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Feature Article

Polymeric materials based on silk proteins

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ABSTRACT

Silks are protein-based fibers made by arthropods for a variety of task-specific applications. In this article, we review the key features of silk proteins. This article initially focuses on the structure and function of silk proteins produced naturally by silkworms and spiders, followed by the biological and technical processing of silk proteins into a variety of morphologies (including capsules, fibers, films, foams, gels and spheres). Finally, we highlight the potential applications of silk-based materials.

1. Introduction – nature's biopolymers

Biopolymers such as polyamino acids, polynucleic acids, polyphenols and polysaccharides have evolved over billions of years to carry out a myriad of tasks such as catalysis, molecular recognition or the storage of energy or information. Biopolymers are synthesized from a very limited number of building blocks and it is their complex three-dimensional structures that are responsible for their highly specialized properties [1–3].

Polyamino acids and polynucleic acids are synthesized in template-directed catalyzed reactions yielding monodisperse linear polymers composed of a specific sequence of monomers; whereas polyphenols and polysaccharides are prepared via untemplated catalyzed reactions yielding polydisperse polymers with a wide variety of potential structures (including linear and branched) depending upon the monomers involved [4–9].

Biopolymers are commonly occurring structural elements of biological systems. Polysaccharides are the most abundant biopolymers on earth, cellulose and chitin serve as structural elements in plant cell walls and animal exoskeletons; polyphenols such as lignins are important structural elements in wood and other plants; and polyamino acids such as collagen and elastin are the main components of blood vessels, connective tissues and skins in animals and humans [4,5,9–20].

Silkworms produce polyamino acid-based (silk protein) cocoons to protect themselves during their metamorphosis into moths, and humans have harvested silk fibers from these cocoons for centuries to produce textiles due to their characteristic luster, moisture absorbance and strength [21,22]. Web-weaving spiders produce silk-based webs (from a variety of different silk protein-based fibers) to capture prey; certain of these silk fibers have mechanical properties surpassing Nylon, Kevlar, silkworm silk, and high-tensile steel [23–26], as summarized in Table 1 [27]. In this article, we will focus on materials derived from silk proteins produced by silkworms and spiders.

1.1. Protein characteristics and structure

Polyamino acids (known as polypeptides or proteins) in higher organisms are synthesized from combinations of up to 19 amino acid monomers (–NH–CHR^1–CO–) and 1 imino acid monomer (–NR^1–CHR^2–CO–), linked via amide bonds (also known as peptide bonds) between the monomers (which are more commonly referred to as residues). In higher organisms, only the L-amino acids are used as monomers, whereas in lower life forms (such as bacteria or lower plants) D-amino acid monomers can also be incorporated [8,28,29].

In vivo, polyamino acids are synthesized in a template-directed fashion: first, DNA is used as a template in the synthesis of messenger RNA (mRNA) via a process known as transcription; mRNA can subsequently be used as a template by ribosomes in the synthesis of a sequence-specific polypeptide, this process is known as translation, because the information stored in a polynucleic acid (genetic code) is translated into information in a polyamino acid (functional code) [30,31].

The sequence of residues in the polypeptide is known as the primary structure. The amino acid residues display different functional groups on the polyamide backbone of the polymer; these functional groups can be categorized as polar, non-polar, aromatic,

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Table 1
Mechanical properties of silk and man-made fibers

Material	Strength, σ_{max}/GPa	Extensibility, ε_{max}	Toughness/MJ m^{-3}
B. mori silk	0.6	0.18	70
A. diadematus MA silk	1.1	0.27	160
A. diadematus Flag silk	0.5	2.70	150
Nylon	0.95	0.18	80
Kevlar 49™	3.6	0.027	50
High-tensile steel	1.5	0.008	6

anionic or cationic. After polymer synthesis, supramolecular interactions (such as hydrogen bonding between the amide bonds in the backbone of the polymer, or π interactions between aromatic groups) determine the local conformation of the polypeptide which is known as the secondary structure – prominent examples of common secondary structures are: α-helices, β-sheets and β-turns (see Fig. 1) [2,32].

