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Review

Disentangling the Web: An Interdisciplinary Review on the Potential and Feasibility of Spider Silk Bioproduction

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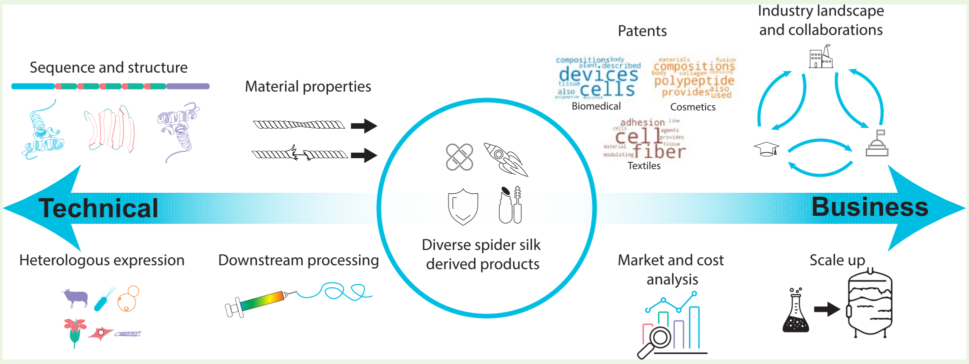

ABSTRACT: The remarkable material properties of spider silk, such as its high toughness and tensile strength combined with its low density, make it a highly sought-after material with myriad applications. In addition, the biological nature of spider silk makes it a promising, potentially sustainable alternative to many toxic or petrochemical-derived materials. Therefore, interest in the heterologous production of spider silk proteins has greatly increased over the past few decades, making recombinant spider silk an important frontier in biomanufacturing. This has resulted in a diversity of potential host organisms, a large space for sequence design, and a variety of downstream processing techniques and product applications for spider silk production. Here, we highlight advances in each of these technical aspects as well as white spaces therein, still ripe for further investigation and discovery. Additionally, industry landscaping, patent analyses, and interviews with Key Opinion Leaders help define both the research and industry landscapes. In particular, we found that though textiles dominated the early products proposed by companies, the versatile nature of spider silk has opened up possibilities in other industries, such as high-performance materials in automotive applications or biomedical therapies. While continuing enthusiasm has imbued scientists and investors alike, many technical and business considerations still remain unsolved before spider silk can be democratized as a high-performance product. We provide insights and strategies for overcoming these initial hurdles, and we highlight the importance of collaboration between academia, industry, and policy makers. Linking technical considerations to business and market entry strategies highlights the importance of a holistic approach for the effective scale-up and commercial viability of spider silk bioproduction.

KEYWORDS: *spider silk, fiber bioproduction, protein biomaterials, industry landscape*

■ INTRODUCTION

Proteins are the foundational building blocks of living systems. These versatile macromolecules fulfill a variety of functions in living organisms and present an opportunity for utilizing and harvesting these functions for human needs.[1−3] While the array of biomanufactured proteins is wide (Table 1), protein-based biomaterials present a rich opportunity for displacing highly polluting, petrochemical-derived materials that are recalcitrant to biodegradation, such as plastics.[4−6]

Unlike their petrochemical synthetic counterparts, protein-based biomaterials can be produced and degraded in benign environmental conditions, with harmless solvents (often water).[7] Their building blocks, amino acids, can be easily fed back into the production process enabling a path toward a circular economy. Due to their high nitrogen content, proteins are less flammable which is ideal for construction and textile applications and reduces the need for toxic flame retardants.[8,9] In addition, proteins can be programmed and engineered to achieve more tunable chemistries (e.g., dye reaction kinetics and retention over time[10]) as well as improved mechanical

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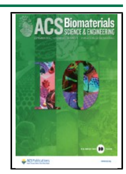

Table 1. Examples of Biomanufactured Proteins Reveal the Wide Range of Applications and Versatility of Proteins

Consumer goods	Industrial goods	Pharma	Agriculture	Food and nutrition
Cosmetics • Keratin • Biotin • Collagen • Spider silk Cosmetics • Skin care (proteases, lipases) • Teeth and gum (glycosydases) • Hair dyes (laccases) Fashion • Silk • Leather • Fur • Synthetic hair • Spider silk Fashion • Leather processing (proteases, lipases, amylases) Performance materials • Spider silk	Wastewater treatments • Organic waste degradation (proteases, amylases, lipases, cellulases, ureases, and xylanases) • Inorganic waste degradation (oxidoreductases, peroxidases, and oxygenases) Waste management • Plastic degradation (PETases, nylonases) Detergents • Carbohydrate stain removal (amylases) • Fat stain removal (lipases) • Protein stain removal (proteases) • Color clarification (cellulases)	Medicine • Bio-compatible implants • Wound dressings (spider silk) • Scaffolds for cell growth Drugs • Insulin Protein Vaccines and vaccine manufacturing • RNA and DNA production for vaccines (polymerases) • Protein subunit based vaccines: (hepatitis B, acellular pertussis) Protein therapy • Monoclonal antibodies • Interferons • Cytokines • CarT	Animal feed • Enhanced starch digestibility (xylanases) • Digestive aid (beta-glucanases) • Feed waste products breakdown (cellulases, ligninases) Crop optimization • Organic fertilizers Pesticides • Neurotoxins against insects	Food-derived proteins • Casein • Whey • Egg white • Pea, hemp, and brown rice protein Dairy processing • Milk coagulation (acid proteinases) • Faster cheese ripening (proteinases, lipases, aminopeptidases) • Cheese processing (catalases) Baked goods • Flour adjustments (amylases) • Dough conditioning and strenghtening (xylanases, glucose oxidases) Beverage production and processing • Haze formation prevention (beta-glucanases, proteases) • Debittering (naringinases, limoninases) Alternative meats • Leghemoglobin

Material · Enzyme · Additives/Food · Drugs

properties.[11,12] The potential for tuning protein sequences through modular blocks also presents opportunities for the development of novel materials and, consequently, a more agile and adaptable economy.[13−15] The promise that biomaterials offer in terms of sustainability, programmability, and improved performance makes their study worthwhile.

Protein-based biomaterials can be broadly classified into four categories:[16] fibrous materials (such as natural silks), bioelastomers (such as collagen[17,18] and elastin[19,20]), hard bulk materials (such as squid beak proteins[21,22]), and adhesives (such as barnacle cement[23,24]). Being able to engineer, express, and produce a wide array of protein biomaterials at scale has been a challenging frontier for bioengineering with very few successfully scaled examples.[25] In this context, spider silk stands out as an example of early exploration in this space. Because of its remarkable material properties which include low density and high tensile strength,[26] spider silk has enticed many over the years, despite the challenges of biomanufacturing it at a cost-competitive scale. Through many attempts, inherent biomanufacturing limitations have been identified, such as heterologous hosts' inability to express the long, highly repetitive sequences found in spider silk, which is one limitation among several others to be discussed throughout this review.[27]

This review emphasizes both technical advances and technical advances as well as market and business considerations for spider silk biomanufacturing. Due to its versatile material properties, spider-silk has potential applications across many industries and sectors, from fashion to the medical field (green dots, Table 1). We will dive into the technical considerations that make spider silk such a desirable biomaterial as well as the feasibility of its production and commercialization at scale. We aim to outline growth opportunities that would further efforts to solve remaining hurdles.

■ DRAGLINE SILK HAS REMARKABLE MECHANICAL PROPERTIES

Many proteins in nature fold into "globular" structures,[28] with hydrophobic residues in their core and hydrophilic, solvent-exposed residues on their surface, making them soluble in aqueous environments (Figure 1a, right). While the majority of proteins take on such conformations, a small minority of proteins fold into "fibrous" conformations (Figure 1a, left). Fibrous proteins are typically elongated and form filamentous, sheet-like structures within the cell. Unlike globular proteins, they are generally water-insoluble (in most pH conditions) and often aggregate in order to form higher order, three-dimensional superstructures.[29−31] Accordingly, fibrous proteins are critical for structural functions, such as mediating cell scaffolding, facilitating cell-to-cell connections, and ensuring other protective roles.[29,31−34] Typically, fibrous proteins contain long stretches of hydrophobic residues (Figure 1b), flanked by globular and water-soluble C- and N-termini (Figure 1b), which results in their elongated structure.[35]

Noteworthy superfamilies of fibrous proteins include keratin, collagen, elastin, and fibrin.[36] Silk proteins (i.e., from spiders and silkworms) are fibrous in nature and constitute the primary building block of silk fibers (Figure 1c, left). Fibrous proteins produced by lepidopteran insects are called fibroins, while spider silk proteins are called "spidroins", a portmanteau of the words spider and fibroin. Spidroins, in particular, exhibit a unique and attractive combination of chemical and mechanical properties of interest to academics and industry professionals. They can undergo hierarchical self-assembly into fibers (Figure 1c, left) which can be spun into yarns (Figure 1c, middle) that can then be woven, knit, or bonded to form useful materials such as fabrics or wound dressings (Figure 1c, right). The quality and properties of single fibers depend on amino acid composition, sample purity, and the parameters of the spinning process. Fibers and other materials made from spidroin are known for their high strength-to-density ratio, which is ∼10× higher than that of steel,[37] making them an ideal candidate for material applications which require lightweight but durable solutions, such as textiles, defense, and aerospace applications.

While most insects produce only one type of silk in their lifetime,[38,39] spiders have the ability to produce seven major types of silk (Figure 1d), each with their unique properties and functions, including cocooning,[40] ballooning,[41] and catching prey.[42] Spiders can adjust the type of silk that they secrete to serve these different purposes.[43−45] Each silk type is produced by a different gland in the spider's posterior abdomen, then pulled from spinnerets, the external part of the gland near the spider's rear legs. Depending on the species' complexity, spiders have 2 to 8 spinnerets, typically occurring in pairs.[40,46] Gland architecture and complexity can also vary by species and silk type. The differing properties of silk types are often utilized and combined by spiders to maximize functionality. For example, within a single spider web, elastic flagelliform silk strands are coated with a sticky aggregate silk glue to aid in prey capture, while dragline silk is stiffer and stronger to provide the web with enhanced structural integrity[47] (Figure 1d).

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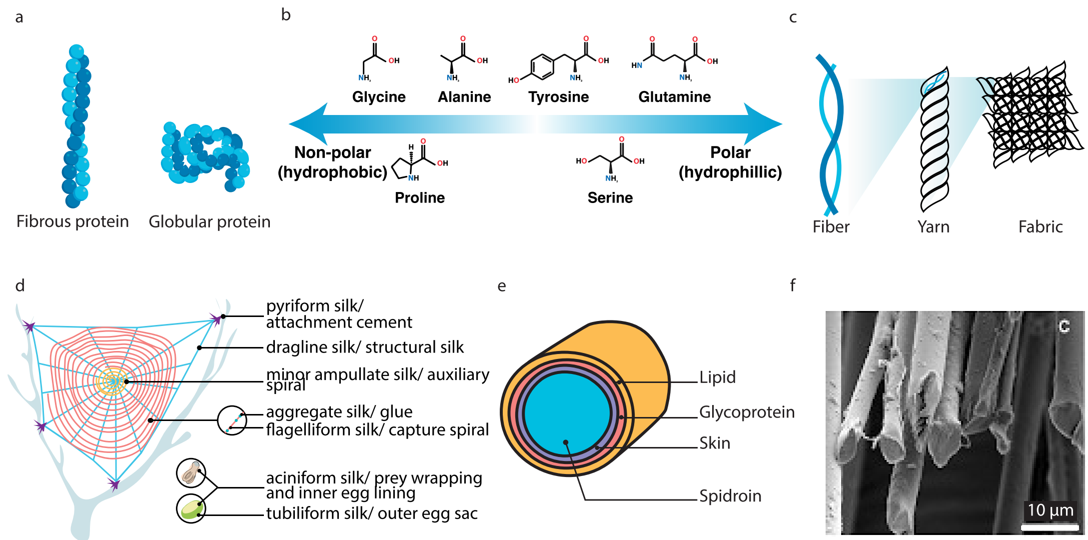

Figure 1. Dragline silk is made from self-assembled fibrous proteins. (a) Cartoon depicting the difference between fibrous and globular proteins. Spheres represent amino acids. While most proteins fold into globular structures, spidroin proteins self-assemble into long fibers. (b) Amino acids can be classified based on their interaction with water. In particular, amino acids such as glycine and alanine, which are most abundant in spidroins, are highly hydrophobic. (c) Protein fibers can be spun into yarns, which are then knit into fabrics. (d) Spiders secrete a variety of silk types which have different functions. Dragline silk has been the type of interest for manufacturing purposes. Adapted with CC-BY license from Ramezaniaghdam et al.[27] Copyright 2022 the Authors, published by Frontiers Media S.A. (e) Natural dragline silk from spiders typically has a circular cross-section.[48] In addition to the spidroin, natural dragline silk is composed of many other components that ensure its structural integrity. (f) SEM picture of spider silk fibers which shows their circular cross-section. Adapted with Elsevier User License from Santos-Pinto et al.[49] Copyright 2016 Elsevier.

Cross-sectional analysis[48] (Figure 1e) coupled with the analysis of SEM micrographs[49] (Figure 1f) uncovers several key features of spider silk dragline fibers. The core fiber is coated by three layers:[50,51] a skin layer, a glycoprotein layer (which protects against bacterial and fungal infections) and a lipid layer. Unlike silkworm silk whose cross-section becomes triangular after degumming,[52] spider dragline silk's cross-section is circular in most cases[53−55] with a diameter that ranges between 3 and 5 μm.

To date, over 41,000 species of silk-producing spiders have been identified,[56] each with their unique silk types.[53,57] The combinatorial nature of silk sequence composition, secretion, and added components results in the remarkable diversity of spider silk types observed in nature.[57] Within this large pool of silks, dragline silk (major ampullate silk, MaSp) from *Nephila clavipes* (golden orb-weaver) and *Araneus diadematus* (European garden spider) has emerged as a model material, well-characterized in the lab and garnering the most interest toward industrial production.[58−60] It constitutes the structural framework of the webs of these two spider species, serving as its outer rim as well as its spokes (the web's radial threads).[61] Dragline silk also plays a role in the spider's ballooning, which is its ability to traverse large distances by becoming airborne and being carried by the wind using silk threads. Additionally, spiders use drop lines for safety during navigation.[61] Accordingly, dragline silk possesses a desirable combination of mechanical properties including high tensile strength, toughness, and elasticity. Besides the material properties of their dragline silk, *N. clavipes* and *A. diadematus* also became model organisms due to their broad ecological range and the ease of collecting and handling them compared to other spider species.

FROM SEQUENCE TO FIBER: TUNING SPIDER SILK'S MATERIAL PROPERTIES

The large diversity of silk types is a result of the unique functions of silk within a spider's life cycle and differing environmental influences, which ultimately give rise to unique mechanical properties from species to species and even within a single species.[62] Significant progress was made in the 1980s and early 1990s when the amino acid sequences of *A. diadematus* and *N. clavipes* spidroins were obtained for the first time.[63−67] These analyses revealed a pronounced bias toward alanine and glycine in the spidroin amino acid compositions. Further advancements were achieved through cDNA cloning of *N. clavipes* Major ampullate Spidroin 1 (MaSp1) and Major ampullate Spidroin 2 (MaSp2), yielding the first partial sequences of dragline silks.[67,68] The first full-length sequence of dragline silk was reported in 2007 upon a complete investigation of *Latrodectus hesperus* (black widow) MaSp1 and MaSp2.[69] Completed full-length spidroin sequences of other silk types soon followed. Currently, minor ampullate,[70] pyriform,[71,72] tubuliform,[73] and aciniform[74] silk spidroins have full-length sequences, illuminating distinct sequence motifs and amino acid compositions among the different silk types.

