Tunable Artificial Spider Dragline Silk Fibers

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Abstract

Spider silk is one of the toughest materials in nature. To fully utilize the mechanical properties of major ampullate spider silk, the origin of the properties must be understood. The structure of major ampullate spider glands, spidroin protein folding and spidroin acquisition will be discussed. Spidroins are composed of 3 main parts, those being the N- and C-terminals and a central repetitive region. The N- and C-terminals play a role in the formation of secondary structures in spider silk, while the repetitive part gives the structure flexibility. The spinning of artificial spider silk is preferably done using wet spinning, as this spinning technique allows for many parameters to be controlled.
The mechanical properties of artificial silk can be partially controlled by pH, reeling speed, relative humidity, and ion concentration. While other parameters are present in the production of natural spider silk, most do not appear to significantly affect the resulting properties of the fibers when created artificially. Reeling speed and relative humidity appear to be useful parameters to adjust the mechanical properties of artificial spidroin fibers, while ion concentration and pH will likely adopt a single optimal value to maximize the desired mechanical properties. While much research has been done to determine the structure of spun silk fibers, the resulting mechanical properties have not yet been thoroughly researched. As such, more of this type of research is required to determine the key parameters to allow tuning of the mechanical properties of resulting fibers.

Introduction

Silk has been used by man due to its fascinating properties for generations[Leng-Duei2015].
A prime example of this is spider silk, which, due to their remarkable mechanical properties, has inspired much research into the origin of the strength of spider silk. Spider silk has been historically used in many applications such as sutures, fishing nets, and ropes. Spider silk is made from proteins called spidroins, which possess a long repetitive domain flanked by two terminals called the N- and T-terminal.
The interplay of these parts of the protein is believed to be the origin of the

mechanical properties of spider silk, though the exact interactions between the parts have yet to be elucidated. The mechanical properties exhibited by spider silk outperform the best man-made materials to this day[Johansson2015] (Figure 1). It is therefore no surprise that many attempts have been made to create fibers to rival or even surpass the abilities shown by naturally created silk. Even to this day, the exact mechanisms behind the secondary structure formation of spider silk fibers are yet to be fully understood.

Material	Strength (N/m^2)	Elongation (%)	Energy to break (J/kg)
Dragline silk	4×10^9	35	4×10^5
Minor ampullate silk	1×10^9	5	3×10^4
Flagelliform silk	1×10^9	>200	4×10^5
Tubuliform silk	1×10^9	20	1×10^5
Aciniform silk	0.7×10^9	80	6×10^9
Kevlar	4×10^9	5	3×10^4
Rubber	1×10^6	600	8×10^4
Tendon	1×10^6	5	5×10^3

Figure 1: Mechanical properties of different types of spider silk and artificial materials. Taken from 'DOI: 10.1146/annurev-bioeng-082719-032747'

For a long time, creation of artificial spider silk fibers has run into the issue of spidroin production. Most spidroins were previously harvested from naturally spun silk, however, due to the low production volume of spiders, this method of procurement is not scalable. Recent advances in recombinant production of spidroins, or the production of spider silk fibroins in host systems, has made major advances. Allowing for possible large scale production of spidroins, which in turn would allow large scale applications of spidroin fibers.
This review will cover the necessary information regarding the natural creation of spider silk and spidroin proteins, the natural environment in which silk is spun, and the interactions inside the fiber that are believed to give rise to the mechanical properties exhibited by spider silk. Additionally, the creation of artificial fibers using spidroins, the effect of mechanical processing parameters, and the effect of environmental processing parameters will be discussed, with the aim of providing an overview of the most important parameters regarding the creation of artificial spider silk.

Spidroins

Spider silk is made from spidroins[Römer2008], shorthand for spider fibroins. Fibroins are insoluble proteins present in most insect produced silks. Depending on the family of spider and on the desired mechanical properties, different spidroins will be used to create the silk.

