

# Spider silk proteins: recent advances in recombinant production, structure–function relationships and biomedical applications

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**Abstract** Spider dragline silk is an outstanding material made up of unique proteins—spidroins. Analysis of the amino acid sequences of full-length spidroins reveals a tripartite composition: an N-terminal non-repetitive domain, a highly repetitive central part composed of approximately 100 polyalanine/glycine rich co-segments and a C-terminal non-repetitive domain. Recent molecular data on the terminal domains suggest that these have different functions. The composite nature of spidroins allows for recombinant production of individual and combined regions. Miniaturized spidroins designed by linking the terminal domains with a limited number of repetitive segments recapitulate the properties of native spidroins to a surprisingly large extent, provided that they are produced and isolated in a manner that retains water solubility until fibre formation is triggered. Biocompatibility studies in cell culture or in vivo of native and recombinant spider silk indicate that they are surprisingly well tolerated, suggesting that recombinant spider silk has potential for biomedical applications.

**Keywords** Spidroin · Protein structure · Recombinant protein production · Biomaterial · Protein self-assembly · Biocompatibility

## Abbreviations

ADF Araneus diadematus fibroin  
LPS Lipopolysaccharide

MaSp Major ampullate spidroin  
Spidroin Spider silk protein

## Introduction

Since ancient times spider silk has fascinated man because of the elegant way in which it combines strength and elasticity, and it has also been ascribed abilities to stop bleeding and promote wound healing [1]. Native dragline spider silk has a higher tensile strength and is stiffer, more extendible and less immunogenic than the commonly used native silkworm silk [2, 3], implying a wider range of applications. While the outstanding mechanical properties of spider silk have been well documented, the suggested utility of spider silk, in particular artificial mimics thereof, still needs to be tested in a rational manner. Spiders are territorial and produce low amounts of silk; therefore, they cannot not be employed for large-scale silk production. This is in direct contrast to the case of the mulberry silk worm *Bombyx mori* (*B. mori*), which is used for the industrial scale production of silk-based textiles and other materials. The practical obstacles to producing spider silk on an industrial scale have been tackled in several ways. One approach is to use *Bombyx* silk and try to make it functionally similar to spider silk. This requires the removal of the immunogenic coat-protein sericin if matrices suitable for biomedical applications are to be obtained. As such, *Bombyx* silk is often dissolved in denaturing solvents, followed by reassembly procedures, to regenerate suitable matrices. Another approach is to dissolve and regenerate silk from non-mulberry silk worms, such as *Antheraea* sp. [4, 5]. *Antheraea* silk is composed of proteins more similar in sequence to spider silk proteins,

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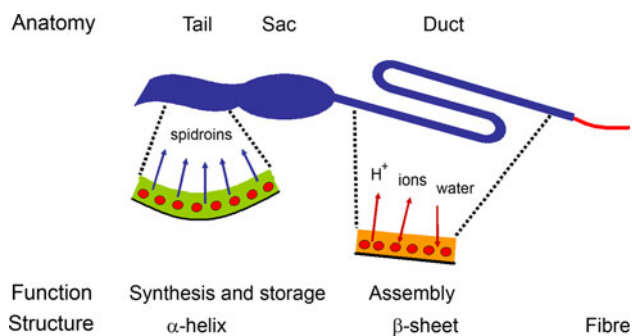
spidroins, than to *Bombyx* silk proteins, and can therefore be assumed to be functionally closer to spider silk. However, although silkworm silk can generate interesting materials from several aspects, this source can not result in truly spider-like silks as the constituent proteins differ. Another alternative is the recombinant overexpression of spidroins or of designed proteins with sequences inspired by the overall properties of spidroins. Often the purification processes in these cases include solubilization steps following precipitation or lyophilization, before the fibres can be generated in similar manners as for dissolved silks. All of these approaches have been subject to recent reviews [2, 6–10].

Yet another approach to produce artificial spider silk has recently been introduced [11]. This method uses recombinant production in *Escherichia coli*, but it differs from previously suggested methods by avoiding the resolubilization steps through the introduction of measures that assure solubility in aqueous buffers throughout the production and purification steps, until silk fiber formation is induced. This method mimics the spider's own way of producing silk, namely, they produce an aqueous solution (dope) that is stored in the glandular sac until insoluble silk fibres are formed in the distal parts of the duct [12, 13] (Fig. 1).

In this review, we will discuss the sequence motifs and structural and functional properties of spidroin domains and how they confer special challenges for recombinant production. We will also address how recombinant spidroins can generate novel functional materials for biomedical applications.

## Spiders and spider silks

All spiders produce at least one type of silk, and some, like orb-weaving spiders, can have up to seven different types



**Fig. 1** The spider's silk production system. A schematic spider silk-producing gland and duct. The main secondary structure adopted by the spidroins in the different locations as well as proposed changes in pH, salt compositions and water contents along the gland and duct are indicated

of silk glands [14] (Table 1), each producing a silk with a specific purpose and unique mechanical properties. Most studies have focussed on the dragline silk, a strong and extendible fibre used for the construction of the framework of the webs and as a lifeline, with a toughness never attained in synthetic or other natural fibres [3]. This silk is mainly composed of proteins, but there are reports of the presence of lipids and glycoproteins in the fibre, although their functions remain to be determined [15–17]. Dragline silk is produced in paired major ampullate glands, each which is connected to an S-shaped secretory duct that leads to the spinnerets on the ventral surface of the abdomen [18] (Fig. 1). The spidroins are produced and exocytosed into the lumen by a single-layered epithelium [19]. The spidroins can be stored in the glandular lumen as a dope until they are converted into a solid fibre in the end of the secretory duct [12, 13]. The silk production machinery of the spider is impressive from several points of view: (1) the spidroin genes are very large, repetitive and rich in guanine and cytosine, which puts high demands on the replication and transcription of these sequences [20–22]; (2) the glandular epithelium may multiply the entire genome without subsequent mitosis [23], and gene multiplicity and/or processing of the transcripts have been suggested [24, 25]; (3) to meet the extreme need of certain amino acids during translation, the epithelial cells of the glands have unusually large tRNA-pools for alanine and glycine [26]; (4) the spidroins are very prone to assemble, yet the spider manages to keep them in soluble helical and coil conformations [27] in the glandular lumen at very high concentrations (30–50%, w/v) [12, 27]; (5) the fibre-forming process is well controlled, enabling almost instantaneous formation of the fibre in a defined segment of the duct, thus avoiding a fatal spread of the assembly process to the dope in the gland [13]. Upon self-assembly, the spidroin's secondary structure converts into mainly  $\beta$ -sheet [28]. This process probably involves shear forces, a drop in pH and changes in the ion composition along the duct [12, 29, 30] (Fig. 1).

