly mechanism.[8–10]

A frequent mechanism for the production of biomolecular structures is liquid-liquid phase separation, also known as coacervation. This can yield droplets of RNA, proteins and other biomolecules, serving to compartmentalize, concentrate or sequester relevant molecules.[11] Coacervates are liquid droplets composed of a high concentration of macromolecules that separate from a diluted solvent phase. Simple coacervation refers to a system containing a single component while complex coacervates involves different types of macromolecules, commonly oppositely charged polyelectrolytes.[12,13] The term coacervate is not unambiguous, partly because confusion can arise when distinguishing it from solid-like polyelectrolyte complexes or when synonyms are used in different fields such as aggregates, nanoparticles or condensates.[11,12]

Coacervation has been observed in other organisms serving as adhesive or structural intermediate. This include the ability of a velvet worm to form fibers from an adhesive slime,[14,15] underwater adhesives from mussels to attach to rocks[16,17] and the production of squid beak.[18] In the context of cellular function, a liquid-to-solid transition of coacervates can be associated with pathological consequences.[19,20] The liquid phase can irreversibly transition into solid structures, usually amyloid fibers, and cells may have designed methods to prevent this from happening.[11] This is contrary to spider silk formation, where spiders have evolved a complex system to be able to transition from a liquid to solid with spatial and temporal precision.[7]

Clearly liquid-liquid phase separation (LLPS) is widespread among biological systems and hence it could play a role in spider silk formation. Understanding whether or how this is the case brings us closer to decipher the processes in spiders, has implications for the concept of proteins that undergo LLPS in general and can help towards the production of strong, versatile, artificial silk. The question is how this compares to the current ideas on spidroin storage in the gland and formation of fibers. Two theories are proposed, which do not necessarily exclude each other, involving the organisation of spidroins into micelles or a liquid crystalline phase.[21,22] This review aims to relate the novel results on liquid-liquid phase separation of spidroins to the observations of micelles and liquid crystals. It will be discussed where differences or discrepancies can arise and what conceptual and experimental aspects can be considered in the future. Before that first the structure of spidroins and the natural spinning system will be introduced. Then the main part of the review will examine LLPS, micelles and liquid crystals of spider silk proteins.

Spidroins and natural processing

Spider silk proteins display a general triblock structure despite the fact that there exists different spider species each with multiple types of silks for different purposes.[7] Generally they are thought to behave comparably due to the similarities although the exact secondary and tertiary structure, assembly mechanisms and properties can vary.[23–25] A spidroin consists of a repetitive domain (RD) flanked by two non-repetitive regions, the N- and C-terminal domains, NTD and CTD, respectively (fig. 1A). The protein has a high M_w of 200-350 kDalton, of which >90% is accounted for by the RD.[26] This region is dominated by the amino acids glycine and alanine, which form into a glycine-rich region of a few repeats of GGX (X = A, Q, Y) connected to a polyalanine stretch of length 4-10 residues, and the combined tandem can repeat up to 100 times.[26] In its soluble state the alanine-rich regions exhibit a random coil and approximately a quarter of the glycine-rich part form polyproline type II helices, the latter being less flexible and thought to be a prefibrillar form to aid rapid fiber formation.[27]

The terminal domains play an essential role in the storage of spidroins in a liquid dope and to form fibers as a respond to chemical and mechanical stimuli. Before listing those, first an overview of the natural spinning system that produces these stimuli. The operation of spidroin production and their conversion to fibers takes place in the spider gland, with separate glands for each type of silk.[7] A schematic of the major ampullate gland, where dragline silk is produced, is shown in fig. 1B. Three regions have been identified with distinct epithelial cells, the first two are within the blue part (fig. 1B-i) and here spidroins are produced and the other is in the green.[28] The green also corresponds to the region where carbonic anhydrase is found which regulates a pH gradient within the gland (fig. 1B-ii).[29] Another important chemical component is the ionic composition in the sac and along the duct, the three-limbed tail to the right of the sac. Notably, in the sac the level of Na^+ and Cl^+ is high and decreases through the duct while K^+ and PO_4^{3-} increase.[30] Mechanical shearing was also noted to be present in the duct.[31]