Hydrogen bonds between the hydrogen atom attached to the nitrogen atom of an amide and the carbonyl oxygen atom of the fourth amino acid on the amino-terminal side of the peptide bond encourage the polymer to coil around an axis into an α-helix; each helical twist contains on average 3.6 amino acids and is 5.4 Å in length. α-Helix formation is encouraged by ion pair formation between oppositely charged residues 3 or 4 amino acids apart, and π-interactions between similarly spaced aromatic amino acids [2,32]. Less common are helical twists containing 3 amino acids (known as 3_1- or 3_{10}-helice [33,34]) which are important in some silk proteins [35–37].

In certain cases, hydrogen bonding between chains (intrachain or interchain) that are side by side causes the polypeptide chain to adopt a zigzag conformation, known as a β-sheet. Amino acids with small side chains such as glycine and alanine allow stacking of β-sheets, whereas bulkier amino acids discourage this sort of assembly process [2].

Turns and loops are also frequently occurring secondary structures in polypeptides. Particularly common are 180° loops, known as β-turns, which consist of 4 amino acids where the carbonyl oxygen of the first amino acid is hydrogen bonded to the hydrogen on the amine of the fourth amino acid. Importantly, the second and third amino acids do not participate in hydrogen bonding [32].

Polypeptides therefore contain regions that are either locally disorganized or locally organized dependent upon their primary structure, and covalent or non-covalent cross-links between different regions within a polypeptide chain determine the overall three-dimensional arrangement of the polypeptide chain, which is known as the tertiary structure. Further interactions (covalent or non-covalent) between individual polypeptide chains (identical or different) determine a protein's quaternary structure [3,38].

The process by which polypeptides assume their secondary, tertiary and quaternary structures after polymerization is known as 'folding' [38,39] and is in some cases aided by accessory proteins. Once the process of folding is complete and the polypeptides are fully assembled into their biologically active conformations, the polypeptides are said to be in their 'native' state [39].

1.2. Silk proteins – structure and hierarchical assembly

Silks are protein-based fibers made by arthropods for a variety of task-specific applications. Silk fibers are typically composite materials formed of silk protein and other associated molecules such as glycoproteins and lipids [40–44].

1.2.1. Silkworm silk

Of all natural silk-producing animals, mulberry silkworms (*Bombyx mori*) are of the most economic importance, because it is possible to rear them in captivity. After the eggs laid by adult moths hatch, the caterpillars are fed fresh mulberry leaves for a month until they are mature enough for metamorphosis into a moth, which requires the construction of a protective cocoon of silk [45–48]. Once the cocoon is complete, silk farmers kill the caterpillar via exposure to hot water/steam, and the silk can be harvested. The crude silk needs both degumming [49–53] (from the glue-like sericin) and processing before it can be dyed and sold.

The fact that there is a readily available source of silkworm silk has facilitated an understanding of its structure and function [22,44,54,55]. *B. mori* silk has a core–shell type structure, with its core composed of a complex of 3 proteinaceous components: a large protein, known as heavy chain fibroin (*H-chain*, ca. 350 kDa) that is linked to a second small protein, known as light chain fibroin (*L-chain*, ca. 25 kDa) via disulfide bonds; and a third small glycoprotein, known as the P25 protein (*P25*, ca. 30 kDa) is associated via non-covalent hydrophobic interactions [56–58]. The molar ratios of H-chain:L-chain:P25 are 6:6:1; the H-chain is hydrophobic and provides crystalline like features to the silk thread, whereas the L-chain is more hydrophilic and relatively elastic, and the P25 protein is believed to play a role in maintaining the integrity of the complex [56–59]. Before fiber formation, a solution of the three proteins is secreted from two glands within the silkworm, assembling into twin filaments that emerge from an exit tube in its head (known as the spinneret) and dry upon exposure to air. The resulting core contains anisotropic β-sheet-rich nanocrystals that are loosely aligned with the fiber axis and dispersed in an unstructured matrix [21,54,55,59,60]. Another pair of glands secretes glue-like **sericins** [61–64] (a set of serine-rich glycoproteins) that coat the fibroin filaments and ensures the cohesion of the cocoon by sticking the twin filaments together. Finally the fiber is coated with a variety of other proteins postulated to protect the cocoon against microbes and predators (see Fig. 2) [65–73].