## Spidroin and spider silk architecture

Spidroin gene and protein nomenclature follows a convention where the first two letters indicate the gland where it is expressed, followed by Sp for spidroin and a number referring to the different paralogues (e.g. MaSp1 for Major ampullate spidroin 1). Notable exceptions include the spidroins corresponding to the MaSp1 and MaSp2 of *Araneus diadematus*, which are referred to as *Araneus diadematus* fibroins 4 (ADF-4) and ADF-3, respectively [31]. Spidroin sequences contain N-terminal signal peptides that direct the protein to the secretory pathway [32] and which are

subsequently removed in the endoplasmic reticulum. Mature spidroins in general have a tripartite composition of a non-repetitive N-terminal domain (approx. 130 amino acid residues in the folded part [33]), an extensive region made up by silk-specific repeat units and a non-repetitive folded C-terminal domain of approx. 110 residues [34]. The repetitive regions of different spidroins are composed of typical amino acid sequence motifs and ensemble repeat lengths (Table 1). It should, however, be noted that not all spidroins conform to these motifs and repeats [24, 25, 31, 35].

The dragline silk is composed mainly of two similar proteins, MaSp1 and MaSp2 [21], which are large (approximately 3,500 amino acid residues long [22]) and can be encoded by several gene loci [24, 25]. In *Nephila clavipes*, MaSp1 is found uniformly in the fibre core, whereas MaSp2 is missing in the periphery and forms clusters in certain core areas [36]. The ratio of MaSp1 and MaSp2 in the fibre differ between spider species and probably also between individuals of one species—and perhaps even in dragline silk from one individual spider spun at different occasions [37].

In the repetitive region, tandem repeat units of a glycine-rich repeat followed by an alanine block ( $A_n$ ,  $n = 6–14$ ) appear consecutively about 100 times. Alanine and glycine residues are by far the most abundant, making up >60% of the entire sequence [22]. Certain motifs, such as  $A_n$ , GGX and GPGXX, have been assigned specific secondary structures in the fibre ( $\beta$ -sheet,  $3_1$ -helix and  $\beta$ -spiral, respectively) that can be correlated to the mechanical characteristics [38, 39]. The alanine blocks form  $\beta$ -sheets that can stack to form crystalline structures in the fibre [28], linking different protein molecules together. The level of crystallinity in the fibre depends on the proportion of alanine blocks in the spidroin and can be correlated to the strength of the fibre [40, 41], although additional factors are likely to be also important [3]. The structures in the more amorphous glycine-rich regions will, on the other hand, contribute to extensibility and flexibility [39]. Thus, the nature of the repetitive region seems to determine the mechanical properties of the fibre [3, 39]. In line with this supposition, *Euprosthenops australis* has one of the strongest dragline silks examined (tensile strength = 1.5 GPa) and also the longest polyalanine blocks [24, 40, 41]. However, a recent molecular dynamics study of polyalanine nanocrystals suggest that the best overall mechanical performance is obtained with crystals containing about five to ten residues [42]. The reasons for these apparent discrepancies between experimental and theoretical studies are not obvious. The now available recombinant spidroins (see below) should make it possible to study the correlation between polyalanine length and mechanical properties in further detail.

## Recombinant spidroin production

Several prokaryotic and eukaryotic hosts are used for recombinant protein production, each with its own pros and cons in terms of cost, ease of use, expression levels and contaminations. Many eukaryotic proteins require post-translational modifications (e.g. glycosylation and phosphorylation) in order to obtain a correct fold and biological activity [43]. Systems for most of these modifications are present only in eukaryotic cells, and the extent, pattern and types of modifications differ between host systems. Phosphorylation of tyrosine and serine residues in spidroins have been reported, but the impact on the processing and/or physical properties of dragline silk remains undetermined [44]. There are several reports on successful recombinant production of partial spidroins in prokaryotes (Table 2). Consequently, the use of eukaryotic production systems may not be crucial. However, several other properties of spidroins complicate recombinant expression. The long, repetitive and guanine/cytosine-rich gene sequences complicate sequencing, cloning work and expression, and it was only recently that all parts of the spidroins were cloned and the first full-length genes sequenced [22, 45, 46]. Genetic instability and undesirable mRNA secondary structures have led to problems, such as truncations, rearrangements and translation pauses [47, 48]. Further on, depletion of tRNA pools, low solubility of the product and suspected proteolysis have been major obstacles [48–50]. Several strategies have been used to overcome these problems, such as codon optimization [48], enriched media [51] and the use of a variety of host systems (Table 2).

Different approaches for recombinant spidroin production have been used depending on the aim in question, ranging from biochemical characterization to the large-scale production of biomaterials. The most widely used host is the Gram-negative enterobacterium *E. coli* (Table 2), which offers a well-controlled, cost-efficient system suitable for large-scale production. Several successful laboratory-scale *E. coli* fermentation processes have been reported [47, 52–62], although unstable DNA fragments [47–49], low yields [47, 49], accumulation in inclusion bodies [63] and generally low protein solubility [52, 54, 59, 64–66] have been reported. The methylotrophic yeast *Pichia pastoris* has been used with the purpose to minimize truncations due to translation stops [67] and to allow extracellular secretion, which has been shown to occur for an amphiphilic silk-like protein [68]. Expression in various plants (tobacco, potato, *Arabidopsis*) has been tried as an alternative approach aimed at efficient and cheap production suitable for scale-up. In order to optimize yields, expression strategies with retention in the endoplasmic reticulum have been used, but large-scale trials have so far only resulted in fairly