A striking feature of spidroins is the high concentration in

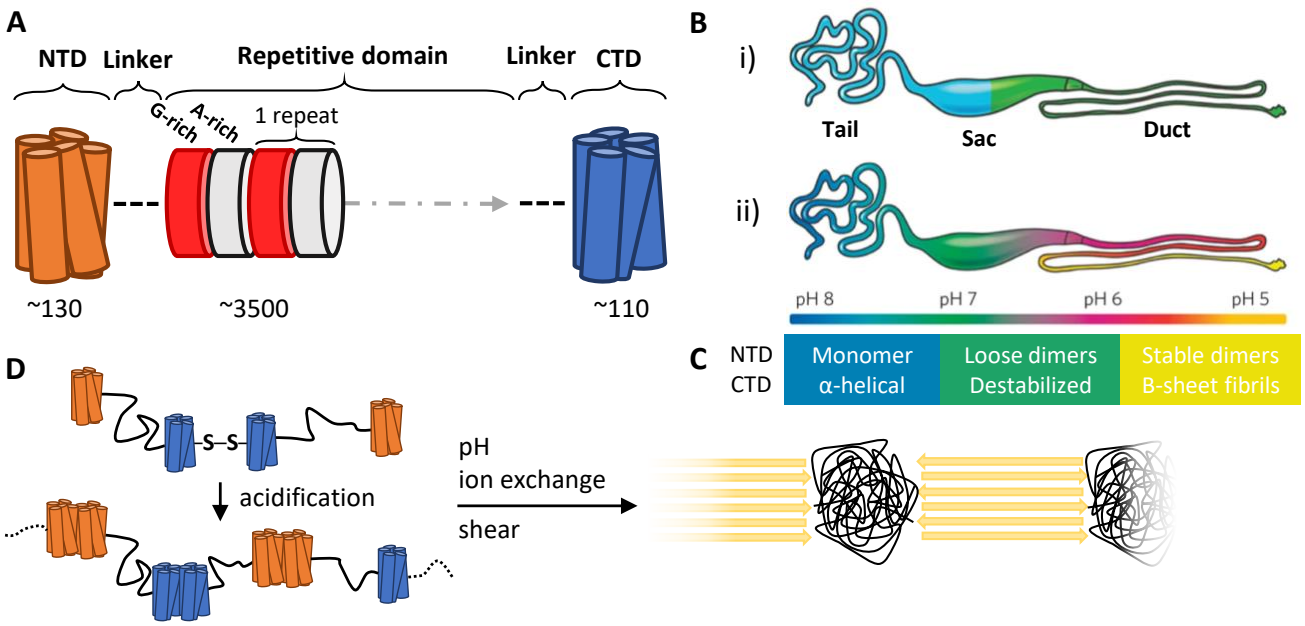

Figure 1. General architecture of spidroins and the organisation of the silk gland. A – schematic of a spider silk protein that consists of a highly repetitive domain containing alanine and glycine rich regions, flanked by two non-repetitive N- and C-terminal domains, NTD and CTD, respectively. B – structure of a silk gland, the blue in i) showing the tail and part of the sac where spidroins are produced and the green the sac and duct where carbonic anhydrase is found which produces a pH gradient through the gland, shown in ii). C – overview of the structure of the terminal domains in a range of pH along the same colour code as in B. D – naturally CTD dimerizes by forming disulfide bonds through their only cysteine residue. Lowering of the pH also causes NTD to form dimers. Chemical or mechanical stimuli such as pH, ion content and shear causes the repetitive domains to align and CTD to unfold forming β-sheets rich in alanine (yellow) embedded in amorphous regions (black). Images B-C taken from [7].

which it is present in the gland, sometimes up to 50%.[32] Two main hypotheses have been proposed. The micellar model suggests that the terminal domains form the surface of a micelle while the repetitive domains sit in the interior.[21] On the other hand, cholesteric and nematic liquid crystalline phases have been identified in the gland and duct.[33,34] A more comprehensive view on these phases will be discussed in further sections.

Now a brief introduction to the terminal domains, which are highly conserved through evolution,[35] and that is why it is thought that they play a large role in the spidroin's properties, storage at high concentration and fast, on demand silk assembly. The NTD and CTD consist of five parallel-oriented helices and their sizes are approximately 130 and 110 residues, respectively.[36–38] The domains of dragline silk do not interact and play their individual role on storage and assembly.[39]

The N-terminal domain of dragline silk has been shown to form antiparallel dimers when pH is lowered from 7 to 6.[37,40] It was observed that this acidification accelerated fiber formation, while on the other hand the presence of NTD at pH 7 slowed down aggregation compared to when it is absent.[37] Hence the NTD seems important both for rapid fiber formation as well as preventing premature aggregation in the gland. Precarious aggregation is also impeded by the high salt concentration in the gland which stabilizes monomers.[23] Dimerization appears to be a three-step process.[41] NTDs can interact through electrostatic interactions. At pH 6.5 weakly associated dimers form which still allow structural rearrangements. Additional protonation at pH 5.5 causes a strong interaction, thereby interconnecting protein chains and facilitating the pulling force to propagate through the spidroin causing structural conversion of the repetitive region. The properties of NTD are commonly accepted to account for its role and behaviour throughout different species and silks.[23] Though it can be noted that this is not necessary a hard fact and other mechanisms can occur for different systems. Recent investigation of the NTD of aciniform silk, used for eggcase and prey wrapping, only shows ∼40% amino acids similarity to dragline silk NTD, and while the domain has a similar morphology in the sense of five α-helices, it can form oligomers due to higher density of surface charges and hence can play a different role in fiber assembly.[24] Another result, now on both terminal domains of tubuliform silk, also used for eggcases, showed no pH dependence because oligomerization is governed by hydrophobic interactions.[25] In both cases higher order assemblies than dimers can form which is distinctly different from the NTD of major ampullate silk.

The CTD is present in a dimeric form through a disulfide bridge. The domain enhances storage of spidroins and ensures correct alignment of β-sheets during shear-induced fiber formation.[38] It assists in the formation of nanofibrils rather than unordered β-sheet morphologies.[42] CTD stability is pH depen-