The two most-well characterized dragline spidroins in *N. clavipes* are MaSp1 and MaSp2,[65,67] while those most well-characterized in *A. diadematus* are ADF-3 and ADF-4. Spidroins typically consist of three distinct domains (Figure 2a):[75] a nonrepetitive N-terminal domain (NR-NTD, shown in blue), an extensive and highly repetitive core domain (shown in red and green), and a nonrepetitive C-terminal domain (NR-CTD, shown in purple).

The NR-NTD usually consists of about 100 amino acid residues, which sequence alignments show are highly conserved across species.[76,77] During silk synthesis within the spider's gland, the N-terminal domains bind to each other and ultimately help control the spidroins' elongation and transport throughout

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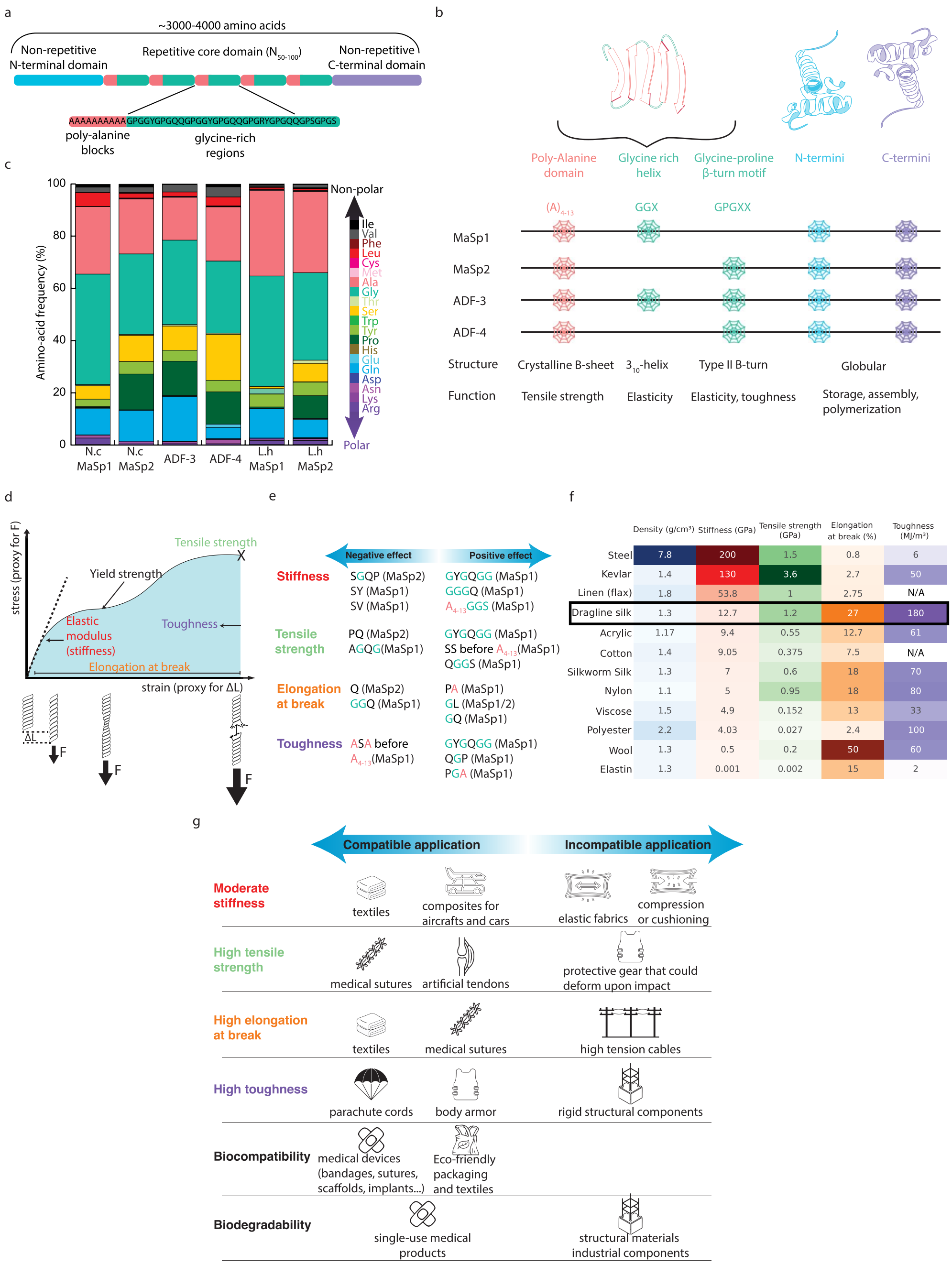

Figure 2. Spidroin sequences determine the material properties of dragline silk fibers. (a) Typical structure of spidroin protein. Natural spidroins are very long proteins with ~3000 to 4000 amino acid residues. They consist of long repetitive regions of polyalanine and glycine-rich domains which make them hard to synthesize in heterologous hosts and handle in solution due to their hydrophobic nature. These long repetitive regions are flanked by nonrepetitive C- and N-terminal domains which allow dragline silk to polymerize into its quaternary fibrous structure. (b) Table summarizing the main domains and motifs present in each of the major spidroins as well as their structure and function. X indicates a placeholder for any amino acid. (c) Frequency of amino acid residues in the most studied types of dragline silk spidroins. The color bars represent amino acid identity and are ordered according to the Kyte−Doolittle hydrophobicity scale. Nc: *Nephila clavipes*, AD: *Araneus diadematus*, Lh: *Latrodectus hesperus*. UniProt IDs: NcMaSp1 (P19837), NcMaSp2 (P46804), ADF-3 (Q16987), ADF-4 (Q16988), LhMaSp1 (A6YIY1), LhMaSp2 (A6YIY0). (d) Cartoon representation of a stress−strain curve for characterizing the tensile properties of a fiber. Main characteristics are indicated on the figure. (e) Specific sequence motifs alter the downstream properties of the synthesized fibers. Adapted from Arakawa et al.[57] Copyright 2022 the Authors, published by AAAS. (f) Table comparing the material properties of dragline silk to other common fibrous or structural materials. Materials are classified based on their stiffness. Notably, dragline silk has relatively high stiffness, tensile strength, elongation at break, and toughness compared to other materials. This is especially remarkable given its low density. Values adapted from Sarkar et al. (2019)[101] and MatWeb (the material property database). (g) Examples of suitable and nonsuitable applications for dragline silk given its material properties. It is important to consider various trade-offs of different applications. For example, the high toughness of spider silk would make it suitable for protective and defense gear. However, its high elasticity suggests that it would largely deform upon any impact, and therefore not be adapted for applications that require stiffness, such as bulletproof vests. We also note that silk proteins can enhance the properties of other composites.

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the gland.[69,78,79] This domain also helps increase the toughness, stress, and stiffness of recombinant spidroins (Figure 2a,b, blue symbols).[80]

As with the NR-NTD, the NR-CTD consists of around 100 highly conserved amino acid residues.[43,69] Additionally, the C-terminal domains bind to each other within the gland, facilitating the alignment of polyalanine and polyglycine motifs of physically adjacent spidroins.[81−83] The alignment of these sequences promotes the formation of microcrystalline structures needed for the assembly of nanofibrils and, ultimately, mature fibers[81] (Figure 2a,b, purple symbols).

The central, extensive, and highly repetitive core domain is responsible for the spidroin's fibrous assembly and noteworthy material properties. Constituting the vast bulk of the protein's length (∼90%), the alanine- and glycine-rich core domain consists of dozens of modular tandem repeats.[45,61,69] Within MaSp proteins specifically, these tandem repeats consist of two key motifs: (1) a polyA or polyAG motif and (2) a polyGGX or polyGPGXX motif.[69,78,84,85] Structurally, the polyalanine motifs form β-sheets, which assemble into hydrophobic crystalline domains that confer tensile strength to the spidroin fiber.[45,86] Conversely, the polyglycine motifs form α-helices and β-turns, which make up the amorphous regions of the fiber that provide elasticity and toughness[45,87−90] (Figure 2a,b, red and green symbols).

Spidroins typically vary in length from 3,000 to 4,000 amino acid residues, corresponding to molecular weights of 250 to 350 kDa.[61] These unusually long proteins present unique challenges when grown exogenously in various heterologous hosts or even handled in solution since they tend to easily aggregate due to their large size and highly repetitive core domains, making purification and crystallization difficult. To our knowledge, no full-length spidroin sequence for dragline silk has been reported for *A. diadematus*, largely owing to the formidable challenge of cloning long stretches of highly repetitive DNA and assembling sequence fragments. Despite this limitation, researchers were able to sequence and annotate the genome of *N. clavipes*, using a combination of short-read and long-read sequencing approaches.[62,91] This milestone marked the first completed genome of an orb-weaving spider, and importantly revealed several full-length sequences of dragline spidroins. Full-length sequences of MaSp1 and MaSp2 from *Trichonephila clavata*, a closely related spider to *N. clavipes*, have also been elucidated.[91]

Amino acid composition analysis of these sequences provides insight into noteworthy amino acid biases and motifs (Figure 2c). A strong bias for alanine and glycine can be observed, consistent with the extensive repetition of tandem repeats throughout the lengthy central domain of the spidroin (Figure 2a). This motif confers a noteworthy level of hydrophobicity in the overall protein. Other amino acid residues also appear to be frequent in sequence composition: serine, tyrosine, proline, and glutamine in particular. Interestingly, biases can vary across the different spidroin types: proline is virtually absent in MaSp1 spidroins but prominent in MaSp2, ADF3, and ADF4 spidroins (Figure 2c), which can be mainly attributed to the lack of the GPGXX motif in MaSp1 (Figure 2b) that the latter three proteins possess. Amino acid ratios vary across the spidroins, and the effect these differing biases have on the fiber's ultimate material properties is an active area of research.[57]

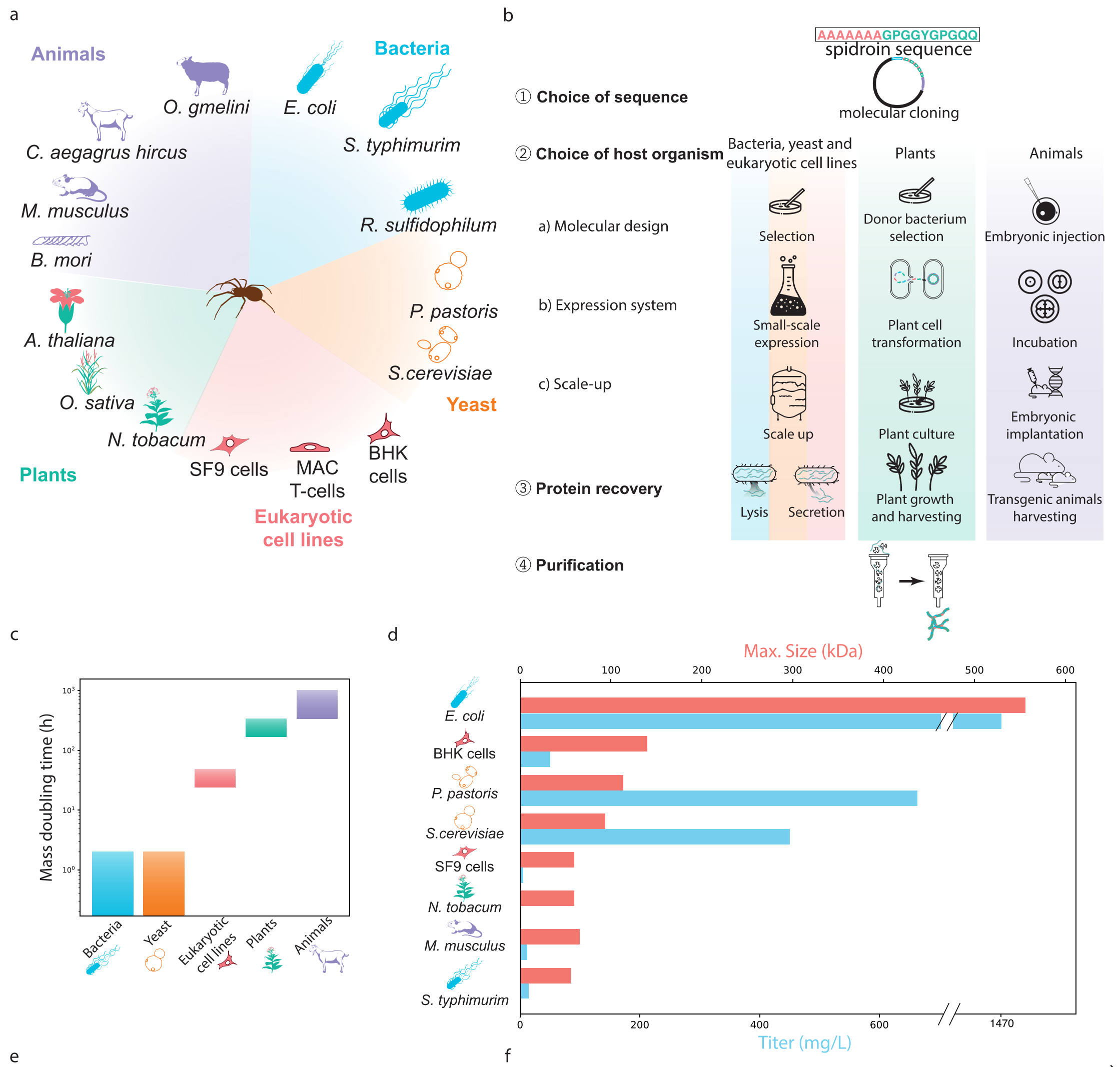
Based on these advances, major companies in the recombinant spider silk space have used sequences from *N. clavipes* or *A. diadematus* as the basis for product design and development. Patents filed by Spiber, AMSilk, and Bolt Threads[92−94] collectively reference ADF3, ADF4, MaSp1, and/or MaSp2, serving as the launching point for initial rounds of design and testing of spidroin-based materials. For example, AMSilk has generated at least 20 unique versions of spider-silk sequences, all originating from *A. diadematus*.[16]

While companies have primarily focused on mimicking the highly repetitive core domain of spidroins, the more complex structure of the NR-NTD and NR-CTD are of major importance in determining the properties of the resulting fiber. These nonrepetitive terminal domains are critical for storage and self-assembly in native spidroins. Incorporation of these nonrepetitive terminal domains to the ends of short recombinant spidroin sequences resulted in fibers with increased strength, stiffness, elasticity, and toughness.[77,95,96] However, it remains to be seen whether NTDs or CTDs offer mechanical benefits to large, native-like recombinant spidroins. While the nonrepetitive N- and C-terminal domains are widely conserved across silk types and spiders, exploring terminal domains optimized for improved material properties could yield promising results.

Silk fibers are known to supercontract, or shrink, when exposed to high humidity or direct wetting, which can pose challenges for subsequent material applications. A notable example where protein sequence design translated into improved properties is silk supercontraction.[97,98] By tuning the composition of the amorphous regions of the protein sequence, scientists were able to alter the interaction of silk fibers with water.[98,99] While much experimentation has relied on trial and error via random mutagenesis in the past, new developments in protein folding models will narrow the space of sequence exploration and fine-tune downstream mechanical properties of silk proteins at the sequence level.[100] However, the available choices of spidroins for companies to utilize have been limited by the availability of sequences which are highly biased toward a few model species of spiders. Broader ecological surveys of the natural properties of other forms of spider silk will reveal new materials to explore and expand the repertoire of known silks with varying mechanical properties.[43]

Advances in sequencing have expanded the known sequences in the global spider "silkome". In 2022, a joint effort between Spiber and numerous academic institutions yielded transcriptome assemblies containing silk sequences of over 1,000 spider species.[57] Dragline silks from over 400 spider species were identified, and their structural and mechanical properties, such as toughness, elastic modulus, tensile strength, and strain at break (defined in Figure 2d), were investigated. The study provided an in-depth exploration of the precise relationship between sequence features of the MaSp repetitive domains and different physical properties (Figure 2e). Specific motifs have positive or negative effects on properties such as toughness, strength, elongation at break, and others. Further investigation of these motifs will clarify how to best design sequences optimized for durability and performance (Figure 2e). This will also allow researchers to train more accurate computational models for generating artificial spidroin sequences.