**Table 1** Characteristics of spider silks and spidroins

Gland	Function	Spidroin	Architecture	Length (residues) <sup>a</sup>	Amino acid motifs	Ensemble repeat length (residues)
Major ampullate	Web frame, life line	MaSp1	NT-REP-CT	3,130	A <sub>n</sub> , GX and GGX <sup>b</sup>	~30
		MaSp2	NT-REP-CT	3,780	A <sub>n</sub> , GGX, GPX, QQ, GSG <sup>b</sup>	~30
Minor ampullate	Web reinforcement, capture silk	MiSp1	U-REP-CT	nd	GGX <sub>n</sub> (GA) <sub>n</sub> , A <sub>n</sub> , spacer (137 residues) <sup>c</sup>	~280, Spacer[(GGX) <sub>n</sub> (GA) <sub>y</sub> A <sub>z</sub> ] <sub>10</sub> ; similar to MiSp1
		MiSp2	U-REP-CT	nd	GGX <sub>n</sub> (GA) <sub>n</sub> , A <sub>n</sub> , spacer (137 residues) <sup>c</sup>	
Flagelliform	Capture spiral	Flag	NT-REP-CT	~5,000	GPGG(X) <sub>n</sub> , GGX, spacer (~28 residues) <sup>d</sup>	~440
Aciniform	Wrapping silk, inner egg case	AcSp1	U-REP-CT	nd	S <sub>n</sub> , TGPSC <sup>e</sup>	200
		AcSp1			S <sub>n</sub> , GPS <sup>m</sup>	~350
		AcSp1-like			GGX <sup>f</sup>	~187
Tubuliform/cylindriform	Outer egg case	TuSp1	NT-REP-CT	2,983	Short A <sub>n</sub> , S <sub>n</sub> , T <sub>n</sub> , (SX) <sub>n</sub> <sup>g</sup>	171–182
		TuSp1			Short A <sub>n</sub> , S <sub>n</sub> , T <sub>n</sub> , (SX) <sub>n</sub> <sup>h</sup>	180, [A <sub>0</sub> B <sub>1</sub> (A <sub>1</sub> B <sub>1</sub> ) <sub>n</sub> A <sub>2</sub> ], A <sub>1</sub> = 139, B <sub>1</sub> = 41
Aggregate	Sticky coating	TuSp1			Short A <sub>n</sub> , S <sub>n</sub> , T <sub>n</sub> , (SX) <sub>n</sub> <sup>ik</sup>	184
		TuSp1			Short A <sub>n</sub> , S <sub>n</sub> , T <sub>n</sub> , (SX) <sub>n</sub> <sup>l</sup>	161, [B <sub>0</sub> (A <sub>1</sub> B <sub>1</sub> ) <sub>n</sub> A <sub>2</sub> ], B <sub>0</sub> = 48, A <sub>1</sub> = 110, B <sub>1</sub> = 61, A <sub>2</sub> = 98
		TuSp1			Short A <sub>n</sub> , S <sub>n</sub> , T <sub>n</sub> , (SX) <sub>n</sub> <sup>m</sup>	~200
		TuSp1			Short A <sub>n</sub> , S <sub>n</sub> , T <sub>n</sub> , (SX) <sub>n</sub> <sup>n</sup>	~300
		TuSp1			Short A <sub>n</sub> , S <sub>n</sub> , T <sub>n</sub> , (SX) <sub>n</sub> <sup>o</sup>	174
		TuSp2	NT-REP-CT	3,218	Short A <sub>n</sub> , S <sub>n</sub> , T <sub>n</sub> , (SX) <sub>n</sub> <sup>h</sup>	180, [A <sub>0</sub> B <sub>1</sub> (A <sub>1</sub> B <sub>1</sub> ) <sub>n</sub> A <sub>2</sub> ], A <sub>1</sub> = 139, B <sub>1</sub> = 41
Pyriform	Attachment disk, joining fibre	ECP-1	Z-REP	932	(GA) <sub>n</sub> , short A <sub>n</sub> <sup>i</sup> in parts of sequence	na <sup>d</sup>
		ECP-2	Z-REP	825	(GA) <sub>n</sub> , short A <sub>n</sub> <sup>k</sup> in parts of sequence	na <sup>d</sup>
Aggregate	Sticky coating	SCP-1	na	36	na <sup>o</sup>	na
		SCP-2	na	19	na <sup>o</sup>	na
Pyriform	Attachment disk, joining fibre	PySp1	U-REP-CT	nd	AAARAQAQAEARAKAE (A <sub>1</sub> ), AAARAQAQAE (A <sub>2</sub> ) and a 78 residue iteration (A <sub>3</sub> ) <sup>p</sup>	[(A <sub>1</sub> A <sub>2</sub> ) <sub>n</sub> A <sub>3</sub> ] <sub>n</sub>

na Not applicable, nd not determined, X any amino acid residue, U unknown N-terminal sequence, Z the ECP N-terminal sequence. Z the ECP N-terminal region is approx.150 amino acid residues long, non-repetitive and contains 16 Cys but is not homologous to other spidroin N-terminal domains

Sources/references for amino acid motifs and ensemble repeat length (residues): <sup>b</sup> [22] (*Latrodectus hesperus*), <sup>c</sup> [116] (*Nephila clavipes*), <sup>d</sup> [46] (*Nephila clavipes*, *Nephila madagascariensis*), <sup>e</sup> [117] (*Argiope trifasciata*), <sup>f</sup> [118] (*Latrodectus hesperus*), <sup>g</sup> [119] (*Argiope aurantia*, *Nephila clavipes*, *Aranus gemmoides*), <sup>h</sup> [45] (*Argiope bruennichi*), <sup>i</sup> [120] (*Latrodectus hesperus*), <sup>j</sup> [121] (*Latrodectus hesperus*), <sup>k</sup> [122] (*Latrodectus hesperus*), <sup>l</sup> [123] (*Nephila clavata*), <sup>m</sup> [35] (*Deinopis spinosa*, *Uloborus diversus*), <sup>n</sup> [124] (*Nephilengys cruentata*), <sup>o</sup> [125] (*Latrodectus hesperus*), <sup>p</sup> [126] (*Latrodectus hesperus*), <sup>q</sup> ECP-1 and -2 are considered to be atypical since they are much shorter than other known full-length spidroins (approximately 800–1,000 amino acid residues) and lack higher ensemble repeat units

<sup>a</sup> Length in amino acid residues. Full-length gene sequences are available for MaSp1 and MaSp2 from *Latrodectus Hesperus*, *Nephila* flagelliform silk and for the cDNAs of CySp1 and CySp2 from *Argiope bruennichi*