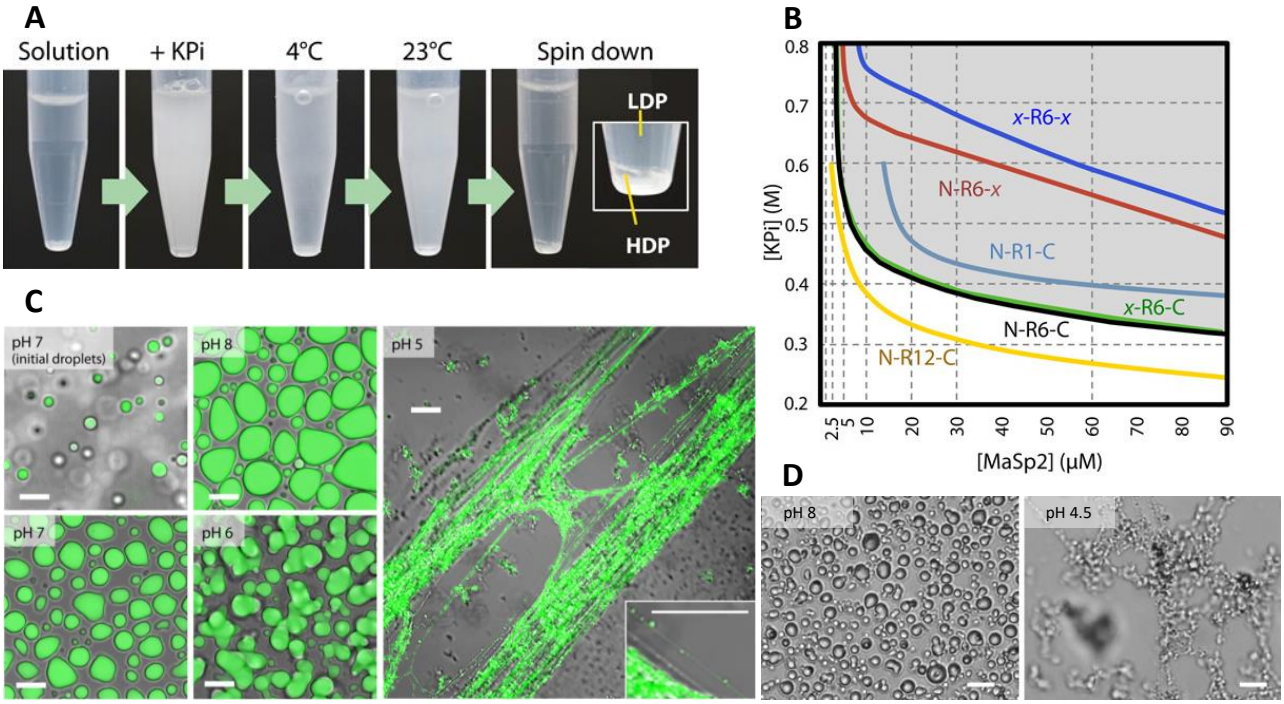

Figure 2. Liquid-liquid phase separation in a MaSp2 derived model spidroin from Malay *et al.*. R1, R6 and R12 refer to the number of repetitive domains and N and C to NTD and CTD while *x* denotes their absence. A – phosphate induced LLPS of N-R6-C showing turbidity increase as a sign of coacervation and a reduction with decrease of temperature. Centrifugation yield a low and high density phase, LDP and HDP, respectively. B – phase diagram of various model protein architectures as a function of phosphate and protein concentration. The area above the curve corresponds to a two phase system. C – pH induced fibrillation of N-R12-C labeled with DyLight 488 in 0.5 M KPi. At pH 7-8 droplets are liquid and fuse easily, at pH 6 they are semi-solid and at pH 5 a fibril network forms. D – natural content of major ampullate gland at 0.5 M KPi and pH 8 and 4.5, showing similarities to model system in C. Scale bars, 10 μm. Images taken from [8].

dent as partial to complete unfolding of the helices occurs at pH 5.5 to 5 and at those pH also β-sheets can form.[29] The pH dependent processes in the terminal domains is summarized in fig. 1C.

Besides dimerization of CTD and, at lower pH, NTD, that induce the formation of fibers (fig. 1D) other stimuli that are found in the gland play a role. The presence of phosphate ions and shear induces aggregation whereas NaCl does not affect stability and even increases solubility.[43] Shearing and extensional flow in the duct cause the formation of β-sheet structures, the formation of which occuring mainly in the third stretch or "limb" of the duct.[31] Fibers are composed of β-sheet structures from the polyanaline region surrounded by an amorphous glycine rich part, which contains to be more precise 3_1-helices and β-turns.[44,45] β-sheet formation is contrary to the propensity of poly-Ala to form α-helices.[46] An explanation could be that destabilization and unfolding/refolding of CTD by pH or phosphate might result in initial β-sheets that are seeds for further sheet formation.[23,29]

Recent development on LLPS

Over the past three years a few observations have been published on liquid-liquid phase separation of spider silk or silk-like proteins, at least to the extent that these papers interpret the formation of condensates as coacervation. A detailed summary is given of the findings from each publication, given to serve as a guideline for further discussion.

Malay *et al.* investigated the effect of phosphate ions and pH on LLPS and fibril formation of various model dragline silk constructs derived from the major ampullate spidroin 2, MaSp2.[8] Recombinant spidroins were used. These are designed proteins made by combining the DNA of relevant parts and expressing it in another organism, usually *E. coli* bacteria. This allows to design the size and sequence of the repetitive domain, to combine domains from different species and to easily fabricate and purify the protein once it is produced in the bacteria. The researchers used recombinant proteins to fabricate various well-defined spidroins and to be able to rigorously study specific functions. Their proteins consisted of either one, six or twelve repeating units (each 29 amino acids) accompanied by either or both terminal domains. Addition of potassium phosphate (KPO_4^{3-}/KPi) to a solution of pro-