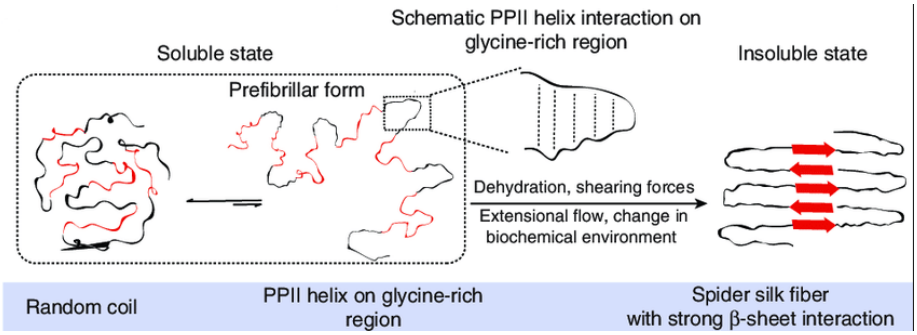

Figure 2: The two main structures of the dope, random coil and PPII helix, are in equilibrium. The PPII helices will transform under external pressure into β-sheet structures. Taken from 'https://www.nature.com/articles/s41467-018-04570-5'

Proteins

The strongest type of silk produced by spiders is Major Ampullate silk, commonly known as dragline silk due to its use in the creation of draglines. Dragline silk is believed to be mainly composed of 2 proteins, Spidroin I[Santos-Pinto2018] and Spidroin II[Santos-Pinto2016]. Both proteins possess different variations, like Spidroin I-a and Spidroin I-b. These variations differ only slightly in their structure[Santos-Pinto2018] The proteins have 3 main regions, namely the C-terminal, a central repeating structure, and the N-terminal. The N-terminal is the end of the protein with a free amine group (NH_2), whereas the C-terminus is the end of the protein with a free carboxyl group (COOH).
It is believed that the C-terminal and N-terminal are of importance when considering protein folding, as many proteins connect their C and N-terminals when forming secondary structures.The C-terminal and N-terminal of Spidroins I, II, and all variations of them, are identical, indicating that these terminals likely also play an important role in the formation of secondary structures.

Protein Folding

The secondary structures of relevance[Beek2002] for spidroins are β-sheets and Poly-Proline II (PPII) helices. β-sheets are responsible for the strength and endurance of many biological materials[Cohen2020], while Poly-Proline II helices give flexibility in the repetitive domain of the spidroins[Adzhubei2013]. β-sheets possess many hydrogen bonds, leading to strong attraction between two parts of the chain. The only way to separate the strands, is to break a significant amount of the hydrogen bonds. If only a small part of the hydrogen bonds is broken, then they will be reformed due to the attraction of the remaining hydrogen bonds.
Due to the large amount of hydrogen bonds present in secondary protein struc-

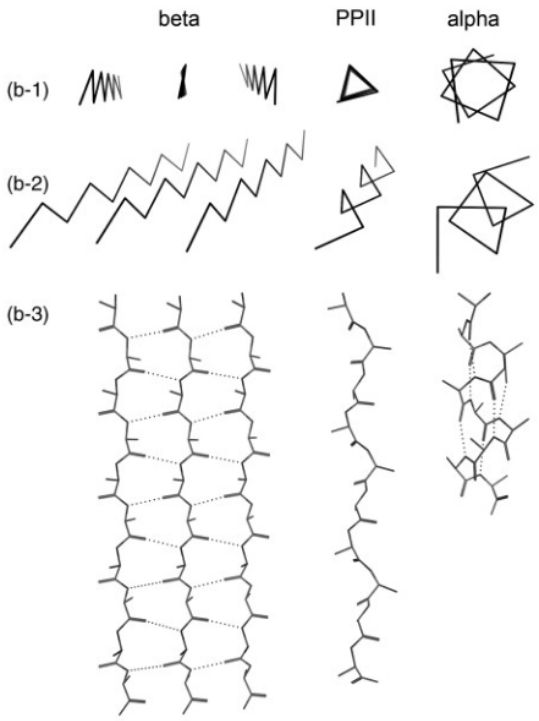

Figure 3: Folding structure of β-sheet, α-helix, and PPII helix, modeled as an axis projection (b-1), a perspective projection (b-2), and a polypeptide chain (b-3) Taken from 'http://dx.doi.org/10.1016/j.jmb.2013.03.018'

tures, a relatively large amount of energy is needed to break the secondary structure. This gives rise to the high tensile strength of materials.
When spidroins are in solution, they form a soluble, transparent and viscous substance known as dope. Dope consists of a high concentration of proteins (30-50% w/v), which due to interaction with surrounding water does not form many hydrogen bonds with other proteins, and as such exhibits mostly a random coil and α-helix structure[Hijirida1996]. When taken out of solution, however, the repetitive domain of the spidroins will partially adopt a Poly-Proline II structure[Oktoviani2018]. Due to the decrease in water in the dope, more hydrogen bonds will be formed between proteins instead of with surrounding water molecules.
PPII helices possesses a unique ability to switch from its PPII structure to another structure, like β-sheets, at a single residue and by shifting only a single dihedral angle[Adzhubei2013]. Therefore, by first adopting the PPII structure, the transition to a β-sheet structure becomes less energy costly. PPII structures have further been shown to provide flexibility[Adzhubei2013], which makes it likely that PPII helices stay as part of the total structure of spider silk.