Table 2 Survey of recombinantly expressed spider protein fragments

Host	Origin	Protein description	Terminal domains	Functionalizations	Protein size (kDa)	Biocompatibility studies	References
<b>Prokaryotes</b>							
<i>Escherichia coli</i>	Synthetic silk like protein	Synthetic repeats	No		76–89	No	[127]
	<i>Nephila clavipes</i>	Repeats from MasP1, MasP2	No		11–41	No	[49]
	Not specified	Repeats from MasP2	No		31–112	No	[79]
	<i>Nephila clavipes</i>	Repeats from MasP1, MasP2	No		65–163	No	[48]
	<i>Nephila clavipes</i>	Gly-rich repeats	No		9–20	No	[52]
	<i>Nephila clavipes</i>	Natural C-terminal part	CT		43	No	[47]
	<i>Nephila clavipes</i>	Repeats from MasP1	No	Met, phosphorylation site for cAPK	25	No	[50, 54, 65, 89]
	<i>Nephila clavipes</i>	Gly-rich repeats from flagelliform	No		25	No	[55]
	<i>Nephila clavipes</i>	Repeats from MasP1, MasP2	±CT		43–56	No	[53]
	<i>Araneus diadematus</i>	Repeats from ADF3, ADF4	±CT		34–60	No	[78]
	<i>Nephila clavipes</i>	Repeats from MasP1, MasP2	±CT		20–56	No	[64]
	<i>Nephila clavipes</i>	Repeats from MasP1	No		Not specified	No	[56]
	Silk-like protein	Gly-rich repeats and Ala-blocks	No		18–36	No	[128]
	<i>Nephila edulis</i>	Natural C-terminal parts, MasP1, MasP2	±CT		10	No	[129]
	<i>Araneus diadematus</i>	Repeats from ADF3, ADF4	±CT		48–106	No	[51, 58, 60, 63, 81–85, 99, 105]
	<i>Nephila clavipes</i>	Repeats from MasP1	No	R5	45	No	[66]
	<i>Nephila clavipes</i>	Repeats from MasP1	No	CRGD	48	hMSC	[59]
	<i>Nephila clavipes</i>	Repeats from MasP1	No	CDMPI	49–60	No	[61]
	<i>Euprosthenoops australis</i>	Natural C-terminal part from MasP1	±CT, ± NT	Cys	10–24	HEK 293 cells, human primary fibroblasts, in vivo (rats)	[11, 32, 33, 90–93]
	<i>Nephila clavipes</i>	Repeats from MasP1, blended with regenerated bombyx	No	CRGD	48	No	[96]
<i>Nephila clavipes</i>	Repeats from MasP1	No		63–71	3T3 cells	[114]	
<i>Arigiope aurantia</i>	Repeats and terminal domains from TuSp1	±CT, ± NT		12–15	No	[9, 62]	
<i>Nephila anipodiama</i>	Repeats from flagelliform	±CT		14–94	No	[130]	
<i>Nephila clavipes</i>	Repeats from ADF1, ADF2, ADF3	CT		30–56	No	[131]	
<i>Salmonella typhimurium</i>	Repeats from MasP1	No		65	No	[67]	
<b>Eukaryotes</b>							
<i>Pichia pastoris</i>	Silk-like protein	Synthetic silk-like repeat	No		28–32	No	[68]
	<i>Nephila clavipes</i>	Repeats from MasP1	No		94–113	No	[86]
	<i>Nephila clavipes</i>	Repeats from MasP1	No		94	3T3 cells	[87]
	<i>Nephila clavipes</i>	Repeats from MasP1	No				

Table 2 continued

Host	Origin	Protein description	Terminal domains	Functionalizations	Protein size (kDa)	Biocompatibility studies	References
Tobacco ( <i>Nicotiana tabacum</i> ) potato ( <i>solanum tuberosum</i> )	<i>Nephila clavipes</i>	Repeats from MaSp1	No	100xELP	13–100	human chondrocytes, CHO-K1	[80, 100]
Tobacco ( <i>Nicotiana tabacum</i> )	<i>Nephila clavipes</i>	Repeats from MaSp1, MaSp2	CT		60	No	[69]
	<i>Nephila clavipes</i>	Repeats from MaSp2	CT	±ELP-tag	65	No	[132]
<i>Arabidopsis</i>	<i>Nephila clavipes</i>	Repeats from MaSp1, MaSp2	No		64	No	[97]
Mammalian cells (MAC-T, BHK)	<i>Araneus diadematus</i> , <i>Nephila clavipes</i>	Natural C-terminal part from ADF3, MaSp1, MaSp2	CT		60–140	No	[70]
Mammalian cells (COS1)	<i>Euprosthenops</i> sp	Repeats from MaSp1	No		22	No	[133]
Insect cells ( <i>Spodoptera frugiperda</i> )	<i>Araneus diadematus</i>	Natural C-terminal part from ADF3, ADF4	CT		3.5–5.6	No	[73]
	<i>Araneus diadematus</i>	Natural C-terminal part from ADF3, ADF4	±CT		50–60	No	[74, 134, 135]
	<i>Araneus ventricosus</i>	Natural C-terminal part from Av Flag	CT		28	No	[136]
Ovary derived cell lines ( <i>Bombyx mori</i> )	<i>Nephila clavipes</i>	Repeats from flagelliform	No		37	No	[137]
Transgenic animals	<i>Nephila clavata</i>	Repeats from MaSp1	No	GFP	70	No	[75]
Mammary glands of mice	<i>Nephila clavipes</i>	Repeats from MaSp1	No		31–66	No	[71]
Salivary glands of <i>Bombyx mori</i>	<i>Nephila clavata</i>	Repeats from MaSp1	No		70	No	[76]

CT C-terminal domain, NT N-terminal domain, *Met* methionine, *cAPK* cyclic AMP-dependent protein kinase, *R5* peptide derived from the silaffin protein of *Cylindrotheca fusiformis*, *CRGD* Cys-Arg-Gly-Asp peptide, *CDMPI* carboxyl terminal domain of dentin matrix protein 1, Cys cysteine, *ELP* elastin-like peptide, *GFP* green fluorescent protein, *hMSC* human mesenchymal stem cells, *HEK* human embryonic kidney, *3T3* mouse embryonic fibroblast line, *CHO-K1* Chinese hamster ovary cell line; *MaSp* Major ampullate spidroin, *ADF* *Araneus diadematus* fibroin

low yields of spidroins [69]. Various mammalian cell systems have also been employed in an attempt to achieve the expression of high-molecular-weight spidroins, the most promising of which is a 60-kDa ADF-3 protein expressed and secreted from two cell lines (from bovine mammary epithelial alveolar cells and baby hamster kidney cells) grown in a hollow fibre reactor [70]. Transgenic production in mammary glands and secretion into milk has been tried in mice [71] and goats [72], but the fairly low yields [7] do not justify the long development times and high production cost. Insect cells have proven to be important tools for the study of assembly properties of spidroin fragments in the cytoplasm [73, 74], but these appear to be less suitable for large-scale production. Motivated by structural similarities between spider and silkworm silk, the expression of spidroins in cell lines and larvae of *B. mori* have been tried, with solubility as the primary limitation [75]. Expression of spidroin fragments in the silk gland epithelium of transgenic *B. mori* resulted in silk with improved mechanical properties [76], although the relative amount of recombinant spidroin in the final silk blend was low [77].

