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Artificial Spider Silk Materials: From Molecular Design, Mesoscopic Assembly, to Macroscopic Performances

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Spider silk is one of the strongest and toughest fibrous materials available in nature. Its superior properties make it a good candidate for applications in various fields ranging from protective body armor to scaffolds for medical implants. However, harvesting a substantial amount of natural spider silk remains challenging because spiders cannot be easily bred. With the development of synthetic biology and its integration with materials science, considerable research has been directed toward engineering and production of synthetic spider silk materials. Here the study overviews general strategies on molecular design, mesoscopic assembly, and macroscopic regulation of artificial spider silk materials. The insights into the correlation between silk protein sequences, mesoscopic assemblies, and macroscopic material properties are provided for guiding de novo design and engineering of next-generation spider silk materials. This review also emphasizes the challenges and future perspectives for advancing the translational research on these designer functional materials for diverse applications.

1. Introduction

Having undergone an extensive evolutionary process, spiders can spin a variety of silk-based protein fibers with exceptional mechanical properties that outperform other natural and man-made polymers.[1,2] Except its admirable tensile strength and toughness, spider silk also exhibits unique physical properties, such as distinctive supercontraction and twist properties driven by humidity,[3,4] endowing it with extraordinary dynamic performance.[5–7] Moreover, spider silk displays superior thermal conductivity,[8] high optical resolution,[9] efficient moisture collection,[10] excellent biocompatiblity and biodegradability.[11] These multifaceted attributes establish spider silk as an inimitable entity among both natural and synthetic fibers, attracting significant attentions in interdisciplinary research fields.[12–14]

The excellent performances of spider silk are primarily attributed to its intricate hierarchical structure—comprising β-sheet crystals, α-helix, and disordered coil configurations.[15,16] The primary driving forces behind the formation of β-sheets are hydrogen bonding, and adjacent β-sheet structures stacked through hydrophobic interactions and van der Waals forces further consolidate β-sheet crystals.[16] From a crystallographic perspective, β-sheets are regarded as 2D crystal planes while β-sheet crystals form more stable 3D arrays.[17] As crystalline regions fracture, new crystals continuously form, thereby enhancing the capacity to dissipate external energy, providing a buffering action against structural breakdown of the silk.[18] The molecular chains within the β-sheet crystals are orderly aligned along the fiber axis, forming highly oriented microfibrils,[19] which leads to the formation of more hydrogen bonds, generating stronger intermolecular interactions within the β-sheet crystals to withstand greater external forces.[16,20] On the other hand, amorphous regions primarily consisting of α-helices and coils allow the molecular chains to extend under stress, bestowing elasticity and ductility to the spider silk. Taken together, the alternating arrangement of highly oriented crystalline and amorphous regions results in fiber structures that can tolerate mutually opposing mechanical forces, which provides an inspiration for the design of strong and tough fiber materials.[20–22]

Despite its exceptional mechanical features and alluring traits, harvesting a substantial amount of natural spider silk is challenging because spiders cannot be easily bred. It was only after deciphering genetic sequences of spider silk, the production of artificial spider silk by recombinant DNA technology became a feasible way for its large-scale production.[23,24] This engineering approach has enabled not only controlling mechanical performances of spider silk but also introducing new functionalities,[25–28] which further expands their applications in the fields of tissue engineering, sensing technology and biorobots.[29,30] However, current research continues to face substantial barriers including low production yield, less reproducible purification process, complicated assembly mechanism, and underexplored fabrication technology.[31–33] Addressing these limitations becomes increasingly urgent to push

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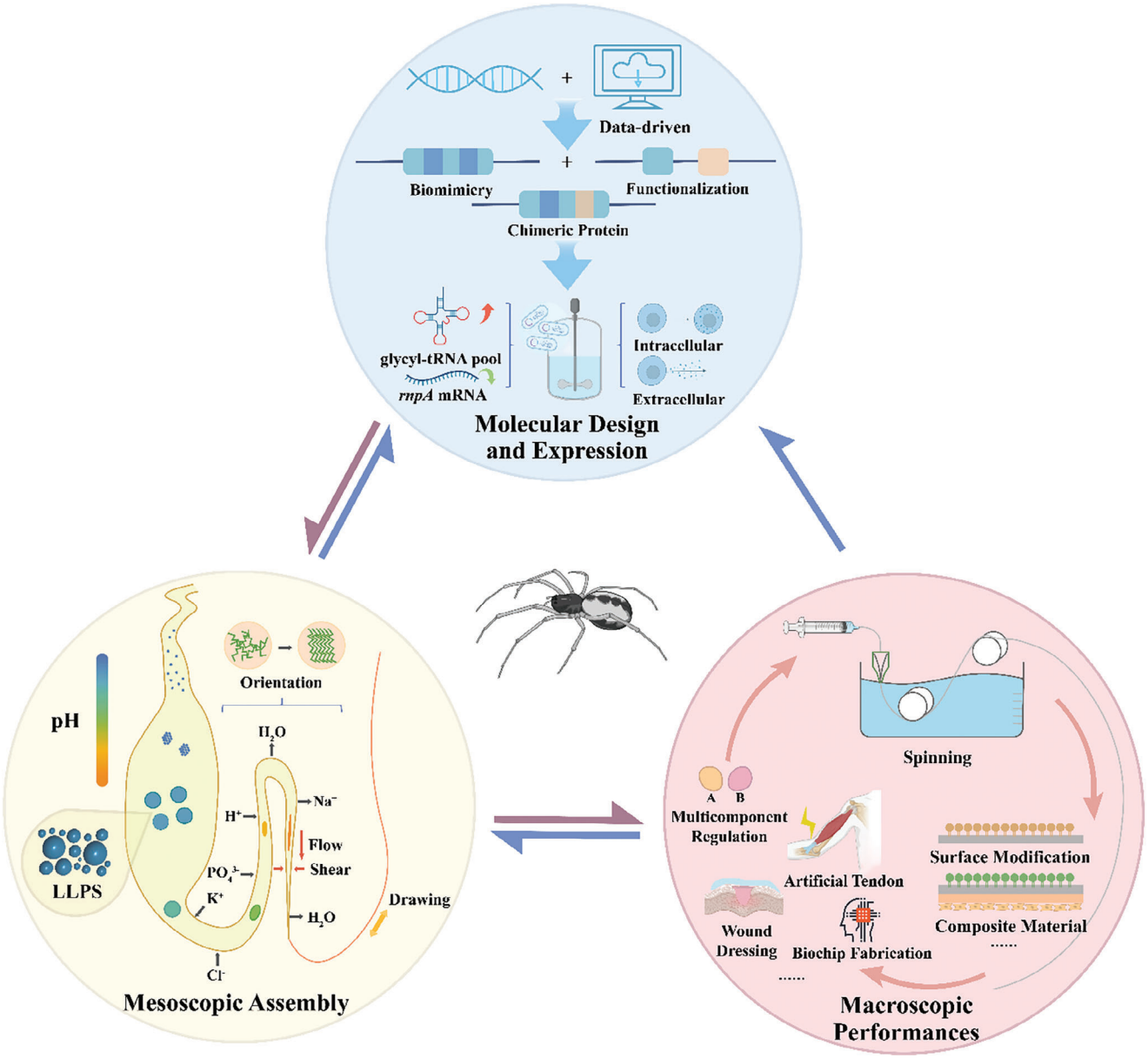

Figure 1. General procedures in generating spider silk protein materials via molecular design, expression, mesoscopic assembly, and macroscopic regulation. The maroon arrows indicate the direction of materials fabrication steps, and the light blue arrows indicate the direction of reciprocal guidance on materials refinements. Recombinant expression of designer spider silk proteins can be greatly elevated in engineered hosts by elevating endogenous supply of glycyl-tRNA to facilitate silk protein translation, and stabilizing the silk mRNA by downregulating a ribozyme (RNase P) protein subunit RnpA. The silk proteins can be produced inside the host cells or translocated to the extracellular milieu to facilitate recovery. Inspired by spidroin storage and fiber spinning in specialized glands and ducts of spiders, the silk proteins transform from a solution into a solid fiber by mesoscopic assembly, possibly via liquid-liquid phase separation (LLPS). Schematics of the spider, wound dressing, artificial tendon, and biochip fabrication are from BioRender's library of icons.

the development and commercialization of the artificial spider silk. Synthetic biology approaches for enhancing the production yield and reliability of artificial spider silk have recently been reviewed.[27,28,31] Moreover, advances in silk fabrication technology were also reviewed to recapitulate natural spider silk and create novel functions.[30,34] However, there is still a lack of efforts to emphasize the orchestration of the three key steps necessary for generating fine artificial spider silk, including molecular design, mesoscopic assembly, and macroscopic regulation.