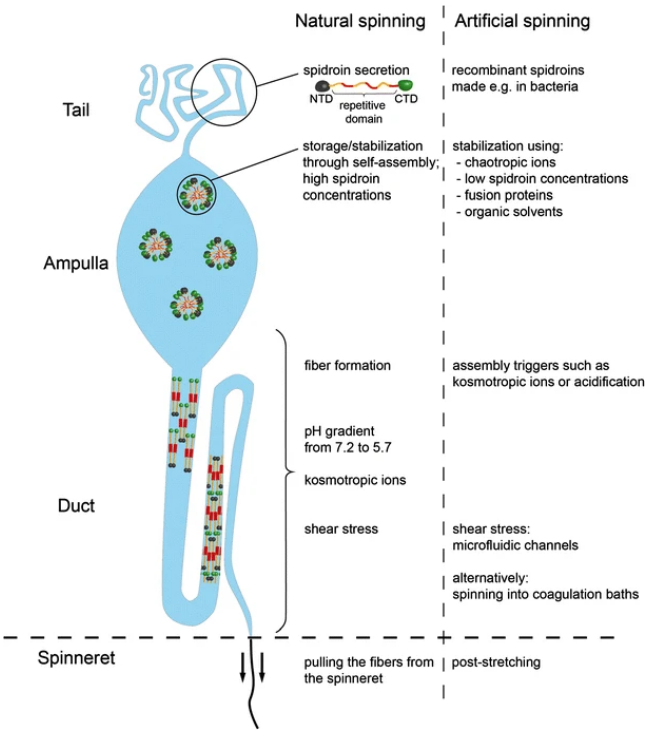

Figure 4: Overview of natural and artificial spinning processes. Taken from 'http://dx.doi.org/10.1007/s00253-015-6948-8'

It has been found that N-terminals assemble into β-sheet structures[Arndt2022]. This leads one to believe that the formation of secondary β-sheet structure in the protein ensemble, is triggered by the β-sheet formation of the N-terminals. The PPII structure adopted by the repetitive domain would thereby partially adopt the β-sheet structure, because it would be energetically favorable to adopt a β-sheet structure due to the low energy needed for this transition. This would lead to the spidroin having a combination of β-sheet, PPII, and α-helix structures. The α-helix structures would arise from hydrogen bonds being formed between different parts of the same protein, instead of hydrogen bonds forming between proteins.

Spidroin Acquisition

There are 3 distinct methods of producing spidroins for use in biomimetic fiber creation. The first is using native dope, which is obtained by dissecting the spider and obtaining the silk protein directly from the silk glands. This method almost guarantees quality, however its time-consuming process and high production costs make it unviable for larger scale production requirements. Currently,

this method is more historical than practical, as the most difficulties of the other two methods have been resolved with time.
The second method is to harvest already spun fibers, and harvest the desired proteins from the fiber itself. While this process does not require the sacrifice of a spider to acquire proteins like the first method, it remains a time-consuming and inefficient process.
The last method, and the only one which can reasonably be upscaled, is to create the protein required for biomimetic applications through genetic manipulation of bacteria[Whittall2021]. The spidroins created using this method are known as recombinant spidroins. E. coli, in particular, has been a popular host system for the creation of spider silk proteins. This is due to the relative ease of genetic manipulation, short generation time, comparative low cost, and potential for industrial scale-up[Chen2012].
An example of the production of genetically manipulated spidroins, is the production of NT2RepCT[Schmuck2021]. NTR2RepCT is a protein possessing the N-terminal and two repeats from MaSp1, connected to the C-terminal from MiSp1, with the name indicating the structure. The major challenge of this production method, however, is that proteins like NTR2RepCT (33 kDa), are much smaller than naturally produced proteins (250-350 kDa).
While longer proteins can be produced in host systems[Bhattacharyya2021], additional difficulties arise when trying to create them. Shorter proteins show relatively weaker mechanical properties compared to their longer counterparts[Venkatesan2019].
Nevertheless, all recombinant spidroin dopes differ from natural dopes in the fact that they do not possess stress-induced phase separation and are not yet able to rival the mechanical properties of natural fibers.