There remains enormous untapped potential in the biomaterial space for recombinant dragline silks from nonmodel spider species, many of which have yet to be systematically explored. Due to the inherent programmability of biomanufactured proteins, any recombinant silk fibers of the future will likely possess increasingly fewer signatures of traditional spider silk sequences over iterative development cycles. Hiroyuki Nakamura, a Research Scientist at Spiber Inc., emphasizes the



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	M. sativa	E. coli	C. aegagrus hircus
Production cost ($/kg)	10	23	54
Production timescale	Seasonal (a few months)	A few days	Gestation period (~3 months). Then daily.
kg of CO2 per kg of product	-0.1	50-500	2-4
Water usage	Intensive for farming	Giga liter tanks	Intensive for feeding and feed production
Land usage	High (crop plantations)	Low (production plant footprint)	High (pasture and feed production)

f

	Type	CO_2 emission (kg/kg)	Energy use (MJ/kg)	Water use (L/kg)	Degradation period
Polyester	Synthetic	3-7	54-109	62-130	~20-500 years
Acrylic	Synthetic	5-5.4	133-175	144-210	~200 years
Nylon	Synthetic	5.5-8	1-139	185-663	~30-50 years
Dragline silk	Synthetic but based on a natural product	-0.1 to 500		~300	~years
Silkworm Silk	Natural	53-81	1460-1860	26,700-54,000	~4 years
Cotton	Natural	0.6-9.5	1.5-113	1.5-35,000	~3-6 months
Viscose	Semi-synthetic	0.2-9	70-100	320-640	~6 weeks
Linen (flax)	Natural	3.8	10	215	~2 weeks

Figure 3. Overview and considerations of host expression systems for the production of recombinant spider silk. (a) Illustrations of various expression systems that have been utilized by academic and industry laboratories. Systems are grouped by kingdom and color-coded. (b) Schematic overview of spidroin production workflow for different classes of hosts. While production specifics vary by host, the overall workflow is largely consistent across platforms and will involve sequence optimization, molecular design, host expression, scale-up, and protein recovery and purification. (c) Bar graph of mass doubling time of various host organisms, ordered from lowest doubling time to highest. Bars represent a general range of observed doubling times for each classification of host. In general, bacteria and yeast have the lowest doubling times, while plants and animals have the highest. (d) Bar graph of maximum titers (top axis, red) and maximum protein sizes (bottom axis, blue) of recombinant spidroins purified from various host organisms as reported in the academic literature, ordered from highest to lowest size. *E. coli* and yeast systems yield the highest titers, consistent with their widespread use in industry. However, it is important to note that not all protein products cited are the same sequence composition or length and some of the highest titers are shorter sequences. (e) Table of estimated environmental and financial constraints associated with several host organisms in optimistic scenarios where production becomes highly optimized.[171] Current costs of production are well above those reported here. (f) Environmental impact metrics of various textile polymers. Dragline silk presents potential opportunities for improvement compared to incumbent fibers, especially in its biodegradability. Values are derived from multiple studies with varying methodologies, but general trends can still be extrapolated.

power of altering protein sequences to tune the material properties of the resulting fibers: "**A lot of Spiber's early work focused on treating the problem of supercontraction, which is a property of natural spidroins. Even though these initial spidroin sequences were at the core of our research, our current Brewed Protein has been engineered away from them to achieve better mechanical properties.**" The ability to control the spidroin sequences has led many other companies, like Spiber, to heavily engineer spider silk proteins. While the starting point of such engineering efforts is the natural spidroin protein, many companies and research groups have significantly drifted from these original spidroins. Sequence optimization can enhance expression, fiber-formation, and spider silk mechanical properties, though there will likely be trade-offs between each of these which should be considered.

Interest in tuning the mechanical properties of spider silk stems from the remarkable tensile properties natural dragline silk possesses (Figure 2f). Despite its low density, dragline silk has moderate stiffness while maintaining a toughness higher than that of many materials.[26] In this domain, comparisons with the tensile strength of steel and with the toughness of Kevlar are often made, though it is worth noting that most of these properties are measured differently across materials. In addition, for dragline silk, small material samples are used (a few inches at most), and the stress−strain curves (Figure 2d) are produced in highly controlled laboratory experiments. These controlled environments assess the effect of specific stresses and strains on the materials tested, which may differ from the stresses they would experience in more "realistic" settings. Despite these caveats, dragline silk outcompetes other fibers since it has a higher tensile strength than most fibers (both natural and synthetic) and can stretch more than most fibers due to its high elasticity. In addition to these mechanical properties, dragline silk is a naturally occurring fiber, making it biodegradable and more likely to be biocompatible. These latter attributes open up a large set of applications where spider silk can compete with synthetic petrochemical-derived fibers. Because of this combination of favorable features, many have imagined that artificial spider silk could replace other materials in their various applications (Figure 2e).

While certain mechanical properties of spider silk may make it suitable for certain applications, other properties may disqualify it from being a viable candidate for those uses. For example, while spider silk's toughness would make it suitable as a shield (or bullet-proof material), its high elasticity would limit its usage in that context since any bullet would deform the material well past the victim's abdomen (this anecdote originates from Prof. Randolph Lewis, USTAR Professor of Biology at Utah State University). Therefore, it is important to take a holistic view of spider silk's mechanical features and consider the many trade-offs involved before choosing a product to manufacture with it (Figure 2g). Additionally, the exploration of spider silk in various product applications involves manufacturing spider silk on a large-enough scale for test trials. Therefore, the early stages of spider silk exploration are limited to small products such as those required for biomedical applications, or composite applications combining spider silk with cheaper and more widely available bulking agents.

Efforts toward mimicking or even improving spider silk's mechanical properties have been at the core of research developments as of late. Professor at Kyoto University and Team Leader at RIKEN, Keiji Numata, summarizes the importance of addressing the question of how spidroin sequence determines its properties, particularly toughness. "**We need to focus on improving the toughness of spider silk. In order to do that, we need to understand the relationship between the hierarchical structure and mechanical properties of this material. This entails bridging our understanding of the many levels that determine its hierarchical structure: how amino acid sequences influence protein composition which itself influences the self-assembly process (for example through liquid−liquid phase separation). We can't run away from improving the toughness of artificial spider silk!**"

■ HOST EXPRESSION SYSTEMS FOR THE PRODUCTION OF RECOMBINANT SPIDROINS

As spider silk's remarkable properties and programmability were recognized along with its numerous potential commercial applications, scaling up the production of spidroins garnered great interest from the research community and industry. Since *Bombyx mori* (domestic silk moth) has been used in silkworm farms for silk production for millennia, primarily in China and India,[102] similar attempts were made to farm spiders en masse. However, because spiders are highly territorial and cannibalistic, housing them in cooperative silk farms proved unfeasible.[103] Furthermore, spider farms are labor-intensive and resource inefficient: for example, producing a single article of clothing (a cape) required farming approximately 1.2 million golden orb spiders, a consequence of the low quantities of dragline silk naturally produced by spiders.[104−106]

Due to the challenges of directly farming spiders, using more efficient host organisms capable of heterologous expression is essential for the mass production and economically viable scale-up of spider silk as a biomaterial. Fortunately, access to and investigation of dragline silk sequences (as discussed in the previous section) enable the engineering of recombinant spidroins in a broad suite of candidate host organisms (Figure 3a). The tunability of the spidroin sequences can allow for modulation of the material properties of the product of interest. This allows researchers to achieve more specific design goals with only minimal changes in the raw material production.

The production of spidroins in heterologous hosts relies primarily on well-established methods of molecular cloning and recombinant protein expression.[107−112] In brief, a spidroin sequence must be selected, designed, amplified, and then inserted into an appropriate expression vector, thereby generating recombinant DNA (Figure 3b). Molecular biology tools such as restriction enzymes, CRISPR-Cas complexes, polymerases, and ligases are integral to this process. The recombinant DNA containing the spidroin of interest must then be introduced into a heterologous host, with the optimal method of introduction varying by organism. Common methods of introduction include transformation, transfection, and transduction, among others. Finally, organisms that successfully incorporate the recombinant DNA must be selected for, typically using a selectable marker such as an antibiotic-resistance gene. The recombinant organism can then be propagated and utilized to conduct pilot expressions, scale-up trials, and optimal protein recovery pipelines (Figure 3b).

Thus far, recombinant dragline spidroins have been produced in bacteria, yeast, eukaryotic cell lines, plants, insects, mammals, and several other emerging platforms (Figure 3a). Various biotechnology approaches (Figure 3b), including molecular cloning,[113] site-directed mutagenesis,[114] directed evolution,[115] and bioinformatics,[116] have been employed to assess the benefits and challenges associated with different host systems and spider

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silk sequences. Below, we describe the properties of each class of hosts, with emphasis on the timescales of growth of each host in particular (Figure 3c), the size and yield of host-extracted spidroins (Figure 3d), and a discussion of pricing and environmental impact analyses (Figure 3e).

Bacterial Systems. Unicellular prokaryotes are a dominant platform for the heterologous production of spidroins. In particular, the Gram-negative bacterium *E. coli* has been broadly employed for recombinant silk production,[117,118] owing to its ease of genetic manipulation, short generation time, high productivity, and industrial scale-up potential.[117,119] Initially, spidroin production in *E. coli* was plagued by low expression levels, due to the genetic instability of the spidroin genes. Because spidroins contain a lengthy, highly repetitive core domain consisting of tandem repeats of polyalanine and polyglycine motifs, their expression in a host is highly demanding on the available cellular pools of alanyl- and glycyl-tRNAs, which, prior to translation, is naturally upregulated in spider silk glands but not in *E. coli*.[120] Expression is further hampered by the natural codon bias of *E. coli* and its mismatch with the preferred codons inherent to native dragline sequences.[65,121] Together, these limitations resulted in premature translation termination (due to tRNA depletion) and accompanying low yields in early pilot expressions.[96] Efforts to address premature translation termination by metabolically engineering elevated pools of glycyl-tRNA in *E. coli* enhanced spidroin titers (500 mg/L) and protein size (∼284.9 kDa).[122] Though this was a big jump in spidroin size and yield, commercialization efforts have not surfaced from this system. One report indicates that for biomanufactured goods to reach a Cost of Goods Sold (COGS) below $100/kg, titers of >20 g/L are required.[123] Molecular engineering techniques show promise in continuing to bolster yields and protein size, demonstrated by the use of split-intein-mediated ligation and DNA part assembly, which facilitated titers and protein size of ∼1240 mg/L and ∼556 kDa, respectively.[124] These studies highlight the power of *E. coli*'s genetic tractability and rationalize its widespread adoption in industry. However, *E. coli* is not without limitations. First, recent studies highlight a potential trade-off between size and titer, wherein significantly improved titers coincide with much shorter spidroins.[125] Moreover, most of the high titers achieved are not of the full-length sequences but usually of multimers of short repeated sequences, resulting in fibers with material properties that underperform compared to natural dragline silk. Additionally, the formation of cytoplasmic inclusion bodies complicates the production of spidroins.[126] Finally, extensive downstream processing (including protein extraction, recovery, and purification) can present cost burdens to large-scale production, which has prompted researchers to study protein secretion-based systems in other bacterial hosts as a potential lower-cost alternative.

Salmonella typhimurium is a Gram-negative bacterium that can export proteins through both the inner and outer membranes. Its native Type III secretion system can export spidroins directly into the culture medium, forgoing the need for cell lysis and downstream purification (Figure 3b), which can lower associated costs. *S. typhimurium* remains an active platform for research, as currently reported titers (14 mg/L) and protein sizes (25−56 kDa) remain much smaller than that of *E. coli*.[127] In addition to *S. typhimurium* and other Gram-negative bacteria, Gram-positive bacterial hosts remain a promising alternative due to their strong secretion capabilities, well-established use in industry, and GRAS (Generally Recognized as Safe) status set by the FDA.[128] Recent studies have demonstrated the ability of *Bacillus megaterium* and *Corynebacterium glutamicum* to express and secrete appreciable yields of recombinant spidroins, with respective apparent sizes of 60 kDa and 43 kDa, as well as reported titers of 100 mg/L and 554.7 mg/L, respectively.[129,130] In both hosts, an N-terminal signal sequence was translationally fused to the recombinant spidroin to direct its secretion into the culture media through the Sec pathway. Because Gram-positive bacteria possess only one cell membrane, the Sec pathway offers the advantage of a single-step secretion event, which can simplify design pipelines and production scale-up. In contrast, extensive engineering of genetic circuits is often needed in Gram-negative bacteria to address the problem of transport through both the inner and outer membranes.[127] However, to our knowledge, these three studies represent the only successful examples of recombinant spidroin secretion in bacteria to date, underscoring the opportunity to further investigate the secretion capabilities of both Gram-negative and Gram-positive bacterial hosts.

Another alternative bacterial host is *Rhodovulum sulfidophilum*, a purple nonsulfur bacterium found in marine environments. Interestingly, *R. sulfidophilum* is photosynthetic, highlighting the potential of sustainable feedstocks (i.e., sunlight, carbon dioxide, and gaseous nitrogen) in its industrial-scale use. In seawater conditions, *R. sulfidophilum* is able to produce MaSp1 in appreciable yields, albeit still lower than that of *E. coli*.[131] In addition, the genetic stability of the spidroin and tRNA depletion remain issues to be addressed within this organism.[131]

Yeast Systems. Yeast systems are an attractive alternative to bacterial hosts and are widely used in industry. In particular, *Pichia pastoris* has been explored by many research groups and companies to express spidroins. Due to its genetic tractability and short generation time, it is well-suited for industrial-scale fermentation. The first published expressions of spidroins in *P. pastoris* yielded 665 mg/L of a 65 kDa protein.[132] More recently, researchers purified the 113.6 kDa 2E12 spidroin, an analogue of MaSp2, from *P. pastoris*, highlighting an increase in the size of purifiable spidroin.[133] Unfortunately, *P. pastoris* is plagued by poor expression in shake flasks, which can hamper pilot expressions and production troubleshooting.

