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Processing of recombinant spider silk proteins into tailor-made materials for biomaterials applications

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Spider silk has extraordinary mechanical properties, is biocompatible and biodegradable, and therefore an ideal material for biomedical applications. However, a drawback for any application is the inhomogeneity of spider silk, as seen for other natural materials, as well as the low availability due to the cannibalism of most spiders. Recently, developed recombinant spider silk proteins ensure constant material properties, as well as scalable production, and further the processing into morphologies other than fibres. Biotechnology enables genetic modification, broadening the range of applications, such as implant coatings, scaffolds for tissue engineering, wound dressing devices as well as drug delivery systems.

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Introduction

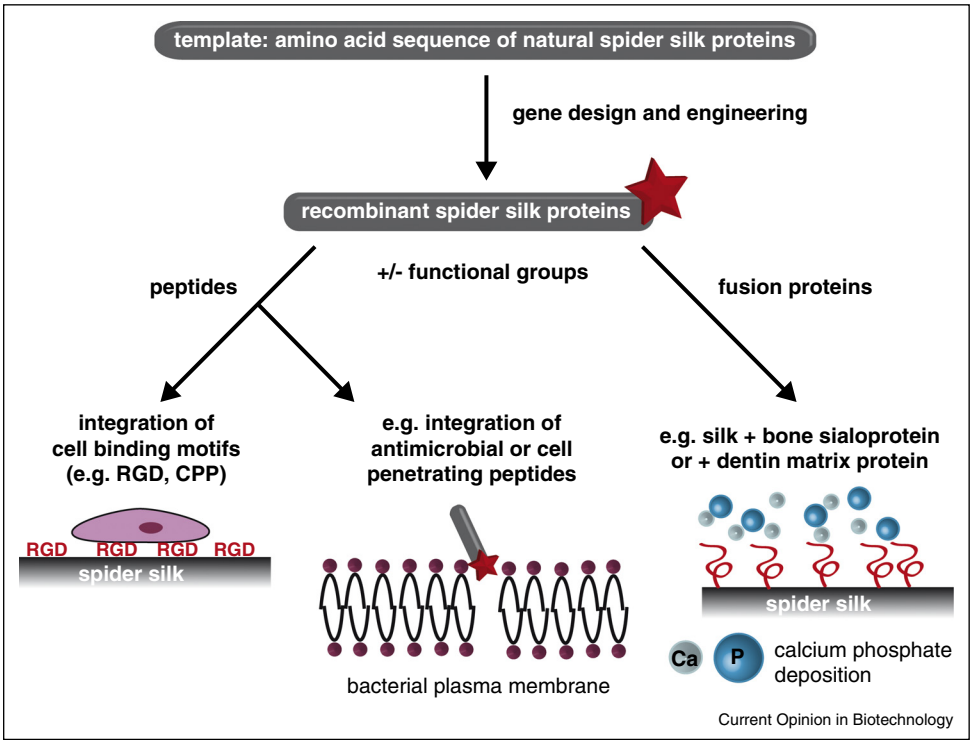
Spider silk fibres fascinate scientists especially due to their extraordinary mechanical properties [1]. The combination of strength and elasticity provides a toughness no other natural or synthetic fibre can achieve [2]. Additionally, spider silk is biocompatible, biodegradable and shows hypoallergenic properties suitable for biomedical applications [3,4].

Importantly, spider silk reflects an entire class of materials with different properties, since spiders can produce several types of silk (for an overview see Heidebrecht and Scheibel [5••]). The best characterised spider silk is the *Major Ampullate* (MA)/Dragline silk, constituting the outer frame of orb webs, serving also as a lifeline for the spider and which will be exclusively discussed herein [6]. Two classes of *Major Ampullate* spidroins have been identified in dragline fibres, called MaSp1 and MaSp2, which differ in proline content and hydrophobicity [7]. All *Major Ampullate* spidroins consist of a highly repetitive core domain, flanked by non-repetitive termini [8]. In the core domain distinct amino acid motifs (glycine-rich repeats and polyalanine blocks) enable secondary structures (random coil/helical and β-sheet) accounting for the mechanical properties of the fibre [8–11]. The terminal domains play an important role during storage of spidroins as a spinning dope in the gland and during the initiation of fibre assembly in the spinning duct [12–15].