Therefore, this review aims to elucidate the relationships between macroscopic properties of artificial spider silk and both molecular design and mesoscopic assembly (**Figure 1**). The state-of-the-art design principle, mesoscopic assembly mechanism, and the advanced fabrication technologies for high-performance silk materials are systematically reviewed. Furthermore, the

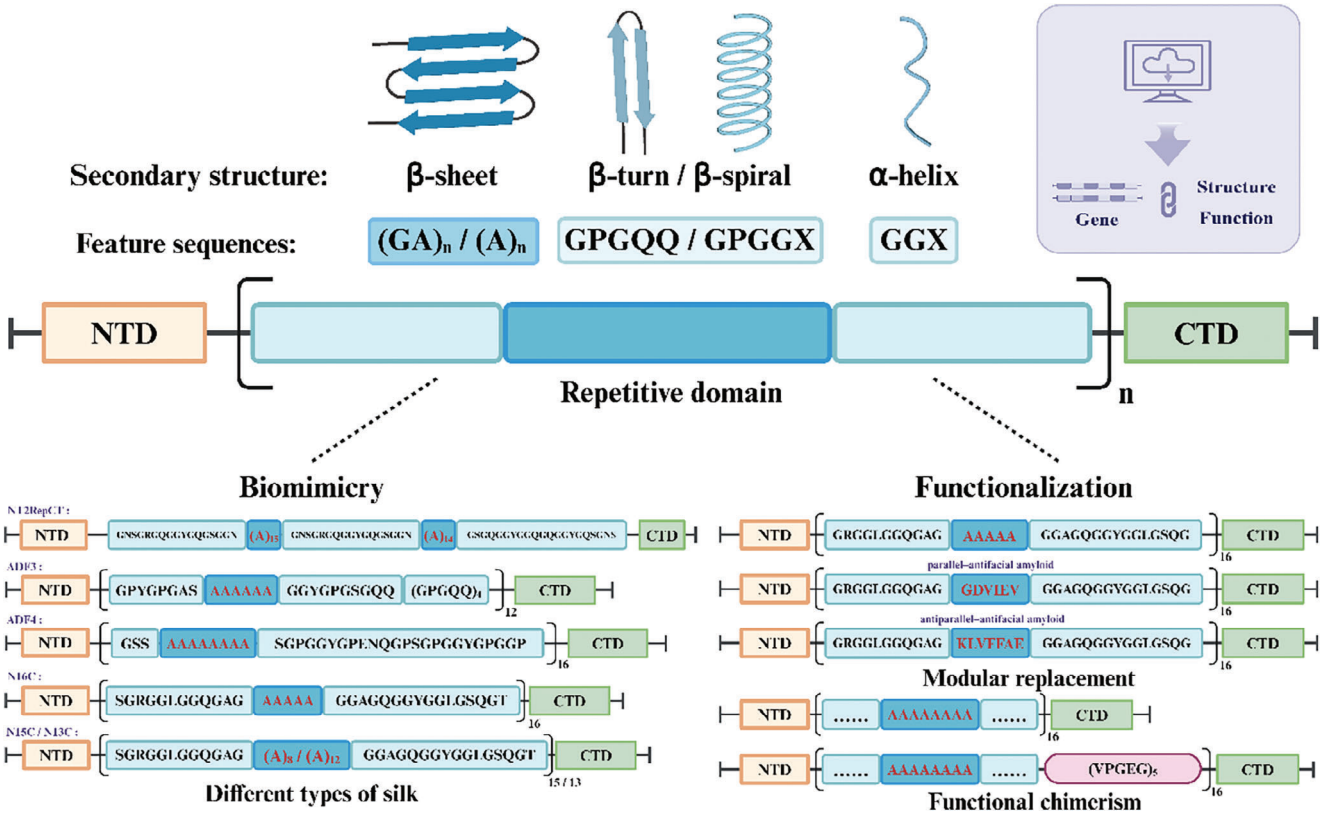

Figure 2. Molecular design of synthetic spider silk proteins by mimicking the natural spider silk sequences (biomimicry)[39–43] or constructing chimeric and modular silk proteins (functionalization)[44–46] which can be guided by computation.[47,48] Created with BioRender.com.

insights into the challenges and future prospects are also provided toward the data-driven design and advanced fabrication technologies that hold for revolutionizing the field of silk materials science.

2. Molecular Design and Expression

In the frontier area of artificial spider silk, the molecular design of silk proteins is not only fundamental to reproducing and enhancing the inherent physical properties of spider silk but also pivotal for achieving scalability and performance optimization in commercial applications. With the continual advancement in synthetic biology, researchers have successfully designed and expressed a series of artificial spider silk proteins (spidroins).[35–38] In the following section, we will review three major molecular design strategies including biomimicry, data-driven, and functionalization design, as well as the expression strategies for high-level production of synthetic spiroions.

2.1. Biomimetic Design

Biomimetic design based on the original feature sequence of natural spider silk is the foundation of all the molecular designs of synthetic spidroin. To date, different types of spidroins secreted by seven types of silk glands have been identified and their partial or complete sequences have been determined. These include the major ampullate, minor ampullate, tubuliform, aciniform, flagelliform, pyriform, and aggregate spidroins.[49] They generally share a tripartite composition with a large repetitive core domain flanked by highly conserved non-repetitive terminal domains – the N-terminal domain (NTD) and C-terminal domain (CTD) (**Figure 2**). The core domain sequences vary across species and silk types, containing alternating crystalline and amorphous regions that largely dictate the fiber's mechanical properties. For example, the most studied dragline silk protein MaSp1's core domain predominantly comprises polyalanine motifs An/(GA)n (where n ranges from 4 to 12) and several GGX (X denotes Ala, Gln, Leu, Tyr) sequences. The An/(GA)n sequences form distinct and orderly β-sheet crystal domains that provide strength and toughness to the fibers. The GGX/GPGXX motifs lead to the formation of disordered PPII helix and β-spiral structures, beneficial for high extensibility of the fibers.[16,32]

Interestingly, the core domains of MaSps typically exhibit low-complexity in amino acid sequences and adopt a mainly disordered structure in silk glands before the spidroins are spun into fibers.[50] The non-repetitive terminal domains NTD and CTD that are composed of five -helix bundles, not only enable the high-concentration storage of spidroin, but also are responsive to altered ion composition and shear forces to induce fiber assembly.[51,52] Compared to core repetitive sequences, spidroin NTD and CTD sequences in various spider species and silk types are highly conserved, and both of them have been demonstrated with conserved dimerization mechanisms.[51–54]