However, *P. pastoris* retains some unique advantages which help explain its current use by some startups. While previous studies reported spider silk yields and sizes that were higher in *E. coli* compared to *P. pastoris*, *P. pastoris* is able to secrete recombinant spidroins directly into the growth medium, a feat yet to be demonstrated in *E. coli*.[132,134] In this regard, the use of *P. pastoris* may reduce downstream processing costs and warrants further investigation alongside the secretion-based bacterial hosts discussed above. Furthermore, unlike *E. coli* and other bacterial hosts, *P. pastoris* and other yeast systems are eukaryotic, not prokaryotic. Accordingly, *P. pastoris* possesses many advantages inherent to eukaryotic protein expression, such as protein processing and post-translational modifications, that bolster its potential in the large-scale production of spidroins, an animal-based protein.[135]

In addition to *P. pastoris*, *Saccharomyces cerevisiae* (brewer's yeast) is a potential candidate for future host expressions. Like *P. pastoris*, *S. cerevisiae* is eukaryotic and possesses export mechanisms that could be optimized for spidroin secretion.[136] Recently, an engineered strain of *S. cerevisiae* was shown to produce 450 mg/L of the recombinant spidroin IF9, a 94 kDa analogue of MaSp1.[137]

Plants. In addition to bacteria and yeast systems, plants have been investigated for their potential and ability to produce spidroins. Compared to bacteria, which often struggle to stably express large, repetitive protein sequences, plant systems possess higher genetic stability during expression of large spidroins, helping to preserve the native length and mechanical properties of purified spidroins.[138] The slower growth of plants (Figure 3c) eases the stress induced by extreme demand for glycine and alanine tRNAs for the production of spidroins and lowers the rate of gene disruption caused by homologous recombination of repetitive sequences, issues that often plague rapidly dividing bacteria.[139,140] Furthermore, the constant exposure of plants to DNA-damaging forces like solar UV radiation has facilitated the evolution of a robust array of DNA repair and genome maintenance mechanisms, which can help preserve the stability of heterologous DNA.[141−143]

Compared to other eukaryotic hosts, like transgenic animals or insects, production costs for protein biomanufacturing in plants are often cheaper[144] largely due to well-established agricultural industries and pipelines that can be efficiently utilized.[145] Additionally, carbon emissions from plant platforms are much lower than those from animal hosts due to plants' ability to photosynthesize. Despite these advantages, one key challenge is the difficulty of plant-specific extraction and purification methods, which is reflected in the comparatively lower yields of plant spidroins. For example, plants can contain toxic secondary metabolites and endotoxins which can be induced by bacterial infection, which is a common mode for spider silk DNA transformation into plants.[146] This may complicate the extraction and purification process from plant hosts. In addition, regulatory challenges related to the strict regulation of genetically modified organisms (GMOs) or living modified organisms (LMOs) in uncontrolled environments such as open fields[147] can pose challenges regarding production scale-up.

Tobacco plants, *Nicotiana tabacum*, have been extensively tested. Researchers were able to purify 60.3 kDa MaSp1 and 58.5 kDa MaSp2 spidroins from *N. tabacum*, employing a strategy where expressed spidroins would be targeted to and accumulate within the endoplasmic reticulum (ER) for downstream purification.[148] The maximum yield for ER-derived spidroin is only 0.0025 and 0.025% of total soluble protein (TSP) of MaSp1 and MaSp2, respectively, within the plant. More recently, the split-intein mediated post-translational fusion method was employed to test if expression of larger protein sizes was feasible.[140,149] A 450 kDa spidroin multimer of flagelliform silk was recovered at 190 mg/kg. Despite the large size of the purified multimer at 37 kDa, the mechanical properties of this spidroin were decreased compared to native flagelliform silk, most notably in measured tensile strength. Importantly, this was not recombinant dragline silk, which remains the most desirable silk type for material applications.

Arabidopsis thaliana, a well-studied model plant system, has also been explored. The DBIP-8p protein, a 64 kDa synthetic analog of MaSp1, was purified from *A. thaliana*.[150] Protein production was targeted specifically to the apoplast, ER lumen, and vacuoles of the plant cells, and protein was extracted from these subcellular compartments during downstream processing. In the apoplast and ER lumen, maximum yield was 8.5 and 6.7% of TSP, respectively, whereas vacuole-derived spidroins were unrecoverable. A less targeted approach to expression wherein 64 kDa and 127 kDa homologues were engineered directly into the seeds was tested, where minor byproducts from the larger homologue were observed in the protein lysate.[151]

In alfalfa plants, *Medicago sativa*, sizes of MaSp2 ranging from 80 to 110 kDa were reported.[152] In this approach, recombinant spidroins did not freeze well due to high levels of insolubility, severely complicating purification. However, modeling and environmental analyses paint a more optimistic picture of alfalfa plants and their production capabilities. If better developed for larger spidroins, alfalfa plants may constitute the most economically viable production host for scale-up, compared with other plants, and even *E. coli* and goats (Figure 3e). Indeed, alfalfa cultivation techniques are well-suited to maintain particularly low carbon emissions associated with scale-up (Figure 3e) and will help promote a more circular economy, as alfalfa waste itself can be resold and reused as animal feedstock. Roberto Velozzi, Chairman and CEO of SpideyTek, champions the use of alfalfa plants as the future of the industry: "**Large-scale fermentation is not the future of spider silk production, alfalfa plants are. Alfalfa plants have very high yields, a robust genetic toolkit, and the ability to consistently produce native-sized spidroins. Net production costs drop to zero due to established agricultural infrastructure and the ability to resell alfalfa waste as animal feedstock pellets to farmers and other buyers**."

Cell Lines. Mammalian and insect cell lines are alternative expression hosts for spidroins. They are attractive systems for further investigation due to their ease of genetic manipulation and scale-up potential compared to their whole organism counterparts. MaSp1, MaSp2, and ADF3 purification has been tested in bovine mammary epithelial cells and baby hamster kidney (BHK) cells, both of which excel at protein secretion into the extracellular medium. Sizes of 60 to 140 kDa and titers of 25 to 50 mg/L for soluble spidroins were observed.[153] Larger spidroin sizes can be observed, though often with lower titers. Furthermore, inefficient mRNA translation can often be limited due to problematic secondary structures and inefficiency of plasmid transfection and host cell machinery.[153]

In insect cell lines, Sf9 cells derived from the armyworm *Spodoptera frugiperda* have been tested for the expression of ADF3 and ADF4.[82,154] Limited protein sizes and titers of 60 kDa and 5 mg/L were observed. Interestingly, as cytoplasmic expression of the proteins accumulated, coiled filaments began to form, but the length of the intracellular spidroin filaments was severely limited by cell size, leading to complications in the testing of material properties.

Animals. Silkworms, i.e., *Bombyx mori*, have been explored as expression systems for spider silk due to their historical use in the mass production of silkworm silk, and the potential to harness their natural spinning apparatus for recombinant spider silk. Multiple genome-editing techniques have been employed to replace the native silkworm fibroin gene with spidroin genes, such as the *N. clavipes* MaSp1 spidroin. Of these, CRISPR/Cas9, TALEN, and baculovirus-based technologies have been the most successful thus far.[58,155,156] In the experiments utilizing CRISPR/Cas9 and TALEN strategies, the inserted MaSp1 genes were translationally fused to the native silkworm fibroin genes (either *FibH* or *FibL*), resulting in the expression of a chimeric spidroin with unique material properties. In the experiments utilizing baculovirus-based technologies, a recombinant spidroin consisting of MaSp1 fused to EGFP was inserted into silkworm larvae. While 70 kDa MaSp1 fusion proteins were observed, the *B. mori* larvae were unable to spin silk fibers.

Many mammals possess mammary glands that secrete large volumes of protein-rich milk, a physiological feature that has been tested and exploited for the production of recombinant spidroin-rich milk in mice, goats, and sheep.[157,158] In transgenic mice, recombinant MaSp1 and MaSp2 were expressed and produced in the mammary glands, although the size and titer of the resultant spidroins were small (40 kDa and 11.7 mg/L).[159] However, the majority of offspring born from transgenic mice were able to produce spidroins, demonstrating the superior genetic stability of the exogenous spidroin gene, especially compared to bacterial hosts. Overall, mammalian hosts are plagued by long reproductive cycles and high production costs. Despite these challenges, some groups have used transgenic goats[157,160,161] and sheep[162] and demonstrated levels of stability and performance of recombinant spidroins similar to those achieved in unicellular organisms.

■ RECOMBINANT SPIDROIN EXPRESSION WORKFLOW AND TRADE-OFFS

As discussed above, many considerations and trade-offs ultimately dictate the choice of a host organism. Despite the dominance of *E. coli* and *P. pastoris* in the recombinant spider silk and broader industrial biomanufacturing spaces, continued interest in optimizing spidroin production bolsters the potential of alternative hosts like alfalfa plants, silkworms, and transgenic mammals. These nonincumbent hosts retain exploitable advantages over their competitors that make their adoption as expression platforms attractive. For example, silkworms possess a naturally evolved spinning apparatus that can be repurposed for recombinant silk, while alfalfa plants benefit from established agricultural techniques and the ability to sell purification waste.

The core pipeline for the design, expression, and purification of recombinant spidroins has been well developed by both industry and academia and retains flexibility for variations based on the choice of host platform (Figure 3b). Historically, MaSp1, MaSp2, ADF3, and ADF4 were the most selected sequences for dragline spidroin expression, largely due to the emphasis on *N. clavipes* and *A. diadematus* as model spider systems. However, recent studies have dramatically expanded the library of known spidroin sequences and their associated mechanical properties.[57] This data will broaden the utilization of spidroin sequences and facilitate investigations into new designs, such as chimeric or silk-inspired fibrous proteins which could improve downstream processing or material properties. With such an extensive repository of available sequence data, several considerations must be taken into account when selecting a specific sequence for heterologous production. As discussed earlier, dragline silk possesses desirable mechanical properties that can vary depending on sequence (Figure 2e). Furthermore, certain sequences may be more amenable for purification from specific host organisms.

There are numerous factors to consider when selecting a host organism to express recombinant spider silk, but all factors must optimize both business profitability and product quality (Figures 3b,c). Genetic tractability, the ease with which an organism's DNA can be modified, is an important criterion to consider when selecting a host organism. Though DNA synthesis and sequencing costs are at an all-time low, some organisms are more readily edited than others, leading to quicker and cheaper DBTL (Design, Build, Test, Learn) cycles. Typically, model organisms like *E. coli, P. pastoris*, or *A. thaliana* are most familiar to researchers and have the most developed genetic toolkits available, making them obvious initial choices. However, interest in non-model organisms has been growing, as researchers trade familiarity and ease of use for untapped metabolic potential. For example, some researchers are exploring organisms capable of highly efficient secretion (e.g., *Trichoderma reesei*) to maximize yield.[163] To further enhance biomimicry, attempts have been made to produce immortalized cell lines from spider silk glands, enabling greater codon optimization, natural post-translational modifications, and continuous secretion and harvesting capabilities.[164,165]

Protein quality is also an important consideration for host choice. Native spidroins are generally over 250 kDa in size and have post-translational modifications (e.g., phosphorylation).[49,166] Certain hosts are more consistently able to produce larger recombinant spidroins than others, which has proven to be necessary for proper fiber formation and preservation of desirable mechanical properties. Researchers have experimented with methods to enhance spider silk protein length, using techniques such as intein-mediated protein fusion[124] or circularized RNA[167] techniques. However, these methods required fine-tuning to prevent cell toxicity and aggregation by the longer proteins. To some researchers, overcoming toxicity is the major stepping stone to improving yield, an opinion championed by Dr. Mattheos Koffas, a professor at Rensselaer Polytechnic Institute. "**In our work we have shown that the increased expression of spider silk proteins results in heightened toxicity effects for the cell, mostly because of the disordered nature of the protein. To scale up spider silk production and achieve competitive titers, we must understand and alleviate the source of this toxicity for the cell. At this stage, toxicity is likely the #1 issue standing in the way of proper scale-up.**"

Furthermore, eukaryotic hosts intrinsically possess post-translational modification pathways, unlike bacterial hosts, which lack such machinery and require genetic modification for this purpose. Currently, reported PTMs in native spidroins include glycosylation, phosphorylation, and hydroxyprolination,[49,166,168,169] which altogether help regulate spidroin solubility and aggregation in native spider environments, though the extent to which these modifications are necessary is still under investigation.[49] Given that not all spider silks are glycosylated, phosphorylated, or hydroxyprolinated, these modifications are likely not strictly required. Furthermore, it is unclear how these modifications affect the solubility, aggregation, landscape, and typology of native spidroins.

For some organisms, protein purification and fiber spinning are laborious, costly processes. Organisms such as *B. mori* and other insects closely related to spiders possess natural fiber spinning apparatuses, an attribute not found in other mammalian, plant, and microbial hosts. In addition, non-microbial hosts often bypass issues such as endotoxin contamination. Purification technologies that are being explored in other biomanufacturing spaces have also been tested here. For example, secretion-based production systems in bacteria, wherein the overexpressed molecule is transported outside of the cell into the growth medium, are a potential cost-cutting strategy that has shown promising development.[127,129,130] Such strategies would forego many downstream purification processes altogether, which would hopefully promote higher yields. However, bacterial secretion of large, repetitive, and hydrophobic proteins remains challenging.

Presently, there is insufficient data and comparative methodologies among the various heterologous hosts used to express spider silks where conditions, purification levels, and optimization procedures can be adequately benchmarked using the same systems. In the future, the best host systems to maximize

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recombinant spider silks may be better matched to the specific silk based on its type, size, and amino acid sequence. Further, scale-up is arguably the most pressing issue afflicting many startups in this space, as scale-up directly impacts the commercial viability of spider silk products. That said, the choice of host organism will also directly influence scale-up potential. Which host organisms will be most amenable to scale-up in the future remains to be determined.

Scale-up must occur across all steps of the manufacturing process from bioproduction, to downstream purification, to postprocessing such as fiber spinning. Although some heterologous systems, such as transgenic silk worms expressing spider silk, take longer to grow and divide, the production, purification, and spinning are accomplished by each individual organism.[170] However, other microbial systems such as genetically engineered *E. coli*, are likely to be more easily transferred to closed bioreactor systems which can scale to larger volumes more quickly. Ultimately, the benefits and limitations related to amounts, purity, and specific use-cases of spider silk production must be weighed during the host organism selection and business model formulation.

Navigating host selection involves a complex balance of trade offs, particularly in goals and strategy. It is important to note that while an ideal host organism will maximize the number of attributes amenable to recombinant spidroin expression, it is unlikely that a single organism will be able to satisfactorily cover all of the production criteria. Sometimes, two or more criteria will come into conflict. For example, within a chosen host organism, prioritizing spidroin size may come at the cost of overall yield. Strengths in some organisms, like innate eukaryotic machinery for post-translational modification, may need to be sacrificed in order to capitalize on other strengths, such as ease of genetic manipulation or scale-up potential. Overall, the ability to implement fast DBTL cycles in a host organism coupled with the feasibility of producing the desired product will dictate which strategy is implemented. This is especially true for academic and industrial projects that seek to engineer novel functionalities and properties into recombinant spidroins and explore their potential downstream applications (which we discuss in further detail below). In this regard, bacteria and yeast have constituted the major molecular workhorses for these exploratory pipelines, due chiefly to their short generation time, genetic tractability, and widespread familiarity among researchers. However, the design of spider silk fibers, the required scale and yield, and the specific downstream applications may eventually guide researchers and companies toward adopting more specialized host organisms in the future.

■ PRICING AND ENVIRONMENTAL BENCHMARKS

While the cost of spidroin production is an important factor in assessing the profitability of a silk product, various other financial and environmental variables can also act as integral guiding principles in determining a host expression platform. Figure 3e provides a case study of fiscal and environmental considerations of specific hosts, and highlights the power of necessary context in these decisions. At first glance, *M. sativa* seems a more attractive candidate for expression than *E. coli* due to its lower cost of production. However, its longer and more limited production time can pose significant challenges from a business perspective, for example if there are fluctuations in demand that require rapid scale-up. We note that the production cost analysis here relies on the assumption of highly optimized, idealistic processing pipelines, which may fluctuate as the industry matures. In a market where consumers are increasingly willing to pay a green premium,[170] measures of environmental impact must also be carefully weighed. *E. coli*, a popular choice for many start-ups and established businesses, currently has a higher estimated carbon footprint than tobacco plants or goats but requires much less land usage than either competitor. Bioprocess optimization is still in its infancy compared to more streamlined processes like traditional plant agriculture, suggesting that initial environmental impact estimates for *E. coli* have much room for improvement. Complete bioprocess optimization for *E. coli* may eventually outcompete plant agriculture in cost. These numerical-based benchmarks also fail to incorporate more qualitative advantages, such as *E. coli*'s notable genetic tractability, which can further complicate the ultimate decision of a host platform.