For centuries, spider's webs have been successfully used to stop bleeding and to promote wound healing [16]. Recently, spider silk has been used as an artificial support for nerve regeneration [17,18]. Defects of peripheral nerves can be repaired by a composite nerve graft made of acellularized veins, spider silk fibres and Schwann cells (SC) mixed with matrigel (a solubilized tissue basement membrane matrix rich in extracellular matrix proteins). In adult sheep, spider silk enhanced Schwann cell migration, axonal-regrowth and remyelination including electrophysiological recovery in a 6.0 cm tibial nerve defect [19••]. Further, native spider silk fibres were tested as a braided microsurgical suture to substitute conventional materials in microsurgery and neurosurgery [20,21]. It was shown that the mechanical properties of braided spider silk sutures were superior to those of nylon, the current clinical gold-standard [21]. However, one major drawback of natural spider silk sutures is the inhomogeneity of the fibres, as seen with other natural materials, since differences in silk properties occur between individual spiders and even within single individuals upon environmental changes. Another drawback is the low availability of natural material due to problems in farming based on the cannibalistic behaviour of spiders [22,23].

Biotechnological production of spider silk proteins, as well as the development of silk processing techniques enabled the supply of engineered silk materials for biomedical applications, such as implant coatings, drug delivery systems or scaffolds for tissue engineering, which are reviewed herein.

Figure 1



Genetic engineering to achieve functional spider silk proteins.

Recombinant production of engineered spider silk proteins

In the last decades, several prokaryotic and eukaryotic hosts have been tested concerning recombinant production of spider silk proteins, as recently summarized in Heidebrecht and Scheibel [5••].

The benefits of recombinant spider silk proteins (RSSP) are the homogeneity of the starting material as well as the controllable processability into different morphologies, like films, hydrogels, particles or non-woven meshes for various applications [24–29]. Further, biotechnology enables genetic engineering to directly incorporate functional groups into the RSSPs (Figure 1) [24,25,30•].

The simplest genetic modification is the incorporation of individual amino acid residues with chemically specific side chains, like cysteine residues comprising thiol groups. A cysteine variant of the RSSP eADF4(C16) (based on the dragline silk protein ADF4 of *A. diadematus*) allowed the covalent coupling of peptides, enzymes or particles before and after silk processing into materials, demonstrating its potential for a broad range of applications [25,30•].

Engineered spider silk proteins comprising functional peptide sequences

For biomaterials applications, specific interactions between cells and the surface of a material are essential. Spider silk proteins can be exemplarily modified with cell adhesive peptides to improve cell binding, such as the integrin-binding motif RGD (Arg-Gly-Asp) (Figure 1) [28,30•,31,32]. Films made of eADF4(C16)-RGD showed a significantly improved attachment and proliferation of fibroblasts (BALB/3T3) in comparison to unmodified eADF4(C16) films [30•]. Another RSSP, 4RepCT, genetically functionalized with RGD or the cell binding peptides IKVAV, naturally found in the laminin α1 chain, or YIGSR, present in the β1 chain, were processed into fibres, foams and films [33,34]. The adhesion of all tested cell types (fibroblasts, keratinocytes, endothelial and Schwann cells) was significantly improved on RGD-modified in comparison to unmodified 4RepCT films. While only Schwann cells adhered better on matrices comprising the IKVAV-motif, no clear effect of YIGSR could be detected on any of the selected cell types [35].

Functionalizing spider silk proteins or silk hybrids with antimicrobial peptides could be a new approach to

achieve multifunctional biomaterials in order to suppress infections in combination with for example, supporting cell growth. Recently, the human antimicrobial peptides human neutrophil defensin 2 (HNP-2), human neutrophil defensin 4 (HNP-4) and hepcidin were fused to an RSSP 6mer, based on the sequence of MaSp1 from *Nephila clavipes* (*N. clavipes*) (Figure 1). The silk hybrids were processed into films showing antimicrobial activity against Gram negative *E. coli* and Gram positive *S. aureus*. In addition, *in vitro* cell culture studies (cytotoxicity/proliferation) with a human osteosarcoma cell line (SaOs-2) demonstrated the compatibility of these films with mammalian cells [36]. *In vivo* studies in mice showed that the silk-hepcidin protein was highly biocompatible, causing a mild to low inflammatory reaction [37]. In a similar approach, a hybrid between spider silk and a silver binding peptide was produced, which could be processed into films. These films nucleated silver ions from a solution of silver nitrate and inhibited afterwards microbial growth of Gram positive as well as Gram negative bacteria *in vitro* [38].