teins turned it turbid, and centrifugation revealed a low and high density phase (fig. 2A) suggesting separation of two liquid phases. The interpretation of coacervates came from the observation of the droplet's ability to fuse readily, a characteristic of a liquid. Also a lower critical solution temperature (LCST) was observed when heating a clear solution. They observed that LLPS was driven by the number of repeating domains with more domains enhancing phase separation; and the C-terminal domain of which in the absence, a higher concentration KPi was necessary for LLPS, while the presence or absence of NTD did not show any differences (fig. 2B). However, NTD being able to dimerize showed an essential role during fibrillation upon acidification as no fibrils were observed for a protein in which the NTD was omitted (x-R6-C) or mutated to make it persistent for dimerization by lowering pH. The effect of CTD seemed to be that it results in tight networks while in its absence only loose, fragmented aggregates were produced. Notably an equimolar mix of two proteins that contained only one terminal domain did not yield fibril structures at pH 5. These findings suggest the importance of the terminal domains in adequate fiber formation, even suggesting the need for them to be in the same chain. The researchers used Raman spectroscopy to analyse the secondary structure of the proteins. It showed that LLPS droplets and pH-induced fibrils showed no change in protein structure whereas strained fibrils showed increased presence of β-sheets, indicating the role of shear in chain alignment and structural transitions. Interestingly, they looked at native spidroin solutions and these showed similar responses to pH (fig. 2C,D). At 0.5 M KPi and pH 8 liquid droplet formed while pH 4.5 caused formation of fibril networks. This means that while the characterization of the droplets being a liquid phase rather than micelles can be up for discussion, the rigorous model proteins do resemble natural spidroins.

Mohammadi *et al.* have considered a few triblock protein architectures.[9] They used either of three repetitive domains of 500-550 amino acids that were based on RD from *A. diadematus* dragline silk and either of two terminal domains that were taken from non-spider species. Most triblock and a few diblock species were able to form "liquid-like" coacervates (LLC) upon increasing concentration. One triblock aggregates and some single domain proteins did not phase separate. Assignment of coacervation came from the dynamic behaviour of the droplets, namely being viscous droplets that grow by coalescence and (dis)assemble based on the concentration. Fig. 3A shows microscopy images of droplets in the dense phase obtained after centrifugation of a phase separated system.

On the other hand, they observed that phosphate induced the formation of "solid-like" coacervates (SLC) which differ to LLC in that the protein structure is more rigid, apparent from a diffusion coefficient that is lower by an order of magnitude. The observation of KPi induced microspheres has been made before on a comparable system although the results were not analysed as thoroughly as in this work.[47] Continuing on the model system of Mohammedi, it appeared that fibers could only been drawn from LLC. However, follow up research on the salt dependence showed that diffusion, a measure for the degree of being liquid-like, is higher for low salt and low incubation times. A transition to a more solid-like phase takes place for high salt or longer incubation time.[48] This shows that the model protein does not necessary fail to be able to form fibers after phosphate is added, as the concentration can be different or it can be added after fiber formation commenced and as such it does not contradict physiological conditions.

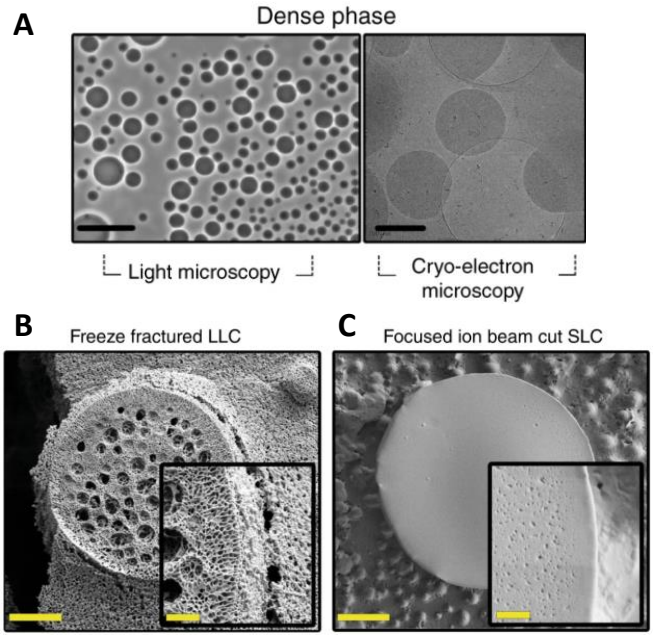

Figure 3. Formation of liquid-like (LLC) and solid-like (SLC) coacervates in a spider silk inspired triblock protein from Mohammadi *et al.* A – light microscopy and cryo-TEM on liquid droplets in the high density phase after centrifugation of a phase separated system. Scale bars, 20 μm for light microscopy and 1 μm for cryo-TEM. B – scanning electron microscopy image of freeze fractured LLC. C – SEM on focused ion beam cut SLC that forms upon addition of KPi. Both LLC and SLC consist of a bicontinuous network. Scale bars B/C, 2 μm, 1 μm for insets. Images taken from [9].