With the development of advanced genomic and bioinformatic methodologies, an increasing number of silk genes from different spider species have been sequenced[24,55,56] which allows us to design and synthesize recombinant spidroins with molecular-level precision. The key factors that we need to consider in the molecular design of a synthetic spidroin are the feature repetitive sequences, repeating numbers, and the presence or absence of terminal domains. In earlier studies, most designs only focused on mimicking the core repetitive sequences aiming to recapitulate the mechanical properties of the native spider silk.[35] Due to the aggregation-prone feature of the repetitive domains,[41] organic solvents are commonly used in the preparation of dope solution and dehydration during the coagulation process for fiber formation. However, this spinning process led to the optimal tensile performance of artificial spider silk by designing a synthetic spidroin containing 96 repeating motifs of spider *Trichonephila clavipes* dragline spidroin followed by a split intein-mediated ligation into 192 repeats.[57] The resulting artificial spider silk exhibited tensile strength of 1.03 GPa and toughness of 114 MJ m^{-3}, comparable to natural spider silk. More recently, a type of protein/peptide pair named SilkCatcher/Tag pair has been developed for isopeptide-bond-mediated protein ligation to generate large synthetic spidroins 4x Silk (259.5 kDa) and 5x Silk proteins (319.5 kDa) with >90% ligation yield and purity.[58]

After deciphering the molecular structures of the NTD and CTD, more studies started designing synthetic spidroins with tripartite compositions for biomimetic spinning. A milestone work was achieved by Rising group, that designed a biomimetic minispidroin NT2RepCT with a tripartite architecture (Figure 2) whose aqueous solubility equals that of native spider silk dope, imitating the molecular assembly mechanisms of the natural spinning process for fiber formation.[39] To further increase the fiber mechanical properties, our group designed a series of new biomimetic spidroins N16C, N15C, and N13C containing longer repetitive sequences and variable alanine residues in the Poly A motif. The resulting N15C spidroin allowed for the formation of stable nanofibril assemblies with a length of ≈200 nm, which enabled spinning of simultaneously strong (623.3 MPa) and tough (107.1 MJ m^{-3}) synthetic fibers.[41] Fusion of different silk domains serves as another effective way to increase silk mechanical performances. A novel two-in-one spidroin was engineered, resembling amino acid sequences of ADF3 and ADF4 proteins from the garden spider *Araneus diadematus*,[43,59] and combining the chemical and mechanical features of both proteins. The resulting fibers spun in a biomimetic, aqueous wet-spinning process, yielded mechanical properties at least twice as high as fibers spun from individual spidroins or blends. Although the biomimetic design and fiber spinning through all aqueous processing procedures have achieved significant progresses, it is still quite challenging to replicate the extraordinary mechanical properties of native spider silk.

2.2. Computer-Aided Design and Modeling

Recent advances in computational models and increased computational power enable more precise simulation and predictive design of complex proteins. However, due to the complex multi-phasic hierarchical structure of spider silk, it is still very challenging to establish a relationship between the primary sequences of various spidroins in spider silk and its mechanical properties.[24,47,60] Buehler group continues working on this field and has developed a coarse-grained model to describe the spider silk proteins as block copolymers, and used this model to reveal the key design parameters and processing conditions that control fiber assembly. The model was also validated by wet experiments including silk protein biosynthesis and characterization, as well as fiber spinning and tensile testing. Based on this integrated approach, they found that intermediate ratios of hydrophobic to hydrophilic block in natural spider silks and longer chain lengths lead to outstanding silk, and further demonstrated that natural silk design is based on the optimal combination of protein solubility, self-assembled aggregate size, and polymer network topology.[48] This study provides a general path toward de novo design of functional materials with enhanced mechanical properties and beyond.

On the other hand, artificial intelligence developments allow deep learning from existing sequence databases for genetic-level adjustments in peptide repetition, peptide arrangement with various characteristics, hence realizing precise and controllable protein design.[60–62] Most recently, Kazuharu and others sequenced 1098 species of spiders and measured the phenotypic properties of silk from 446 species, such as mechanical performance, thermal degradation, and hydration properties, thus creating an extensive "sequence-material property" database. More encouragingly, their analysis of the sequences offered insights into amino acid sequence features of underlying MaSp repetitive domains that have varying effects on different physical properties of spider dragline silk.[24] This provides a solid foundation of data and concepts for future de novo design of artificial spider silk. Leveraging this dataset, a spidroin sequence generation modeling was developed to design new spider silk protein sequences for achieving targeted mechanical properties. The model enables the generation of novel spider silk sequences featuring combinations of traits not found in nature, and through detailed analysis of sequence patterns and related properties, allows deeper understanding of the mechanistic role of sequence patterns in achieving overall key mechanical properties (elastic modulus, strength, toughness, failure strain).[63] This study lays out a promising blueprint for using deep learning algorithms to establish reliable models that predict the mechanical performance of spider silk fibers from spidroin sequences.

Furthermore, with a thorough understanding of spidroin structural characteristics, design strategies can be optimized to fine-tune attributes across all levels of spider silk, including mechanical strength, solubility, biodegradability, and biocompatibility.[61] This enables the de novo assembly and synthesis of silk fibers endowed with specific functions and performances, allowing precise control over spidroin sequences and structures to meet the demands of various domains. While limitations currently exist, such as inaccuracies in data correlation and difficulties in expressing some artificially designed sequences, the deepening understanding of spidroin allows us to refine models with more accurate relationships. Continuous supplementation of datasets will enhance the predictive and generative capabilities, contributing to the advancement and innovation within the field of artificial spider silk.

2.3. Functionalization Design

In addition to biomimicry design of feature sequences in spidroins, researchers also impart functional properties to them through selectively engineering the sequences or the integration of functional domains (Figure 2). The advances in engineering spider silk proteins for tailored functions at the molecular level have been previously reviewed, especially for modular incorporation of functional domains.[27,64] Here, we focus on the recent progress encompassing amino acid/peptide replacements and design of chimeric proteins.

By introducing hydrophobic or hydrophilic residues/peptide sequences within synthetic spidroins, researchers can effectively regulate the surface charges of artificial spider silk materials, thus influencing their stability and biocompatibility. Based on this principle, Weiss and colleagues designed different eADF4 proteins by genetically fusing with polyanionic/polycationic peptide tags, and fabricated them into silk materials with varying surface charges. They clarified the significant roles of surface charges of materials on the biomolecular corona composition when in contact with blood, which in turn affected macroscopic effects such as fibrin formation and blood clotting.[65] In another example, anionic supercharged polypeptides were genetically fused with spidroin module sequences to create membrane materials, which exhibited robust proton conduction and impressive mechanical stability.[66]

This design strategy not only aids in tailoring spider silk-based materials to specific biomedical applications, but also increasing the mechanical properties of spider dragline silk. Arndt and colleagues designed a series of mini-spidroins with designed modifications of the poly-Ala blocks using more hydrophobic residues like Val, and Ile which show a higher β-strand propensity.[25,34] These purposeful modifications indeed resulted in synthetic fibers with increased tensile strength and toughness, rendering them attractive for industrial applications.[25,34] Thus, the design and modification of critical motifs in original sequences of spider silk proteins have led to dual optimization of silk performance and production levels, offering promising perspectives for the commercialization of high-performance spider silk. Similarly, Zhang and others replaced the polyalanine sequences within the core repetitive region of spidroin with various more hydrophobic amyloid protein sequences, designing a series of recombinant spidroins.[67] These proteins, once spun into fibers, exhibited a higher β-sheet crystal content than their counterparts with similar molecular weights, demonstrating enhanced strength and toughness. This design strategy decreased sequence repetitiveness of recombinant spidroins, which conferred advantages in biosynthesis and purification.