Silica binding peptides were fused to a consensus sequence of *N. clavipes* MaSp1 introducing silicifying properties [39]. Films thereof promoted osteoblast development with the upregulation of key markers associated with bone formation [40,41].

Other peptides, such as cell penetrating and cell membrane destabilizing peptides (CPPs) are useful candidates, for example, for promoting gene transfer. In this context, recombinant spider silk–polylysine fusions with a ppTG1 peptide, a lysine-rich cell membrane destabilizing peptide to bind plasmid DNA (pDNA), were designed as a highly efficient gene carrier. The newly generated fusion proteins showed useful transfection efficiency, comparable to the transfection reagent Lipofectamine 2000, and at the same time the possibility to control gene release by controlling enzymatic degradation rates of the complexes [42].

Engineering of chimeric spider silk proteins

Chimeric proteins consisting of the RSSP 6mer and the bone sialoprotein (BSP) were processed into films as a support for bone regeneration. Compared to the control (6mer silk films), the Young's modulus of films made of the chimeric silk protein was significantly increased. Furthermore, the chimeric protein retained the ability to form supramolecular aggregates, and in the presence of Ca^{2+} ions these aggregates generated networks [43–45]. *In vitro* studies have demonstrated that human mesenchymal stem cells proliferated and differentiated into the osteogenic lineage on 6mer + BSP films. The presence of cell binding domains in BSP (such as RGD) may be responsible for the cell response when compared to silk alone. For the plain 6mer films, osteoblast-like morphology was not as evident, and a reduction on viability/proliferation was observed after 3, 7 and 14 days in osteogenic medium [46,47].

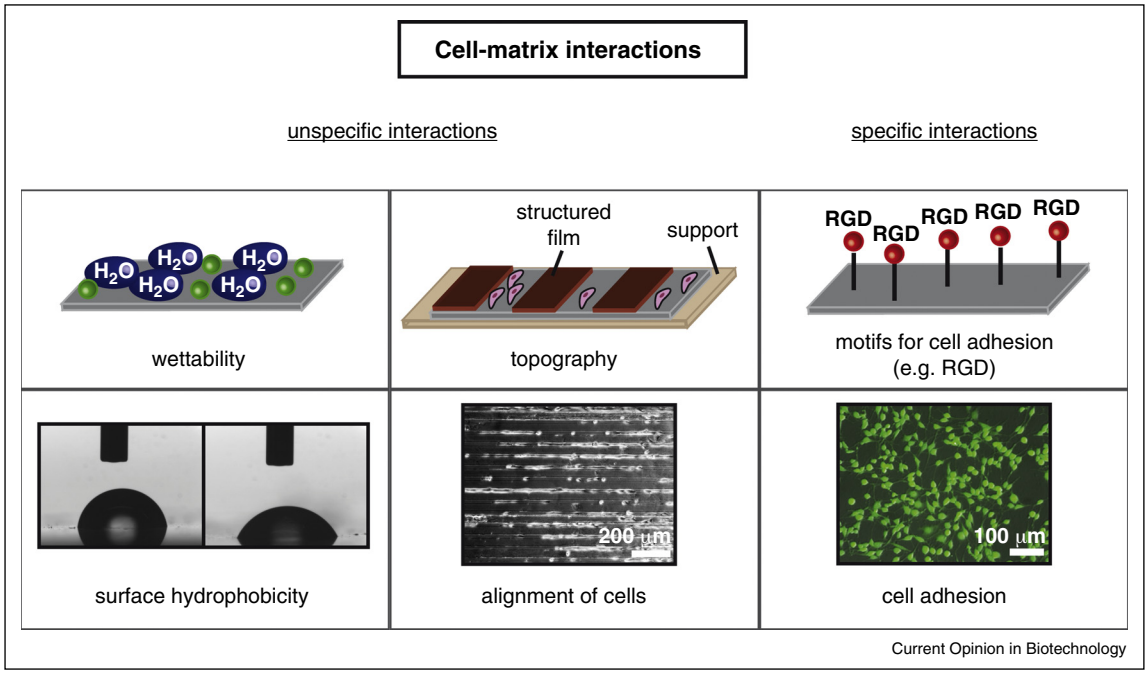
Chimeric proteins based on *N. clavipes* MaSp1, and the dentin matrix protein 1 (CDMP1), which provides controlled nucleation and crystallization of hydroxyapatite, targeted self-assembled silk structures with controlled hydroxyapatite (HA) mineralization. Upon processing into films, mineralization was initiated using simulated body fluids (SBF) [48]. Mineralized silk films mediated and promoted bone regeneration around wounds and promoted osteoblastic differentiation in a three dimensional scaffold [49–52].

