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Strategies for Making High-Performance Artificial Spider Silk Fibers

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Artificial spider silk is an attractive material for many technical applications since it is a biobased fiber that can be produced under ambient conditions but still outcompetes synthetic fibers (e.g., Kevlar) in terms of toughness. Industrial use of this material requires bulk-scale production of recombinant spider silk proteins in heterologous host and replication of the pristine fiber's mechanical properties. High molecular weight spider silk proteins can be spun into fibers with impressive mechanical properties, but the production levels are too low to allow commercialization of the material. Small spider silk proteins, on the other hand, can be produced at yields that are compatible with industrial use, but the mechanical properties of such fibers need to be improved. Here, the literature on wet-spinning of artificial spider silk fibers is summarized and analyzed with a focus on mechanical performance. Furthermore, several strategies for how to improve the properties of such fibers, including optimized protein composition, smarter spinning setups, innovative protein engineering, chemical and physical crosslinking as well as the incorporation of nanomaterials in composite fibers, are outlined and discussed.

1. Introduction

Spider silk is nature's high-performance fiber.[1] Despite being made in fractions of a second from renewable components and under ambient conditions, it displays an impressive combination of tensile strength and extensibility. These features make spider silk highly attractive for many technical applications.[2] Examples of such applications include fibers for the production of textiles for clothing, furniture, and the automotive industries, high-performance sports gear, or for making durable components for robotics.[2,3] Thus, spider silk could replace many of the petroleum-based fibers we use today that are becoming a major environmental problem. For example, the textile industry uses >60 million tons of primarily produced plastics annually to make products with a lifetime of only about 5 years,[4,5] and the use of plastic fibers such as polyester, polyethylene, and polyamide (nylon) prospects to increase even further in the coming decades.[5] Most textiles have limited biodegradability and release plastic microfibers, which accumulate in our environment throughout the product's lifetime. Therefore, novel sustainable materials that can replace these fibers are urgently needed.

Among the more than 51 000 spider species[6] some are capable to spin up to five different types of silk fibers and two adhesive substances from silk glands located in the opisthosoma (abdomen).[7] The different silk types have diverse mechanical properties, some are characterized by high tensile strength, for example, the major ampullate silk, while others are more extensible, for example, the aciniform silk.[1] Spider silk fibers are composed of spider silk proteins (spidroins) that, in general, are large and dominated by an extensive central repetitive region.[8,9] The repetitive region is capped by small and globular N-terminal and C-terminal domains (NT and CT, respectively).[10,11] The terminal domains mediate high solubility of the spidroins during storage and induce polymerization and fiber formation when the pH is lowered along the silk gland.[10–15] The repetitive region, on the other hand, confers the mechanical properties to the fiber.[1,16] Accordingly, the primary and secondary structures of the repeat region differ in different types of spidroins.[17] In this review, we focus on the major ampullate silk, which can have a tensile strength of 1–2 GPa and 30% strain at break, depending on the species.[18] The major ampullate silk is mainly composed of major ampullate spidroins (MaSps), whose repetitive region is dominated by characteristic blocks of poly-Ala residues interspersed with Gly-rich repeats.[19] There can be up to 100 poly-Ala repeats in a single MaSp, and these segments mainly adopt a β-sheet

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conformation in the fiber.[8,9,19–21] Through the stacking of several β-sheets, nano-sized crystals are formed in which the side chains from β-strands from neighboring sheets are interdigitated in structures that resemble zippers. Using computational methods the size of such a nanocrystal should be <6.5 nm to maximize the strength of the silk fiber,[22] since larger crystals may lead to uneven stress distribution in the material which in turn results in earlier fiber fracture. For the same reason, the degree of orientation of the crystals relative to the fiber axis has been shown to correlate to the strength and toughness modulus of the fiber.[23] Experimental studies have revealed that the β-sheet crystals are indeed in the range of $3 \times 4 \times 7$ nm[23–27] and are likely made up of β-strands from many different individual proteins. Thus, the protein network in the fiber has been suggested to take on the form of a fishnet structure in which the β-sheets crystal serve as crosslinking points between the individual protein chains, explaining the high toughness of silk.[28] In particular, the crystals are believed to be important for the fiber's tensile strength while the Gly-rich regions, that are found between the poly-Ala blocks, are more important for the extensibility of the fiber.[21] These parts of the spidroins will adopt helical and random coil conformations that form an amorphous matrix in which the crystals are embedded.[21,23] When the fiber is stretched, the Gly-rich regions are gradually extended before the load is transferred to the crystals. If the load is high enough, the crystals will ultimately fail via a stick-slip mechanism in which individual β-strands are being pulled out of the crystals.[22]

Because of the territorial and cannibalistic nature of spiders, they are unsuited as production animals.[2] Thus, bulk-scale production of spider silk must involve the production of the silk proteins in heterologous hosts followed by purification of the proteins and fiber spinning in artificial spinning devices.[29,30] However, producing artificial spider silk has turned out to be a formidable challenge, mostly due to the large size and aggregation-prone nature of the spidroins.[29–32] There are two main strategies for producing artificial silk fibers.[30] One tackles the commonly encountered solubility problem by allowing the spidroins to form insoluble aggregates during expression and subsequently use organic solvents for solubilization and fiber spinning.[29] This approach enables the expression of large spidroins that can be spun into fibers with high tensile strength, but the protein yields are far from what is required for industrial production.[33,34] Furthermore, the use of harsh solvents makes this approach less attractive from a sustainability perspective. The second approach is a biomimetic method that involves only aqueous solutions in the purification and spinning process.[30] To ensure solubility during expression in heterologous hosts, mini-spidroins have been designed that are composed of the terminal domains and a central repeat region that has been substantially shortened compared to the natural protein template.[35–44] One of these mini-spidroins can be produced at high expression levels in bioreactor cultivations (>20 g L^{-1}), which makes the process economically feasible, and they can be concentrated to the same extreme concentrations as seen in silk glands.[35,36,38,45] Furthermore, since the mini-spidroins carry both terminal domains and are kept natively folded throughout the production process, they respond properly to a lowered pH. This means that the mini-spidroins assemble into fibers using similar mechanisms that control native spider silk spinning when wet-spun spun into low-pH spinning baths.[36,46,47] However, fibers spun from such mini-spidroins are inferior to spider silk in terms of tensile strength.[35,36]

The situation has brought the field to a point where novel strategies for producing high-quality artificial spider silk at bulk scale have to be outlined (**Figure 1**), which is the focus of this work. At this point, it is worth distinguishing between the various spinning techniques which are used in industry to make continuous synthetic filaments,[48] that is, wet-spinning,[49] dry spinning,[50] melt spinning,[51] and gel spinning.[52] For making artificial spider silk fibers, wet spinning methods are currently standard.[53] In the broader sense, wet spinning is defined as extruding a dissolved polymer into a coagulant where the polymer solidifies,[49] but within the scope of artificial spider silk the wet-spinning method can be further classified by the type of extrusion device used. Typically, artificial silk spinning is accomplished by extrusion of the spinning dope containing recombinant proteins through a nozzle or needle, but there are examples of more elaborate setups, for instance the use of a coaxial devices[37,54] or microfluidic chips.[40,55–57] The latter two extrusion devices are used only in few studies despite that they could be key in mimicking the gradual pH decrease and/or the shear forces in the natural spinning apparatus,[13,56] and with that make stronger and tougher artificial spider silk. Nevertheless, due to the increased complexity of the microfluidic chips compared to standard spinnerets commonly used in industrial settings,[48] spinning with microfluidic chips is not easily scalable[58] and thus might be more suited for producing micro- and nanoscale scaffolds for, for example, tissue engineering applications.[59] For these reasons, we herein focus on wet-spinning methods, and direct readers that are interested in different types of extrusion methods to a recent review on microfluidics to fabricate protein-based fibers by Su et al.[60]

One possibility to improve the mechanical properties of artificial spider silk would be to use protein engineering of small spider silk proteins to increase the intermolecular contacts in the fiber.[36,61,62] This could enable the production of small proteins at high yields and at the same time result in fibers with good mechanical properties.[61,62] Other methods for improving the fiber properties would be to optimize the post-spin stretching of the fibers,[39,63,64] to make composite fibers that incorporate nanomaterials,[65,66] to screen for optimized spinning conditions and coagulants,[63,64] and to cross-link the protein chains in the as-spun fiber.[67,68] In addition, recent studies using next-generation sequencing technologies have suggested that the classical spidroins are far from the only proteins that make up the silk, such as spider silk constituting elements (SpiCE proteins),[9,43,69] that are defined as low-molecular weight non-spidroin proteins, some of which could be instrumental in the silks' properties. In this review, we go through the strategies that have been tested for producing artificial spider silk until the present day and give future directions on the most promising strategies.