Functional peptides, generally consisting of 2–50 amino acids with biological activities such as antimicrobial, cell adhesion, and targeted binding, can be incorporated into artificial silk coding sequences to obtain customized spidroin materials for diverse applications.[64] For example, incorporating RGD motifs into cysteine knot loops significantly enhanced the cell adhesion properties of recombinant spider silk with promising prospects in cell culture and tissue engineering.[68] As an emerging trend, various synthetic biology tools have been developed for the design of standardized, easily assembled peptide modules, and assembly into functional protein materials.[27,28] This synthetic biology approach endows spider silk materials with excellent properties even beyond the nature, propelling silk field toward a controllable and standardized design and fabrication process.[27,69]

2.4. Recombinant Expression Strategy

Synthetic spider silk proteins typically have long and highly repetitive sequences, which poses great challenges for recombinant expression such as genetic instability, high demands for certain tRNAs, premature termination of transcription or translation, and potential degradation of large protein products.[31,35] Thus, an appropriate expression strategy is essential to produce these synthetic spidroins. Selecting suitable expression hosts is the first step to ensure the quality and yield of the silk protein. A recent review has thoroughly overviewed and compared all the host systems for the production of synthetic spidroins,[31] including microbial cells, plant cells, transgenic plants, and animals. The microbial hosts are generally regarded as the most commonly used and industrializable hosts because of their fast growth and facile genetic manipulation. Since 2010, Xia and colleagues have metabolically engineered bacterial *E. coli* cells to produce native-sized dragline silk protein MaSp1 of spider *T. clavipes*. By increasing the supply of glycyl-tRNA within cells, the expression level of native-sized MaSp1 (284.9 kDa) was significantly increased more than tenfolds, and no obvious truncation was observed by western blot analysis of the expressed spidroins.[35] Building upon this, Yang and colleagues combined this host engineering strategy with low-temperature induction (16 °C) to express high molecular weight dragline silk protein MaSp2 (201.6 kDa) in *E. coli*. This combined strategy alleviates the nutrients and energy competition between cell growth and protein expression, which enables the production titer of target protein reaching 3.6 g L^{-1}, the highest level reported to date for the high molecular weight spidroins.[70] In addition to augmenting the intracellular supply of glycyl-tRNA, employing an RnpA knockdown system to boost mRNA stability emerges as an effective strategy for the expression of large spidroin fragments. Owing to RnpA's role in degrading rRNA and mRNA, Lee and colleagues strategically developed a synthetic small regulatory RNA-based knockdown system to reduce the expression level of RnpA, which subsequently increased mRNA stability and promoted the expression of large spidroins.[71]

The relatively low production level of large spider silk proteins is one of major limiting factors for the fabrication of high-performance materials and industrial applications. To address this problem, Rising and others designed a miniaturized spider silk protein NT2RepCT (33 kDa) containing two tandem repeat units and terminal domains. After high-density fermentation, the yield reached 14.5 g L^{-1} with excellent water solubility,[72] enabling the biomimetic spinning of high-performance silk fibers.[73] Another important attempt was to initially express low molecular weight spidroins with high yields and then employ appropriate protein ligation/interaction strategy in vivo or in vitro to increase the molecular weights. For example, fusion of split Mfp5 fragments to the N- and C- termini of a repetitive spidroin (≈54 kDa) endows the resulting protein fibers with a toughness comparable to natural spider silk, due to the end-to-end intermolecular interactions between bi-terminal fused Mfp5

fragments. Most encouragingly, the production level of this relatively small recombinant spidroin reached ≈8 g L^{-1}.[74]

Currently, most recombinant spidroins are intracellularly expressed in the cytosol of microbial hosts, which would require cell lysis to purify the recombinant silk proteins. To simplify the purification process, our research team recently established an efficent platform for the extracellular secretion of spider silk proteins in gram-positive bacterium *Corynebacterium glutamicum* through signal peptides screening and metabolic engineering.[75] After high cell-density fermentation and one-step precipitation purification, a yield of 2.2 g L^{-1} of recombinant MaSp1 (43.36 kDa) was achieved. The purified spidroin showed extremely high solubility (66%) and after wet spinning, yielded artificial spider silk fibers with a toughness of 70 MJ m^{-3}. This secretion method not only simplifies the purification process but effectively avoids endotoxin issues related to gram-negative bacterial hosts, providing a prerequisite for the application of artificial silk materials in the biomedical field. In fact, the exploration of secretion expression of recombinant spidroins has never ceased. Stephen and colleagues have also tried to secrete recombinant spidroins by another gram-positive bacterium *Bacillus subtilis*. Surprisingly, they found the translocation mechanism of *Bacillus subtilis* drove the silk proteins to assemble into fibers spontaneously on the cell surface, which provided a facile route to create living materials from cells.[76]

In summary, design of artificial spider silk is mainly based on spidroin feature sequences and modular functional domains which are tailored for various applications. The design strategies include specific amino acid modifications, functional peptide incorporation, chimeric protein fusion, and de novo computational design. After molecular design, the selection of appropriate expression strategies is to ensure both protein quality and yield, which covers host selection, post-translational protein modifications, cellular and metabolic engineering, etc. In the following section, we will shift the focus toward understanding how these spidroin molecules experience intricate assembly processes at the mesoscopic level to remarkable macroscopic performances. In fact, designs at the molecular level dictate the biophysical properties of spidroins, which are presented and amplified through complex self-assembly at the mesoscopic level. This mesoscopic assembly endows artificial spidroins with a high degree of controllability and potential for forming diverse materials with excellent properties.

3. Mesoscopic Assembly

The assembly of materials at the mesoscale level involves not only how proteins interact and build higher-level structures but also how to utilize these structures to realize and optimize specific properties such as mechanical strength, elasticity, and biocompatibility. However, a lack of understanding of assembly at the sub-micron level—commonly referred to as the mesoscale—creates a disconnect between micro and macro views. We aim to delve deeper into these assembly processes and their critical roles on the final performance of artificial spider silk, emphasizing strategies on mesoscopic assembly.

Typically, the spinning of synthetic polymer fibers requires high temperatures or toxic solvents through either melt or wet spinning methods, whereas spider silk is spun under remarkably mild conditions, merely extruding aqueous spidroin solution through spinning duct into air at ambient temperature and pressure, forming fibers with excellent mechanical properties (**Figure 3a**).[77] To elucidate the assembly process of spidroins, several models such as the liquid-liquid phase separation (LLPS) model,[78–80] the liquid crystalline model,[49] and the micelle model[81] have been proposed to interpret the underlying mechanisms (Figure 3b). Although each model emphasizes different aspects, they can be unified throughout the stages of spidroin stock solution, intermediate states, and fibers. The distinction lies in the mesoscale assembly of the spidroins, with each model corresponding to highly dynamic droplets (condensates), rod-like structured liquid crystals, and micelles, respectively.[32,80]

Inspired by the natural spinning processes of spiders, several strategies are provided for controlling mesoscopic assembly of artificial spider silk: 1) adjusting pH, ionic environment, or dope concentration of synthetic spidroins to tune their inter- or intramolecular interactions for mesoscopic assembly into micelles, nanofibrils or condensates; 2) applying effective shear force and elongational flow to dope solutions (with or without mesoscale structures) to enhance their molecular chain orientation and alignment.