Processing of engineered spider silk proteins for biomedical applications

Spider silk proteins can be processed into coatings which can be used to improve the biocompatibility and the surface properties of biomaterials, such as medical grade silicone implants. Silicones are highly resistant against hydrolytic and enzymatic degradation, otherwise they are considerably hydrophobic. Thus, adhesion of unspecific proteins and cells as well as proliferation of inflammatory and pro-fibrotic cells is promoted, all of which trigger foreign body-associated fibrosis [53,54]. The most frequently identified complication is capsular fibrosis, which occurs in up to 27% of the patients in the first year after surgery, and no modification of the silicone surface tested showed beneficial effects so far [55–57]. Coatings of eADF4(C16) yielded reduced fibrosis and contraction upon implantation in rats, since the silk coating inhibited fibroblast adhesion, proliferation and collagen I synthesis. Additionally, significant reduction in capsule thickness, post-operative inflammation and re-modeling of extracellular matrix were observed for silk coated implants in comparison to uncoated ones [58•].

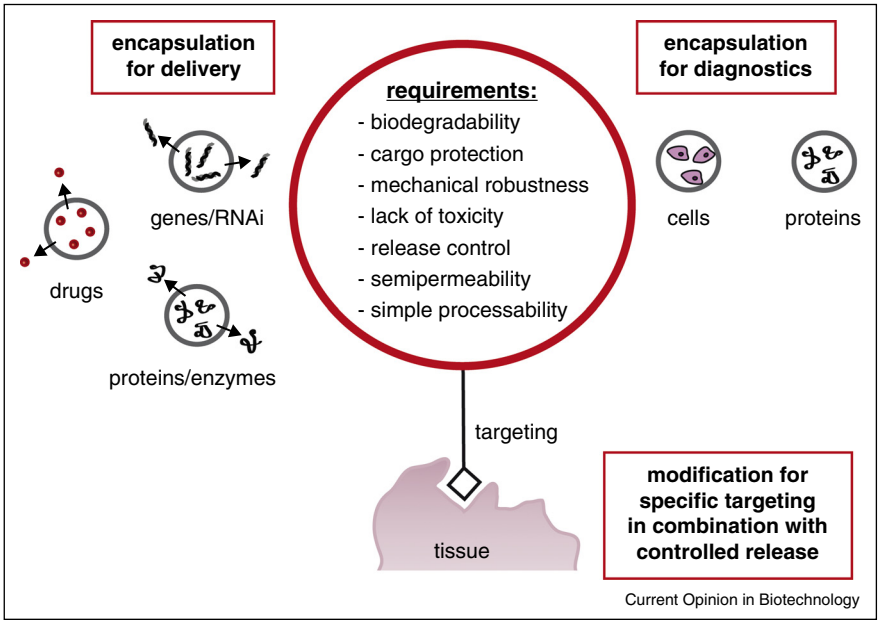
Non-woven meshes made of natural (e.g. collagen, fibroin) or synthetic (e.g. PLGA, PCL) polymers have a great potential as an artificial extracellular matrix (ECM) useful for wound healing and tissue engineering [59]. Electrospinning of eADF4(C16) yielded a mesh enabling the adhesion of fibroblasts (BALB/3T3) as well as their proliferation [28]. In contrast, fibroblasts did not adhere and proliferate on flat films indicating that the topography of a silk scaffold influences cell-matrix interactions [32]. Therefore, structured films were analysed to learn more about the influence of topographies on cell attachment [32,60]. Patterned silk films were made of two different silk proteins (RSSP and recombinant lacewing silk protein) using a Polydimethylsiloxan (PDMS) stamp. Both, fibroblasts (BALB/3T3) and myoblasts (C2C12), preferably adhered and aligned on the ground layer (RSSP) and not on the ridges (recombinant lacewing silk protein) [60]. In general, it can be stated that in the absence of specific cell adhesion domains (such as RGD), charge, wettability and topography of a surface