Environmental impact considerations made by policy regulators and businesses extend beyond the choice of the host organism. The environmental impact of recombinant dragline silk, compared to other established fibers and materials, is a key consideration to highlight when considering the viability of the enterprise altogether. Currently, as seen in Figure 3f, recombinant dragline silk has a mixed record on benchmarks such as carbon footprint, energy usage, and water usage. It performs better than some materials on certain benchmarks (i.e., less water usage than silkworm silk or cotton) and worse than some materials on other ones (i.e., higher carbon emissions than many other fibers). It is important to note that these values are estimates and can vary widely based on the methodology and analysis paradigm of the data. Additionally, biomanufacturing scale-up and processing is a nascent, decades-old technological paradigm with vast space for improvement, compared to the millennia-old production methods for incumbent materials like cotton and silk. One key advantage of recombinant spider silk is its inherent biodegradability and overall low degradation period, which synthetic polymer-based textiles generally do not possess. However, this advantage alone may not be enough to sustain further investment and growth of the recombinant dragline silk industry if other environmental and financial benchmarks are not improved. This will inevitably require sustained cooperation between governmental, academic, and industry sectors, and continual advancements in our scientific understanding of and engineering capabilities surrounding dragline silk. Leading figures in the space, such as Dr. David Kaplan, Professor at Tufts University, agree: "**To fully unlock spider silk's potential as a biomaterial, we need a colossal cross-disciplinary collaboration on a similar level to that of the Manhattan Project or Human Genome Project: the Silkhattan project!**"

■ DOWNSTREAM PROCESSING OF SPIDER SILK INTO MATURE FIBERS

Having delved into host organism considerations such as yield, environmental impact, and cost, we now move on to discuss the crucial phase of downstream processing. Downstream processing involves all the steps after the fermentation including extraction, purification, and formulation of the spider silk protein. Depending on the selected host organism, the ease of extraction and purification can vary significantly. For example, an organism capable of protein secretion will alleviate challenges in protein extraction given the ease of collecting protein in the supernatant, and the relative purity of this extracellular medium.[172] Once the spidroin proteins have been purified, they must be spun to produce a fiber. Given the environmental concerns associated with incumbent materials and the initial

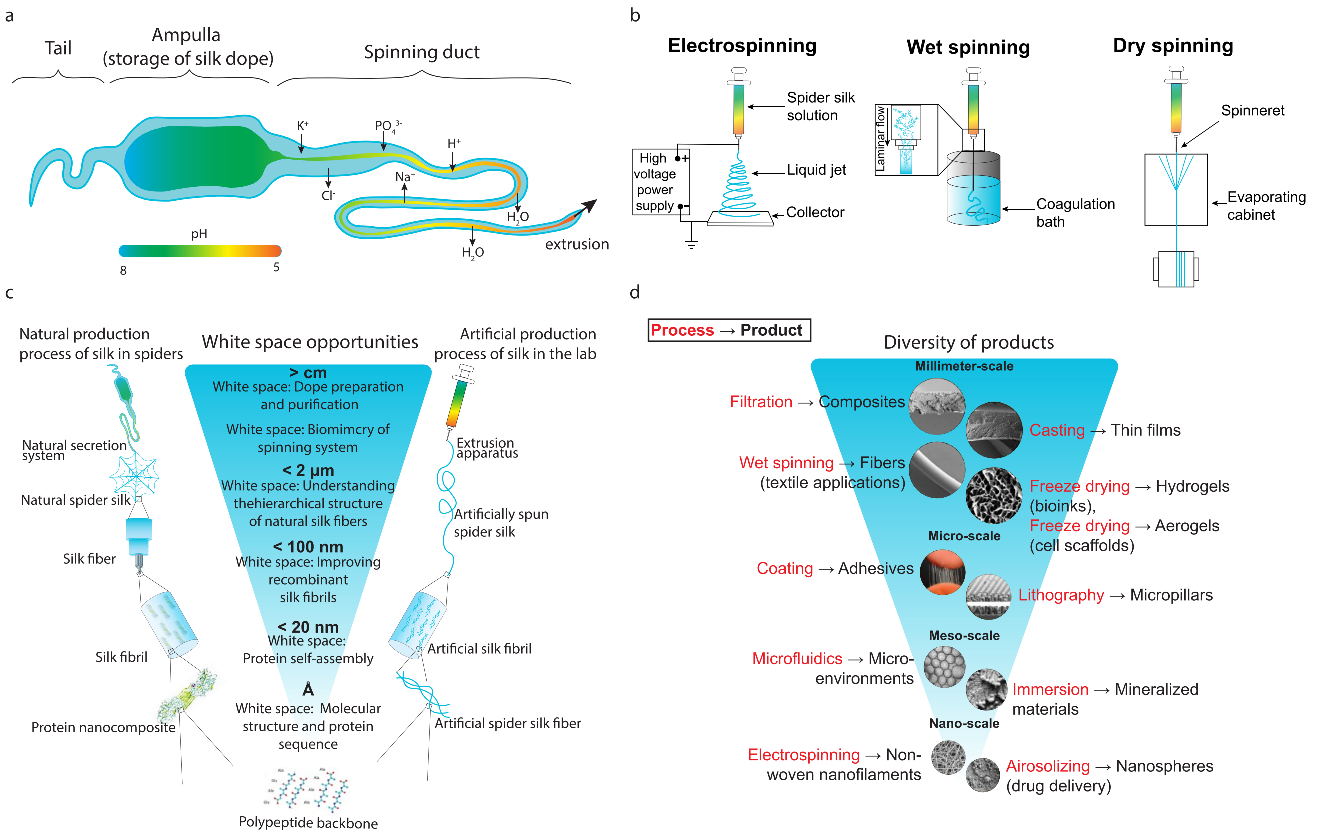

Figure 4. Preparation of spider silk into fibers and other products. (a) Natural spider silk spinning across a spider abdomen. The spinning dope is stored in the ampulla and sent through the spinning duct which tapers off and forms a pH gradient that concentrates and assembles the spider silk protein. Adapted with CC-BY license from Whittall et al.[208] Copyright 2020 Elsevier. (b) Different methods for spinning spider silk protein into fibers including electrospinning (left), wet-spinning (middle), and dry-spinning (right). The choice of a spinning method will depend on the desired properties of the spun product. (c) Comparison of natural vs artificial spider silk spinning across various length scales ranging from angstrom scale to centimeter scale. White space research opportunities are included at different levels of hierarchy. (d) Alternative nonfibrous forms of spider silk protein products across scales of underlying structure from nano- to millimeter scale. Structures include composites, fibers, films, hydrogels, adhesives, micropillars, microenvironments, mineralized materials, nonwoven nanofilaments, and nanospheres. In red text are the various processes which can form these products. Micrographs are adapted with CC-BY-NC-ND from Mohammadi et al.[196,209,210,211] as well as Hummerich et al.[154] Copyright: 2018 American Chemical Society, 2020 Elsevier, 2019 AAAS, 2018 Springer Nature, and 2004 Elsevier, respectively.

promise by early spider silk companies to deliver high-performance textile fibers made from spider silk, we mainly focus on the intricacies of spinning methods and their applications. However, researchers have also explored numerous alternative uses for spider silk, including adhesives, films, and coating in various applications.[173,174]

The natural process of spider silk production and spinning is highly complex and remains an active area of research. Understanding the natural spinning process has the potential to inform principles of artificial spinning that would maintain the integrity of the spider silk fibers (Figure 4a). First, spidroin proteins are secreted into the tail of the gland by epithelial cells, before traveling to the gland's ampulla, or storage vesicle.[175] Here, dragline spidroins begin to accumulate in a highly concentrated (up to 50% weight by volume) aqueous solution known as the spinning dope.[176] In the dope, spidroins self-assemble into spherical micelles, featuring a surface composed of the hydrophilic N- and C-terminal domains.[177,178] As discussed in a previous section, the globular N- and C-terminal domains play critical roles in spidroin stability, solubility, and oligomerization.

The spidroin micelles in the dope then migrate toward the spinning duct, a long, S-shaped duct that gradually tapers toward its terminal end.[179] Throughout the tightening duct, shear and elongation forces compress the spherical micelles, leading to a gradual transformation into extended crystalline β-sheets with corresponding amorphous regions (liquid crystals).[180,181] Structural reassembly into insoluble fibers is facilitated during passage through the duct by ion exchange and water recapture. Increasing acidification is also essential, with a continuous lowering of pH from about 8 to 7.5 in the ampulla and reaching an approximate pH of 5 at the end of the spinning duct.[77,175,179,182]

At the end of the duct, microfibers are spun out of the spider's body via external microtubules in the spinneret, the external part of the gland.[183] This process is typically regulated by a valve at the end of the duct, which controls the timing of ejection. The bundle of extruded microfibers forms the cylindrical core of the macrofiber, which is then packaged and coated by glycoproteins and lipids.[26,184] The mature, complete fiber is then finally pulled out from the spinneret by the spider's rear legs.

The process of spider silk secretion is highly complex, with numerous physical and biochemical forces playing key roles in fiber stability and formation. In theory, artificial spinning methods must capture the main features of the natural process in order to recreate the mature silk fiber's properties. This would entail the preservation of factors such as a high spidroin concentration in the dope, a narrow passageway resembling the spinning duct to create shear and elongation forces, and the maintenance of biochemical elements like ion exchange, acidification gradients, and water recapture.

Silkworm silk can be harvested within the time scale of a silkworm's life cycle, which typically spans 5 to 8 weeks from egg to cocoon.[185] In contrast, the bioproduction and harvesting of spidroin protein can be accomplished within a few days.[186] Once the spidroin solution is extracted and purified, it needs to be spun into fibers. In the spider gland, spider silk can transition from being highly soluble to becoming a highly structured, high-performance, solid fiber in just a second. This change of state is achieved through progressive changes in pH and temperature as the solution traverses the spider's glands. These environmental changes help the peptide chains change their conformations and acquire structural integrity. The C- and N-terminal domains act as pH switches: at high pH ∼ 8 (similar to the gland sac), spidroin solutions are highly soluble whereas at low pH ∼ 5 (similar to the duct) the peptides become more structured.[187] In particular, the aggregation of the highly repetitive regions organized in β-sheets make the material hydrophobic at low pH.

There are multiple synthetic methods that try to mimic this natural process and spin spider-silk fibers out of solution (Figure 4b). Therefore, optimizing in vitro spinning methods is necessary for ensuring the integrity of the mechanical properties of synthetic spider silk. Three historically popular in vitro spinning methods are electrospinning, wet-spinning, and dry-spinning. Electrospinning involves aligning charged particles along an electric current to form fibers, which typically results in short nanometer scale strands which have weaker tensile properties compared to natural silk and other spinning methods.[188] Electrospinning is an efficient way of producing nonwoven materials and mats. Wet-spinning is a method that involves solubilizing a polymer in a doping solution, then extruding it through a nozzle into a coagulation bath. Compared to electrospinning which produces fibers on the nanoscale, wet-spinning can be used to produce micrometer-thick fibers.[189] Polymer strength and integrity are preserved in wet-spinning contexts. Dry spinning is a third popular method that involves sending polymer solution through a spinneret capable of evaporating the solvent to produce a dry polymer comprising a solid fiber. This method is typically chosen when there is risk of thermal degradation of the polymer or to enable specific surface characteristics.[190] The caveats of dry spinning include difficulty in controlling the fiber cross-section, slower processing, and high requirements for heat and energy. However, dry spinning can produce long fibers that can be minimally processed after spinning.

One of the most promising spinning methods is "biomimetic" spinning, also known as straining flow spinning, which seeks to recapitulate the environmental changes that the spider silk solution is exposed to in a synthetic setting.[95] It is a variation of wet spinning with a more intricate interaction between the dope jet and the focusing jet (solvent). This method, which can be done using environmentally friendly solvents, maintains the integrity and high performance of spider silk fibers.

The natural and artificial production of spider silk share numerous parallels, presenting various opportunities for improvement and active research at each production step which can improve biomimicry, perhaps even surpassing natural spider silk capabilities (Figure 4c). White space areas encompass optimization across all scales of a fiber, ranging from the angstrom to the centimeter scale. At the angstrom scale, molecular structure is influenced heavily by amino acid sequence interactions. Altering the combination of alanine and glycine repeats and polyalanine stretches can also affect the nanoscale interactions which give rise to self-assembly. This sequence optimization can clarify the biophysical phenomena for enhanced protein self-assembly on the nanoscale. Within the microscale, the hierarchical structure of spider silk can be better mimicked by applying coatings and layers and incorporating composite formulations, including glycoproteins or lipids. Lastly, at the macroscale, there is great opportunity to enhance mechanical properties using various spinning methods, adjustment of fiber extrusion parameters such as speed, temperature, doping solutions, die shape, as well as post-spinning coatings and treatments.

Though wet-spinning has been done with promising results, the mechanical properties of artificially spun spider silk have struggled to match those of natural spider silk,[191] and the diameters of artificially spun spider silk are often larger than natural versions.[192] Analysis and understanding of the sequence−structure−function relationship of spider silk proteins can eventually enable materials which can surpass the material properties of nature.[26]

■ DIVERSE PRODUCT APPLICATIONS FOR RECOMBINANT SPIDER SILK

Recombinant spider silk has great utility outside of textile fibers and can be used to fabricate a versatile range of materials, each with their own nanostructure and downstream mechanical properties. Alterations in production methods, as well as different environmental conditions such as pH, temperature, protein concentration, composition, and postprocessing can lead to vastly different materials each with a specific structural length scale and different ultimate applications.

Aside from composites, recombinant spider silk has been proposed in additional nonfibrous or nonwoven use cases including coatings, films, adhesives, hydrogels and aerogels, nonwoven nanofibrils, nanospheres, and filaments (Figure 4d). Each of these alternative product types has demonstrable potential for improvement of material properties and optimized use cases, especially in biomedical applications, which currently hold the highest promise for these silk forms and are described in further detail below.