3.1. pH and Ionic Environment-Induced Mesoscopic Assembly

It has long been regarded that factors such as pH and ionic environment uniquely affect the assembly of native spidroins into silk fibers. In 2010, the effect of pH and/or ionic environment on the assembly of spider silk was revealed for the first time from the point of molecular structures, emphasizing the critical role of terminal domains in the meso-scale assembly process[51,52] The decrease of pH induces dimerization of NTD, which contributes to fiber assembly by strengthening inter-spidroin molecular interactions.[82] Recent mechanistic studies with the NTDs from different silk types have shown that this pH dependency is conserved, but the key residues that mediate this dimerization might differ.[83,84] For the CTD, its structure tends to loosen as pH drops, and expose hydrophobic regions, thereby undergoing a β-sheet transition under shear forces, acting as nucleation sites to rapidly induce more β-fold crystals.[85,86] Furthermore, the pH dropdown-triggered NTD dimerization has been reported to be related to microfibrillar formation, while the CTD and central repetitive domain are related to LLPS of the spidroins.[78]

Based on the spidroin assembly mechanism, Rising group developed for the first time a biomimetic spinning process to assemble a chimeric minispiroin into artificial fibers. Notably, the spidroins had aqueous solubility which equals that of native spider silk dope and assembled into micelles that fused and elongated upon shear.[39] Recently, our group designed a biomimetic spidroin composed of N- and C-terminal domains bracketing 16 consensus repeats of the core region from spider *Trichonephila clavipes*. Interestingly, the spidroin assembled into fibril-like (rather than canonical micellar) nanostructures in concentrated aqueous dope, and ordered alignment of these nanofibrils was observed upon extrusion into an acidic coagulation bath (Figure 3c). Notably, the presence of both termini was essential for this self-assembly.[42] We further modulated the polyalanine (polyA) motifs in repetitive region of the spidroins, and the

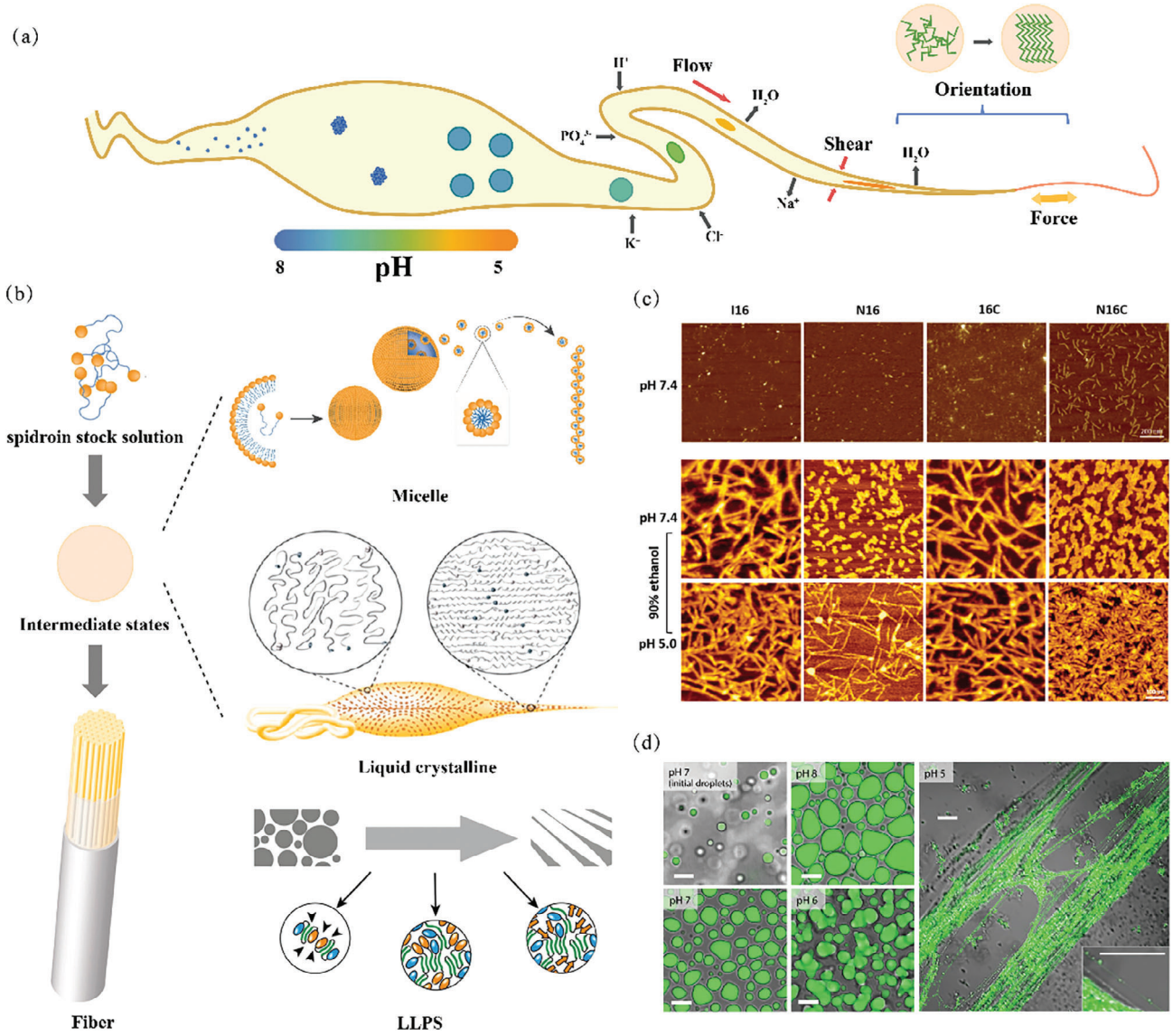

Figure 3. Nature-inspired mesoscopic assembly and regulation of synthetic spidroins. a) The assembly of native spider silk proteins is influenced by pH, ions, and shear forces. b) Three mesoscopic assembly states (adapted from refs. [32,79,80] under the creative commons attribution license) proposed to mediate the transition of spidroins to fiber. c) The presence of both termini in the synthetic spidroin N16C induces its self-assembly into fibril-like nanostructures in concentrated aqueous dope (upper panel) and ordered alignment of these nanofibrils upon extrusion into an acidic coagulation bath (lower panel) (adapted from ref. [42] under the creative commons attribution license). d) Acidification triggers rapid self-assembly of synthetic spidroin condensates into aligned fibril networks (adapted from ref. [78] under the creative commons attribution license).

morphology and stability of pre-assemblies in the aqueous dope solution could be tuned correspondingly, thus controlling the structural and mechanical properties of the resulting fibers.[41]

It should be noted that the control over mesoscopic assembly structures and processes plays pivotal roles not only in macroscopic mechanical properties but also biocompatibility. Central repetitive regions of spidroins share structural similarities with amyloids, both forming nanoscale fibrillar aggregates with high levels of order.[87] However, amyloid-like fibrils produced from spidroins are not significantly toxic when compared to amyloid fibrils derived from human pathogenic amyloidogenic peptides. The fundamental differences between the two types of nanofibrils are their primary structures and the mechanisms underlying their fibril formation. Spidroins use a non-nucleation-dependent isodesmic aggregation pathway, whereas pathogenic amyloidogenic peptides use a nucleation dependent amyloid forming pathway.[88] Therefore, it is possible to control the self-assembly process of synthetic spidroins and the resulting mesoscale structures to improve their biocompatibility.[45]