Figure 2



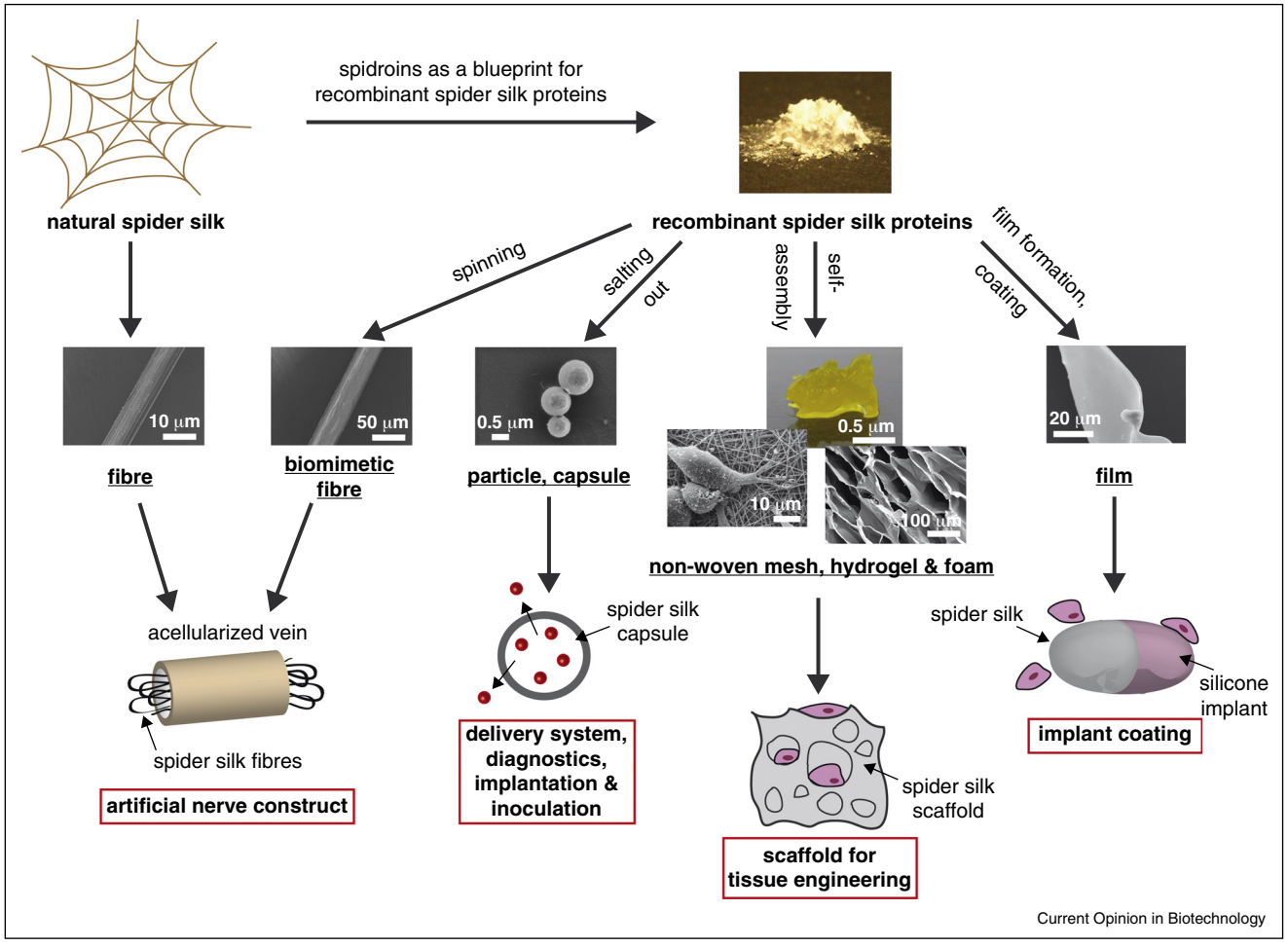
Overview of unspecific cell–matrix interactions determined by wettability and topography, as well as specific cell–matrix interactions mediated by motifs for cell adhesion.