Spider Silk Spinning in Nature

Silk Gland Environment

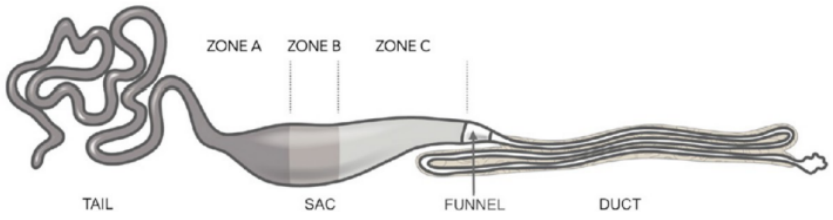

Figure 5: A schematic representation of the major ampullate gland. The gland consists of a tail, sac or often called ampulla, and a three part duct. The three zones of the sac are indicated as zone A to C. Taken from 'https://doi.org/10.1021/bm400898t'

The Major Ampullate silk gland present in certain spiders[Andersson2013] con-

sists of 3 main parts: a tail, an ampulla, and a duct. The tail synthesizes the spidroins used in the creation of the silk. The spidroins form a dope inside the tail of approximately 30-50% w/v. Currently, the mechanism by which the dope inside the tail can stay in solution at such high concentrations is believed to be due to the formation of micelle nanoparticles[Parent2018]. The ampulla stores the spidroin dope and consists of 3 parts[Andersson2013].

The 3 parts differ in cell type and show clear separation between the segments. The cells in the duct region possess microvilli that likely allow for efficient extraction of water from the spidroin dope. Lastly, the duct is the part where the liquid silk is transformed into fiber. The duct is connected to the sac via a funnel and decreases in diameter from the funnel to the spinneret. The dope is believed to be either liquid crystalline, micelle like, or a combination of the two, inside the major ampullate gland and the first and second parts of the spinning duct.

Along the spinning duct, the pH decreases from the end of the ampulla, to the end of the spinning duct[Knight2001]. The pH is the highest at roughly 8 in the tail, with the pH decreasing to around 6 at the end of the spinning duct. It is difficult to determine the exact pH values, as measuring the values must be done using either dyes, which have low resolution, or micro-electrodes. The pH has influence on the solubility of proteins, with a high pH level exhibiting much greater protein solubility than low pH levels.

Additionally, the concentrations of sulfur, potassium, and phosphorus increase along the spinning duct, while the concentrations of sodium and chlorine decrease[Knight2001]. Sulfur, potassium, and phosphorus are part of chaotropic salts, while sodium and chlorine are osmotic salts. Chaotropes show better protein solubility, while kosmotropes show better water absorption[Adebowale2007]. The capacity of water absorption shown by both salts decreases as the concentration is increased above 0.1 M. Apart from water absorption, chaotropic ions also inhibit intra- and intermolecular interactions of the repetitive domain[Oktaviani2019].

The effects of pH and the type of salts present on the solubility of the proteins, is linked to the structural changes of the N-terminal domain[Gaines2010]. The N-terminal adopts a formation which removes solvent from the structure, this effect was seen to depend on a combination of pH and ionic strength. Higher concentrations of salts and higher levels of pH stabilized the protein solution, while lower concentrations of salts and a more acidic environment promoted aggregations of proteins.

The assembly of silk fibers has two proposed theories. The first is based on crystalline alignment, viewing the spidroin dope as a liquid crystal, while the second considers the spidroin dope as small micelles.

These two theories are, however, not necessarily mutually exclusive. The liquid crystalline theory assumes the dope to adopt a liquid crystalline state, due to the combination of high protein concentration and directional alignment. This state would allow for the formation of intermolecular interactions, which, coupled with the removal of water by decreasing pH and change in ionic strength, would allow for the formation of secondary structures. The micelle theory considers the silk to be made from a large collection of small (100-200 nm) micelles,

which together would form globules that are elongated and turned into fiber along the duct.