Fibers were formed by stretching a droplet of concentrated LLC between the tips of a pair of tweezers.[9] Fibers only formed from LLC, not SLC, and in particular only those consisting of triblock proteins. The molecular structure of fibers was measured by wide-angle X-ray scattering. At 0% strain, polypeptide chains were randomly oriented. At 100%, β-sheets and α-helices formed, the latter packing together, and both increased in numbers at 150%. This is comparable to the results from Malay *et al.* and the general observation on shear induced spider silk formation.[8,31]

An interesting consideration is the formation of a bicontinuous network in the droplets, both in LLC and SLC although more dense in the second (fig. 3B-C). Combined analysis with molecular dynamics simulations revealed that strong interactions between terminal domains are responsible for the stabil-

ity and cohesion of the condensate.[49] The repetitive units are compact and show weaker α-helical bundling that break and reform easily on deformation and this is denoted as a "spring-like" part. When the condensates are pulled upon, the TD act as mechanical load-bearing connections while the RD can extend and restructure into for instance β-sheets as already observed by the same researchers in [9]. This structural balance can connect to natural spidroins as the terminal domains can form strong bonds through disulfide bridged (C-terminal) or pH induced (N-terminal) dimerization while the middle part is more flexible and shows no preferred interaction sites.
Finally, Shen *et al.* showed a generic propensity of biomolecular liquid phase separated droplets for a shear-mediated liquid-to-solid transition (LST).[10] Five unrelated proteins were used including human RNA-binding and membrane-binding proteins but also the silk protein from the silkworm *Bombyx mori*, and all showed identical LLPS and LST behaviour. FTIR indicated the formation of hydrogen bonds and β-sheets upon fiber formation. Another sign for hydrogen bonding came from an analysis of a proline-arginine repeat peptide that was capable to LLPS but did not form fibers because of its lack of ability to form backbone-backbone hydrogen bonds. The most striking feature of this work is that LLPS and shear inducing fiber formation can occur for a range of proteins, virtually irrespective of their primary structure, apart from the hydrogen bonding capabilities, and thereby can suggest similar phenomena to occur for spidroins, although these have many specific interactions, for instance in the terminal domains, as well.

Micelles, liquid crystals and LLPS

One of the main questions that motivated this review is how the new observations of LLPS relate to the current theories of silk protein storage and its conversion to fibers. This work tries to put it into context and find aspects to take into consideration when comparing different ideas. Currently it is thought that spidroins are stored as micelles. Also it has been observed that liquid crystals form in the sac and duct which contribute to protein chain alignment during spinning. First, these ideas are explained more and compared to the concept of liquid-liquid phase separation. Then a few more thought are presented, and all subsections are in the hope to see what should be considered to clear up apparent discrepancies.

Micelle theory

The prominent theory for spidroin solubility in the spider gland and the subsequent transition to fibers is that spidroins assemble into micelles with the hydrophilic terminal domains on the surface while the hydrophobic repetitive domains are shielded from the solvent (fig. 4A).[21] A number of micelles aggregate into globules, explained by that the terminal domains are still also hydrophobic, the loss of water and increased concentration.[21,50] These deform when shear is applied, thereby allowing interactions between hydrophobic repetitive domains, and transforming into a fiber. This idea is originally based on the sequence of the silkworm but is now generally applied to spider silk as well. The amino acid sequence of silkworm proteins show a large difference in hydrophobicity between the repetitive and terminal domains, the first being more hydrophobic.[38] This is not apparent from the sequence of spidroins with the terminal domains scoring higher on a hydrophobicity scale compared to the RD.[38] In the same work they explained that for CTD the hydrophilic amino acids are located at the surface while the hydrophobic part is buried, effectively making the domain more hydrophilic. Surface analysis of many NTD shows highly charged regions on the surface so it is sensible to also regard them as hydrophilic.[37] Transmission electron micrographs on native dragline silk reveal a hierarchical structure where a few proteins form a ∼30 nm domain and multiple of these assemble into a "micellar" structure of several hundred nm.[51] This is then different from the common spherical micelles as there is no selective positioning of domains at the outside and inside of the "micelle".[51] Shear causes initial stretching of the individual domains and leave the larger structure intact.[51]

The conventional micelle model considers hydrophilic parts in the hydrophobic repetitive domain that combine into solvent patches within the micelle. In silkworm silk protein these regions are apparent,[21] but for spider silk this might not always be the case. The micelle presented in [38] is based on the repetitive domain of dragline silk of *A. diadematus* which has relatively more of the polar amino acid glutamine in the glycine rich region than the sequence of *L. hesperus*.[26] This can alter the extent to which these patches form. This is not particularly relevant as the underlying principle of a hydrophobic RD retains but I would have liked to note it to show that amino acids sequence differences can play a role. Alanine and glycine are generally neither very hydrophobic nor hydrophilic when it comes to biological hydrophibicity scales based on membrane interfaces.[52,53] This can mean that the amphiphilic nature of the spidroin is not very strong. The repetitive domain is largely unstructured in that it is mainly random coil or helical structures.[27,32,54] Hence, it is not likely for there to be an unforeseen consequence of the structure on the (a)polar nature of RD. Setting this aside, there seems to be a structural basis of an amphiphilic protein that could be able to form micelles.

The terminal domains are not always included in model spidroins. A single repetitive unit of tubuliform (eggcase silk protein) can form nanometer sized spherical objects that were called micelles.[55] This is possibly the result of that the 161 amino acids show two clear hydrophilic and hydrophobic sections. Another small protein of 191 residues based on the repetitive domain of major ampullate 1 silk was completely miscible due to its small size and charged residues.[56] Both examples mention the micelle theory in their publication but I would argue that it does not compare to more realistic spidroins. Experiments on small engineered proteins are very valuable regarding development of artificial silk spinning but cannot be used to validate micelle theory.