2D structures have numerous clinically relevant applications, especially in cell scaffolding functions. Electrospinning has been used to manufacture 2D nonwoven meshes with various filament orientations from engineered ADF3 spidroins, with unique biomedical applications. Silk filaments of diameters ranging from 700 to 900 nm in mesh form were able to support the immobilization of fibroblasts and other cells, a feat not achieved by flat films of the same engineered ADF3 spidroins in other formats.[193] Additionally, 2D films are also a powerful tool for screening the response of interacting cells: surface charge has been demonstrated to alter cell attachment in biological scaffolding contexts. For example, silk-derived films manufactured with a positively charged, engineered ADF4 proved a capable substrate for cardiomyocyte binding, unlike their negatively charged counterparts.[194] In addition to meshes and films, spider silk-derived coatings of synthetic implants can modify and improve interactions with native cells. Coating silicone breast implants with spider silk improves biocompatibility and can help dissuade the formation of unwanted fibrous tissue which can be observed in traditional synthetic implant

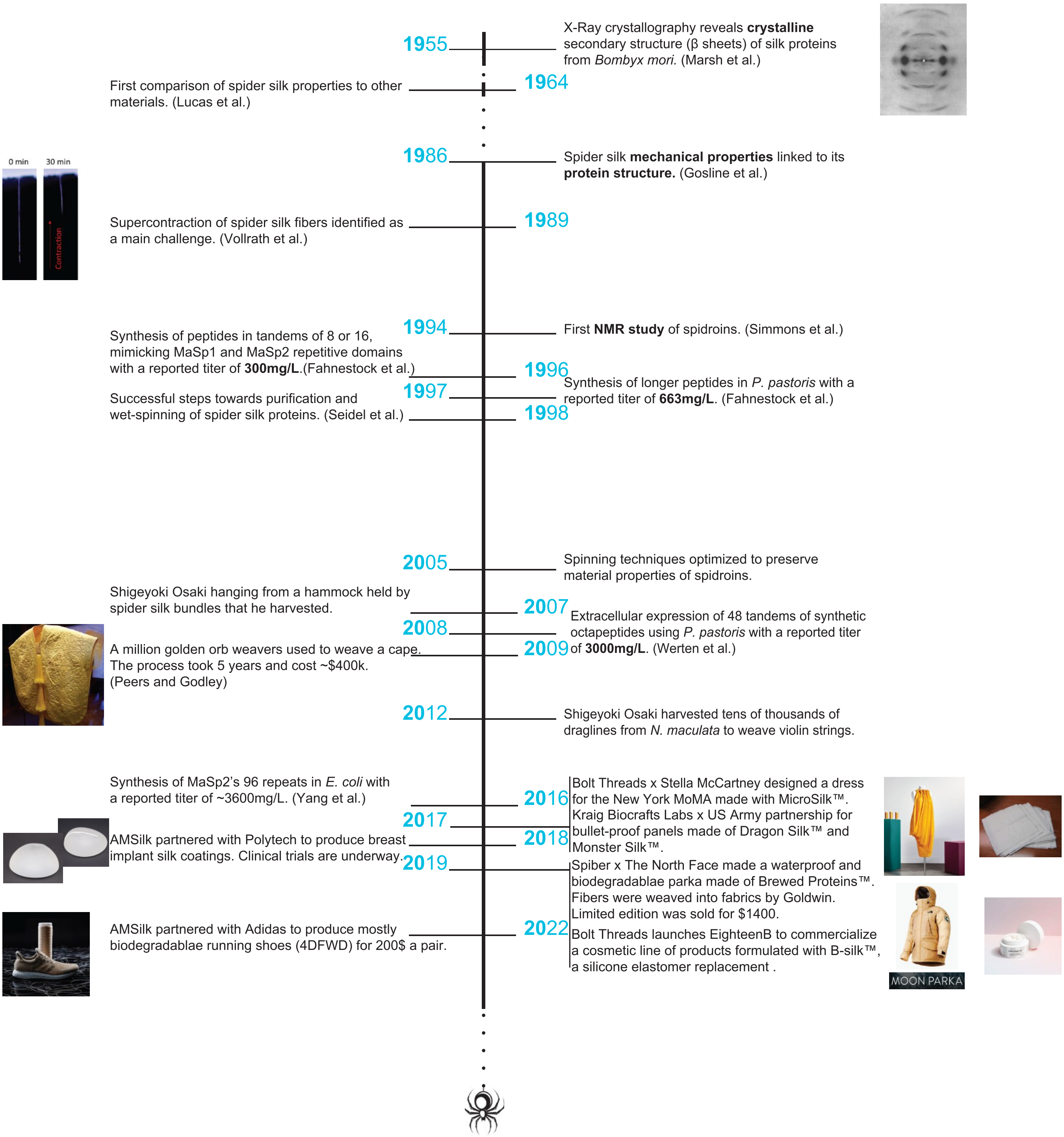

Figure 5. Timeline of key discoveries and innovations that have enabled the investigation and industry of recombinant spider silk production. Left: Illustration of supercontraction reproduced with CC-BY license from Greco et al. Copyright 2021 Springer Nature. Golden cape reproduced with CC-BY-SA license. Copyright: Cmglee, 2008. Breast implant silk coatings and running shoes reproduced with permission from AMSilk. Copyright: AMSilk, 2017, 2019. Right: X-ray image reproduced from Marsh et al. (1955), Elsevier license number: 5712310087210. MoMA dress and B-silk advertising images reproduced with permission from Bolt Threads. Copyright: Bolt Threads, 2016, 2022. Picture of bullet proof panels reproduced with permission from Kraig Biocraft Laboratories. Copyright: Kraig Biocraft Laboratories, 2016. Picture of MoonParka reproduced with permission from Spiber. Copyright: Spiber, 2019.

contexts.[195] Finally, the use of silk-derived adhesives materials has also been explored.[196]

In addition to these 2D structures, recombinant spider silk has promising clinical applications in the form of 3D structures, such as porous foams, hydrogels, and nanospheres. Foams with numerous, interconnected pores hold promise as efficient cell scaffolds, supporting adhesion of a wide variety of cell types, e.g., adult precursor cells and human embryonic stem cells, when designed and constructed with the appropriate cell adhesion sequence motifs.[197,198] While foams are investigated for cell scaffolding functions, hydrogels are investigated for their potential as bioinks in biofabrication, an additive manufacturing

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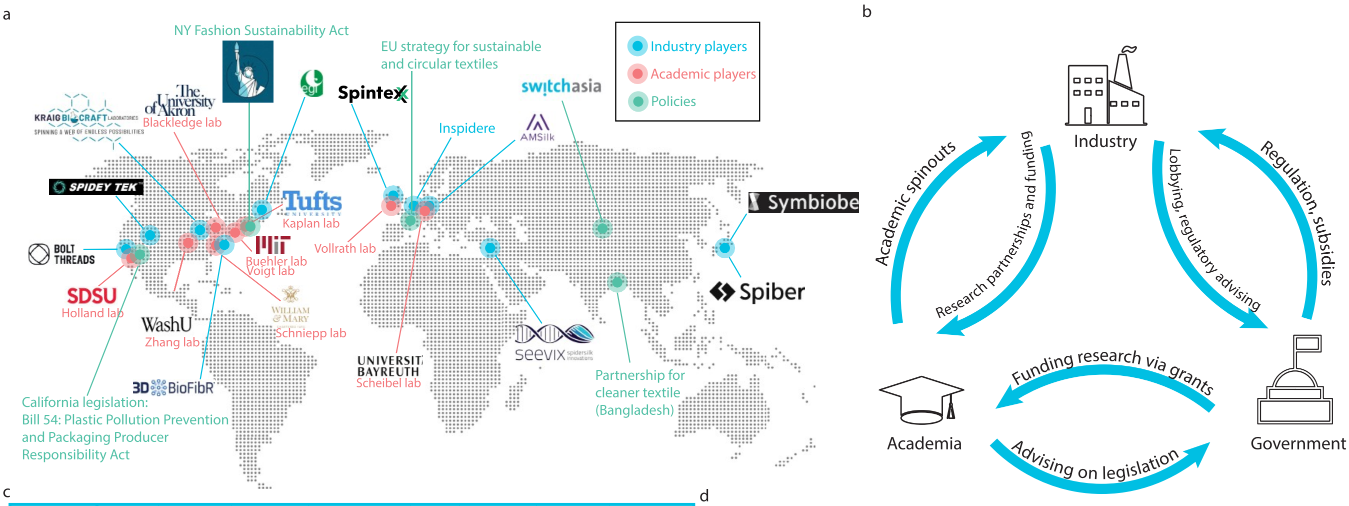

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Company	Year Founded	Location	Industry	Product name	Capital raised	Funding stage	Ownership	Host organism
KRAIG BIOCRAFT LABORATORIES	2006	Lansing, MI, USA	Textiles, Defense	Dragon Silk™, Monster Silk®, SpiderPillar™	17.5M$	Post-IPO Equity	Public: OTC:KBLB	Silkworm
Spiber	2007	Yamagata, Japan	Textiles	BrewedProtein™	910M$	Private Equity	Private	Bacteria (*e.g. E. Coli*)
AMSilk	2008	Neuried, Germany	Textiles, Medical, Cosmetics	Silkbeads (microparticles) Silkgel (hydrogel) Biosteel® (fibers)	54M$	Series C	Private	*E. Coli*
BOLT THREADS	2009	Emeryville, CA, USA	Textiles, Cosmetics	B-silk (silicone elastomer alt. in cosmetics), Microsilk (fiber)	334M$	IPO via SPAC	Public: Nasdaq: BSLK	*P. Pastoris*
Inspidere	2011	Eindhoven, Netherlands	Textiles, Composites	-	-	-	Private	Goats
seevix	2014	Jerusalem, Israel	Biomedical, Cosmetics, Composites	SVX™, additive to materials	18M$	Series A	Private	SF9 cells *E. Coli*
SPIDEY TEK	2015	Los Angeles, CA, USA	Semiconductors Aerospace, Defense, Transportation, Textiles, Cosmetics	Fibers, adhesives, composites	1.5M$+	Beyond seed	Private	Alfafa plant *E.Coli* Silkworms
Spintex	2018	Oxford, UK	Textiles, Medical	-	1.1M$+	Seed	Private	Unknown (trade secret)
3D BioFibR	2020	Halifax, Nova Scotia	Tissue engineering	SpidRFibR™	4.5M$	Seed	Private	Unknown
Symbiobe	2021	Kyoto, Japan	Textiles, Composites	AirSilk™	2M$	Seed	Private	Marine purple photo-synthetic bacteria

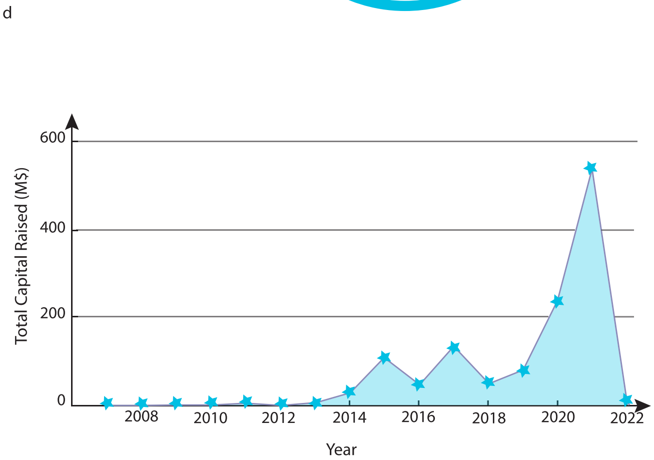

Figure 6. Landscaping the spider silk industry. (a) World map indicating the main players in spider silk research, production, and development. Blue dots indicate companies, red dots indicate academic laboratories, and green dots refer to the location of various policies. Most activity is in North America, though Japan and Europe have a few important players. We note the geographic proximity between academic laboratories and startup companies. The University of Akron logo reproduced with permission from the University of Akron communications office. Copyright: University of Akron. Tufts University logo reproduced with permission from the Tufts University communications office. Copyright: Tufts University. MIT logo reproduced with permission from the MIT communications office. Copyright: MIT. SDSU logo reproduced with permission from the SDSU communications office. Copyright: SDSU. William and Mary logo reproduced with permission from the William and Mary communications office. Copyright: William and Mary. Kraig Biocraft Laboratories logo reproduced with permission from Kraig Biocraft Laboratories. Copyright: Kraig Biocraft Laboratories. Spiber logo reproduced with permission from Spiber. Copyright: Spiber. AMSilk logo reproduced with permission from AMSilk. Copyright: AMSilk. Bolt Threads logo reproduced with permission from Bolt Threads. Copyright: Bolt Threads. Seevix logo reproduced with permission from Seevix. Copyright: Seevix. Spidey Tek logo reproduced with permission from Spidey Tek. Copyright: Spidey Tek. Spintex logo reproduced with permission from Spintex. Copyright: Spintex. 3DBioFibr logo reproduced with permission from 3DbioFibr. Copyright: 3DbioFibr. Synbiobe logo reproduced with permission from Synbiobe. Copyright: Synbiobe. (b) Cartoon illustrating the main interactions between academia, industry, and government. The development of a healthy and thriving ecosystem requires the interaction between all of these components. (c) Table landscaping the main, active spider silk companies. Information was compiled from publicly released data on company Web sites and confirmed through personal correspondences with the firms. (d) Fundraising amounts as a function of time. Data was compiled using CrunchBase. The drop in funding in 2022 is correlated with a wider drop in biotech funding during that year.

technique that combines living cells with hydrogels to generate tissue-like structures. ADF4-derived hydrogels were recently explored as promising bioinks, and the cell compatibility of these recombinant hydrogels was tested with various cell types, such as osteoblasts, HeLa cells, myoblasts, among others.[199,200] Finally, silk-derived nanospheres have been explored for their promising uses in drug delivery technologies.[201] Different variants of MaSp1 were used to create the first class of these nanospheres,

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and the mechanical properties of these spheres could be directly modulated.[202]

Another major frontier for spider silk innovation is that of spider silk chimeras with other proteins in order to confer novel properties absent in native spider silk. In some cases, highly soluble regions of spidroin can be combined.[95] Strength motifs can be combined with elastic motifs inspired from different species of spiders, flanked by the N- and C-terminal domains of silk fibroin to favor expression,[203] Furthermore, chimeras can provide novel functionalities to spider silk via the fusion of enzymes[204] or affinity domains targeting antibodies, minerals (e.g., biotin)[205] or metals such as uranium.[206] These chimeras expand the sequence space for spider silk tremendously and therefore widen the repertoire of potential product applications due to the flexibility of designing chimeric spidroins for specialized functionalities.

Whether for medical or industrial applications, the inclusion of spider silk protein would bolster the recyclability or biodegradability of materials, as opposed to glass, metallic, or synthetic fibers, and their addition would result in lower density and weight with increased tensile strength. Spider silk has great potential in the composites industry because it can confer high strength to weight ratios, stiffness, and elasticity. Additionally, spider silk is nonmagnetic and has increased strength with decreasing temperature.[207] These unique property combinations provide spider silk with great potential in aerospace, defense, and construction applications. These properties may also confer unique advantages or negate disadvantages associated with incumbent materials, as is the case with silk-coated breast implants improving biocompatibility. Further academic and industry investigations will help optimize the inclusion of silk proteins in specific products and will likely expand the repertoire of possible product applications altogether.

■ A RICH AND DIVERSE ECOSYSTEM FOR SPIDER SILK PRODUCTION

The acceleration of discoveries related to the structure and properties of spidroins resulted in an ushering of companies attempting to utilize this technology for industrial applications (see timeline, Figure 5). Significant diversification of host organisms,[27,131,208,212] increases in production yields (Figure 3d and timeline, Figure 5), developments in understanding how sequences affect material properties (Figure 2e)[180,213] and optimization of downstream processing techniques[61,214−219] have all allowed for companies specialized in the production of synthetic spider silk to begin their activities. Even though such companies have multiplied over the past 20 years (Figure 6a), this nascent field still has room to grow, transform, and develop, and new startups proposing to produce spidroins at scale emerge every year. These early companies, many of which spun out of academic laboratories, target sectors ranging from defense to sustainable fashion and are striving to make the bioproduction of spider silk commercially viable. As a first step, many of these companies began releasing pilot projects and demos of products demonstrating their potential for the at-scale production of spidroins in heterologous hosts. Some of these examples have ranged from the production of garments and shoes to coatings for breast implants (see timeline, Figure 5). In addition, notable partnerships have been made with consumer brands such as The North Face,[220] Archer Daniels Midland,[221] and Toyota[222] (Spiber); Airbus,[223] Mercedes Benz,[224] and Adidas[225] (AMSilk); and Kering, Lululemon, Adidas,[226] and Stella McCartney[227] (Bolt Threads), indicating the future potential for scale-up and strong interest in spider silk.