2. Replicating the Major Ampullate Spinning Dope

To produce artificial silk fibers with properties that match those of the pristine fiber, it seems likely that both the protein composition and the hierarchical architecture of the fiber must be

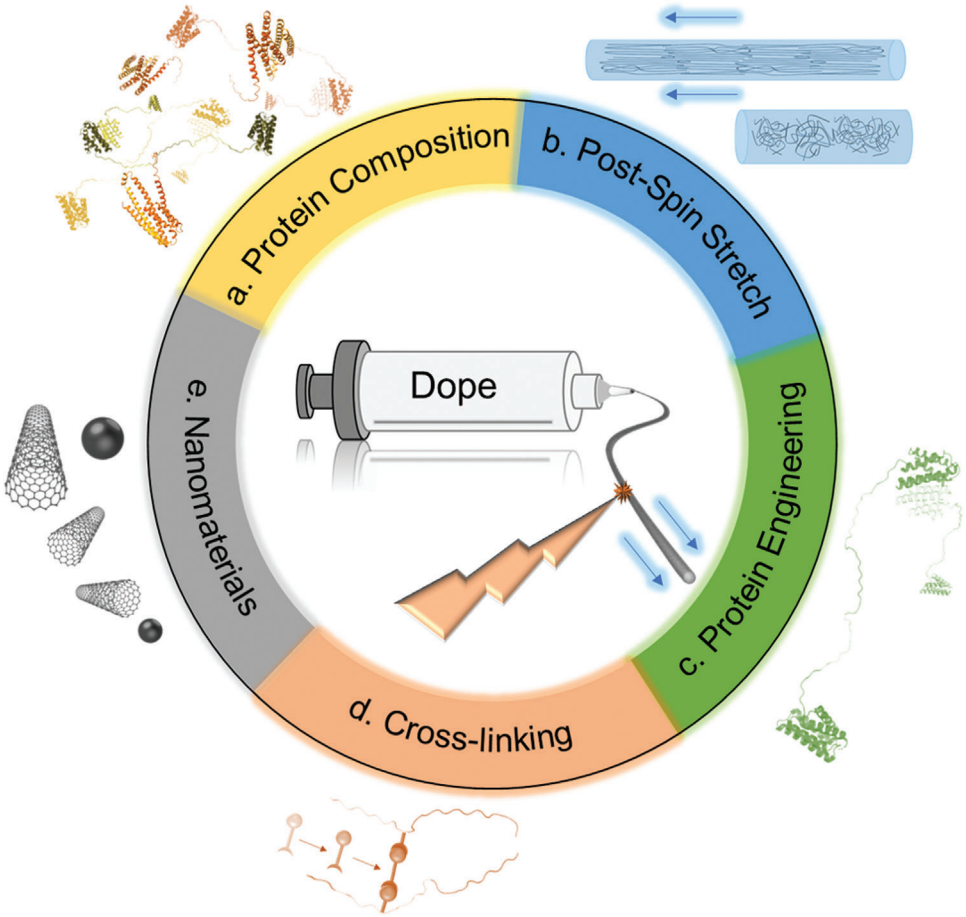

Figure 1. The different routes proposed in this review for improving the mechanical properties of biomimetic artificial spider silk fibers: a) To prepare mixtures of different silk proteins in order to mimic the composition of the natural spider spinning dope, b) post-spin stretch of the fibers to increase the alignment of the protein chains and improve the intermolecular interactions, c) the use of protein engineering to increase the intermolecular contacts in the fibers, d) crosslinking the protein chains, and e) the design of a silk-nanomaterial composite fiber. Protein structures in a) represent a recombinant mini-spidroin (adapted from reference[36] under the creative commons attribution license) and the spider silk constituting element NMA1 from *Trichonephila clavata*.[46] The sequence for NMa1 was obtained from the National Center for Biotechnology Information (GFR3246.1) and the structural predictions were obtained using AlphaFold2 in ChimeraX.[315,316] The protein structures shown in c) were adapted under the terms of CC BY license.[36] Copyright 2022, The Authors, published by Wiley-VCH.

mimicked.[70] Despite that the spider major ampullate silk fiber has been studied extensively over the past decades, we still lack a thorough understanding of its composition and structure. Several reports that provide morphological characterizations of the fiber have suggested a skin-core structure.[71–77] The core likely consists mainly of spidroins and is therefore assumed to contribute most to the fiber's mechanical properties.[72,78] The skin is a thin outer layer of the fiber that is composed of glycoproteins and lipids, and has been proposed to protect against antimicrobial degradation and/or play a role in the regulation of fiber water content.[72,73,79] However, there are additional proposed models of the fiber that suggest a more complex multi-layered structure[72,80] including three,[71,77] four,[80] and five layers.[72] In the hope to shed light on the fiber structure, composition and spinning mechanisms, several studies have investigated the silk gland morphology, cellular composition, transcriptomics and physiology.[7,13,75,81] The major ampullate gland is composed of a long and winding tail, a slightly wider part called sac, and a duct[82] where protein polymerization and fiber formation take place. The tail and sac are lined by a single layered epithelium that produces and secretes the silk feedstock into the lumen of the gland.[82] Histological examinations of the major ampullate gland have shown that there are three distinct cell types in the sac and the tail, localized to three separate zones (A, B, and C).[13,75] These cells produce and secrete substances in the gland lumen that stain differently by histochemical dyes and stay separated as the dope travels into the duct where the conversion into a fiber takes place. This implies that the silk fiber has at least three layers.[75] A recent study using single cell and spatial transcriptomics of the major ampullate gland reported the presence of six cell types in the tail and sac of the gland but their distribution in relation to the zones remains unclear.[75,83] However, the results show that the cells in the tail and sac likely produce secretions with somewhat different compositions,[83] which further attests to the presence of layers in the fiber.

Table 1. Motifs occurring in the repeat regions of the MaSp subfamilies. The table was compiled using data from selected publications.[9,43,69] $(A)_n$, $(GA)_n$ represent poly-A and poly-GA motifs, respectively. Residues in the square brackets separated by | imply occurrence of either of those two residues/motifs.

MaSp subfamily	Variant name	Motifs			Reference
		Gly-rich	Ala-rich	Other	
MaSp1	MaSp1		$(A)_n$, $(GA)_n$		[9]
	MaSp1A1	GGX, GGYGGL	$(A)_n$		[43]
	MaSp1B1	GGX, GGYGGRF	$(A)_n$		
	MaSp1C1	GPGXX	[A|S]AS	XQQ, SSX, TTX	
	MaSp1D1	GGX, GGYGGL	$(A)_n$		
	MaSp1E	GYPGQ	[$(A)_n$|SSSVAISL]		[69]
	MaSp1F	GQGGYGSG	$(A)_n$		
	MaSp1G	GSGGYGGR			
MaSp2	MaSp2A	GGX, GPGGX, GPGQQ	$(A)_n$	XQQ	[9]
	MaSp2B				
	MaSp2A1	GPGXX, GPGQQ			[43]
	MaSp2B1	GGX, GPGXX, DGGR, GGYGGL	[A|S]AS	SSX, TTX	
	MaSp2C	GPGSQ	$(A)_n$		[69]
MaSp3	MaSp3	GGX, GPGGX	$(A)_n$	SSX	[9]
	MaSp3B1	GGX, DGGR, GGYGGL			[43]
	MaSp3C1				
	MaSp3D	SGGRGGY			[69]
MaSp4	MaSp4A	GPGPQ	[V|I]SVVS[A|T]VS, VSVVS[A|T]VS		
MaSp5	MaSp5A	GGLGGSG, GSGGR			

With recent developments in sequencing technologies and the cumulative availability of spider genomes and transcriptomes, it has become clear that the major ampullate silk is a multicomponent material, but the bulk of the fiber is composed of MaSps.[43,69,83,84] These are grouped into MaSp1–5 subfamilies based on the occurrence of characteristic motifs in their repeat regions (**Table 1**). The first full-length sequences of genes encoding MaSp1 and MaSp2 were reported for *Latrodectus hesperus*,[19] and homologs to these two genes have since then been found in all spider genomes reported to date.[9,43,69,83,85] The repetitive regions of both MaSp1 and MaSp2 proteins contain poly-Ala regions interspersed with Gly-rich repeats.[17] The MaSp1 and MaSp2 can be distinguished by that the MaSp1 is rich in the motif GGX (where X = A, L, Q, Y, R), GXG (where X = L, R, Q, Y, P), and poly-GA,[9,86,87] whereas MaSp2 has, in addition to GGX and GXG, abundant GPGXX and characteristic QQ motifs.[16,43,86,87] The GPGXX motif has been shown to adopt a β-turn conformation in the fiber and is therefore assumed to contribute to the extensibility of the fiber.[88,89] Recent transcriptome and proteome analyses of the gland and fiber have revealed the presence of three additional MaSp paralogues (MaSp3–5 subfamilies).[9,43,69,85] The nature of the amino acid motifs in the repeat region of MaSp3 seems to differ between species. For example, in *L. hesperus*, MaSp3 lacks the poly-Ala motifs typical of MaSp1 and MaSp2, as well as the GPG motifs typical of MaSp2, and contains more polar and more acidic amino acids than the MaSp1 and MaSp2 from the same species.[90] However, several other reports show that MaSp3 in other species contain poly-Ala[9,43,69,87] and GGX motifs.[9,43] MaSp4 and MaSp5 were first reported in *Caerostris darwini*,[91] and later also identified in *Caerostris extrusa*.[69] Both MaSp4 and MaSp5 lack poly-Ala motifs, and MaSp4 is rich in Pro with up to 52% of the repetitive region made of GPGPQ motifs, while MaSp5 is abundant in GGX motifs. The specific functions of the MaSp3-5 remain to be elucidated, but the presence of MaSp3 and 4 are suggested to be correlated with higher fiber toughness.[18,85]

In addition to the MaSps, the major ampullate silk contains other types of spidroins, as well as non-characterized proteins.[43,83,85,92–94] Transcripts encoding multiple spidroin types have been found in the major ampullate glands from several species[8,83,85,95,96] some of which have also been identified by proteomics analyses.[92,93,97] This promiscuous and variable expression of different spidroins could be a means for the spider to adapt to what dietary sources are available,[98] but could also be related to an ability to tune the mechanical properties of the fiber according to the spider's need.[83] Recent reports have shown the presence of numerous non-canonical spidroin proteins in the fiber, termed spider silk-constituting elements (SpiCE). This is a versatile group of proteins, loosely defined as low molecular weight non-spidroin proteins,[9,43,69] that has low sequence similarity and hence could lack a common function.[43] A subset of the SpiCE proteins are Cys-rich[93,94] and form complexes via disulfide bonding, which could be important for the mechanical properties of the fibers.[94] There is only one study in which the mechanical properties of artificial silk fibers made from recombinant spidroins and a SpiCE protein have been reported, and in contrast to the SpiCEs' suggested function, fibers spun from a mixture of a mini-spidroin and a SpiCE protein were inferior

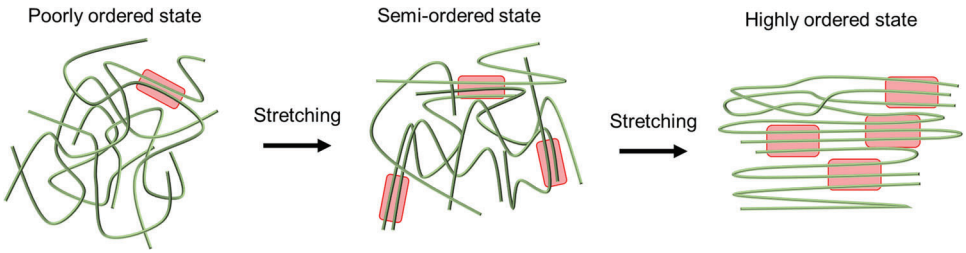

Figure 2. Stretching a synthetic polymer or protein fiber, respectively, leads to increased orientational order and intermolecular interactions, which is why a post-spin stretched fiber will improve in tensile strength and Young's modulus.

to pure spidroin-based fibers.[43] Clearly, additional research is required to obtain a more comprehensive understanding of the functions of these proteins.