1.2.2. Spider silks

Spiders have evolved to be able to produce a variety of task-specific silks for catching prey (via trapdoors and webs), protection and preservation of their offspring and prey (in cocoon-like structures), as a lifeline to escape from predators and even as a means of transport (parachute-like structures) [23,74–81].

More than 34,000 different species of spiders have been identified, of which approximately half catch their prey in webs (of which there are over 130 catalogued designs). Orb webs are a particularly interesting example of web design and they are constructed from four different types of task-specific silks (see below) [23,24,76,82,83].

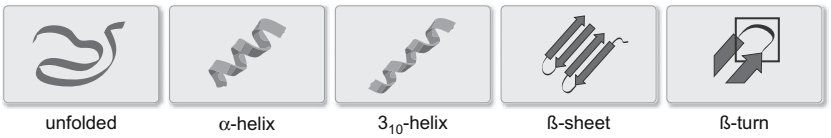

Fig. 1. Common secondary structural motifs in proteins.

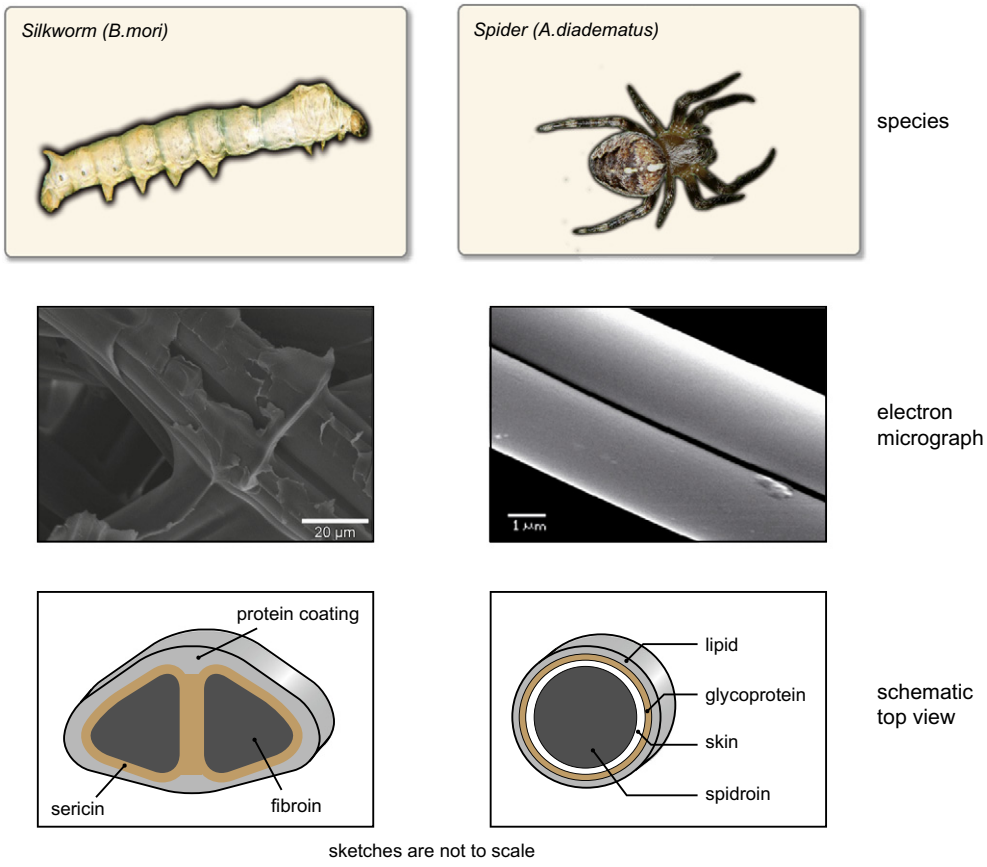

Fig. 2. Examples of silk fibers produced by silkworms and spiders and a schematic illustration.