Figure 3



Processing of recombinant spider silk proteins into colloidal systems for various biomaterials applications, such as drug, gene or enzyme delivery or cell and protein encapsulation for diagnostics.

Figure 4



Possible applications of spider silk materials in biomedicine. Recombinant spider silk proteins can be processed into morphologies other than fibres, broadening the spectrum of possible applications.

play an important role for cell attachment (Figure 2) [28,32]. Interestingly, in case of the patterned silk films the presence of the RGD-cell binding motif did not significantly improve cell binding, indicating the importance of topography [60].

In vitro studies demonstrated that three-dimensional porous scaffolds made of the RSSP rS1/9 allowed mouse fibroblast adhesion and proliferation. Within one week, cells migrated into the deeper layers of the porous scaffolds [61]. Next, porous scaffolds made of rS1/9 and natural silkworm fibroin were compared concerning microstructure and physiological behaviour. There was a detectable difference in the microstructure with the walls of the rS1/9 scaffolds being thicker and containing specific micropores in contrast to fibroin-based ones [62••]. The vascularization and intergrowth of the connective tissue, penetrated with nerve fibres, was more prominent in Balb/c mice 8 weeks after subcutaneous implantation using the rS1/9 scaffolds. Further, after implantation into bone defects of Wistar rats, the regeneration, accounting the number of macrophages and multinuclear giant cells, was better in case of the rS1/9 scaffolds.

Foams and fibre-based matrices made of 4RepCT supported growth, attachment and collagen type I production of human primary fibroblast. Further, macroscopic 4RepCT fibres were well tolerated when implanted subcutaneously in rats. In contrast to the control using silkworm silk (Mersilk^{TM}) fibres, 4RepCT-fibres supported ingrowth of fibroblasts and formation of capillaries in the centre of 4RepCT fibre bundles, indicating that 4RepCT is superior in supporting the physiological migration of fibroblasts and angioblasts [63,64]. Yang et al., 2010 used supersaturated simulated body fluids to deposit calcium phosphate coatings on 4RepCT spider silk fibre bundles, yielding a homogeneous and thick crystalline calcium phosphate (CaP) layer, which was a perfect template for bone marrow-derived hMSCs [65].

RSSP can further be used to encapsulate active ingredients including drugs, proteins, genes and cells for delivery or diagnostics (Figure 3) [66–68]. Low molecular weight drugs as well as proteins can be loaded into eADF4(C16) particles, which makes these slowly biodegradable particles a promising drug carrier system, with uptake and release of water-soluble substances being controlled by processing conditions and crosslinking [66–68].

Spider silk particles can also be used as gene carriers for tumour cell-specific delivery. Importantly, pDNA complexes of RSSP containing poly(L-lysine) and the tumour-homing peptides (THPs) F3 and CGKRK showed significantly higher target specificity to tumour cells and transfection efficiency in comparison to pDNA complexes of recombinant spider silk proteins without THP [69••,70].

Microcapsules made of eADF4(C16) resemble an enclosed reaction chamber with a semipermeable membrane, in which reactions can be initiated from outside. Further, the capsules can protect the enzyme (β-galactosidase was used as a model) against proteolysis. Recently established eADF4(C16) capsules are therefore promising tunable as well as protective enzyme reaction containers for technical and medical applications [71•].

Conclusion

In the past five years, the establishment of engineered recombinant spider silk proteins has led to a steadily increasing number of putative applications of spider silk materials (Figure 4).

The benefits of recombinantly produced spider silk proteins include the high quality and homogeneity of the raw material. Biotechnological production is further easily scalable, and processing as well as functionalization through genetic engineering are controllable. By incorporation of individual amino acids, functional peptide sequences or even proteins, novel spider silk hybrid proteins can be designed with a combination of natural and non-natural silk features, opening the road towards novel multifunctional (bio-) materials.

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