3.2. Mesoscopic Assembly through LLPS

It is reported that protein solutions are enriched in the silkworm silk gland as micron-sized spherical assemblies, providing a reversible intermediate state for the generation of silk

fibers.[80] This state plays a dual regulatory role: one is to enable the storage of a highly unstable and aggregation-prone protein solution by decreasing local viscosity, another is to regulate protein crystallization under applied shear during the spinning process.[80] Although this study did not claim these micron-sized spherical assemblies were formed via LLPS, the morphology and structural characterization most probably proved that. In addition, Numata group provided another line of evidence by extracting native spidroins from spider major ampullate gland for characterization.[78] They proved that native spidroins exhibited liquid-phase condensation upon exposure to phosphate ions at neutral pH, and spontaneously assembled into fibril structures responsive to phosphate ions at acidic pH (Figure 3d). Although it is challenging to elucidate the actual structural form of the proteins inside the liquid condensates when stored in the gland, and the process by which this precursor solution is transitioned into a hierarchical crystalline structure when spun through the duct. However, an increasing number of in vitro reconstitution studies have demonstrated that synthetic spidroins could form liquid condensates driven by LLPS via controlling the factors such as solvent type, concentration, pH, temperature, and flow rate.[78,89–91]

Recent studies have provided new insights into the role of protein sequences on LLPS, where tyrosine (Tyr) and arginine (Arg) residues in synthetic spidroins might also be critical molecular "pins" propelling LLPS except the previously proposed role of polyalanine (PolyA) sequences.[89,91,92] In addition, the CTD is considered as a folded sticker that mediates LLPS, and its destabilization drives the conversion of droplets to fibers by nucleating β-sheet aggregation upon external stimuli.[79] Not only does this study enhance our understanding of the driving forces behind LLPS, but it also provides a strategy to regulate the LLPS process by engineering the key amino acid residues. It is noted that LLPS mechanism is spidroin sequence dependent, and different molecular designs of spidroins might result in inconsistent conclusions.[78,79,89,90] Therefore, careful characterization should be performed to study the LLPS process of respective synthetic spidroins to control their mesoscopic assemblies and the resulting spider silk properties.

With the development of advanced imaging and analytical techniques, scientists will be gradually unlocking the molecular mechanisms and pathways involved in the LLPS process of spidroins in vivo,[80,93–95] providing a robust scientific foundation for the development of highly customized biomaterials. Built upon this, the mesoscopic assembly achieved through LLPS might allow artificial spider silk in the future to closely match or even surpass natural spider silk in terms of mechanical strength, structural stability, and functionality, thus significantly expanding their potential applications.[96,97]

3.3. Flow Shear-Mediated Mesoscopic Assembly

Flow shear serves as a major factor influencing mesoscopic assembly of spidroins because it not only disrupts the protein's hydration layer, allowing molecular chains to contact and form secondary structures such as β-sheets, but also promotes the formation of oriented β-sheet nanocrystals that impart outstanding strength to the fibers. It is commonly believed that natural spinning involves flow and chain alignment, however, its connection with spidroin mesoscopic assembly remains obscure. A series of recent studies have supplemented the understanding of how flow affects protein chain deformation, orientation, and phase separation on both energetic and molecular levels, filling a key gap in our grasp of spidroin assembly pathways.[98–101]

Microfluidic technology, as a means to precisely control the experimental conditions such as pH, ions, and shear, has significantly increased the understanding of the mesoscopic assembly of spidroins.[102] A recent study designed a finely controllable microfluidic device composed of LLPS region, pH drop, and fiber assembly region.[93] Initially, LLPS of the spidroin solution is induced by citrate-phosphate buffers. Further, the microfluidic system adjusts ion strength and lowers pH to 5, prompting the LLPS state of spidroins to form preliminary microfibers. Finally, narrowing spinning orifices coupled with applied shear force promotes the formation of artificial spider silk. Compared to conventional wet-spinning or dry-spinning methods, this process substantially replicates the natural spider silk spinning process, recreating the LLPS transformation at the early stages of spidroin spinning.

Computational simulations have also revealed the conformational changes of spider silk proteins upon hydrodynamic flow, validating that flow stretches the spidroin molecules, and pushes alanine residues into β-sheet and poly-proline II conformations.[103] Coarse-grained simulations of the assembly process further reveal the spidroins aggregate faster but into low-order assemblies when they are less extended. At medium-to-large peptide extensions, assembly slows down and becomes reversible with frequent association and dissociation events, whereas spidroin alignment increases and alanine repeats form ordered regions.[98] The exploration of these mechanisms inspires us to find the critical point for the required self-assembly and employ controllable flow shear to induce spidroin assembly, enhancing the performance of artificial spider silk materials simultaneously as the spinning process is optimized.

After studying the details of mesoscopic assembly of artificial spider silk, we will explore how these assembled structures can be converted into the macroscopic performances required for practical applications. The finesse of the mesoscopic assembly ensures the formation of spidroin microstructures and their highly oriented arrangement, which forms the basis for optimizing macroscopic mechanical properties. The following section will focus on the mechanisms and techniques of physical performance regulation at the macroscopic level for artificial spider silk, such as improving key performance indicators like mechanical strength, extensibility, and durability through various post-treatment technologies.

4. Macroscopic Performances

It is essential to integrate our knowledge of mesoscopic assembly into macroscopic engineering to optimize the performance of artificial spider silk for applications. Researchers have long endeavored to replicate the mechanical properties of native spider silk by developing a suite of spinning and post-treatment techniques (e.g., wet-spinning, dry-spinning, electro-spinning, microfluidic spinning). With other advanced techniques, artificial spider silk proteins can also be fabricated into other forms of materials