A global survey of spider silk-focused companies, academic laboratories, and local policies encouraging the development of sustainable materials reveals many interesting trends (Figure 6a). Three major geographic poles drive innovation in this space: North America, Europe, and Japan. This finding can be explained by the availability of funding to derisk the early stage research necessary for the commercialization of spider silk. It is also worth noting that these companies, though based in geographically distinct innovation hubs, expand their production beyond these locations, often with the purposes of reducing production costs. For example, Kraig Biocraft Laboratories purchased a subsidiary in Vietnam,[228] while Spiber built a mass production facility in Rayong, Thailand,[229] with the purpose of scaling up their activities. Such interactions are bound to export interest in sustainable biomaterials beyond the three hubs identified above. Local governments can encourage the exportation of such sustainable industries within their territories by promoting legislation and implementing fiscal benefits that may help recombinant spider silk reach price parity with incumbent materials.

The global analysis (Figure 6a) also reveals that companies often colocalize their activities with academic laboratories and operate in areas with favorable policies for their activities. This indicates the importance of cross-institutional support and collaboration for achieving a thriving ecosystem that would enable the development and success of the spider silk industry (Figure 6b). In particular, links between academia and industry are of paramount importance. For example, several companies were founded out of direct associations to academic institutions. In 2008, AMSilk was cofounded by Dr. Thomas Schiebel, who was a Biomaterials professor at the University of Munich working on recombinant spider silk research.[230] Similarly, Bolt Threads resulted from the interdisciplinary collaboration of three graduate students who met through their common interest in biomaterials.[231] Finally, Spidey Tek was cofounded by Roberto Velozzi and Prof. Randolph Lewis, a world expert in spider silk research. Conversely, companies can partner with academic laboratories and fund research projects within them to outsource some of the risky basic science necessary to optimize the production processes. For example, Spiber has had close ties with the University of Kyoto, and current Spiber employees are also cross-listed as graduate students at local universities. Models where academics can advise and contribute to the industrial R&D process should be encouraged. These examples highlight the potential of bridging mismatches in professional cultures, timeframes, and specific interest between academia and industry.

In addition to close ties between academia and industry, local governments can also enable a thriving ecosystem in the sustainable biomaterials space. By proposing legislation that deters using incumbent petrochemical materials and encourages more sustainable (though often pricier) alternatives, governments can encourage investments in early stage companies and accompany the transition toward more sustainable and biodegradable materials. Several government policies have emerged which will increase the adoption of protein-based materials and textiles. One example is the EU strategy for sustainable and circular textiles (2023) which states that "by 2030, textile products placed on the EU market [will be] long-lived and recyclable, to a great extent made of recycled fibers, free of hazardous substances and produced in respect of social

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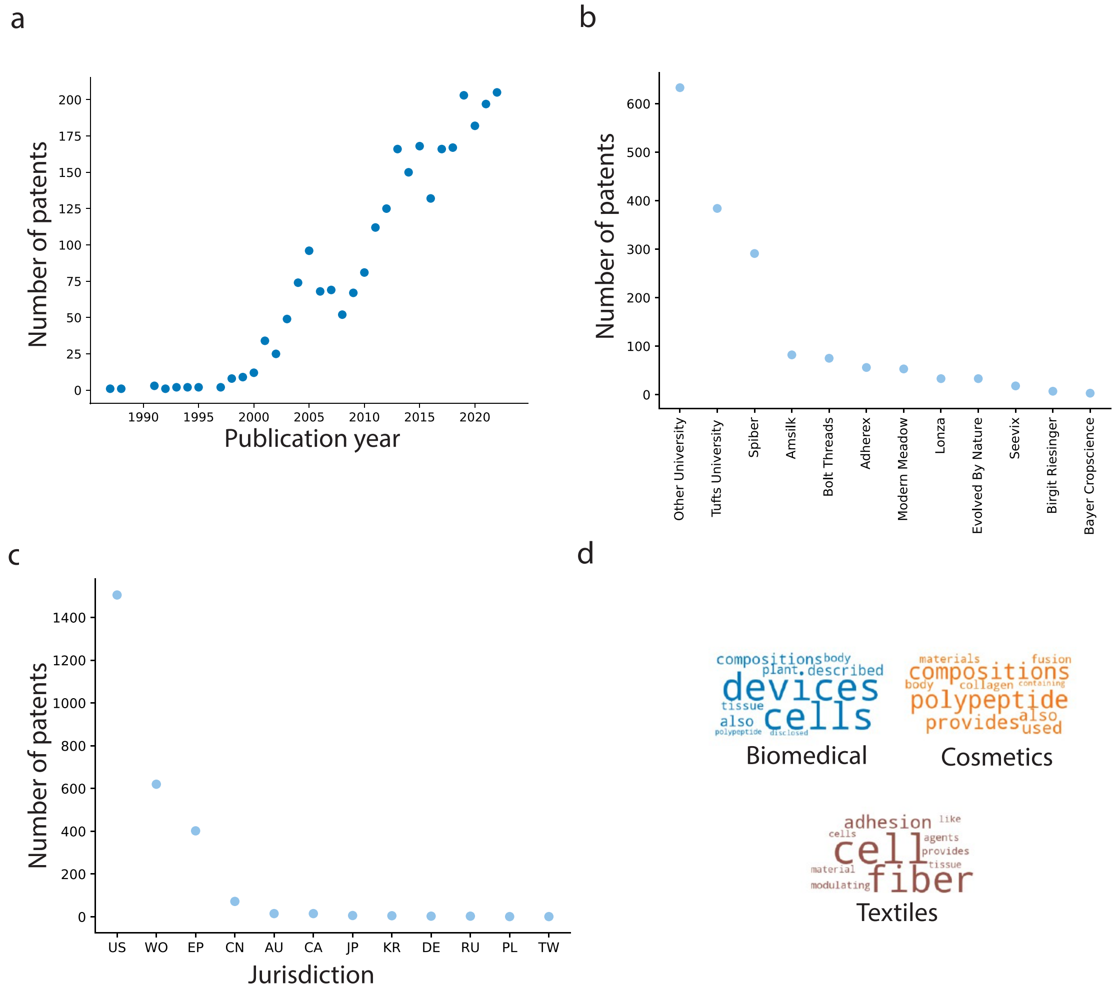

Figure 7. Analysis of spider silk patents. Database of spider silk patents was assembled using search results for "recombinant spider silk" in TheLens.org[242] on 03/12/2023. The database consisted of 2467 patents. (a) Number of published patents as a function of time. Interest in spider silk has been steadily increasing since the early 2000s, with about 200 patents published every year. (b) Main patent authors. Tufts university and Spiber are the main patent authors. "Other University" comprises various universities mostly based in the US and China. (c) Patent number for multiple jurisdictions. The US dominates the jurisdictions in which patents related to spider silk research are filed and published. US: United States, WO: World, EP: Europe, CN: China, AU: Australia, CA: Canada, JP: Japan, KR: Korea, DE: Germany, RU: Russia, PL: Poland, and TW: Taiwan. (d) Semantic analysis of topics present in the abstracts of our patent database. Topics were identified by performing LDA analysis on a corpus of texts composed of all patent abstracts. Word clouds of these topics were then obtained and interpreted as belonging to three major sectors for spider silk research: biomedical, fashion, and cosmetics. We note that sectors such as defense or aerospace materials were lacking from our analysis.

rights and the environment".[232] In the US, a succession of governmental initiatives and executive orders targeted at developing biomanufacturing have emphasized the importance of the biomanufacturing industry to the US economy and national security. A recent Executive Order by President Biden[233] (EO #14801) promoting government investment and cooperation in the bioeconomy and biomanufacturing space is one prime example and hopefully signals greater participation by government bodies to come.

Though protein-based textile fibers and other material applications may not be cost-competitive compared to dominant fibers currently, global policies and trends put added pressure on companies to switch to more sustainable raw materials.[234,235] Government policies that encourage adoption of durable, biodegradable, renewable, recycled, recyclable, or flame-resistant materials will inevitably promote the adoption of protein-based materials which meet these needs. As Environmental Social and Corporate Governance (ESG) reporting becomes more mainstream and expected of corporations, we can expect there will be a forced change toward more sustainable raw materials and manufacturing practices. ESG reporting is now mandatory for all corporations who wish to be listed on European exchanges, and similar expectations are predicted to occur in the U.S. with added pressure from environmental organizations.[236] In addition, many companies with ESG mandates have the prerogative to avoid animal-derived products. Biomanufactured spider silk either through microbial or plant-based processes then becomes an ideal match, providing a competitive advantage to companies using such hosts.

Given the early-stage, risky nature of spider silk production , spider silk companies have mostly been funded by private venture capital. Ethan Mirsky, Ph.D., cofounder of Bolt Threads concisely summarizes the challenge: "**The production of recombinant spider silk fiber is a very hard problem. I don't know if there has been any magic solution yet! The question to answer is: can you make enough volume with a low enough price with good enough properties to warrant the**

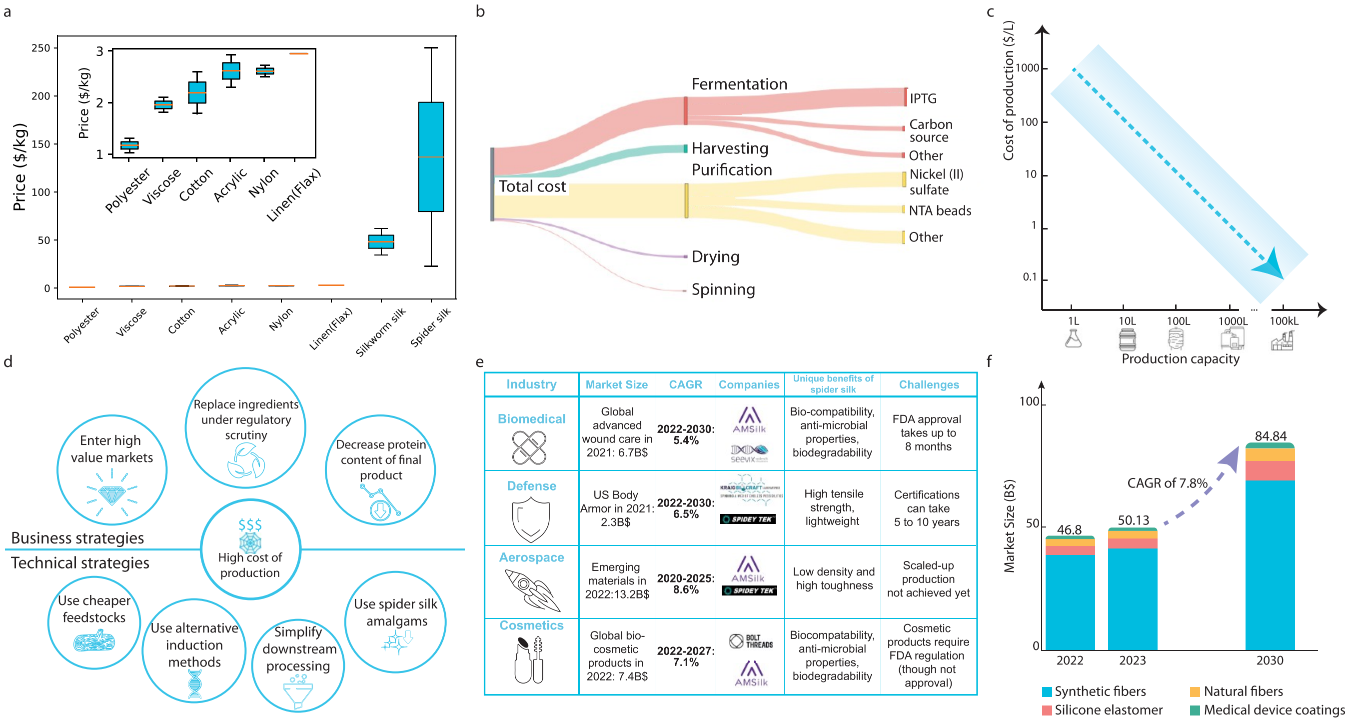

Figure 8. Market outlook of spider silk products. (a) Price range of various fibers used for textile applications compared to silk. It is clear that silk (from silkworm and spider origins) is more expensive than other synthetic and natural fibers. The price range of spider silk was estimated from private correspondence with various companies and scientists. The lower range of \$5/kg corresponds to what protein bioproduction experts view as the theoretical limit for bioprocess optimization (personal experience of KOL interviewees). While the current price of spider silk is prohibitive for textile applications, we note that its bioproduction is still being optimized. (b) Sankey diagram representing main cost contributors to spider silk bioproduction through a fermentation process. Information adapted from Edlund et al. (2018).[171] Main contributors to cost and research avenues for their optimization are discussed in detail in the main text. (c) Schematic illustration of how the cost of protein bioproduction logarithmically decreases as the production capacity increases. The blue band represents general ranges in the cost of production. Estimated price ranges for each fermentation capacity were obtained from private correspondence with executives in the industry. (d) Various market entry strategies to mitigate the current high cost of production of spider silk. Both business and technical strategies are presented. (e) Table depicting alternative high-value markets for recombinant spider silk production. Included in the table is market sizes determined from publicly available market research. The unique benefits of spider silk to each industry are also shared, along with the unique barriers to entry for each market. AMSilk logo reproduced with permission from AMSilk. Copyright: AMSilk. Seevix logo reproduced with permission from Seevix. Copyright: Seevix. Kraig Biocraft Laboratories logo reproduced with permission from Kraig Biocraft Laboratories. Copyright: Kraig Biocraft Laboratories. Spidey Tek logo reproduced with permission from Spidey Tek. Copyright: Spidey Tek. Bolt Threads logo reproduced with permission from Bolt Threads. Copyright: Bolt Threads. (f) Market potential of biomanufactured spider silk. We picked a few segments where bioproduced spider silk can have direct applications. The CAGR was computed as the weighted average of individual sector CAGRs, as determined from market research (sources available in raw data file).

production of your material? It's all about effectively and economically turning your technology into an economically viable product." Significant private investment since the mid-2000s has funded most of the spider silk ventures to date, with a majority of companies currently in the Series C or D, pre-IPO, phase of their fundraising (Figure 6c). A few companies have been able to become publicly traded. Kraig Biocraft IPO'd in 2008, two years after its inception, with stock prices staying relatively flat since, besides a notable spike in 2019 likely due to the company's Vietnamese subsidiary, Prodigy Textiles, raising its first batch of production silkworms, a significant technical milestone.[228] More recently, in 2023, Bolt Threads announced plans to go public via a SPAC (Special Purpose Acquisition Companies) deal with Golden Arrow.[237] However, despite these few developments most spider silk companies remain tied to private investment interest, often through collaborations with VCs.

A close analysis of private investment trends in the field shows a steady-increase in funding with a peak in 2021 which corresponds to the global biotech spike (Figure 6d). The sharp decrease in funding in 2022 is correlated to a general market downturn during that year and lower VC investment.[238] Sydney Gladman, Ph.D. CSO of MII, a veteran of the biomaterials space, says: "**Trends are the way the fashion industry works. It is what people and brands care about. This is why interests for sustainability or next generation products such as spider silk may come in cycles, with punctual drops of interest from the industry. However, the fundamentals and properties of these materials help garner renewed interest and excitement. Regardless of the economy, brands are still clamoring for next-gen materials. There might be drops in funding, but there is no decrease in demand.**"

Due to this currently unfavorable market landscape, many companies have had to either scale back or pivot away from spider silk research and production. For example, despite successful pilot projects in the textile[227] and cosmetics spaces,[239] as well as the ability to garner investor interest, Bolt Threads has pivoted away from spider silk research and production owing to difficulties in fundraising and scale-up operations. Generally, due to the difficulties inherent in producing large, native-like spidroins and spinning them as robust fibers, many companies such as Seevix have geared their efforts toward producing shorter

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spidroins that can be used as additives or merged with other materials as composites or chimeras.[240] Importantly, there are other applications where the spidroin need not require long lengths. This has been demonstrated in academic contexts as well, as Venkatsen and colleagues utilized a 32 kDa spidroin for use in water collection.[241] Other companies such as 3DBiofibr have had to pivot away from spider silk fibers due to high costs of production that are not balanced with market demand and leveraged their experience in fibrous materials to pivot toward new markets such as collagen fibers for tissue engineering (private correspondence). It must also be noted that companies still pursuing the textiles market such as Spiber have had to heavily engineer the native sequences to achieve their desired material performance metrics[221] to the point where they no longer refer to their proteins as "spider silk".