Several proteomics studies have identified and semi-quantified different proteins in the major ampullate silk.[9,43,69,83,92–94] In general, all studies confirm that the most abundant proteins in the silk fiber are MaSps but the relative abundance of different MaSps varies between species. In the *Araneus ventricosus* major ampullate silk fiber, MaSp3 was found as the most abundant protein.[9] Using the intensity Based Absolute Quantitation in shotgun proteomics, Kono and coworkers later quantified the ratios of the MaSp, SpiCE, and other proteins in the major ampullate silk of four *Nephilinae* spiders.[43] The results reveal that MaSp1–3 proteins together constitute more than 90% w/w of the major ampullate silk but the relative abundance of each MaSp subfamily varies significantly between these species. Additionally, the SpiCE proteins accounted for 1% or less, and around 2–9% of the fiber content was represented by proteins with no functional annotation. Similarly, in *C. darwini* and *C. extrusa*, the MaSp1–5 account for more than 80% of the total proteins in the dragline silk whereas SpiCE proteins account for 1–5%.[69] Since the relative abundance of MaSp proteins in the major ampullate silk is so variable between different spider species, it is difficult to correlate the MaSp abundance with their role in the mechanical properties of the fibers.

With this knowledge at hand, it is clear that current models of the structure–function relationship of the major ampullate fiber are over-simplified since the models only consider the repeat region of MaSp1 and 2, while the contributions from the terminal domains, other protein components as well as the hierarchal layering of the fiber are largely ignored. It is plausible that a multicomponent composition is crucial for the native spider silk fibers' mechanical properties, hence, a spinning dope with a composition that more closely mimics the natural one may result in fibers with better and more native-like properties. In support of this, the Scheibel group showed that the presence of MaSp heterodimers in the spinning dope could increase the tensile strength or the extensibility, respectively, under some spinning conditions.[41,99] This is an interesting avenue for future studies, but it should also be noted that bulk-scale production of a multitude of proteins will make the production of artificial silk fibers more expensive and complex. Finally, the structural hierarchy of the native fiber could be important for obtaining better mechanical properties, which could be achieved, for example, by the use of microfluidic chips or coaxial devices that allow simultaneous spinning of dopes with different compositions. However, although this approach is interesting from a scientific perspective, scaling up such a complex spinning method to an industrial scale will likely be very challenging.

3. Post-Spin Stretching

An important feature of native silk spinning is the pultrusion of the silk fiber,[100] which means that the fiber is not primarily extruded from the silk glands, instead, it is pulled out.[101] Thus, after it is formed in the duct the fiber will be stretched, which is analogous to the post-spin stretch applied in industrial production of synthetic polymer fibers. During stretching, the polymer chains align and become more ordered which leads to a higher fiber tensile strength (**Figure 2**),[102,103] and in the case of silk also a higher Young's modulus.[103–107]

To get a better understanding of how post-spin stretch in general affect the mechanical properties of artificial spider silk fibers (spun from recombinant proteins) and fibers spun from regenerated silkworm silk, we compiled most of the currently available literature on the topic (**Figure 3**). Even though the fibers investigated in Figure 3 were made from different proteins, spun under different conditions, and post-spin stretched by hand, which introduces uncertainties and questions about reproducibility, it is

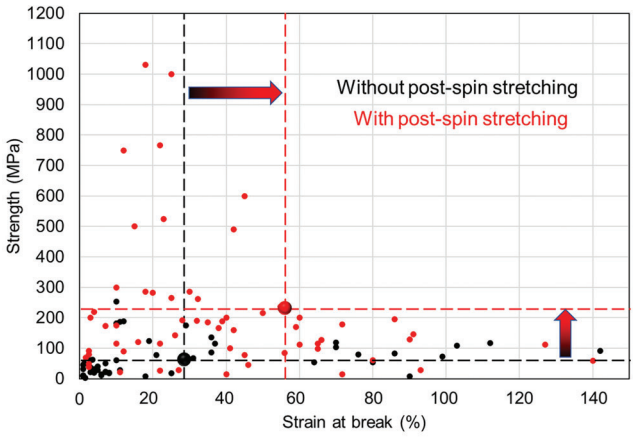

Figure 3. Scatter plot of the mean values of strength and strain at break of regenerated *Bombyx mori* silk and recombinant spider silk fibers. The mean values of the strength and the strain at break (dashed lines and big dots) are in general higher for fibers that were subjected to post-spin stretch. Data was obtained from refs. [33,34,36–39,44,54,56,64,107–131] and is listed in File S1, Supporting Information.

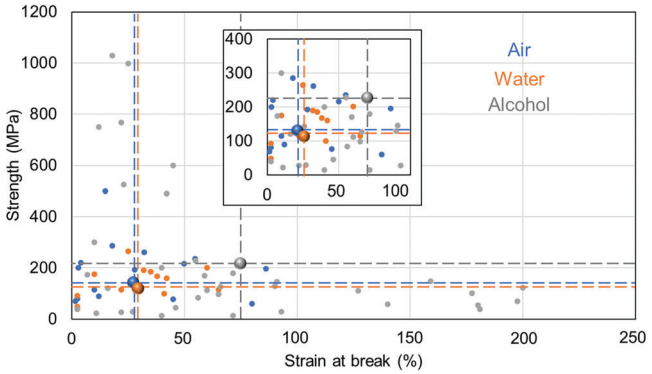

Figure 4. Scatter plot of the average values of the strain at break and strength of the different artificial silk fibers (recombinant spider silk and regenerated *Bombyx mori* silk) post-spin stretched in different solutions with a focus panel of the same graph. Dashed lines and big dots indicate the averages of all the values for fibers processed in the respective solutions. In this plot, methods in which post-spin stretch was applied sequentially in different solutions were not considered. Data points obtained from refs. [33,34,36–39,44,54,56,64,107–131] are listed in File S1, Supporting Information.

clear that post-spin stretch improves the mechanical properties of regenerated and artificial silk fibers. In further support of the positive effect of post-spin stretch, a recent report shows that out of 93 different spinning conditions investigated, post-spin stretch improved the strength and the Young's modulus of recombinant spider silk fibers the most.[63]

Post-spin stretching can be performed with the fiber submerged in a solution or suspended in air, as well as during or after the fiber spinning. We therefore sought to find answers in the literature as to whether the conditions under which the procedure is performed have an impact on the resulting mechanical properties of silk fibers. As can be seen in **Figure 4**, post-spin stretch in alcohols in general generate fibers with higher strength and strain at break compared to fibers that have been stretched in air or water, but also here, caution should be used when interpreting the data since the fibers are produced from different proteins by different groups, and post-spin drawn by hand. In general, alcohols are known to promote β-sheet formation in regenerated *Bombyx mori* (*B. mori*) silk,[132,133] recombinant spider silk,[134,135] and native spider silk fibers,[136] which could contribute to the improved tensile strength while it is more difficult to explain the increased strain at break. In conclusion, the effects of post-spin stretching on artificial silk fibers' mechanical properties should be further investigated since on a global level, the procedure increases the mechanical properties, but currently available data from the silk field is too variable and too diverse to allow detailed conclusions to be drawn.

4. Protein Engineering

Spiders have evolved over at least 400 million years and can spin fibers with impressive characteristics, but does this mean that spiders have found the most optimal amino acid sequence for making strong and extensible silk fibers? In other words, is the only way to make artificial replicas with the same mechanical properties as native spider silk to produce exact copies of the full-length spidroins? We argue that this is not necessarily the case. Spiders have evolved under certain constraints, for example, the amino acid sequence of the spidroins cannot contain segments that are hydrophobic according to the biological hydrophobicity scale since such segments are inserted in the endoplasmic reticulum membrane during translation.[137,138] Furthermore, the extensive length of the spidroin repetitive region could indeed be necessary for maximized intermolecular interactions and thereby high tensile strength, but could also be a consequence of genetic mechanisms that propagate repeats along a gene.[139] We suggest that it may be possible to engineer spidroins that are shorter, but that form stronger local intermolecular interactions and thus could represent the best of two worlds—efficient production of soluble proteins in heterologous hosts and formation of fibers with increased intermolecular interactions. Below, we first discuss sequence motifs and their effect on artificial spider silk fibers and then propose three approaches to overcome current challenges: protein engineering to increase the steric zipper formation, the introduction of non-spidroin repeats, and the incorporation of non-natural amino acid residues.