Silk Spinning Forces

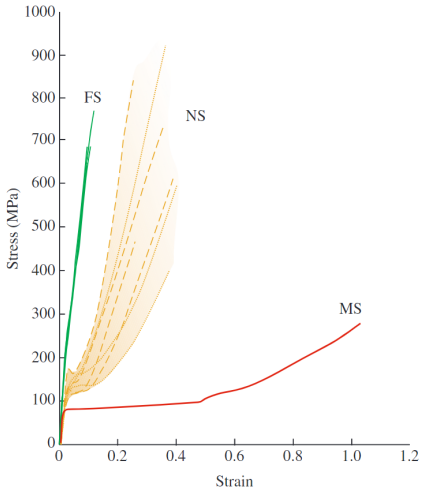

Figure 6: Typical stress–strain curves of forcibly silked (FS), naturally spun (NS) and maximum supercontracted fibers (MS) tested in air. Taken from 'DOI: 10.1242/jeb.01701'

Stress-induced phase transitions, reeling speed, and shear forces are believed to additionally play a role in the mechanical properties of spider silk. Shear forces promote aggregation of proteins[Gao2013]. Nevertheless, there appear to be exceptions, as forcibly spun dragline fibers from the web-building spider Trichonephila clavipes have shown similar mechanical properties regardless of spinning speed and humidity[Yazawa2021]. The reeling speed used by spiders has been shown to greatly affect the mechanical properties of the resulting fiber of Nephila edulis spiders[Vollrath2001]. At higher reeling speeds, the diameter of the fiber and the strain at break decrease. The Initial Modulus and Tensile Strength of the fiber, however, seem to possess a maximum, with the properties decreasing when the reeling speed is either increased or decreases with respect to the optimal value[Young2021].
According to the theory established by Brookes et al.[Brookes2008], the structure of spider silk is made from parallel reinforcing units with amorphous regions between them. However, the mechanism by which these regions are formed have yet to be elucidated. Lastly, the hydrodynamic flow inside the spider duct also affects the β-sheet formation of fibroins. Uniform flow inside the spider duct promotes the adoption of β-sheet and Poly-Proline II structures by alanine residues[Herrera-Rodriguez2022]. The shear and drawing forces applied during the creation of silk fibers further facilitate the adoption β-sheet and Poly-Proline II

structures compared to the random coil structures[Xie2006]. The amount of random coil structures increase when no shear and drawing forces are applied. Depending on the silking force 3 types of silk fibers can be created[Pérez-Rigueiro2005]. Using high force, around the yield limit, will create so called forcibly strung (FS) fibers. Lower forces will create fibers with properties similar to naturally spun (NS) fibers. Very low forces will result in fibers that act comparably to maximum supercontracted (MS) fibers. Greater silking force leads to stiffer silk fibers, with FS fibers being noticeably stronger and less strainable than NS fibers and far stronger and far less strainable than MS fibers.

Artificial Spider Silk Spinning

The spinning of artificial spider silk is done through two main methods, namely wet spinning and dry spinning. Other notable methods include, electrospinning and rotary jet spinning[*Rogalski*2017]. Wet spinning possesses the most controllable parameters of these, however has the main disadvantage of requiring a cleaning step to remove residues from the bath. While this makes wet spinning a very favorable technique for academia, it is also more costly to produce. For the purposes of this review, only straining flow spinning, a subsidiary of wet spinning, will be explained as it allows for the control of the most processing parameters.

Straining Flow Spinning

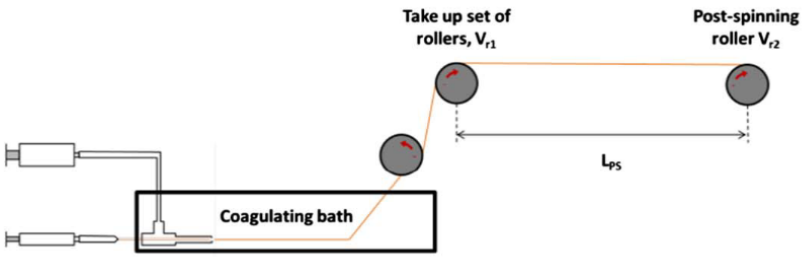

Figure 7: Schematic of the structure of a straining flow spinning setup. Taken from 'https://doi.org/10.1016/j.eurpolymj.2017.09.037'