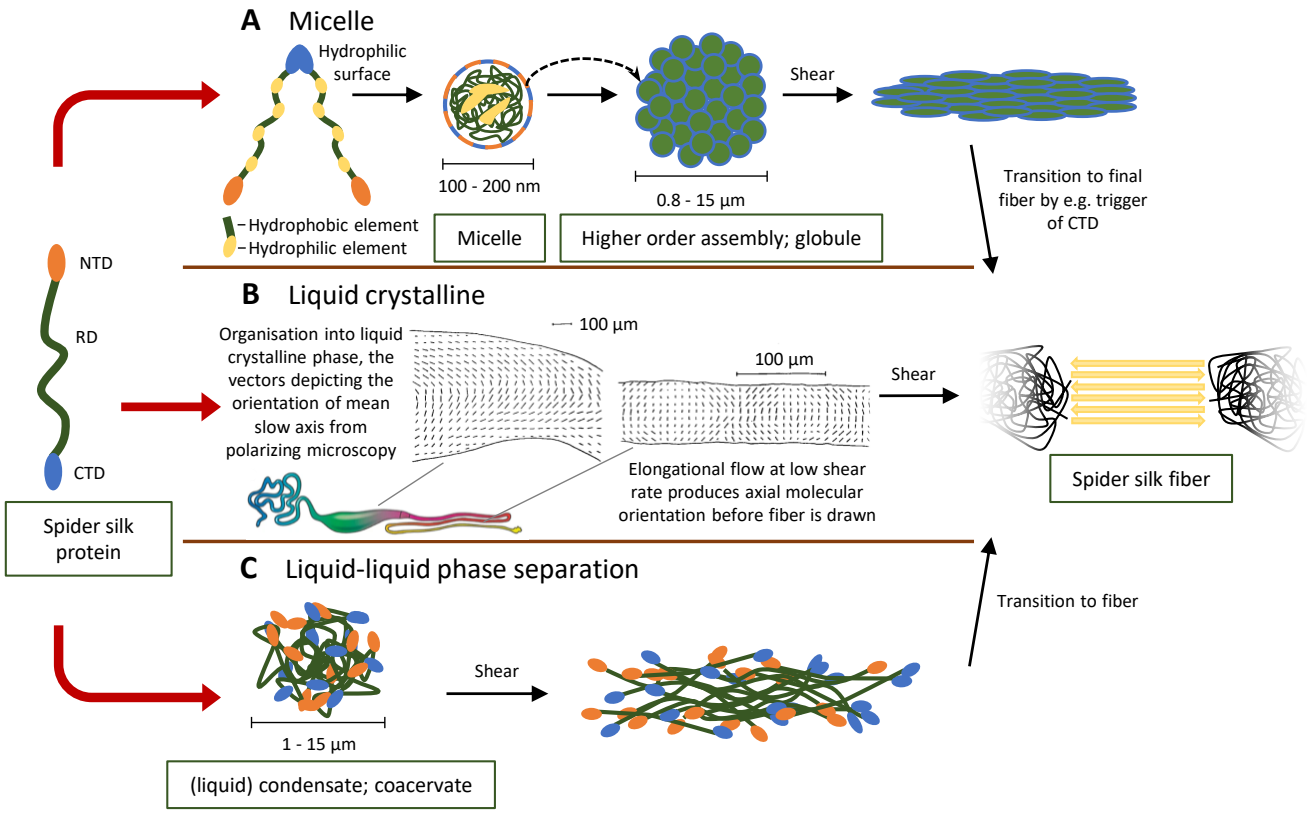

Figure 4. Three ideas for spidroins on their liquid storage and mechanism to form solid fibers. These do not mutually exclude each other. Only the effect of shear is included since the pH and phosphate stimuli, especially on the terminal domains, are presumed to have the same effect regardless of the organisation of proteins. A – spidroins are considered to contain hydrophilic terminal domains and a hydrophobic repetitive domain that comprises hydrophilic regions. They form into micelles with the terminal domains at the exterior while the hydrophilic repetitive regions form solvent patches in the interior contributing to solubility. Micelles pack together into globules that deform under mechanical stress during which protein chains align into solid fibers. Design of micelle model taken from Jin and Hagn ([21] and [38]). B – at sufficiently high concentration, spidroins form into a liquid crystalline phase. Elongational flow at low shear rate guide the alignment of chains before a fiber is eventually formed towards the end of the duct. Liquid crystalline description and image taken from [34]. C – a two phase system is formed where spidroins partition into concentrated condensates through liquid-liquid phase separation. Flow can deform the droplet which is thought to assist in chain alignment. CTD dimerization in schematic droplet is not included for clarity. Coacervate size is based on observed condensates in [8] and [9].

An interesting feature from the original work of Jin *et al.* on silkworm silk is that the shear induced fibers showed a skin-core structure with strong protein alignment at the outer side of the fiber while having a more rough morphology in the core.[21] The core morphology was explained based its physical appearance as coming from the micelles/globules in which the proteins were organized. A very similar observation has been made in the work on LLPS of a model spidroin by Mohammadi *et al.*, namely a more pronounced β-sheet structure formation on the outer side.[9] This is remarkable as the terminal domains of the model triblock protein are not even derived from spiders. While this is not sufficient to point out a definite theory on spidroin storage and assembly, it leaves the possibility that the spherical micrometer sized structures observed in both systems are the same in nature but are either coined micelles or coacervates.