A major aim in the recombinant production of spidroins has been to express as many continuous repetitive regions as possible (Tables 2, 3). Two strategies have been most commonly used: (1) to express parts of the native sequences (see for e.g. [47]) or (2) to generate iterated consensus repeats (see for e.g. [59, 78]). Overexpression of such recombinant spidroins has often resulted in water-insoluble products in the host and/or during downstream processing. For some applications, it is advantageous to obtain the product in an aggregated state, as the yield can be very high (up to approx. 50% of total *E. coli* protein content) and the aggregates can easily be isolated by centrifugation or filtration. Solubilization of aggregates is often achieved using urea, guanidine hydrochloride, LiBr, hexafluoroisopropanol or formic acid [9, 47, 48, 52, 53, 55, 57–60, 63, 64, 66, 67, 70, 78–88]. However, if the intended use of the spidroin involves biological applications, these compounds have to be removed in the final product. After removal of the solvents used for aggregate solubilization, it has generally been difficult to keep the spidroins in solution and to control assembly. Several strategies to avoid these problems have been tried, such as the introduction of methionine residues for controlled oxidation/reduction to prevent and promote assembly, respectively [50, 65, 89], kinase recognition motifs for controlled assembly via enzymatic phosphorylation [54] or solubility-enhancing fusion partners that can be released enzymatically [11]. In the latter case, spontaneous assembly into macroscopic, sterile, pyrogen-free and biocompatible silk-like fibres is possible, with the use of only physiologically compatible solvents [11, 90, 91] (Fig. 2).



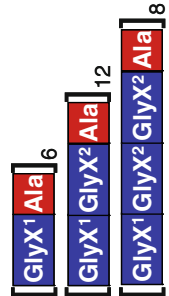






Spontaneous self-assembly into silk-like fibres (in terms of structure and stability) has so far been observed after recombinant production in the cytosol of insect cells [73] and after production and purification under non-denaturing conditions [11, 92, 93]. Solubilization of these fibres in denaturing solvents apparently destroys the ability to reassemble into silk-like fibres ([73] and unpublished observations). Huemmerich et al. [73] showed that solubilization of ADF-4 filaments in guanidinium thiocyanate followed by dialysis or dilution resulted in aggregation, but no fibrillar structures. In a similar manner, we have observed that spontaneously assembled fibres from recombinant miniature spidroin (4RepCT, see further below) dissolved in formic acid or hexafluoroisopropanol are not able to spontaneously reassemble into fibres (unpublished data). This is not surprising as not even the respinning of dissolved native spider silk results in fibres that match native silk [94, 95]. For recombinant spidroins that do not self-assemble spontaneously, different methods to convert them into fibres have been used, such as wet spinning [9, 53, 62, 70, 79, 86, 96, 97], hand drawing [82, 96], spinning through microfluidic devices [83] and electrospinning [56, 59, 66, 86, 98]. A few recombinant spidroins that generate fibres stable and long enough to allow mechanical testing have been reported [11, 70, 86, 92, 93, 96]. However, none of these fibres have replicated the extraordinary mechanical properties of the spider's dragline silk, regardless of spidroin length. For some applications, such as scaffolds for cell culture and drug delivery, other formats than fibres are preferred. Therefore, materials have been produced by casting into films [50, 52, 53, 58–61, 65, 66, 81, 89, 99, 100], formation of microbeads [63], microspheres [57, 84], and microcapsules [101]. Three-dimensional porous scaffolds have also been produced using salt-leaching techniques [87]. Most of these techniques require post-treatment in dehydrating or salting-out solvents to increase the  $\beta$ -sheet contents and stability.

Collected experience from studies on the recombinant production of spidroins implies that several factors have to be taken into account to obtain silk-like materials, including which parts of the spidroins are to be expressed, structures and solubility of the recombinant protein, and assembly and spinning conditions.

### Structure and function of spidroin domains

From studies at the molecular level, it is becoming increasingly clear that the three different spidroin parts are structurally and functionally distinct. The mechanical properties of the final silk fibre have traditionally been regarded as the most important functional parameter. However, it should be emphasized that the production,

**Table 3** Architecture of selected recombinant spideroin fragments

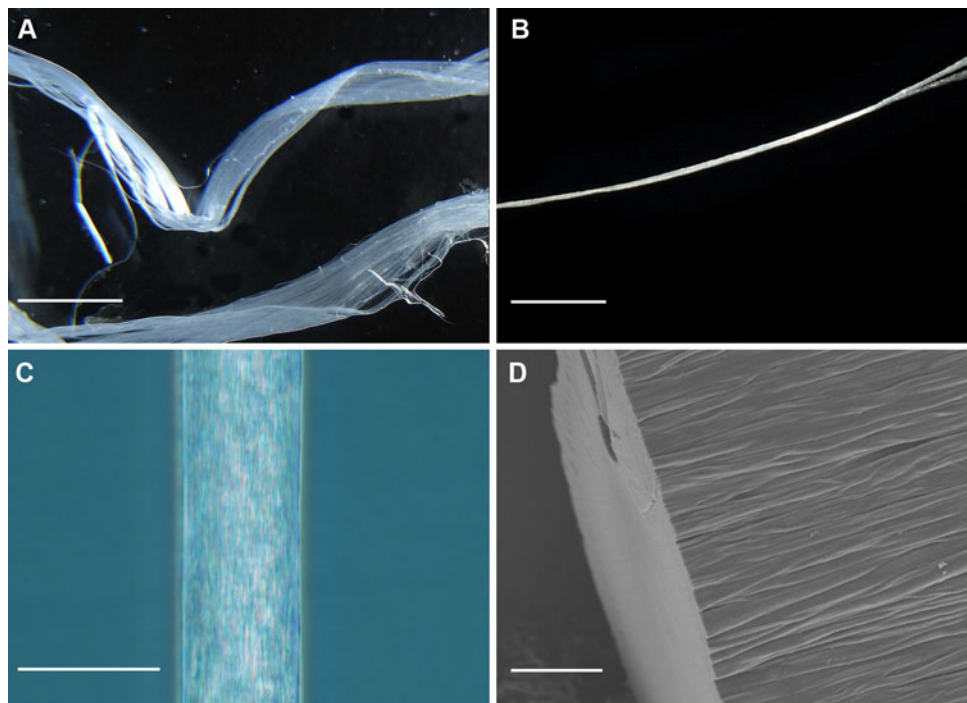
Schematic description of construct <sup>a</sup>	Example (construct name)	Block length (residues)	Protein size (kDa)	Reference
	DP1B	Ala: 7; GlyX: 15–20	65–163	[48, 56, 67, 97]
	SP1	Ala: 5; GlyX: 10	25	[49, 50, 54, 59]
	IE 2E 3E	Ala: 8; GlyX: 28	63 71 67	[62]
	IF9	Ala: 5–7; GlyX: 20–25	94	[9, 86, 87]
	SO1 (ELP)	Ala: 6; GlyX: 20–30; 5 different GlyX	51	[80, 100]
	CRGD15	Ala: 6; GlyX: 20–30	55	[59, 66, 114]
	AQ12 (NR3); AQ24 (NR3); C16 (NR4)	Ala: 6–8; GlyX: 6–20	48 (60); 94 (106); 48 (58), respectively	[57, 58, 63, 78, 81, 82, 83, 84, 85, 99, 105]
	ADF3	Ala: 6–8; GlyX: 6–20	60	[70, 73]
	(NT)4RepCT	Ala: 12–15; GlyX: 13–19	12 (21)	[11, 33, 90, 91, 93]