4.1. Artificial Spider Silk Production Using Native Major Ampullate Spidroin Amino Acid Sequences

The recombinant spidroins that are used to produce artificial spider silk fibers differ in their molecular weight, number, and length of poly-Ala repeats, number, length, and composition of the Gly-rich region, as well as the absence, presence, and origin of the terminal domains (Table 1). These differences may influence the mechanical properties of the spun fibers. In theory, large silk proteins would have a higher number of intermolecular interactions which could be beneficial for fiber mechanical properties, and this has been verified in several studies where artificial spider silk fibers have been spun from proteins of different molecular weights. For instance, the tensile properties of synthetic pyriform spider silk increase with larger repetitive regions,[140] fibers made from ≈284 kDa protein has a higher strength (550 MPa) compared to fibers made from a smaller 50 kDa protein (<100 MPa),[34] while a fiber made from a 556 kDa silk protein reaches an impressive strength of 1031 MPa.[33] Even when using non-natural amyloid peptides to replace the poly-Ala segments, the same trend is manifested; ≈47 kDa proteins can be spun into fibers with a strength of up to 250 MPa, while an ≈378 kDa protein forms fibers reaching 980 MPa.[122] Pearson correlation analysis of currently available data on the mechanical properties of artificial spider silk fibers (**Table 2**; Figure 6) confirms that a higher number of poly-Ala repeats, and thereby molecular weight of the recombinant spidroin, is associated with increased fiber strength, but only for post-spin stretched fibers (**Figures 5** and **6**). Interestingly, for as-spun fibers the correlation is negative, suggesting that post-spin stretch is necessary for the poly-Ala regions to orient and align correctly, in line with the theory of molecular alignment in relation to mechanical properties that is discussed in the previous section. Increased number of residues in the poly-Ala blocks correlates with increased strain at break for as-spun fibers but not for post-spin stretched fibers. A possible explanation for this observation could be that the poly-Ala segments are in α-helical conformation in the as-spun fiber

Table 2. Recombinant spidroins based on MaSp sequences, their origin, properties, and the mechanical properties of fibers spun thereof. Based on refs. [33–37,39–44,56,63,64,109,110,113,119,120,125,127,131,140–151]. Studies producing artificial silk but not performing mechanical tests were excluded. Fibers with the highest strength or toughness modulus are shown if several fibers were reported in one study. "Preserving native structure" refers to the production method, i.e., if the proteins were kept natively folded in the purification process and the spinning bath did not include alcohol. Mw: Molecular weight. YM: Young's modulus. N.s.: not stated. (-): not applicable or not performed. § titanium oxide was incorporated in the fibers and fibers were cross-linked. Grip et al.[143] state that comparisons with the literature should be made with caution. Mechanical properties from Kono et al. were estimated from stress–strain curves.

Reference	Silk type	NT	CT	Mw [kDa]	Number of poly-Ala regions	Poly-Ala length	Preserving native structure	Strength [MPa]	Strain at break [%]	Toughness modulus [MJ m^{-3}]	Diameter [µm]	YM [GPa]	Strength [MPa]	Strain at break [%]	Toughness modulus [MJ m^{-3}]	Diameter [µm]	YM [GPa]
Lazaris, 2002 (145)	MaSp2			60			+	n.s.	n.s.	n.s.	n.s.	n.s.	227	43	106	20	5
Teule, 2007 (151)	MaSp, Flag			62	20	8	–	50	15.8	11	16	1.1	–	–	–	–	–
Stark, 2007 (148)	MaSp1		+	24	4	13.5	+	n.s.	n.s.	n.s.	n.s.	n.s.	200	8	–	80	7
Grip, 2009 (143)	MaSp1		+	24	4	13.5	+	–	–	–	–	–	110	1	n.s	40 to 90	12
Xia, 2010 (34)	MaSp1			285	96	5	–	n.s.	n.s.	n.s.	n.s.	n.s.	508	15	82	n.s.	21
An, 2012 (141)	MaSp1			70	24	6	–	36	3.1	0.9	40.9	2.8	133	23	24	17	5.7
Teule, 2012 (149)	MaSp2, Flag			58	14	8	–	28	1.7	0.3	68.3	1.1	128	52	55	28	4.4
Albertson, 2014 (64)	MaSp2			87	8	8	–	14	1.2	0.1	62.1	1.9	39	181	59	36	1.6
Heidebrecht, 2015 (39)	MaSp2		+	60	12	6	–	50	7	2	39	2.0	148	159	172	26	3
Heidebrecht, 2015 (39)	MaSp2	+	+	134	12	6	–	12	6	0.3	155	0.5	123	200	189	27	4
Jones, 2015 (110)	MaSp1, 2			62.5	n.s.	n.s.	–	n.s.	n.s.	n.s.	n.s.	n.s.	192	28	34	n.s.	8.3
Copeland, 2015 (109)	MaSp1, 2			65	n.s.	n.s.	–	33	1.1	0.2	60.6	3	222	56	103	29	n.s.
Peng, 2016 (56)	MaSp1			47	16	5	–	n.s.	n.s.	n.s.	n.s.	n.s.	286	18	38	14	8.4
Lin, 2016 (146)	MaSp, AcSp		+	64	4	8	+	–	–	–	–	–	21	28	5	3	0.8
Thamm, 2017 (113)	MaSp1s	+	+	43	–	–	–	31	4	0.9	77	0.9	101	177	144	49	1.5
Lyda, 2017 (147)	MaSp1	+	+	60	8	5	–	148	4	3.5	85	4.6	–	–	–	–	–
Lyda, 2017 (147)	MaSp2	+	+	n.s.	n.s.	n.s.	–	67	2	8.1	85	1.2	–	–	–	–	–
Andersson, 2017 (35)	MaSp1, MiSp	+	+	33	2	14.5	+	162	37	45	12	6.0	–	–	–	–	–
Bowen, 2018 (33)	MaSp1			556	192	5	–	n.s.	n.s.	n.s.	n.s.	n.s.	1031	18	114	6	13.7
Zhou, 2018 (150)	MaSp1, AcSp1, MiSp	+	+	104	–	–	–	245	280	51	1.5	6.7	–	–	–	–	–
Gonska, 2020 (37)	MaSp1, MiSp	+	+	33	2	14.5	–	8	90	6	38	0.3	29	20	4	27	0.5
Zhang, 2020 (119)	MaSp1, MaSp1s, MiSp	+	+	42	–	–	–	53	97	3.6	92	1.7	–	–	–	–	–
Zhu, Rising, 2020 (140)	MaSp1, PySp, MiSp			92	–	–	–	119	70	62.7	7.4	3.9	–	–	–	–	–
Zhu, Sun, 2020 (120)	n.s.			93	n.s.	n.s.	–	48	30	94	9	3.1	–	–	–	–	–
Zhu, Sun, 2020 (120)	n.s.			93	n.s.	n.s.	–	167	197	249	8.9	6.3	–	–	–	–	–
Xu, 2020 (42)	MaSp1, Flag	+	+	43	4	8	+	149	18	21	2.2	7.3	–	–	–	–	–
Greco, 2020 (131)	MaSp1, MiSp	+	+	33	2	14.5	+	80	37	40	15	2.4	–	–	–	–	–
Finnigan, 2020 (142)	MaSp1	+	+	35	3	6.5	+	40	3	3.9	28	1.8	–	–	–	–	–
Hu, 2021 (40)	MaSp1	+	+	65	16	5	–	n.s.	n.s.	n.s.	n.s.	n.s.	289	47	101	34	3.8

(*Continued*)

Table 2. (Continued).

Reference	Silk type	NT	CT	Mw [kDa]	Number of poly-Ala regions	Poly-Ala length	Preserving native structure	Strength [MPa]	Strain at break [%]	Toughness modulus [MJ m^{-3}]	Diameter [µm]	YM [GPa]	Strength [MPa]	Strain at break [%]	Toughness modulus [MJ m^{-3}]	Diameter [µm]	YM [GPa]
Kono 2021 (43)	MaSp2	+	+	50	9	8.5	–	200	15	25	n.s.	n.s.	–	–	–	–	–
Saric, 2021 (4	MaSp1, 2	+	+	60	–	–	–	–	–	–	–	–	605	38	143	27	5
Kvick, 2021 (144)	MaSp1		+	23	4	13.5	+	60	1	–	40-75	2-8	–	–	–	–	–
Cheng, 2022 (44)	MaSp1	+	+	65	16	5	–	65	9	4.3	28.2	3	68	82	45	n.s.	2.7
Jin, 2022 (125)	MaSp1			43	16	5	–	n.s.	n.s.	n.s.	n.s.	n.s.	169	52	70	n.s.	4.5
Jin, 2022 (125)	n.s.			169	64	5	–	n.s.	n.s.	n.s.	n.s.	n.s.	170	59	75	n.s.	4.5
Cheng, 2022 (44)	n.s.			n.s.	16	5	–	–	–	–	–	–	218	94	160	n.s.	4.9
Asakura, 2022 (127)	MaSp			210	73	6	–	–	–	–	–	–	286	31	n.s.	35.3	n.s.
Arndt, 2022 (36)	MaSp1, MiSp	+	+	33	2	14.5	+	132	160	146	4.16	3.5	–	–	–	–	–
Schmuck, 2022 (63)	MaSp1, MiSp	+	+	33	2	14.5	+	110	113	90	8.8	2.2	196	86	129	n.s.	4.5
Schmuck, 2022 (63)	MaSp1, MiSp	+	+	33	2	14.5	+	–	–	–	–	–	261	32	68	6.6	4.7

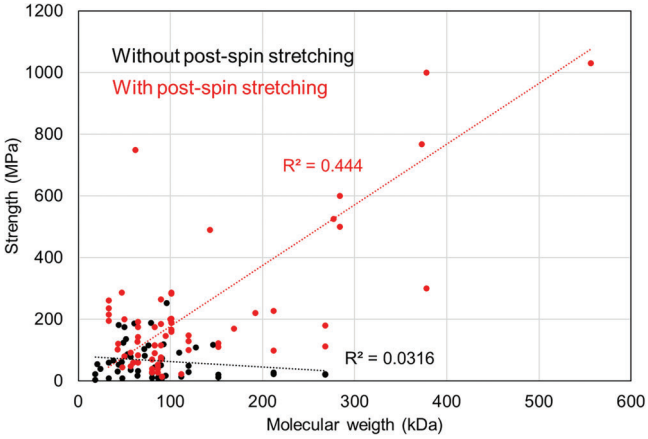

Figure 5. Strength versus molecular weight of recombinant spidroins, using average values for stress at break obtained from the literature. Data was obtained from refs. [33,34,36–39,44,54,56,64,107–131].

and thereby a bit more extensible and less ordered than the corresponding β-strand which presumable could form during post-spin stretch of the fiber.[46] Thus, for the production of high tensile strength fibers, large recombinant proteins are well suited, however, long and repetitive proteins are difficult to produce in heterologous hosts which may prevent this strategy from being developed into an industrialized process.[29,31]

As described above, the terminal domains play a crucial role in the assembly of native silk fibers, but in theory, could also influence the mechanical properties of the fibers. However, in the published literature, we found no correlation between the mechanical properties and spidroin architecture (Figure S1, Supporting Information), with the exception of as-spun fibers made from spidroins without the terminal domains which tend to have higher strength than those containing both terminal