Straining flow spinning is a wet spinning technique that uses two flowing liquids to create fibers[Madurga2017]. The first fluid, the so called focused fluid, is surrounded by another fluid, the so called focusing fluid. The focusing fluid allows for control over the shearing forces that are applied during spinning. Using this method, the chemical parameters of the focusing fluid, the dope solution, and if desired a coagulating bath can be controlled and adjusted.
This allows for optimization of many production parameters previously found to

play a role in the creation of spider silk in naturally produced silk. Furthermore, the flow rate and capillary size of the outlet nozzle can be adjusted similar to any other spinning technique. The use of the focusing liquid and a coagulating bath can allow the mimicking of the natural gland environment, by having different pH levels, ions, and ion concentrations. Lastly, using the rollers at the end of the setup, the reeling speed of the fibers can be adjusted.

Reeling Speed

Another key parameter of rotary jet spinning, but also of other production methods, is the reeling speed of the fiber. Increasing the reeling speed increases the Young's Modulus, Toughness Modulus and Engineering Strength, however decreases the strain at break according to Schmuck et al.[Schmuck2022]. This is in-line with the theory established by Du et al.[Du2006] and the natural silk used by Pérez-Rigueiro et al.[Pérez-Rigueiro2005], which gives an increasing stress and decreasing strain at greater reeling speeds. The increase in reeling speed creates greater forces on the protein structure, inducing shear forces that promote the aggregation of proteins and the formation of β-sheet structures.
However, Young et al.[Young2021] appears to indicate that higher reeling speeds above a threshold, 1.9 mms^{-1} in this case, actually lower the Young's Modulus and Tensile Strength of the fiber. Just like Schmuck et al., they have also observed a decreasing strain at break with increasing reeling speed. Schmuck et al. used reeling speeds of 17-69 cms^{-1}, while Young et al. used reeling speeds of 0.5-128.6 mms^{-1}. It is, however, important to mention that both groups used different proteins, with Schmuck et al. using NT2RepCT and Young et al. using major ampullate MaA and minor ampullate MiA proteins.

Additional Processing Parameters

pH

While much research has been done on the effects pH has on the creation of secondary structures, little measurements have been done regarding the consequences on mechanical properties[Schmuck2022]. Schmuck et al. did measurements on the mechanical properties of NT2RepCT at pH values between 5.0 and 4.0. They found that at a pH < 4.0 the structure of the fibers could not be maintained. Additionally, no increase in mechanical properties was found for pH < 5.0 , as the fibers created at pH 5.0, 4.8, and 4.5 gave similar mechanical properties apart from some outliers. This indicates that pH is not a relevant factor for the mechanical properties of small spidroins.

Relative Humidity

The presence of water on spidroin fibers affects the strength of the β-sheet hydrogen bonds. This means that the mechanical properties depend on relative humidity not only during the spinning process, but also during the measuring

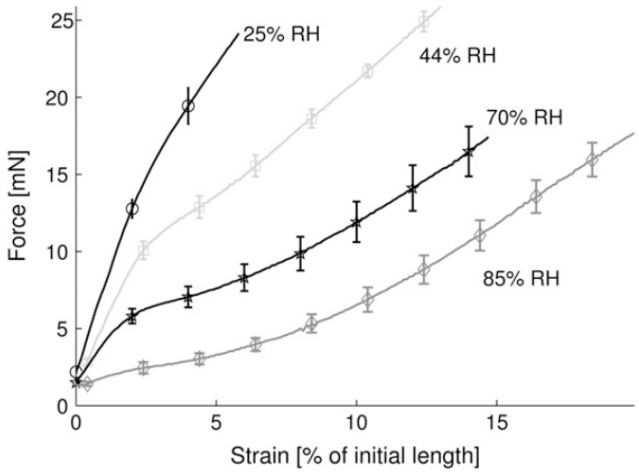

Figure 8: Averaged force-strain curves for Nephila senegalensis ensembles measured at 25%, 44%, 70%, and 85% relative humidity. For clarity, error bars and symbols are shown only at every 20th data point Taken from 'DOI: 10.1529/biophysj.106.099309'

process. When using higher relative humidities, the β-sheet content and degree of crystallinity of the resulting fibers decreased[Peng2015]. As a result, the toughness of the fibers increase. Lower relative humidity give rise to different levels of toughness, with lower relative humidity giving less strain at break and a greater Young's modulus[Vehoff2007].
Below a certain relative humidity, however, the overall strength of the fiber decreases. The likely cause of this is that below a certain humidity, the proteins cannot reorient themselves sufficiently to create a crystalline β-sheet structure. Above a certain relative humidity, the fiber will undergo supercontraction[Greco2021]. This supercontraction depends on the amino acid groups that make up the fiber, thereby allowing the response of fibers to relative humidity to be tuned.