CT C-terminal domain, NT N-terminal domain

<sup>a</sup> The superscript indicates the different nature of the repeat, the subscript indicates the number of bracketed repeats. For longer repeats, internal segments are indicated with dots. Domains within parenthesis are optional



**Fig. 2** Fibres of recombinant spider silk. **a** Photograph of a wet fibre (*scale bar 2 mm*), **b** photograph of a dried fibre (*scale bar 2 mm*), **c** light micrograph of a dried fibre (*scale bar 100  $\mu$ m*), **d** scanning electron micrograph of a dried fibre with a fracture surface obtained after rupture during tensile testing (*scale bar 10  $\mu$ m*). All fibres were made from the 4RepCT miniature spidroin (see Tables 2 and 3)



secretion, storage and, in particular, the controlled conversion of spidroins from soluble to insoluble states pose significant challenges. It is likely that the different domains are specialized to function during one or several—but probably not all—of these stages. The fact that the N-terminal domain is lacking in several recombinantly expressed constructs (Tables 2, 3), but that these are still able to polymerize and even form macroscopic silk-like fibres in vitro, supports this concept. Spiders manage to keep the spidroin dope at a high concentration, similar to the total protein concentration in some intracellular compartments (30–50%, w/v) without precocious aggregation; even more impressively, they manage to convert the same dope into solid fibres during the rapid transit through the extrusion duct. It is conceivable that studies of the molecular mechanisms that control spidroin solubility versus polymerization will generate insights which are of interest to the understanding of protein folding and misfolding in a general context. Given the proposed amyloid-like properties of spider silk [102], structural transitions in amyloid fibril formation may be particularly relevant.

Early studies focussed on the repetitive regions and their importance for fibre formation. For example, the absence of proline in one of the constituent proteins was suggested to be essential for fibre formation [103]. Later, an ADF-4 fragment (including the C-terminal domain) was found to form filaments in the cytosol of insect cells, although the ADF-3 counterpart stayed soluble, despite similar proline contents [78]. A significant difference in hydrophobicity has been suggested as the major cause of the different

solubility properties. Biochemical studies of recombinant ADF-3 and ADF-4 fragments that have been lyophilized, resolubilized in guanidinium thiocyanate and dialysed revealed that the presence of the non-repetitive C-terminal domain affects aggregation behaviour [78]. Upon expression in the cytosol of insect cells, ADF-4 fragments lacking the C-terminal domain do not self-assemble into nano fibers, as do their counterparts containing the C-terminal domain, but instead form aggregates [74]. The presence of amphipathic helices in the C-terminal domain has been proposed to be important for fibre formation [104]. In the recently determined folded structure of a C-terminal domain, the nonpolar parts of the amphipathic helices form the buried core; however, under certain experimental conditions nonpolar parts of the protein can become exposed [34]. It is possible that the C-terminal domain can adopt conformations that promote spidroin assembly (see below). Recombinant production in *E. coli* followed by recovery under nondenaturing conditions has generated structural and functional data of spidroin domains individually and in different combinations [11, 32–34, 92]. A construct comprising four polyalanine/glycine-rich repeats and the following C-terminal domain from MaSp1 of *E. australis* (4RepCT; see Tables 2, 3) and its two constituent parts have yielded information on the requirements for solubility and fibre formation [11, 92]. The repetitive part alone is unable to form fibres but aggregates into amorphous structures. Covalent linkage of the two parts (4RepCT) results in a miniature spidroin that spontaneously form metre-long macroscopic fibres under ambient

conditions. Combined, these results indicate that (1) the repetitive regions mediate intermolecular contacts, (2) the C-terminal domain governs spidroin polymerization so that ordered fibres are formed rather than amorphous aggregates and (3) the N-terminal domain is not a prerequisite for in vitro fiber formation, but seems to regulate fibre formation in vivo.

The N-terminal domain is the most conserved of the spidroin domains and has evolved according to silk type [32]. Like the other spidroin domains, the N-terminal domain lacks known homologues, even in fibre-forming proteins from silkworm silk. These observations suggest that the N-terminal domain fulfils a function that is unique to spidroins and which requires structural features that can only be obtained by a folded polypeptide chain. The N-terminal domain from MaSp1 of *E. australis* is highly soluble and cooperatively folded [33, 92]. The recently determined high-resolution X-ray structure of recombinant N-terminal domain from the MaSp1 of *E. australis* [33] reveals several properties that point to specific functions. The inclusion of this N-terminal domain in miniature spidroins (NT4RepCT; see Tables 2, 3) accelerates charge-dependent self-assembly at pH values below approx. 6.4 (as observed in the spinning duct), but it delays aggregation above pH 7 (as observed in the dope). The X-ray structure analysis also revealed a homodimer of dipolar, antiparallel five-helix bundle subunits, and the overall dimeric structure and observed charge distribution are expected to be conserved through spider evolution and in all types of spidroins. These results suggest a relay-like mechanism through which the N-terminal domain regulates spidroin assembly by inhibiting precocious aggregation during storage and by accelerating and directing self-assembly as the pH is lowered along the spider's silk extrusion duct [33]. A solution structure of a fragment of the N-terminal domain from an egg case spidroin, which after refolding forms a monomer in the presence of detergent micelles, has been reported [88]. This structure is also  $\alpha$ -helical, but is otherwise quite different from the crystal structure of the N-terminal domain of MaSp1 of *E. australis*. It remains to be determined whether these differences reflect an inherent structural flexibility of the N-terminal domain, the different ways the recombinant proteins were obtained and investigated and/or other factors.