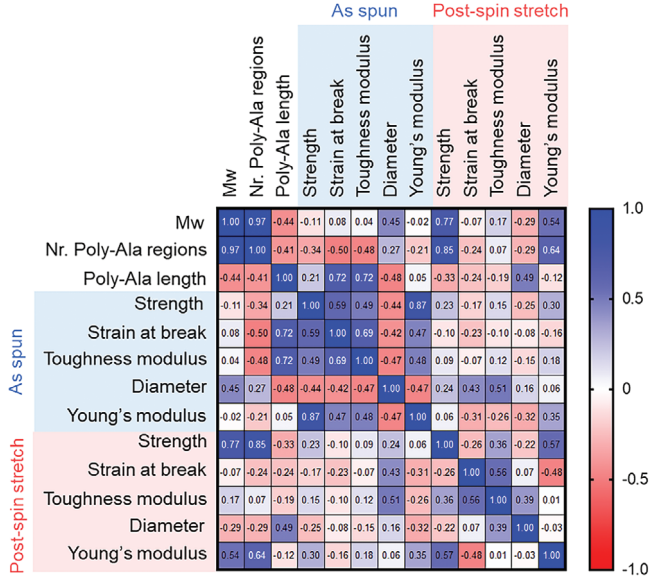

Figure 6. Pearson correlation matrix of recombinant spidroin sequence properties and the resulting fiber mechanical properties. Data was extracted from Table 2.

domains or only the C-terminal domain (Figure S1A, Supporting Information). The lack of a clear effect of the presence of the terminal domains on the fibers' mechanical properties could be due to that these indeed have a minor impact on fiber mechanics and that their main function lies in the control of spidroin polymerization, but it could also be a result of the diverse and sometimes denaturing spinning conditions used, which would prevent proper functionality of the globular terminal domains.

4.2. Increasing the Propensity to Form Steric Zippers

As mentioned above, the poly-Ala segments of the spidroins form crystals that are held together by steric zippers.[17,22,24,152,153] Steric zippers have primarily been studied in protein nanofibrils (amyloid fibrils) that spontaneously self-assemble from amyloidogenic proteins and peptides. The protein nanofibrils are composed of tightly packed β-sheets that can reach several μm in length,[154] where the side chains of two β-sheets are interdigitated, which means that the side chains of one sheet fills the gaps around the α-carbons of the neighboring sheet and create a dry, tight packing.[154,155] Examples of amino acid residues that can be involved in interdigitation are unbranched residues such as Ala, Asn, and Gln, as these enable tight packing due to their relatively small size and their ability to form hydrogen bonds.[156–158] On the other hand, branched, non-polar side chains such as Val, Leu, Ile, and the aromatic Phe may contribute to the formation and stabilization of steric zippers as they not only maximize the hydrophobic effect and support close contact in which van der Waals interactions are enabled,[157] but also have a higher propensity to form β-sheets.[159] This notion is supported by the fact that amyloid fibrils assembled from protein segments containing residues with hydrophobic side chains are more stable than those assembled from segments composed of more polar residues.[160,161] For instance, non-disease related functional amyloids are known to contain residues with charged and polar side chains, which makes fibrillation controllable, and in some cases even unstable and reversible.[162,163] The high content of interdigitated β-sheets makes protein nanofibrils both stiff and strong,[164–167] and thereby interesting from a protein engineering perspective.[168–171] For instance, a Young's modulus of 3.7 GPa was reported for fibrils made from β-lactoglobulin,[164] a corresponding value of 14 GPa was reported for fibrils made from a 10 amino acid segment from transthyretin,[165] and HET-S (a prion of the fungus *Podospora anserina*) makes nanofibrils with a strength of almost 2 GPa and a Young's modulus of 10 GPa.[166]

Structurally, however, there are several differences between silk and protein nanofibrils. First, the silk contains both nanocrystals made of β-sheets, but also less ordered amorphous regions. Second, the β-sheets are aligned parallel to the fiber axis instead of perpendicular as is the case in protein nanofibrils.[172,173] Third, as already discussed, the height of individual stacked β-sheet crystals is substantially smaller in the silk fiber, reaching only a few nm, and the entire silk fiber is several micrometers thick instead of a few nm as is the case for protein nanofibrils.

Nevertheless, the similarities between the structures is the basis for the spidroin-amyloid "mash-up" concept where the poly-Ala blocks in spidroins are replaced by amyloidogenic peptides (**Figure 7**) to increase the β-strand propensity and favor interdigitation of the side chains.[21] This approach could in theory make use of both the high strength and stiffness of amyloids and the extensibility and flexibility of the silk fiber. Recently, macroscopic fibers were spun from such 378 kDa hybrid proteins containing 128 amyloidogenic peptides linked via spidroin Gly-rich regions.[122] The fiber had impressive mechanical properties with a tensile strength of ≈1 GPa and a strain at break of >20%. The fibers were spun by first dissolving the lyophilized protein in hexafluoroisopropanol and then extruding it into 95% methanol, followed by post-spin stretching the fiber 4–6 times its original length in a methanol bath. This method of spinning and the strength of the resulting fibers is virtually identical to the methods used for producing artificial silk fibers in Bowen et al.[33] (0.98 ± 0.08 GPa vs 1.03 ± 0.11 GPa). Interestingly, Bowen et al. used recombinant spidroins encompassing 192 repeat segments (556 kDa), which is 47% larger compared to the spidroin-amyloid hybrid. Noteworthy these two papers are the only examples in the literature where fibers made exclusively from recombinant proteins reached a tensile strength close to or exceeding 1 GPa. These results also suggest that long protein chains may be needed to generate fibers with high tensile strength, as already discussed in Section 4.1. To assemble long recombinant spidroins, recently described innovative strategies to improve the end-to-end protein–protein interactions could be employed. For instance, Li and coworkers designed a 60 kDa amyloid-silk hybrids that were N-terminally and C-terminally tagged with self-interacting mussel foot protein fragments.[62] By doing so, fibers with an average tensile strength of 481 MPa were obtained. Another impressive strategy was developed by Fan and coworkers, who used covalent protein conjugation orthogonal Catcher/Tag pairs to assemble a 319 kDa spider-silk-like protein.[174]

An alternative but similar approach to the spidroin-amyloid "mash-up" is to increase the strength of artificial spider silk is to use rational protein engineering to increase inter-sheet interactions and steric zipper packing.[36,61] Ala may not be the amino acid residue of choice if the aim is to form strong β-sheet crystals since Ala inherently possesses a low preference for adopting a β-strand conformation.[159] Interestingly, if Ala is replaced by Val or Ile, the propensity to form both ß-strands and steric zippers would be increased according to predictions by the Zipper database.[36,61] This idea was investigated by Arndt and coworkers by making a set of 15 mutant mini-spidroins carrying Ala to Ile, Val, or Thr substitutions, respectively.[36] It was shown that fibers spun from the mutants indeed had higher tensile strength, but that the strain varied. Fibers spun from two of the mutants carrying three Ala to Ile mutations in the poly-Ala blocks showed increased strength and strain at break, and the toughness modulus of the fibers reached the same level as the native major ampullate silk.[36]

The use of amyloidogenic or engineered peptide segments as promoters for β-sheet crystal formation and as a tool to increase the strength of artificial spider silk should be investigated further, especially if the amyloidogenic peptides can improve the tensile strength of fibers spun from water soluble and small

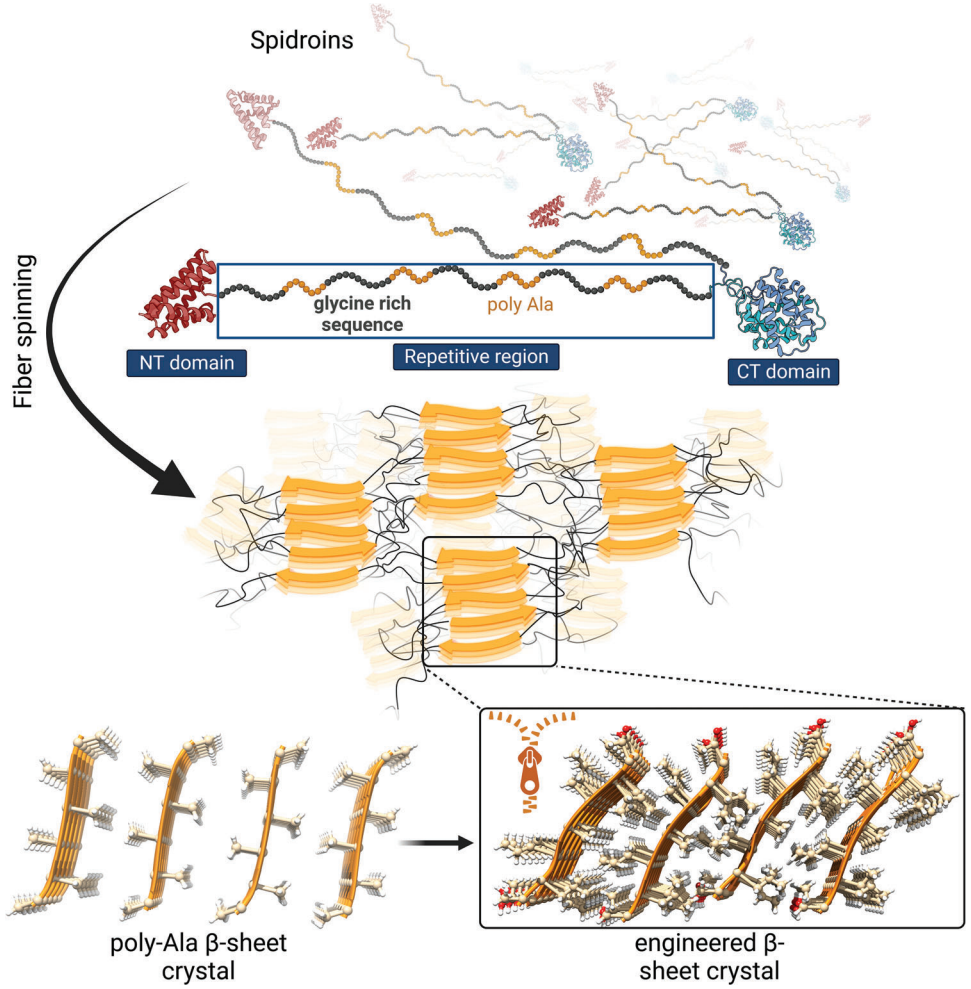

Figure 7. Schematic representation of a strategy to increase the strength of artificial silk fibers by engineering the poly-Ala segments of spidroins. Here, the poly-Ala is replaced with peptides that have a much higher propensity to form β-sheet crystals where they should adopt a steric zipper packing arrangement that increases the inter-sheet interactions in the mature fiber. By doing so, the strength of the β-sheet crystal could potentially be increased. NT domain PDB entry number: 4FBS; CT domain PDB entry number: 2MFZ. The steric zipper is represented by an amyloidogenic peptide from Transthyretin with the PDB entry number: 6C4O. The poly-Ala zipper was obtained from the Zipper database. The protein structures were illustrated with ChimeraX and the final image was assembled with BioRender.com.[315]

recombinant spidroins. In addition, replacing poly-Ala blocks with such segments may not only render stronger fibers but could potentially also solve the problem with the low yields obtained when expressing large spidroins since it would reduce the demand for alanyl-tRNA during translation.