PATENTS REVEAL A DYNAMIC FIELD DOMINATED BY A FEW PLAYERS AND GEOGRAPHIES

While reviewing scientific articles reveals academic interest and discovery leaps in the heterologous production of spider silk, patents are an important indicator of industrial Research and Development (R&D) and have the potential to reveal future products and developments in the field. To understand the main trends in scientific advances in industry related to heterologous spider silk production, we conducted a comprehensive analysis of all patents published in the field starting from 1987. Using TheLens.Org[242] as our search engine, we compiled a database of about 2500 unique patents.

Interest in spider silk production has been steadily rising over the past few decades, with currently about 200 patents published every year (Figure 7a). Universities across the world (mostly in the US, Japan, and China) regularly patent new findings related to the heterologous production of spider silk. Tufts University, in particular, has been a pioneer of spider silk research. Universities are closely followed by the major companies we identified above in their interest in spider silk production (Figure 7b). In particular, Spiber, AMSilk, and Bolt Threads continually publish patents to maintain the intellectual property accumulated through their R&D research. These patents comprise a variety of findings: novel sequences identified, optimized downstream processing methods, new product applications, etc.

Analyzing the jurisdictions in which patents for spider silk are filed reveals a global landscape consistent with our innovation mapping analysis in Figure 6a. The US dominates the jurisdictions in which patents for spider silk are filed, with the majority of patents filed by universities (Figure 7c). World and European patents closely follow. Surprisingly, jurisdictions such as Japan or China lag behind, indicating that patent filers in these areas prefer to patent their findings within broader jurisdictions.

To identify the main topics of these patents, we performed a semantics analysis of all abstracts in our data set. Using Large Language Models and semantic analysis on the contents of these abstracts reveals the themes that have dominated the technological development of heterologous spider silk production. By clustering patents by their common themes and producing word clouds, we are able to obtain a snapshot of the main topics within our data set. Figure 7d shows some of the themes that emerged from our analysis. We attribute each word cloud to a specific application of spider silk research: biomedical devices, textiles and fashion, and cosmetics and composites. These represent the major industries that dominate patents and, by extension, industry interest. Interestingly, fields such as defense and aerospace applications that are often referred to in conversations about spider silk are not represented in our corpus.

THE PRODUCTION OF SPIDER SILK COMES AT A HIGH PRICE POINT

Given the many similarities between silkworm silk and spider silk, one of the most obvious markets for spider silk applications is that of textiles.[208] This is because spider silk has the potential to replace water-intensive silkworm silk as well as other synthetic fibers and to be used in a variety of applications such as clothing, bedding, and furniture. Perhaps unsurprisingly, this is why many of the early spider silk companies have focused on textile applications for their products (Figure 6d).

Given the colossal size of the textile industry (estimated at about a trillion dollars[243]), this offers a potentially important opportunity for the adopters of spider silk for textiles. However, given the high price point of spider silk production compared to cheaper synthetic fibers such as polyester (Figure 8a), a more realistic market share that can be considered is within premium luxury goods.[244] For example, some have considered that spider silk could be competitive with silkworm silk[245] while others see it competing with cashmere wool.[246]

Even within these luxury segments in textiles, spider silk production will need streamlining if its production price is to decrease. While the average price of existing synthetic and natural fibers is below \$3.00 per kilogram, artificial spider silk can currently easily cost upward of \$761.00 per kilogram[171] (Figure 8a), with a current optimistic price range around ~\$500/kg, disclosed to us from private correspondences.

The main contributors to the high production cost of spider silk were identified through a techno-economic analysis[171] and are illustrated in Figure 8b. In that analysis of a typical liquid fermentation setup for spider silk production, the production process was segmented into five main steps: fermentation, harvesting, purification, drying, and spinning where the total production cost was on the order of hundreds of dollars per kilogram. Not unlike many other precision fermentation processes for the production of proteins, the major contributors to cost were the induction system for protein expression (IPTG), the carbon source, as well as downstream purification methods for protein recovery.

Each of these contributors to cost are the subject of extensive research and some of them have been remediated to some extent. For example, instead of the use of expensive synthetic molecules such as IPTG for induction, certain engineered systems have relied on heat induction[247] or quorum sensing.[248] Other systems utilize clever genetic constructs for the induction of expression systems as well as metabolic engineering for the optimization of yields within existing manufacturing processes.[249,250] To defray carbon source expenses, extensive research (including by the US Department of Energy) is underway to replace sugar-based feedstocks with lignocellulosic materials.[251] Some laboratories and companies (Figure 6c) have resorted to using autotrophic photosynthetic organisms only reliant on light and CO_2.[131] Other companies have transitioned from glucose to glycerol, which is cheaper and more readily available and has shown promise in increasing dry cell weight and improving yields.[252] The purification step is being made more accessible by exogenously tagging the proteins for easier recovery or bypassing this step altogether by implementing secretion systems for simpler harvesting that obviate the need for bacterial lysis and complicated purification strategies.[130]

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While the analysis in Figure 8b shows cost contributions that are solely related to the production and recovery of spider silk, it omits capital expenditures that are necessary for scaling-up the production process. Because large fermentation facilities require investments of the order of $300M, many companies initially elect to relegate their large-scale production to CMOs (Contract Manufacturing Organizations) and CDMOs (Contract Development & Manufacturing Organizations) that are already well-positioned with experience and infrastructure. Keiya Masuno, Executive Officer at Spiber Inc., discusses the advantage of their in-house fermentation facility, "**While many other companies have been successful at producing spider silk at the lab and pilot scale, our strength at Spiber has been our ability to scale up production to 500 tonnes of Brewed Protein annually in part thanks to our Thailand plant. This has been possible through the constant feedback between R&D efforts on the biotechnology side and the operational and engineering needs for such a large operation. Our strength has been to integrate talent across both technical developments and engineering and has allowed us to achieve the high productivity we have today in only a few years.**" Scaling up production plays a crucial role in improving unit economics and facilitating widespread adoption of fermentation products such as spider silk. Consequently, the primary long-term objective of any nascent company should be to increase bioprocess volumes to achieve further cost reductions (Figure 8c). In addition, highly trained labor with technical aptitude is crucial for the proper operation of fermentation facilities and optimization of the development of production processes.[253] Many companies are optimistic that with proper technology development and production scale-up as well as proper governmental initiatives and policies, the price point of spider silk will significantly decrease.

A database on microbial fermentation efforts worldwide, called Capacitor, indicates that more than half of the companies poised for scaling up require bioreactors with capacities exceeding 20,000 L, yet only a mere 23% of existing facilities can fulfill this requirement.[123] This significant mismatch between the supply and demand for large-scale bioreactor facilities is anticipated to keep production costs high until there is substantial investment in infrastructure. Efforts to increase the supply include building greenfield or brownfield fermentation infrastructure at new sites or retrofitting existing fermentation infrastructure such as breweries, wineries, or ethanol plants.[254] The cost of spider silk production is contingent upon advancement in technology, the upscaling of production, and the implementation of supportive government initiatives and policies. These developments may unfold over several years or possibly longer.

Despite the current high price point of production, spider silk can respond to consumer demand for more sustainable materials in their consumer goods. In particular, its short timescale for biodegradability compared to recalcitrant synthetic fibers such as polyester (Figure 3f) may constitute a desirable response to the textile waste problem.[255] However, the "green premium" alone may not allow spider silk to be competitive within this market since textile manufacturers and brands look for properties beyond "sustainability" in making their choices, such as safety, inflammability, supply consistency, dependable properties, durability, color retention, and size retention. Governmental initiatives and regulations for more sustainable biomaterials in textiles (Figure 6b) will also be key in enabling the development of spider silk as a sustainable textile alternative.

Even with years of optimization of recombinant protein production, it would be near impossible to break past the theoretical limit of highly optimized recombinant protein production, which many recognize to be around $5/kg (private correspondence with past executive at Bolt Threads which has been confirmed by many others in the field). This means that despite highly streamlined and scaled-up production processes, spider silk is unlikely to compete within the textile industry. This is why many companies have considered implementing their businesses within other markets, such as luxury or high-value goods or even transitioned to the exploration of other more readily scalable biomaterials such as mycelium.

HIGH-VALUE MARKETS CAN HELP OFFSET THE HIGH COST OF SPIDER SILK PRODUCTION

To mitigate the high production cost of spidroins, companies can resort to various strategies for entry into new markets (Figure 8d). In addition to implementing technical cost reduction measures, companies can also adjust their business goals. From a business perspective, they can choose to enter high value markets (such as those discussed below and in Figure 8e), identify and replace antiquated ingredients such as silicone elastomers which are being phased out by the EU starting in 2022,[256] or decrease the utilization rate of spidroins in the final products. This last strategy is particularly relevant for applications where spidroins can be used in composite materials, produced via co-purification with polysaccharides, other proteins, or lipids. Such composites which possess unique material properties are useful in a wide range of applications, from cosmetics[239] to car armatures.[222,257]

In particular, entry into high-value markets would allow a recombinant spider silk product to be sold at a price premium. Spider silk may be particularly well-suited to some of these markets which require high quality products with remarkable material properties (Figure 8d). For example, materials used in medical devices, which require higher standards for biocompatibility, antimicrobial properties, and biodegradability tend to have a higher price range than fibers used for textiles. From breast implants[258] to medical coating devices,[259] a few companies have attempted to enter the biomedical space (Figure 8d).

In addition, the cosmetics industry, which requires similar biocompatible properties in addition to consumer safety, has been another promising avenue. A few products[260] where spider silk serves as a drop-in replacement for silicone elastomers have already populated cosmetic store shelves. The advantage of the cosmetics space over biomedical applications resides in lower FDA approval standards with shorter timescales. In this vein, Lindsay Wray, Director of Beauty and Personal Care at Bolt Threads, provides the following advice: "**Look for chemistries that are undergoing 'regulatory scrutiny'. For example, in the beauty and personal care industry, the European Union is looking to put intense regulatory pressure on long chain silicones because they persist in the environment. Unlike silicones, silk is readily biodegradable and biobased**". Furthermore, cosmetics are an attractive product class because they only require small protein segments rather than fully folded proteins.

Because of the high toughness and low density of spider silk, many have advocated for its applications in defense, aerospace, and automotive-related products.[261] As a composite with other materials, spidroins can increase their toughness. For example, Kraig Biocraft, collaborating with the US Department of

Defense in 2018 was researching the production of bulletproof materials.[262] Spiber in collaboration with Toyota[222] as well as SpideyTek with Velozzi[257] are already promising a few pilot products within the next few years. In the words of Shinya Murata, Business Development executive at Spiber: "**The automotive industry will become a key business direction in Spiber's future. Contributions for spider silk include leather upholstery for seats, and composites for structural components of automobiles. We are currently researching how incorporating spidroins in composite materials can greatly improve their mechanical properties. Concept cars were exhibited at the Japan Mobility Show in collaboration with Toyota and Goldwin, and we expect our first products to be released shortly.**" Additional industries not included in Figure 8e are construction, infrastructure, performance/luxury textiles, and composites.

The commercialization of spider silk into economically viable products is still in its infancy, with a current potential market of $50 billion. We estimated that the size of the potential market can significantly grow with a CAGR (Compound Annual Growth Rate) of 7.8% (Figure 8e). In our analysis, we included a few promising market segments for spider silk: synthetic and natural fibers, silicone elastomers, and medical device coatings. Since these markets are far from exhaustive, the actual potential markets as well as the CAGR may be higher.

■ CONCLUSION

This review highlights the many remarkable aspects of the bioproduction of spider silk as well as the growing excitement and interests for its commercialization. Despite the recent commotion around spider silk, we also point out many outstanding research areas that, if adequately addressed and explored in the future, will help advance its research and production. As a self-assembled biopolymer with a highly ordered hierarchical structure, spider silk holds many favorable mechanical properties that are ultimately controlled by its genetic sequence. Advances in computational models of proteins will inevitably help explore the large space of sequences and potential chimeras that will optimize certain properties of the fibers. Linking macroscopic properties of fibers to the genetic sequences and identifying specific motifs remains a hot topic of research. Generally, most spidroin sequences have high toughness despite a low density, allowing for their use in a wide range of applications.

Advances in synthetic biology have made the heterologous, at-scale production of spider silk accessible in many host organisms. *E. coli* and *P. pastoris*, due to their many favorable qualities, have dominated production processes in many of the current startups. However, other startups are betting on the cost-effectiveness of alternative hosts, particularly plants. The choice of which host to use should be closely tied to the final application. Production systems that permit large amounts of protein product, such as bacteria and yeast, are most suited for bulk applications, such as textiles, whereas more complex organisms such as transgenic silkworms, plants, and goats may be better suited for specialized products, such as biomedical and cosmetics applications. Regardless of the host, downstream processing methods of the recovered proteins allow for the production of different kinds of materials, from fibers to sponges to gels. As with the host platform, the choice of such techniques should also be closely tied to the final application. In addition to the economic considerations of titers and yields, other aspects should be weighed when picking a host organism such as the energy demands of the host system, the timescale of production, the adaptability of the system to changes (e.g., new sequences), and the downstream processing techniques that the host will require.

Through an extensive survey of the spider silk industry landscape, we identified key global hubs leading spider silk research and innovation, as well as its commercialization. We have also identified a few key aspects of cross-institutional collaborations between industry, academia, and government and highlighted many examples where such collaborations were central to the success of both research and industry. By analyzing patents related to the heterologous production of spider silk, we uncovered the main jurisdictions leading in translational work, as well as the dominant themes and trends in innovations.

Given our analysis, it is clear that the market potential for spider silk is still vast and largely untapped. While early companies have positioned themselves as leaders in the space, initiating large-scale collaborations and spearheading research developments of spider silk, there are many unexplored opportunities both in research and business innovation. The breadth of potential applications, from textiles to biomaterials, highlights a market that is only beginning to realize the full scope of its possibilities. However, due to the monumental challenges inherent to successfully producing materially desirable silk at commercial scale, sustained funding and interest in the space is not indefinitely guaranteed. Recently, fluctuations in funding have underlined the precarious nature of spider silk's industrial future. At this critical moment in the development of the recombinant spider silk space, we highlight the urgent importance of synthesizing insights from both the academic and commercial sectors, which will be increasingly necessary for the success of the wider industry. The synthesis and bridging of insights, which we hope to catalyze with this review, will help address white spaces in the broader industry, such as sequence design exploration, host optimization, and material property enhancement. Ideally, progress in these areas will broaden the range of production applications and market capitalization strategies, which would solidify the adoption of recombinant spider silk and its future as an industry.

By integrating both technical considerations regulating the heterologous production of spider silk and business considerations such as the economic constraints of bioproduction, we present a comprehensive view of the challenges of scaling up spider silk production. We underscore some of the hurdles and white spaces remaining for scaling up production and targeting relevant spaces. Such integrative approaches are necessary for guiding future research, investment, and policy making to increase the capabilities of spider silk.

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Notes

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