The presence of the C-terminal domain in recombinant miniature spidroins is a prerequisite for the formation of macroscopic fibres and fibrous aggregates in the test tube [92, 105]. A solution structure of the C-terminal domain from ADF-3 has recently been determined by nuclear magnetic resonance (NMR) spectroscopy [34]. This revealed a dimer of two five-helix bundles with no known structural homologues. Also, a solution structure of a monomeric C-terminal domain from an egg case spidroin,

in detergent micelles, has been reported [88]. This C-terminal domain consists of a five-helix bundle but with a different topology than the ADF-3 domain. The C-terminal domain is rather well conserved at the amino acid sequence level and should consequently show a similar fold independent of origin. The reasons for the observed different folds in crystals or in solution [33, 34], on the one hand, compared to those in detergent micelles [88], on the other hand, are not clear at the present time. The ADF-3 C-terminal domain lacks surface-exposed charged residues but is quite polar due to an abundance of serines and glutamines [34]. Hagn et al. [34] further suggested that the structural state of the C-terminal domain can be regulated by chemical (in particular, the surrounding salt concentration) and mechanical stimuli and that this is a means to promote solubility versus aggregation in spidroin storage and fibre formation, respectively. The C-terminal homodimer is stabilized by a disulphide bridge. In miniature spidroins, the cysteine that forms the disulphide can be exchanged for serine without loss of ability to form fibres in the cytosol of insect cells [74] or in the test tube [93].

The N- and C-terminal spidroin domains are not related at the level of amino acid sequences, and although both fold into five-helix bundles, the topologies are different [33, 34]. There are, however, some apparent overall similarities between the N- and C-terminal domains, such as conformational flexibility and quite polar surfaces. The symmetric homodimeric nature of both domains may be important for aligning the repetitive segments [33, 34]. It has been suggested that the terminal domains form the shell of micellar structures that help to keep the spidroin repetitive segments in solution [34, 88, 106]. Micellar structures have indeed been observed in aqueous solutions of silkworm silk proteins reconstituted by polyethylene oxide treatment [106], but whether micelles exist in the spidroin dope remains to be studied. As the pH and salt composition are changed along the extrusion duct, the N- and C-terminal domains may trigger polymerization by bringing spidroins together [33, 34], but this alone does not explain what makes the many polyalanine blocks convert from helices/random coil to  $\beta$ -sheet. It can be speculated that once the N- and C-terminal domains have brought spidroins together in the narrowing extrusion duct, shear forces will trigger concerted conversion into  $\beta$ -sheet, explaining how fibre formation can occur in fractions of a second.

In summary, salient molecular determinants that currently are thought to affect spidroin solubility and conversion into fibres include: (1) the storage of a highly concentrated dope in the sac, possibly favoured by electrostatic repulsions involving the N-terminal domain and folding of polyalanine segments into  $\alpha$ -helices; the polar nature of the serine-rich C-terminal domain and micelle formation may also contribute to high water solubility; (2)

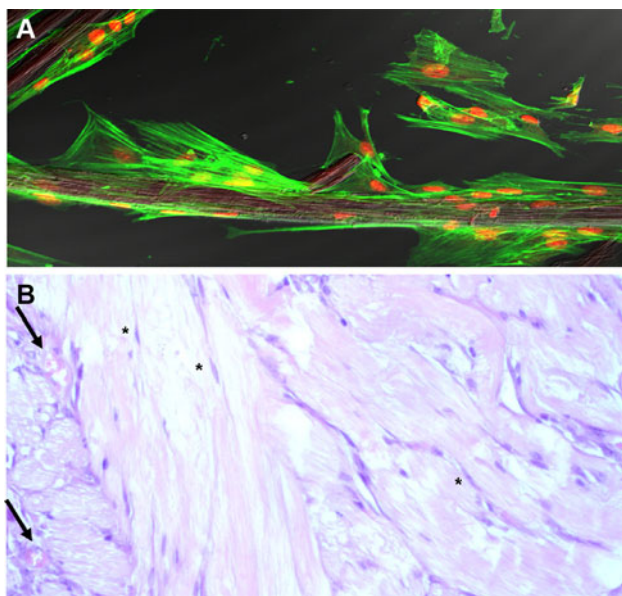
as the pH is lowered below approx. 6.4 and the salt composition is altered along the extrusion duct, the N- and C-terminal domains turn into mediators of assembly, thus bringing the spidroins together; (3) shear forces in the narrowing duct induces  $\alpha$ -helix to  $\beta$ -strand conversion of polyalanine segments, which is rapidly propagated throughout the crowded spidroin population. These events eventually turn the spidroins into fibres made up of crystalline  $\beta$ -sheets surrounded by more flexible glycine-rich regions.

### Spider silk for cell support and implantation

Even if it is not possible to use native spider silk at a larger scale, it is interesting to study its biocompatibility. The cytocompatibility of native dragline and egg-sac silk fibres has been demonstrated in a few human cell culture systems [107, 108]. Primary chondrocytes from articular cartilage were shown to attach to and survive for several weeks when grown on native dragline or egg-sac silk [108]. A study of in vitro proliferation using an endothelial cell line, however, showed that spider egg-sac silk had an inhibitory effect, although not as pronounced as that of non-mulberry silk [109]. Allmeling et al. [107] showed that in vitro human primary Schwann cells adhere quickly to and elongate along native dragline silk fibres. Later, artificial nerve constructs consisting of acellularized veins, dragline silk fibres, Schwann cells and, in some cases, extracellular matrix obtained from a tumour cell line (Matrigel; BD Biosciences, San Jose, CA) were used to replace a 2-cm deficit of the sciatic nerve in rats. These constructs promoted the regeneration of peripheral nerves with high functionality, while constructs consisting of vein and Matrigel alone resulted in nearly no myelinated nerve fibers and distinctive muscle degeneration [110]. Interestingly, successful nerve regeneration was obtained with constructs of veins and spider silk alone, which promoted the migration of autologous cells. Furthermore, no signs of inflammatory response or foreign body reaction were found, indicating the biocompatibility of the spider silk used [110]. In vivo biocompatibility of native spider silk has also been demonstrated by subcutaneous implantation of dragline silk in pigs [111] and enzyme-treated egg-sac silk in rats [112]. These results suggest that spider silk is well tolerated in vitro and in vivo.