4.3. Incorporating Reactive Amino Acids into Spidroins

Covalent bonds are present in natural spider silk, for instance as disulfide bridges connecting two C-terminal domains in order to form stable dimers of MaSps.[175,176] Recent results also suggest that a large number of Cys are found in the repeat region of aciniform spidroins from *A. ventricosus*,[177] and that Cys residues are common in the SpiCE proteins.[43,94] To understand the contribution of disulfide bonds, but also to reinforce artificial spider silk, a single Cys residue was introduced into a CT domain that naturally lacks Cys.[108,143,178] When such engineered Cys-containing spidroins were wet-spun, the obtained fibers were stiffer and stronger, but less extensible compared to reference fibers.[108] Crosslinking of spidroins has also been achieved by replacing Ala with Cys in the repetitive region, which resulted in fibers that were 37% stronger than fibers made from reference proteins.[143] Fibers with an improved mechanical performance were also obtained by electrospinning spidroin constructs that consisted of three MaSp repeats in which a single Ser was mutated to Cys.[179] Apart from these examples, there are comparatively few reports that attempted to introduce Cys into spidroin constructs for strengthening artificial silk fibers. The reason may be attributed to the challenging expression and purification of spidroins carrying Cys residues, which usually lead to the formation of inclusion bodies,[180] lower expression yields, or disulfide mispairing.[181] Even though a wide selection of strategies exists for tackling these challenges,[181,182] for instance, the use of Origami or SHuffle T7 cells for recombinant protein expression which promote correct disulfide bond formation in the

cytoplasm, no effective production method for these proteins has been reported so far.

Another amino acid residue that could be utilized for crosslinking the proteins in artificial silk fibers via covalent bonds is Tyr. Dityrosines are structural elements in several natural materials, such as resilin,[183,184] collagen,[185] and silkworm silk, where they reduce lateral chain fluctuations to aid β-sheet formation,[186] and they are present in native major ampullate silk.[187] Interestingly, the location of the Tyr in the repeats is conserved, which points to an important function, but there is still scarce experimental work that validates the presence of dityrosines in the native spider silk fiber, and their potential contribution to the mechanical properties remains to be elucidated.[70] Intuitively, though, the formation of dityrosines in the silk fiber would increase the intermolecular interactions and thereby the strength of the fiber. Tyr residues are frequently occurring in the repetitive region of many spidroins[43,86,91] such as MaSp1, MaSp2, and MaSp4, and thus protein engineering is likely not needed to introduce this residue. Surprisingly, despite the potential of using dityrosine crosslinking, there are no examples in the literature where this strategy has been attempted to improve the mechanical properties of artificial silk fibers. However, in other protein-based materials dityrosines have been chemically crosslinked in order to improve the mechanical properties, which is reviewed in Section 5.2.

Beyond the limited selection of reactive proteinogenic amino acid residues, an unexplored method for crosslinking proteins for making artificial spider silk fibers is the use of non-natural amino acids.[188] Typically, non-natural amino acid crosslinking reactions are induced with light which gives good spatiotemporal control. The method is compatible with physiological conditions and has successfully been used to analyze transient biomolecular interactions in living cells.[189–191] However, there are also examples in the literature that employ photochemical non-natural amino acids for making materials. For instance, intrinsically disordered peptides that have been engineered to contain photoreactive non-natural amino acid residues, self-assembled into nano- to micrometer-sized spheres depending on the conditions and could be converted into solid beads by photochemical crosslinking.[192] An obvious disadvantage of using this strategy is that the process is probably not scalable due to the high cost of the non-natural amino acid residues. For price-sensitive applications, like, for example, the textile industry, this approach of crosslinking to strengthen artificial silk fibers would not be feasible.

Another caveat to the approach of internal crosslinking with reactive amino acids is that it might be important where in the sequence the individual proteins are cross-linked with each other. For instance, if the amorphous region is randomly crosslinked one would expect a brittle material that is not necessarily stronger. Therefore, a crosslinking strategy must be carefully planned and based on structural information, which is why an in-depth discussion about this topic will be provided in the next section.

5. Crosslinking

To obtain artificial spider silk fibers with improved mechanical properties, post-spinning treatments that crosslink the proteins can complement post-spin stretching and protein engineering approaches. In this review, crosslinking will be defined as the process of linking proteins by the formation of intramolecular or intermolecular non-covalent and covalent bonds.[193,194] Physical crosslinking (non-covalent) refers to the formation of molecular interactions (e.g., hydrogens bonds, electrostatic and hydrophobic interactions, etc.) that are triggered by changes in temperature, pH, solvation state, or mechanical forces. Chemical crosslinking, on the other hand, refers to covalent bonds formed through a chemical reaction between reactive functional groups in proteins. These reactions can be triggered by ionizing radiation or a crosslinking reagent (crosslinker), which may or may not be part of the final product.[68,195–197]

Crosslinking is a common strategy to improve the mechanical properties of biopolymers for various applications, for instance in scaffolds for tissue engineering, bioinks for 3D printing, and biodegradable plastic materials.[198–201] Inspired by previously used crosslinking strategies for protein and carbohydrate-based materials, we will explore possible applications of crosslinking to improve the properties of artificial spider silk fibers. In line with the main scope of this review, crosslinking examples were selected for having specifically attempted to improve the mechanical and/or textile properties of silk fibers or fibrous material. Aspects as mild reaction conditions and scalability were also taken into consideration. Examples from studies on *B.mori* silk will also be included since the methods used are likely applicable to both types of materials.

5.1. Physical Crosslinking

Labile intermolecular interactions are the main driving force behind physical crosslinking. Examples include hydrogen bonding, ionic and hydrophobic interactions, π–π stacking, stereocomplex formation, and metal ion coordination.[196] When considering silk-based materials, the formation of β-sheet nanocrystals among the repetitive regions of fibroins and spidroins is the most prominent physical crosslink holding the polymeric chain together.[25] Thus, factors that promote spidroin self-assembly into β-sheet crystallites (mechanical forces, pH changes, alcohols, water removal, etc.) are used to fabricate different materials such as gels, films, and fibers.[68,202]

As previously described, spider silk proteins are particularly sensitive to pH changes. In the major ampullate gland, exposure to a lower pH leads to dimerization of the NT domain,[11,12,15] and thereby the dimeric spidroins are linked together in networks. Acidification also promotes unfolding of the CT domain and the formation of β-sheet fibrils.[13] It is hypothesized that these fibrils could act as nuclei for the formation of β-sheets crystals in the repetitive region, promoting fiber solidification.[30] In this sense, pH changes could be regarded as a natural dual-mechanism physical crosslinking method. Spinning artificial fibers under acidic conditions has been reported for methods using aqueous recombinant spidroin solutions as feed-stock and is appealing due to the similarity to the native silk spinning in that the process is void of toxic chemicals. pH induced polymerization requires natively folded terminal domains and therefor works for mini-spidroins that can be purified in native conditions and concentrated to 30% w/v or more in aqueous buffers.[35,36,119,203]

Methanol and ethanol are commonly used to induce conformational changes in silk proteins.[204,205] Different alcohols are known to stabilize certain types of secondary structures of proteins in solution[206] and improve the mechanical properties (notoriously, the strength) of both artificial[207] and native silk fibers[208,209] (Figure 4). Methanol and ethanol are used when spinning from regenerated *B. mori* silk, while isopropanol is preferred for regenerated spider silk or recombinant spidroins solutions in hexafluoroisopropanol[29,210] (HFIP). It is argued, however, that alcohol treatment fails to fully restore silk's native hierarchical assembly, which could explain why fibers spun from regenerated dope usually do not replicate the strength and extensibility of native fibers. A proposed solution by Yazawa et al.[211] was to spin fibers in a coagulation bath of tetrahydrofuran (THF) that does not promote β-sheet formation. Crystallization was then induced in a controlled manner by a combination of post-spin stretching and a second coagulation bath, containing either THF or water. Compared to native silk, the resulting fibers displayed higher extensibility and improved toughness, despite lower strength. Interestingly, a lack of clear correlation between crystallinity, strength, and spinning conditions highlights the need for further understanding of how protein hierarchical assembly in the fibers correlates to the resulting properties.

Alcohols are frequently used as coagulants for spinning dopes made of aqueous recombinant spidroin solutions.[29,210] However, the resulting fibers differ significantly in terms of mechanical properties which could depend on the diverse spinning conditions used[39,56,110] (Table 2). And, as previously discussed, alcohol treatment is often combined with post-spin stretching, which makes it difficult to properly pinpoint the factors influencing fiber properties. Being such a popular strategy for silk spinning, it would be interesting to investigate the effect of alcohols on their own and in combination with stretching to align the proteins before crosslinking. This method would also be a cost-effective and non-hazardous post-spin treatment.