On the other hand, a thorough analysis from Lin *et al.* on tubuliform supported the presence of micelles.[57] Repetitive domains containing either one of the terminal domains were His-tagged on both sides separately, so on the terminal domain or the open end of the repetitive side. Only those actually at the terminal domain were immunodetected and could be cleaved with thrombin, suggesting that the terminal domains are on the surface of a micelle and the repetitive part is buried in the core. However, as mentioned before, assembly of terminal domains of this type of silk is different compared to dragline silk showing no pH dependency.[25] In fact, Wang *et al.* concluded on the oligomerization of terminal domains through hydrophobic interactions which forms what could be a shell structure of a micelle.[25] It seems that the formation of such a micelles is the result of preferred assembly of terminal domains that subsequently lead to the repetitive domains moving to the interior. This would then be markedly different than the premise of hydrophilicity/phobicity for the terminal/repetitive domains that form a conventional micelle.

An example showing a possible basis for different views based

on perception is from [58] and [38]. In [58] they examined the effect of NaCl and pH on a solution of an engineered derivative of spider silk from *A. diadematus*. This is from the same natural protein on which the repetitive domain of the model in [9]) was based on and lead to the micelle model in [38]. The RD was 24 repeat units and was either combined or not with an C-terminal domain.[58] They observed a low and high density phase, irrespective of the presence of a terminal domain. Upon increasing the NaCl concentration the phase separation disappears. It is proposed that this can account for the spidroin solubility in the gland as there the NaCl concentration is also high.[58] They named the behaviour liquid-liquid phase separation. If it is coacervation, it is expected that high salt swells the droplets till it combines with the diluted phase to be one phase.[13] At least, this is the case when the interactions are governed by electrostatic interactions, the degree of salt dependence relating to the type of interaction at play, descending from charge-charge, cation-π, dipole-dipole to π-π.[59] Glycine is assessed as a polar residue,[59] and glutamine is polar, so it expected that NaCl enhances mixing. This is also observed for the P granule protein LAF-1 that has a arginine/glycine RGG low complexity, disordered domain, that is responsible for LLPS and the phase separation disappears at high concentration of NaCl.[60] Thus, a spidroin coacervate system at high concentration and NaCl concentration can result that the entire system is a highly concentrated macromolecular phase that can roughly be imagined as a large "coacervate" phase. What makes this a striking example for the discussion is that a subsequent work ([38], much used in this review) uses the same protein construct, does additional experiments on the terminal domain, and then presents the results in a micelle model. This is accompanied with little argumentation for its use and does not pay notice to the observation of a low and high density protein phase in [58]. The new work is widely cited, mainly for its seminal work on CTD but partly as a basis for the micelle theory, and this might not be appropriate as spidroin assembly/phase behaviour seems more complex and based on the protein structure, also discussed later.

Liquid crystals in silk glands

Analysis of spider glands let to the conclusion that spidroins in the sac and duct are organized in liquid crystalline phases, mainly nematic phases.[22] A cholesteric phase has also been observed in the duct but this can untwist into a nematic phase under the influence of the flow in the duct.[33] The benefits of liquid crystals is that the molecule align before fiber formation causes fewer defects and that the spinning force required is small.[22] The cholesteric phase was characterised by TEM and AFM, and other indications for the existence of liquid crystals have been found, for instance from rheology.[22,33] Polarizing microscopy on the sac and duct from different directions yielded the image of a nematic phase in fig. 4B.[34] Slow flow allows the proteins to align while it prevents premature crystallization.[34] The evidence for a liquid crystalline phase seem plentiful and was already established more than two decades ago. To my impression it is not considered much after that. Possibly it is not observed, either because the concentration is lower than in the natural system or an experimental bulk solution does not compare to the μm to mm sized spider gland. Either way, usually microspheres are observed, if characterised at all, the identification of which is the subject of this review. How LLPS relates to liquid crystals is a bit different now. Due to the high concentration of protein it is not unlikely that a gland solution in a typical phase diagram is shifted past LLPS into a one phase system that is a concentrated phase.[13] Then it is not expected to observe two phases in the gland. Thus, it is plausible that if coacervation occurs for spidroins, it is not observed in the gland and that it forms a liquid crystals, implying that both hypotheses do not exclude each other.

Considerations on spidroin behaviour

Determining the mode of storage and transition to fibers for spidroins seems like a laborious task. There is more to the eye than putting its behaviour in one group such as micelles, liquid crystals or coacervates. The assembly seems to depend on various factors of which some have been mentioned throughout the previous sections. More aspects will be added in order to find (potential) considerations on this topic.

Sato and Takahashi have modelled an amphiphilic diblock copolymer on its possibility to form micelles and/or coacervates.[61] The model is fairly simple and only considers a diblock. Nevertheless it can be argued that the qualitative results hold for spidroins or can at least be used when constructing a rationale to spidroin assembly. They found that an amphiphilic diblock polymer can form either coacervates or micelles depending on the strength of interaction of the solvent with each monomer type in the polymer. Only if the amphiphilic character of the polymer is high enough or the solvophilicity of the surface group is high, micelles will form, otherwise the system is one phase or biphasic. Increasing the length of the polymer blocks would cause the LLPS phase to occur less and only micelles or one phase to remain. In case of spidroins the amphiphilicity is not distinctly large, especially compared to silkworm, as discussed in *Micelle theory*.[38] Also among different types and species the sequence of the repetitive domain can differ influencing the difference in hydrophilicity/phobicity of the terminal and repetitive domains. Also noticeable is that the length of the spidroin studied can have an impact on the behaviour observed. The model systems in *Recent development on LLPS* are usually smaller than natural spidroins with $\sim$10 repeat units rather than $\sim$100.