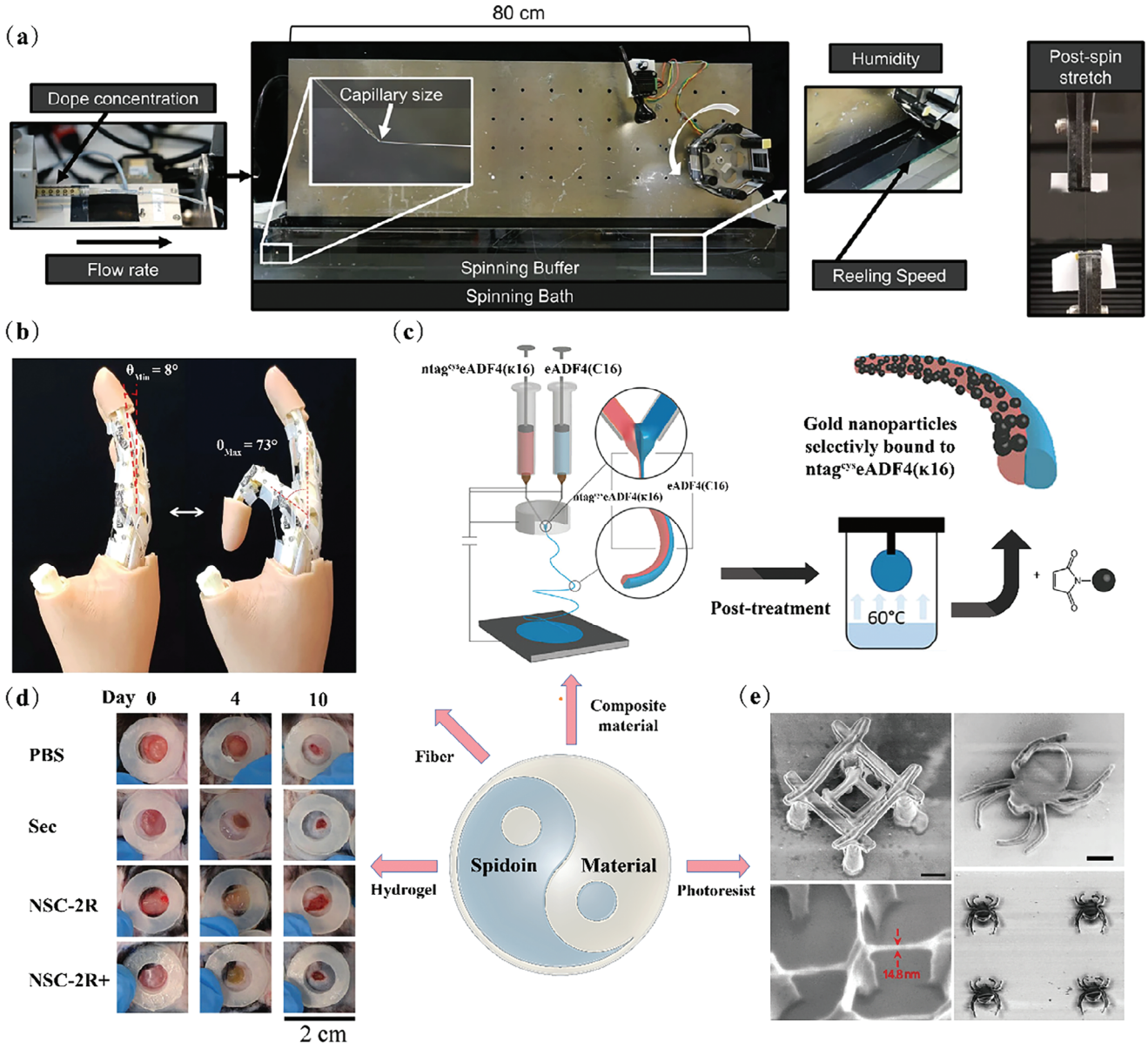

Figure 4. Macroscopic regulation of spider silk materials for diverse applications. a) Spinning setup for artificial spider silk production with the controlling parameters highlighted (adapted from ref. [104] under the creative commons attribution license). b) Photograph of the finger bent to the maximum position pulled by a supertough electro-tendon based on spider silk composites (adapted from ref. [105] under the creative commons attribution license). c) A side-by-side electro-spinning strategy to produce Janus fibers that combine two different spider silk proteins, and site-specifically functionalize one phase by covalent binding of AuNPs (adapted from ref. [106] under the creative commons attribution license). d) Selected diabetic wound closure images indicate the secretome-laden hydrogel of chimeric spider silk fusion protein (NSC-2R+) exhibiting enhanced wound closure compared to the control group (adapted from ref. [107] under the creative commons attribution license). e) 3D nanostructures fabricated by electron beam lithography using recombinant spider silk proteins as the resist (adapted from ref. [108] under the creative commons attribution license). Scale bars in the SEM images: 1 µm.

except fibers and endowed with unnatural properties for diverse applications.

4.1. Biomimetic Spinning

To fabricate artificial spider silk with structures and properties comparable to natural silk, researchers have continuously explored fabrication methods inspired by the natural spinning process of spiders. Wet-spinning is one of the most commonly used and effective means of producing artificial spider silk. During the wet-spinning, concentrated spidroin dopes are extruded directly into a coagulation bath through spinnerets, solidifying into nascent fibers (**Figure 4a**). The properties of resulting fibers are seriously affected by various spinning conditions such as dope concentration, dope flow rate, the diameter of the capillary opening, humidity, presence of NaCl, reeling speed, buffer composition/temperature, post-spin stretching, etc.[104,109–111]

A recent study has systematically compared the effects of these spinning conditions on the mechanical performances of fibers spun from artificial NT2RepCT spidroins. It revealed that excessively high or low protein concentrations, high flow rates, and presence of NaCl adversely affected the performances of artificial spider silk.[104] Under conditions of increased relative humidity, the toughness of artificial spider silk fibers was enhanced but tensile strength decreased,[112] and the addition of acetonitrile and polyethylene glycol to the collection bath resulted in an increased β-sheet content in the fibers, thereby improving the mechanical properties.[113] In addition, reeling speed for fiber collection and post-spin stretching was identified as one of the most critical factors in augmenting the mechanical tensile strength of the fibers. Higher reeling speed resulted in smaller diameter and higher polarized light birefringence of fibers, while enhancing the silk strength. For example, artificial NT2RepCT silk strength increased from 30 to 114 MPa when reeling speed was raised from 17 to 58 cm s^{-1}. Post-stretching further strengthened the silk, reaching a tensile strength of 261 MPa at 80% stretching ratio,[104] which might be due to the improved molecular order and crystallinity of fibers.[111,114]

Dry spinning has attracted much attention because it comes closest to natural spider silk spinning.[2,111] However, this method needs extremely high concentration of spidroin dopes for fiber spinning which makes it challenging to keep the dopes stable in vitro and form continuous fibers. A recent study reported that dry-spinning could be successfully achieved using the regenerated spidroin dope via the addition of zinc acetate, which yields artificial spider silk with a Young's modulus of up to 14 GPa and toughness of 51 MJ m^{-3} after the post-stretching treatment.[115] Beyond this organic salt-assisted dry spinning, an anisotropic (liquid crystal)-based dry-spinning process offers a more promising potential. A highly viscous and stable nematic silk microfibril solution was first prepared via partially dissolving cocoon silk fibers, and then spun into regenerated silk fibers by direct extrusion in the air. This method offers a useful route to generate polymorphic and hierarchical regenerated silk fibers with a modulus of 11 ± 4 GPa, even higher than natural fiber.[116]

If the spinning process is divided into two phases based on before and after the dope solution leaves the spinaret, traditional wet- and dry-spinning methods try to mimic the latter phase, whereas microfluidic spinning aims to mimic the transition of dope solutions within the gland (the former phase).[102,117] It remains rather challenging to effectively piece these two phases together to simulate the entire biomimetic spinning process. Most recently, Numata group attempted to achieve this by designing an integrated microfluidic device that simulates the native-like chemical and physical gradients within the channel. Although this device incorporates three important assembly processes including ion-induced liquid-liquid phase separation, pH-driven fibrillation, and shear induced β-sheet formation, the resulting fibers still exhibit inferior mechanical properties.[93] The major reason might be due to the lack of post-drawing and treatment process to optimize β-sheet crystallization and alignment. Therefore, the combination of micofluic chip and post-drawing process is supposed to be a good strategy that has been demonstrated previously, endowing the artificial spider silk with favorable performances.[41,117]

4.2. Multi-Component Engineering

Unlike protein design at the molecular level, composites engineering involves blending spidroin with other types of spidroins or materials during the spinning process. This strategy aims to emulate the complex structure of natural spider silk, utilizing the molecular interactions and complementary properties of different spidroins to yield composites with outstanding performance. The natural dragline silk is mainly composed of two spidroins MaSp1 and MaSp2, with MaSp1 uniformly distributed within the silk's core, and MaSp2 clustered in the most interior part of the silk core.[15] However, the presence of the MaSp3B spidroin as well as several nonspidroin spider-silk constituting element (SpiCE) in dragline silk of golden orb-weaver spiders were also revealed by a multiomics study, combining genome sequencing, silk gland transcriptomics, and dragline silk proteomics.[55] Although it is still unclear how this composition of spider silk is achieved and what role it plays concerning the mechanical properties of the resulting fibers, it implies that varying the proportions of these different proteins might produce fibers with different mechanical properties.[32]