To the best of our knowledge, treatment with alcohols is the most investigated physical crosslinking method used to improve the mechanical properties of artificial spider silk fibers. Several additional strategies to physically promote and accelerate the formation of β-sheets in silk proteins have been reviewed/described, but are mostly used for the fabrication of other non-fiber materials (i.e., hydrogels or films).[212–214] Such strategies include: increased temperature; vortexing and sonication; electrogelation, addition of surfactants or salts (e.g., Ca^{2+}), and water removal.[68] Nonetheless, as silk hydrogels and fibers are fundamentally different materials, any extrapolation on the application of these methods to prepare improved artificial fibers by wet spinning process is highly speculative and requires further investigation.

5.2. Chemical Crosslinking

The most common crosslinking agents that target biopolymers (carbohydrates and proteins) include glutaraldehyde (and other reactive aldehydes such as formaldehyde), carbodiimides, genipin, acrylates, and polycarboxylic acids. In addition, methods employing enzymatic (e.g., horseradish peroxidase) and photoactivated reactions (e.g., ruthenium complexes) are examples of chemical crosslinking strategies.[67] Crosslinking requires the presence of functional groups that can react with the crosslinker. In proteins, these are the nucleophilic side chains of amino acid residues, from which the most reactive representatives are the ϵ-amino groups from Lys and the thiol group from Cys.[215] The occurrence of these residues is generally low in spidroins and fibroins, but residues with other reactive groups, that is, carboxylates (Asp and Glu) and hydroxyl-containing amino acid residues such as Ser, Thr, and Tyr are more common.[216,217] Therefore, to efficiently target silk proteins, crosslinking strategies should consider the amino acid sequence and structural information, regarding the availability and accessibility of reactive residues. In this section we will discuss suitable crosslinking strategies based not only on their feasibility from a chemical-biology point of view, but also consider reported attempts to crosslink protein-based fibrous materials.

5.2.1. Reactive Aldehydes

Aldehydes are known to form crosslinks with amino groups from Lys residues. However, depending on the reaction conditions they could also target hydroxyl groups,[194] making them suitable candidates for crosslinking silk proteins. Glutaraldehyde is the most widely used reagent to crosslink proteins, but other reactive aldehydes include formaldehyde and glyoxal.[67,68,218]

Glutaraldehyde has been used as a chemical crosslinker in preparing different materials from regenerated *B. mori* silk, such as hydrogels,[219] films,[220] and electrospun silk fibroin/gelatin nanofibers mats.[221] In the later examples, crosslinking was associated with the structural transition from random coil to β-sheets. Glutaraldehyde vapor has also been described for the crosslinking of recombinant spider silk fibers prepared by electrospinning.[222] The authors reported improved mechanical properties (tensile strength and Young's modulus) and increased β-sheet content for fibers that were treated for 6 h while retaining biocompatibility for stem cell culture.

Formaldehyde is also a reactive, but toxic, aldehyde with protein crosslinking properties. It has been used for wet-spinning a recombinant minispidroin solution (in HFIP) into a methanol/formaldehyde coagulation bath.[120] Including the crosslinker in the coagulation bath increased the strength of the fiber by a factor of four (from 48 to 211 MPa), compared to when the fibers were spun into a methanol bath without the crosslinker. Despite a three-fold reduction in strain at break (≈3% to 1%), the toughness modulus was increased from 94 to 163 MJ m^{-3}. Fiber characterization by Fourier Transform Infrared Spectroscopy (FTIR) revealed no differences in β-sheet content. On the other hand, an increment in crystallinity, observed by wide-angle X-ray diffraction, was associated with increased strength in fibers spun in formaldehyde.

Glyoxal is considered less toxic than glutaraldehyde and formaldehyde, but it retains similar crosslinking properties.[223] Glyoxal has been deployed as a crosslinking agent in the preparation of various protein films.[224–226] Regarding silk-based materials, glyoxal has also been effective in crosslinking fibroin, collagen, and chitosan into composite scaffolds that were cytocompatible.[218] Glyoxal is also reactive toward hydroxyl groups, making it a suitable crosslinker for polysaccharides such as poly(vinyl alcohol)[227] and galactoglucomannans.[228] Thus, it

is reasonable to speculate that it should crosslink spidroins, due to the abundance of hydroxyls from Ser and Thr residues. Despite the potential reactivity and application of glyoxal in crosslinking artificial silk fibers remain to be investigated.

To address the health hazards associated with synthetic aldehydes, Mu and co-workers[229] describe a sucrose-based crosslinking method for silk fabric. The oxidation of sugarcane sucrose with sodium periodate results in reactive formyl-saccharides that can act as crosslinkers for the fibroins' hydroxyl groups. The addition of diols (e.g., ethylene glycol) also proved beneficial as these act as crosslinking extenders and stabilizers, by the formation of more water-resistant acetal bonds. Silk fabrics crosslinked using this method have improved water resistance while retaining the desired textile properties.

5.2.2. Targeting Carboxylates

The carboxyl groups from Glu and Asp residues, and the C-terminus, display low reactivity in aqueous solutions. To overcome this problem, the activation of carboxylates using carbodiimides is a widely used approach to target these residues in protein chemistry.[230] Carboxylic acids react with carbodiimides, as 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC), to form O-acylisourea intermediates, which can, in turn, react with unionized primary amines yielding stable amides.[231]

Water-soluble EDC is an appealing crosslinking agent for structural proteins, such as collagen. Reactions yield a "zero-length" crosslink in the form of an isopeptide bond between adjacent carboxylates and Lys residues. The resulting urea derivative can be removed by rinsing and no toxic by-products are left behind.[232,233] However, compared to other reagents (e.g., glutaraldehyde), EDC crosslinks yield only a modest increase in fiber strength and strain at break.[234,235]

Data on carbodiimides crosslinking of silk fibers is scarce, but reports on EDC crosslinking of silk fibroin electro-spun nanofiber scaffolds (for cell growth and tissue engineering) share similar trends.[236–238] Using regenerated *B. mori* silk solutions, three studies describe the fabrication of nano-fibrous matrices by electrospinning, followed by crosslinking using EDC and N-hydroxysuccinimide (NHS), and report on the generation of fibrous materials with improved mechanical properties in terms of both strength and extensibility. However, the lack of information on how the tensile parameters were calculated makes any extrapolation to individual fibers highly speculative.

In summary, despite being a popular class of crosslinking reagents for proteins, the feasibility, effectiveness, and cost of carbodiimide crosslinking applied to artificial spider silk fibers remain to be investigated.

5.2.3. Targeting Hydroxyl Groups

Due to their low nucleophilicity and the presence of water, Ser and Thr residues remain elusive targets in selective reactions.[239] Thus, taking inspiration from crosslinking methods for polysaccharides could open opportunities to find reagents that target the abundant hydroxyl groups in silk proteins. Apart from reactive aldehydes, other crosslinking reagents for polysaccharides include polycarboxylic acids (PCAs), boric acid, sodium trimetaphosphate, and ammonium zirconium carbonate.[67,240]

Among these alternatives, PCAs stand out for being safe, cheap, and reactive toward several types of proteins. The carboxylic groups in citric acid (CA) and 1,2,3,4-butanetetracarboxylic acid (BTCA) can react with hydroxyl groups under anhydrous conditions, to form ester bonds, releasing water as a by-product. Thus, PCA crosslinking usually requires a curing step with temperatures above 120 °C.[67,201] Despite this limitation, crosslinking with citric acid has been successfully applied to improve the mechanical properties of regenerated protein fibers (e.g., collagen, gliadin, and casein), with results depending on the protein type and reaction conditions.[241–243]

Citric acid and BTCA crosslinking have been attempted in silk fabrics to increase the wrinkle-recovering angle in wet conditions.[244–246] Interestingly, the need for an acid catalyst (sodium hypophosphite) and a curing step was considered to be important for the resulting mechanical properties of the material. Upon CA and BTCA crosslinking using sodium hypophosphite and curing temperatures varying from 130 to 175 °C, the silk fabric displayed reductions in breaking strength of 14% for CA[245] and up to 30% using BTCA.[244] On the other hand, CA crosslinking was shown to increase silk fabric strength by 15%.[245] To avoid the reduction in breaking strength, Reddy and co-workers[246] described an alkali-catalyzed CA crosslinking for silk fabric under low temperatures (below 50 °C). The crosslinked silk did not only exhibit the intended increase in wrinkle recovery angle but also displayed improved breaking strength (20%) and tear strength (15–20%).

PCA crosslinking is appealing for being safe, cost-effective, and likely compatible with large-scale production. Evidence suggests it can improve the textile properties of silk fibers, however, a significant challenge lies in optimizing reaction conditions.

5.2.4. Targeting Tyrosines

Compared to other hydroxyl-containing residues, the phenol group of Tyr is a more reactive nucleophile, particularly in its ionized phenolate state.[217,247] Various strategies to chemically label Tyr residues (diazonium salts, palladium complexes, boronic acids, ruthenium photocatalysts, etc.) have been reported, but a Tyr-specific crosslinking reagent was only recently described, for applications in protein mass spectrometry.[217,248,249]

On the other hand, proteins naturally form Tyr-Tyr crosslinks (referred to as dityrosines), due to the residue propensity to form tyrosyl radicals upon proton abstraction. Reaction among tyrosyl radicals, from adjacent oxidized Tyr residues, yields a stable dityrosine crosslinked product.[250,251] Dityrosine formation to enhance the properties of protein biomaterials has been attempted and achieved using enzymatic, oxidative, and photochemical methods. Enzymes such as peroxidases (e.g., horseradish peroxidases), tyrosinase, and laccases are metal-containing oxidoreductases that can oxidize Tyr into tyrosyl radicals.[184] Fenton-like redox reactions yielding hydroxyl radicals also can promote tyrosyl radical formation and tyrosine crosslinking.[252] Finally, oxidized Tyr residues and dityrosines can be formed using photocatalysts (e.g., ruthenium complexes, riboflavin, and its

derivatives) upon ultraviolet (UV) and visible light irradiation.[253] Both enzymatic and photochemical methods have been successfully applied to prepare silk fibroin hydrogels[254,255] and matrices for 3D printing.[200]

Liu and co-workers (2022)[256] have described a method to induce dityrosines crosslinks in native *B. mori* silk fibers using a ruthenium photocatalysts, tris(2,2′-bipyridyl)dichlororuthenium(II) (Ru-bpy). Degummed silk fibers were soaked in Ru-bpy and ammonium persulfate in the dark, followed by visible light irradiation for different periods. Treated fibers displayed an increase in Young's modulus, but no significant change in strength. Combining crosslinking and fiber stretching resulted in stronger fibers (60% increase, in dry conditions), however without a stretch-only control group, it is not possible to tell apart the influence of the two treatments.