Ion Concentration

The type of ions, and the concentration of them, plays a role in the removal of water from the fiber structure. Ion concentration therefore functions similarly to relative humidity when considering the effects on the mechanical properties of artificial fibers. High concentrations of salts like sodium chloride weakens bonding and results in less stable secondary structures[Gronau2013]. Finnigan et al. determined that pH alone does not trigger assembly of proteins with both terminal domains.[Finnigan2020] Instead, a salting-out effect was needed to trigger fiber formation. Salting-out is a purification technique that makes use of the reduction in solubility of proteins or molecules when in a solution of high ionic strength. Salting-out of the dope would accomplish the same thing as the spider

natively does, which is to bring the molecules out of solution.

Discussion

A lot of the research done on spidroin fibers is done on different proteins. Some is done using recombinant spidroin, which often do not possess the N- and C-terminals. These terminals are believed to play a role in the formation of β-sheet structures. It has been shown that proteins with longer repetitive domains, and without either the N-terminal, C-terminal, or with neither the N- and C-terminal, each have different structures and as a result mechanical properties. With a more reliable production process, as mentioned by Schmuck et al., I hope that more research can be done using one type of spidroin protein. Additionally, very little research has been done on how the structural changes of the proteins affect the mechanical properties of the fiber. While many papers research the structure of resulting fibers, few actually measure the effects these structures have on the mechanical properties of the fiber.

From what has been researched, however, some estimates of the importance of different processing parameters can be made. The results of current research suggest that while ion concentration does play a role in the formation of fibers, this can be limited to precipitating the proteins out of the dope solution. This parameter appears to inhibit the formation of β-sheet structures due to the interactions between the protein and water hindering the interactions between the proteins with themselves.

It is my belief that ion concentration will likely not be tuned to achieve different desired properties, and instead will have a range in which the resulting fibers will have the optimum mechanical properties. The relative humidity of the environment during spinning, as well as reeling speed, appear to allow a selection of mechanical properties. High reeling speeds, or low relative humidity, give rise to tough fibers. These fibers often possess slightly lower stress at break values, however the stress at break values are not low enough as to make these fibers unusable. Medium reeling speeds and average relative humidity give rise to fibers that are comparable to naturally spun fibers. Low reeling speeds and high relative humidity give rise to fibers that behave similarly to supercontracted fibers. These fibers possess much lower stress at break values, but much higher strain at break values, making these fibers weaker but more flexible.

Lastly, pH serves a similar role to ion concentration, bringing the proteins out of solution. The pH appears to have a distinct optimal range, as a pH values that are either too low or too high will not allow the proteins to form a stable fiber. It is not clear whether changes in the pH of the dope or focusing liquid can give better mechanical properties, as not enough research has been done on the topic. Considering the fact that most natural silk spinning systems use almost the same pH values however, it could be assumed that pH will also have an optimum value, with the tuning of these parameters only decreasing the performance of the silk fiber.

Conclusion

Spider fibroins are composed of 3 main parts, those being the N-terminal, repetitive regime, and the C-terminal. The N- and C-terminal play a role in the creation of secondary structures of fibroins. The mechanical properties of silk fibers are likely due to the interplay between PPII and β-sheet structures, with PPII structures playing a role in the formation of β-sheet structures as well as providing flexibility to the resulting structures. Straining flow spinning can be used to control the most important parameters for the creation of fibroin silk fibers. Of these parameters, pH, reeling speed, and relative humidity allow for tuning of the parameters of resulting fibers. There has been little research on the effects of different ion compositions and concentration on the mechanical properties of silk fibers.
Additionally, the research that has been done on artificial silk fibers is often done using different processing parameters or using different proteins. Nevertheless, it is my belief that the reeling speed and relative humidity used during the creation of fibers, can tune the mechanical properties of the fibers and allow for the creation of specifically engineered fibers. While the effects of pH on the resulting fiber are as of yet uncertain, I believe that, considering the pH values of natural systems are all identical, pH is unlikely to be a key tuning parameter for the creation of artificial silk fibers.

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