The techniques that have become available to recombinantly produce spidroins in large quantities, and to fabricate various scaffolds, provide opportunities for new biomaterial applications. To date, few in vitro studies have been carried out on the biocompatibility of recombinant spider silk, and the materials that have been studied vary considerably in terms of amino acid sequence, mode of

production and format. However, the results are mainly encouraging. Agapov et al. [87] recently examined the capability of porous three-dimensional scaffolds prepared from recombinant spider silk to support mouse fibroblast ingrowth and proliferation. These scaffolds were produced by salt leaching after the recombinant 1F9 protein (Table 3) had been dissolved in formic acid containing 10% LiCl. Cells were found to attach to the scaffolds, proliferate and migrate into the deeper layers of the scaffolds within 1 week, suggesting that recombinant spider dragline silk can be used to prepare scaffolds that are stable and biocompatible and show good interconnectivity. It would be interesting to assay survival quotes and growth rates on this kind of scaffolds and to obtain more detailed information on their mechanical properties. It would also be relevant to study the ability of other cell types to grow on the scaffolds, as well as to determine their in vivo compatibility. The ability of recombinant spider silk to support cellular differentiation has also been tested in various systems; for example, human mesenchymal stem cells grown on recombinant dragline spider silk in the presence of osteogenic medium showed increased calcification compared to tissue culture plastics, indicating differentiation into osteoblast-like cells [59]. However, the authors of this study suggested that the increased calcification of spider silk films could be a result of an inflammatory response induced by lipopolysaccharide (LPS) from the expression host *E. coli*. LPS is an integral component of the outer membrane of Gram-negative bacteria, which is endotoxic and can activate the innate immune system even at low concentrations. After recombinant spidroin production, all traces of potentially toxic or immunogenic compounds from the host cells must be removed before usage in vivo, as well as for some in vitro applications. Affinity purification usually yields recombinantly produced proteins with over 90% purity, but a significant amount of endotoxins is retained. A production route that combines purification of a minispidroin (4RepCT; Table 3) and endotoxin removal by using a simple cell wash procedure, protein affinity purification and LPS depletion has recently been developed [91]. Applying this method, it was possible to produce fibres with in vitro pyrogenicity corresponding to less than 1 EU/mg; the fibres could be sterilized by autoclaving, and human primary fibroblasts were shown to grow on them [91] (Fig. 3). Moreover, in vivo data demonstrate the biocompatibility of recombinant spider silk, since fibres of the miniature spidroin 4RepCT subcutaneously implanted in rats are well tolerated and allow the infiltration of fibroblasts and ingrowth of capillaries [90] (Fig. 3). This is the first in vivo study to be carried out on the biocompatibility of recombinant spider silk, and the results are intriguing as they expand on the possibilities for the use of recombinant



**Fig. 3** Cell interactions with recombinant spider silk. **a** Human fibroblasts grown in vitro for 3 days on 4RepCT fibres, stained for F-actin (green) and DNA (red). **b** Histological section stained with haematoxylin and eosin of a 4RepCT fibre bundle after 1 week of subcutaneous implantation in rat. The fibres are infiltrated by elongated cells (*asterisk*), likely fibroblasts and angioblasts. *Arrows* indicate the presence of newly formed capillaries in the implant

spider silk in tissue engineering and regenerative medicine. Another recent in vivo study using recombinant spider silk in a rat model of wound healing likewise shows promising results [113]. However, the proteinaceous nature of spider silk and its recombinant counterparts calls for closer immunological studies of, for example, specific antibody responses.

One obvious benefit of recombinant silk is the possibility to introduce motifs with various effects on cell growth, differentiation and migration, which can make it possible to make customized matrices. Such functionalization of spider silk has been explored to some extent; for example, Scheller et al. [100] introduced a fusion protein of an elastin motif (elastin-like polypeptide) in combination with the MaSp1-derived SO1 protein (Table 3) in order to enhance the growth of chondrocytes. This fusion protein was produced in transgenic tobacco and potato, and extraction from the leaves was achieved in a process that included heat treatment and salt precipitation. The obtained protein coated onto cell culture plates was biocompatible and supported growth of human chondrocytes in a similar manner as collagen. Chondrocyte-specific morphology was also preserved, suggesting that the spider silk-elastin protein is able to prevent dedifferentiation. However, the lack of controls using the constituent proteins separately makes it unclear whether the positive effects are a consequence of the elastin or the spider silk part, or both. Another approach

is the introduction of the integrin-binding motif RGD (Arg-Gly-Asp) into sequences derived from spider silk, resulting in biocompatible materials that support cell growth [59, 114, 115]. When human mesenchymal stem cells were cultured on RGD functionalized spider dragline silk films in the presence of osteogenic medium, however, no positive effects on calcification could be observed, compared to the control without RGD [59]. Morgan et al. [114] developed RGD-containing blends of regenerated *Bombyx* silk and recombinant RGD-spidroin dissolved in hexafluoroisopropanol. The films were then spin-coated onto cover slips and shown to support differentiation of a murine undifferentiated osteoblastic cell line. The authors concluded that around 10% RGD-spidroin provides optimal attachment and differentiation into osteoblasts. Li et al. [115] combined RGD-containing recombinant spider silk of unspecified origin and polyvinyl alcohol polymers to prepare scaffolds supporting the growth of cells from a mouse embryonic fibroblast cell line. No comparison with non-modified silk was presented, making it difficult to estimate the effect of the introduced RGD motif. Provided that proper surface exposure of introduced motifs can be achieved, functionalized recombinant spider silk may be useful for advanced cell culture systems and implantables.

## Conclusions

During evolution, spidroins have developed properties that allow the swift generation of silks with outstanding mechanical properties. Moreover, although not required for its biological functions, spider silk, as well as recombinant spider silk-like fibres, appear to be biocompatible as they support the growth of mammalian cells in vitro and can be implanted without being rejected. These features make artificial mimics of spider silk attractive alternatives for cell culture and regenerative medicine. Spidroins contain several repetitive segments capped by folded domains, all with apparently unique sequence features and specific functions. Recent developments in techniques used for the recombinant production of miniature spidroins enable the generation of sterile, pyrogen-free and biocompatible artificial spider silk with the use of physiological solvents only. This approach, in combination with an increased understanding of the structures and functions of spidroins at a molecular level, make it realistic to believe that rationally designed artificial spider silk can be used for biomedical applications in the future.

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