Tyrosine reactivity and occurrence in the repetitive region of spidroins make them particularly suitable targets for crosslinking strategies. Compared to some other crosslinking methods, dityrosines can be formed under milder conditions, without the need for hazardous chemical reagents. However, despite being a natural component of other protein fibers, the effects of dityrosines crosslinks on the mechanical properties of natural and artificial spider silks are yet to be fully investigated.

5.2.5. Non-Canonical Reactive Handles

Suitable strategies for crosslinking silk fibers discussed so far included employing crosslinkers to bridge together naturally reactive residues found in silk proteins. As an alternative approach, using chemical modification methods to insert new reactive handles on these proteins to promote crosslinking in spun fibers could provide better selectivity and control over the reactions. An example would be the modification of silk proteins to insert methacrylate groups which can undergo radical polymerization (i.e., crosslinking) in the presence of a chemical (e.g., ammonium persulfate) or photo-initiator (UV light).[257–259] Moreover, this strategy has been attempted to modify silk fabric[260] and individual fibers[261] for material functionalization. Chemical modification approaches have also been reported for the fabrication of hydrogels using orthogonal click chemistry.[262] In this context, Ryu et al. described a composite hydrogel prepared by crosslinking chemically modified silk fibroin and polyethylene glycol, using a thiol-ene photo-click reaction.[263] Otherwise, regarding silk proteins, click chemistry approaches have being mainly applied for preparing site-selective bioconjugates[264] and further developments are required to adapt these techniques into new crosslinking methods.

At last, it is important to emphasize that the application of chemical modification strategies to improve the mechanical properties of artificial silk fibers is still speculative and based mainly on non-fibrous materials. Nonetheless, it will be interesting to see how further developments in orthogonal and click chemistries will translate into new tools to modify and improve silk protein fibers.

In summary, reported strategies to crosslink silk fibers are few, especially for artificial spider silk. Evidence from attempts to crosslink silk fabric suggests that, depending on the selected method, this approach can yield improved materials. However, a deeper understanding of the protein structural features and the fiber molecular arrangement is required to further develop this concept. A successful crosslinking method would not only require targeting available reactive residues, but also be compatible with the fiber hierarchical organization. Otherwise, as discussed in previous sections, random or excessive crosslinking could hamper the exact same molecular interactions responsible for the fiber outstanding mechanical properties. In addition, the crosslinking method should fulfill requirements of safety, scalability, and cost-effectiveness if a commercial application is to be achieved.

6. Protein Composites Containing Nanomaterials

Another promising strategy to improve the mechanical properties, in particular strength and Young's modulus, of artificial silk fibers revolves around designing composites with nanomaterials, which should act as nano-sized reinforcing elements.[65] The nanomaterials can be made from different materials, for example, carbon (such as nanotubes or graphene), cellulose, or metals. The reason for the interest in nanomaterials stems from their often-encountered superior mechanical properties. For example, carbon nanotubes are one of the strongest materials (up to 80 GPa in strength),[265] metal nanoparticles, such as magnetite, possess high stiffness (up to 300 GPa in Young's modulus[266]), and nanocellulose is a strong and stiff material that is currently used to improve structural and mechanical properties of many composites.[267–273] Among these different materials, carbon nanotubes are the most extensively used to produce silk fiber composites.[276] Below we address how to manufacture silk-nanomaterials composites by wet-spinning. For other types of processing the reader is referred to work of Wang et al.[276]

The nanomaterials can be added both after the fibers are spun (post-spinning techniques) or to the spinning solution (in-spinning techniques). Post-spinning techniques usually require the exposure of the silk fibers to sputtering, vapor, or solutions containing nanomaterials,[274,277–279] which are relatively easy to perform but may plasticize the fibers and reduce their strength. Moreover, post-spinning techniques also lead to a non-uniform distribution of the nanomaterials, which locally can form agglomerates that reduce the mechanical properties of the fibers.[280,281] Despite these challenges Steven et al. immersed native spider silk fibers in a dispersion of carbon nanotubes obtaining an increase in strain at break of 25%.[274] In contrast to post-spinning treatments, in-spinning techniques usually lead to a more uniform distribution of the nanomaterials in the protein matrix.[282,283] This requires the spinning dope (mixture of proteins and nanomaterials) to remain soluble, which can be challenging if the dope is aqueous based since nanomaterials in general are hydrophobic.[284–287] For example, in order to suspend carbon nanotubes or nanoparticles, organic solvents that are incompatible with natively folded proteins are usually required.[44,275,288–295] However, Fang et al. showed that wet-spinning of regenerated silk fibroin fibers containing multi-walled carbon nanotubes and sodium dodecyl sulphate is possible and renders fibers that are 30% stronger than plain regenerated silk fibroin fibers.[275] Another method for making the nanoparticles more hydrophilic is to engineer the nanomaterial surface, as elegantly shown for iron-oxide nanoparticles that can

be coated with dextran or meso-2,3-dimercaptosuccinic acid to keep them stable in aqueous solutions.[296,297] In addition, Arabic Gum, polydopamine, and dextrin have been successfully used to coat carbon nanotubes to disperse them in aqueous media.[107,298]

Even if the nanomaterials are well dispersed in the spinning dope, the mechanical properties of the outcoming fibers can still be inferior to fibers spun without nanomaterials. In fact, there are several reports of protein-nanomaterial composites in which the mechanical properties are lower than the pure protein material.[55,107,120,129,275,283,288,289,299] This can be due to i) poor alignment of nanomaterials (for those with high aspect ratio) or ii) poor mechanical communication between nanomaterials and protein matrix.[66,280,281,300,301] These two phenomena and methods to avoid them are described in detail below.

If the nanomaterials are longitudinally aligned in the fiber, they contribute more to the mechanical response of the fiber under traction.[302] To have more aligned nanomaterials, the concentration of nanomaterials in the dope can be optimized, that is, lower concentration improves processability and reduces the viscosity of the dope, which makes it easier to align the nanomaterials during the spinning.[303] Alternatively, one can induce higher shear/stretch forces during the spinning to promote the alignment.[304,305] Mohammadi et al.[306] achieved this by increasing the length of the capillary from which the fibers were extruded. Hence, both the concentration of the nanomaterials and the degree of applied shear/stretching forces must be optimized to achieve the best mechanical properties of the composite fibers.[55,307–311]

To improve the mechanical communication between the protein matrix and the nanomaterials, one can target the interface between the two material types. Indeed, if the interaction between the nanomaterial and the protein matrix is weak, the load transfer from the matrix to the nanomaterial will be low, which will make the nanomaterials act as defects rather than reinforcing agents and reduce the mechanical properties of the fibers.[280,300,301] There are a few reports on the design of the interface between carbon nanotubes or inorganic nanoparticles, and silk proteins. A possible way forward could be to coat the nanomaterials with polydopamine,[312] which will interact via hydrophobic interactions with, for example, carbon nanotubes, while still allowing hydrogen bonds to form between the protein chains and the polydopamine.[107] Another method is to covalently link the nanomaterial and dextran,[296] whose hydroxyl groups can form hydrogen bonds to the surrounding protein matrix.[313] When making recombinant silk and nanocellulose composites, a clever way to improve the interaction between this nanomaterial and silk proteins is to use cellulose binding domains.[314] For example, Mohammadi et al.[305] made a composite fiber from a recombinant spider silk protein flanked by two cellulose binding domains and nanocellulose. This approach resulted in fibers that were stronger (+50%) and stiffer (+75%) compared to fibers spun from nanocellulose alone.

7. Conclusion and Outlook

Production of artificial spider silk fibers with properties that match those of the native fiber in a process that is sustainable, economically feasible, and scalable remains an unmet goal in the field of material science. Increased understanding of the natural fiber and its composition, recent success in producing large amounts of recombinant spidroins, and the development of biomimetic spinning processes have resulted in manufacturing methods that are compatible with bulk-scale production of artificial silk fibers. However, these fibers' mechanical properties have to be improved. Here, we review and suggest several strategies to address this challenge, by i) exploring the impact of protein composition, ii) optimizing the spinning methods, iii) employing rational protein engineering spider silk proteins, iv) developing crosslinking methods to increase the intermolecular interactions in the fiber, and v) making a composite fiber. Implementing these strategies individually will most likely not be enough to generate bulk-scale fibers that beat the strength and toughness of native major ampulate spider silk. Instead, the field should focus on generating systematic studies that could lay the foundation for a process design in which the native spinning dope composition is mimicked by engineered spidroins, the spinning and stretching conditions have been optimized, and cross-linking and/or nanomaterials could be used as the final tool to create super-strong fibers. Thus, by continued efforts from the scientific community, the generation of silk fibers with properties that would make them valid replacements for many petroleum-based fibers, is within reach.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

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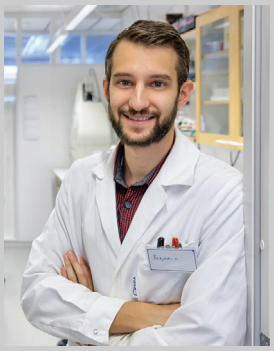

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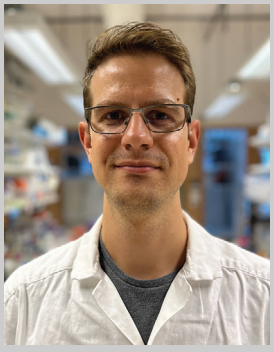

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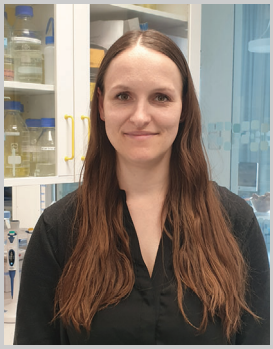

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