The impact of SpiCE was tested by producing composite films of recombinant MaSp and SpiCE, which demonstrated the incorporation of SpiCE enabled MaSp-SpiCE mixed film achieving twice the tensile strength of MaSp film. However, composite silk fibers with SpiCE did not show an increased tensile strength, which might be because SpiCE disturbed the strain-induced crystallization of silk molecules.[55] Molecular dynamics simulations further revealed that SpiCE protein contained more amino acids that could act as hydrogen bond acceptors/donors and salt bridge partners, which significantly amplified the interchain interactions with MaSps to obtain increased Young's modulus.[55,118] In spider eggcase, the role of the minor spidroin TuSp2 was also demonstrated to significantly promote the self-assembly and chain alignment along the fiber's long axis of the main component TuSp1. Artificial fibers spun from the mixture of TuSp1 and TuSp2 spidroins exhibited strength and Young's modulus superior to its native counterpart.[119]

To study the possible interplay of two different spidroins ADF3 and ADF4 from *A. diadematus* and effects on the mechanical properties of fibers, Scheibel group co-produced these two spidroins in *Escherichia coli* for fiber spinning. Intriguingly, they found the heterodimerization of CTDs in these spidroins resulted in fibrillary network structures upon assembly. In combination with biomimetic spinning, the resulting fibers exhibited mechanically native-like performances with strength (834 ± 34 MPa), toughness (143 ± 6 MJ m^{-3}) and extensibility (32 ± 1%).[59,120] The major bottleneck for this strategy is the potential batch-to-batch variations of cross-linked networks due to the co-production of multiple spidroins. To address this issue, Li and colleagues produced separately two engineered spidroins AQK5-CTD and CK5-CTD, and then mixed them together for dope preparation. Notably, the natural spidroin modules, designated as AQ and C, are derived from ADF3 and ADF4, and K5 consists of repeatable sequence $(VPGKG)_5$ for soluble expression. Wet-spinning of these two proteins in a 3:2 mixture resulted in dual-component spidroin fibers with minimal batch-to-batch variation and high toughness.[121]

To bestow artificial spider silk with performance and properties beyond nature, researchers began to focus on the doping of spider silk with nanomaterials, such as graphene, carbon nanotubes, iron oxide, cellulose, and more.[122–124] For nanomaterial doping, pre-doping (solution doping) and post-doping (fiber doping) are considered. Post-doping is straightforward but might lead to uneven nanomaterial distribution within the fiber, adversely affecting performance,[123,124] and pre-doping allows for more uniform distribution within the fibers but the nanomaterial might affect assembly of the spidroins.[125] As a successful case of post-doping, natural dragline silk thread was coated with a layer of a magnetostrictive FeCo alloy which secured excellent mechanical strength of silk and imparted both electrical conductivity and stress-sensitive magnetic properties to it.[126] The doping of spider silk is not limited to a single material, but can also be multi-components. An "electro-tendon" based on artificial spider dragline silk fiber was recently reported by coating a conductive layer containing both PEDOT:PSS and single-walled carbon nanotubes (SWCNT).[105] Because of hydrophilic PSS, PEDOT:PSS has good adhesion with the processed spider silk to form a conformal conducting layer. On the other hand, the introduced SWCNT improved the toughness of fibers. The resulting fibers can withstand more than 40000 bending-stretching cycles without changes in conductivity (Figure 4b), which is promising for the development of robots and various applications in advanced manufacturing and engineering.[105] More interestingly, a complex bi-functional structure with spatial resolution was developed by electrospinning a cysteine-modified spidroin protein, ntag^{Cys}eADF4(k16), and "non-functional" eADF4(C16) together into Janus fibers in a side-by-side arrangement (Figure 4c). Gold nanoparticles were selectively deposited only on the cysteine-modified spidroin side to form a conductive gold layer, which was potentially useful in the field of nerve regeneration.[106] Taken together, multi-component regulation not only enhances the mechanical properties of artificial spider silk, but it also opens up new possibilities for fabricating multi-functional fibers with specific applications.[30,127]

4.3. Other Material Forms and Frontier Applications

With deeper insights on the meso- and macro-assembly of artificial spidroins, the researchers have developed diverse material forms based on the synthetic spidroins and explored their frontier applications. The unique 3D porous structure and commendable biocompatibility of artificial spidroin-based hydrogels have rendered them ideal for 3D printing and tissue engineering.[46,128,129] During study on the function of CTD structure in fiber spinning, our group occasionally found that recombinant CTD proteins were sensitive to both low (≈4 °C) and high temperature (≈65 °C) to form a novel dual-thermosensitive hydrogel.[130] The fusion of elastin-like polypeptides could tune the dual transition temperatures of CTD to a physiologically relevant window, which is envisioned for on-demand cell preservation and delivery.[131] Interestingly, NTD was also observed to form self-supporting and transparent hydrogels at 37 °C, which provides a protein immobilization platform to protect the fused proteins with intact functions.[132] Due to their contribution to the high solubility and assembly of artificial spider silk dope, NTD and CTD of spidroins were fused with the suckerin peptides to generate a fusion protein. This protein is capable of thermal gelation at physiological temperatures for encapsulation and subsequent release of the stem cell secretome, which has been demonstrated as a promising strategy to enhance healing in chronic wounds of diabetic mice (Figure 4d).[107]

We and collaborators have creatively used recombinant spider silk protein as a resist for the high-fidelity manufacturing of functional, arbitrary 3d nanostructures by ion and/or electron beam lithography (Figure 4e). Genetic or mesoscopic modification of spidroins provides the great opportunity to impart diverse biological functions within as-fabricated 3d nanostructures for biomedical applications.[108,133] This study not only enriches the diversity of spider silk forms, but also provides an advanced technology for the fabrication of functional silk materials. In addition, the exceptional fabrication of synthetic spidroins has also led to the development of antibacterial spider silk films as bioselective coatings,[134] tough and elastic nanomembranes for tissue engineering,[135] implantable optical waveguides for sensing and therapy.[136]

Overall, novel spider silk materials with diverse forms and functionalities have been successfully fabricated and demonstrated to be useful across various biomedical fields. Despite persistent challenges in material processing and performance improvement, continued technological innovation and interdisciplinary collaboration will offer new routes to address these challenges, enabling artificial spider silk as a significant force in the high-tech materials field of the future.

5. Conclusion and Future Perspectives

This review extensively explores the progress in the design and fabrication of artificial spider silk materials, from molecular design of spidroins at the microscopic scale to mesoscopic assembly and macroscopic regulation of materials. It is worth to note that precisely controlling over the mesoscale structure and assembly process of spider silk proteins can effectively broaden performance boundaries and applicability of the resulting protein materials. Indeed, a variety of prototype and commercial artificial spider silk products are entering the markets as cosmetics, clothing, medical biomaterials, and even leather alternative in automotive vehicles.[137] Nonetheless, production efficiency, consistency, and the cost-benefit ratio remain critical challenges for the successful commercialization of artificial spider silk materials.

Looking to the future, the focus should be on the computation-aided design of novel synthetic spidroins and comprehensive optimization of the production process, in pursuit of high-throughput, large-scale, and high-quality output while ensuring sustainability and economy. Establishing a "sequence-structure-performance" design system and deeply understanding the impact of each amino acid residue on mesoscale assembly, overall performance and functionality will guide the future research trajectory for artificial spider silk materials. Further investigations into the mesoscopic assembly dynamics and LLPS mechanisms of spider silk proteins will provide theoretical and technological foundations for developing a new generation of high-performance biomaterials. Moreover, strengthening interdisciplinary collaborations across fields such as materials science,

bioengineering, nanotechnology, and computational science will be crucial to advancing artificial spider silk research.

The potential applications of artificial spider silk materials in the modern technological landscape are boundless because of the unique physicochemical properties and adjustable functional features. With the continuous advancement and innovation in design and fabrication technologies, artificial spider silk materials are expected to play important roles in shaping material science and engineering in the near future.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

liquid–liquid phase separation, protein materials, self-assembly, silk fiber, spider silk proteins, synthetic biology

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