Other considerations are regarding the characterization of coacervates or micelles. The works of Mohammadi *et al.*, Malay *et al.* and Shen *et al.* assess visible droplets in optical microscopy or TEM as being liquid condensates due to their dynamic nature where droplets readily coalesce,[8–10] and this is proposed as a valuable indication for liquid droplets.[62] Fusion of micelles is less usual and are expected to form by unimer

addition.[63] However, direct fusion of micelles is observed and also the tubuliform assemblies discussed before were shown to fuse.[57,64] Another method to assess coacervation is to measure the concentration of the dilute phase (and/or saturated phase, but that faces practical difficulties). This should not change for varying concentration of protein and remain equal to the saturation concentration where phase separation occurs.[60,62]

An aspect that is not considered, perhaps due to its lack of scientific accountability, is the size of the droplets. My impression is that micelles are in the 100 nm range,[21,57,64] which is expected due the molecular nature of micelles where one protein that is at most a few hundred nm occupies a space from the surface to the center of a sphere. Liquid phase separated droplets, also from other biomacromolecules, commonly range from 1 to 20 μm.[8–12] This is distinctly different from the tubuliform protein spheres from Lin.[57] These were 50-100 nm which would corresponds to micelles, and their results and from others also suggest the formation of micelles.[25,57] Another result of micelles is from a model dragline protein containing just two repetitive units corresponding to only 25% of the total number of amino acids.[65] Spherical particles were visible in cryo-TEM and measured 5-40 nm in size, behaving similarly to the micelles in [57].[65] Similarly, a uniform distribution of particles of 122 nm were observed from a small engineered dragline construct.[50] It was not further characterized (e.g. coalescence, phase diagram), and thus could be liquid phase separation but from its size it is most likely micelles. This is contradictory to the idea posed before that a smaller protein can be more inclined to phase separate rather than form micelles. More surprising is that some of the constructs from Malay *et al.* contained only one repetitive unit and nevertheless showed LLPS,[8] the explanation of this with regard to [65] and [50] is not apparent to me. This leaves room for rigorous structure-function analysis on well-defined proteins where small sequence and domain alterations can help to find where differences originate from.

The formation of biomolecular liquid condensates is usually connected to intrinsically disordered proteins.[11,13] These have high conformational flexibility, accessibility to posttranslational modification, multivalency or low sequence sequence complexity.[66] They have low hydrophobicity, which would favour aggregation, and higher content of polar or charged groups.[67] These also include glycine and glutamine,[68] of which glycine constitutes a large portion on the repetitive domain and also glutamine is frequent.[26,58] The similarity in sequence to LAF-1 was mentioned before, and this also phase separated due to the disordered domain.[60] Thus given that RD is mainly random coil and a highly repetitive, low complexity sequence, it means that liquid-liquid phase separation is reasonable. Variety in LLPS propensity might arise between different silk types that have different residue composition. As discussed before, the repetitive domain from *A. diadematus* is more polar than from *L. hesperus*. This is then a factor to play with when designing a protein sequence for artificial spinning procedures for spider silk.

Conclusion

This review looked into the current ideas on the storage of spider silk proteins, called spidroins, in solution and the subsequent mechanism by which these spidroins can transform to solid fibers. Till now, many efforts have led to a large understanding of the terminal domains of the protein, such as their structure and structural responses to chemical and mechanical stimuli, mainly acidity, ion content and shear stress. Meanwhile the structure and behaviour of terminal domains from different spider species and silk types are still being unravelled. To a smaller extent this is also (being) done for the repetitive domain. On the other hand, there is a clear view on the silk gland responsible for the production of spider silk, including the pH and ionic composition along the sac and duct and where and how shear forces are generated. However, there is still a gap between the picture of the individual molecules and the macroscopic system. This is concerned with the exact processes that take place at the microscopic scale at which storage and rapid fiber formation occurs. The conventional ideas is that spidroins assemble into micelles that combine into globules and also liquid crystalline phases have been identified in the natural system. Recently it has been observed that model spidroins can undergo liquid-liquid phase separation, or coacervation, forming droplets that behave as liquids.

The reconciliation of the micelle and coacervate hypotheses is not trivial. Some aspects that were touched upon in this work is that larger proteins can favour micelle formation; the amino acid composition of the repetitive domain can enhance the tendency for coacervate formation due to a reduced amphiphilicity with respect to the terminal domains or an increased polar or charged nature contributing to the intrinsically disordered nature of the repetitive domain; the mechanism of assembly of the terminal domains can favour micelles as a result of oligomerization, rather than only dimerization; and that the general amphiphilicity of spidroins is not sufficiently pronounced to yield the same assembly by definition for all proteins. Is has been noted that there are differences between different types of spidroins and between different species which could have an effect on assembly whereas usually the effect and purpose of certain domains is assumed to hold throughout spidroins.

This review has not necessarily managed to cover all aspects that go into deciphering the differences between coacervates and micelles. One aspect that might prove useful is rheological behaviour and response since plenty of research approach protein solutions and silk formation with polymer physics but this is not considered here at all. Hence no definite conclusion is reached on whether one idea is favoured over the other. Nonetheless, the discussion might have shown where differences can arise and it indicates that possibly all types of assemblies appear depending on the parameters. This also leaves opportunities. A system might be tuned to display either mi-

celles, liquid crystals or coacervates, and they bring unique properties that can be played with. Consolidating a theory of liquid-liquid phase separation into the realm of spider silk research adds new ideas that can lead to better understanding of complex protein systems in general and can aid to produce high quality, adjustable artificial spider silk for applications.

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