In contrast to silkworms, it is impossible to farm most spiders in large scale due to their cannibalistic nature, therefore spider silks are typically obtained via harvesting of the silk at its point of application (in the case of spider webs great attention to detail is required to obtain uncontaminated samples); anaesthetization of single spiders followed by forcible silking of the spidroin from its source gland (in the case of major and minor ampullate and cylindriform silks [84]), or extraction of the spidroin directly from the gland in which it is produced (after killing the spider) [85].

In order to circumvent such complicated procedures, identification of the protein sequences has allowed the recombinant production of genetically engineered analogues in sufficient yield and purity for application as high performance biomaterials. A comprehensive review of these biotechnological developments was recently published by Vendrely and Scheibel [86].

1.2.2.1. Major ampullate silk. Silk fibers made of proteins produced in the **major ampullate** silk gland (MA silk, >300 kDa [87–91]) have a very high-tensile strength (comparable to Kevlar) and moderate elasticity [27,92–99]. MA silks are used as a scaffold upon which to attach other silks during the construction of a web and as a lifeline when it is necessary to escape from a predator [23]. MA silks have diameters between 1 and 20 μm (depending upon spider species) and have a core–shell type structure. The core contains two major proteins (Major Ampullate Spidroins 1 and 2) that are composed predominantly of glycine, alanine and proline (although the quantity of the latter varies significantly between species) [85,91,100]. Major ampullate spidroins of *Araneus diadematus* and *Nephila clavipes* spiders are reminiscent of block copolymers containing blocks of polyalanine and either $(GGX)_n$ (where X is typically tyrosine, leucine or glutamine) or GPGXX [101]. The alanine-rich blocks are known to form β-sheet stacks [93] that are responsible for the high-tensile strength of MA silks; whereas the blocks of $(GGX)_n$ presumably form 3_{10}-helices [35], and the blocks of GPGXX form β-turn spirals imparting elasticity/flexibility to the proteins [102–104]. MA proteins assemble in the spinning duct are secreted as a solution from a valve located at the end of the spinning duct, and dry upon exposure to air, resulting in a core spidroin filament containing isotropic β-sheet-rich nanocrystals dispersed in a matrix [42–44]. This core filament is coated in another layer of MA spidroin-like protein, a glycoprotein coat, and finally a lipid coat (see Fig. 2) [40,42–44,105].

1.2.2.2. Minor ampullate silk. Silk fibers made of proteins produced in the **minor ampullate** silk gland (MI silk, >250 kDa [89]) have similar mechanical properties to MA silk, and are used to build a spiral that temporarily stabilizes the scaffold of the web during its construction [106–108]. MI silks are also mainly composed of two major proteins (Minor ampullate spidroins 1 and 2) that are similar in composition to MA silk, but with two significant differences: first there is virtually no proline present in MI silk; and second the glutamate content is markedly reduced [85]. Minor ampullate spidroins from *A. diadematus* and *N. clavipes* spiders comprise blocks of polyalanine, $(GA)_n$, $(GGX)_n$, and multiple copies of a highly conserved serine-rich 130 amino acid non-repetitive sequence that acts as a spacer, breaking up the repetitive regions (see Fig. 3) [37,101,108,109].

1.2.2.3. Flagelliform silk. Silk fibes made of proteins produced in the **flagelliform** silk gland (Flag silk, ca. 500 kDa) are highly elastic and are used to produce the capture spiral [80,101,104,108,110,111]. In comparison to MA silk, Flag silk is composed of only one major

protein that contains greater amounts of proline and valine, and reduced amounts of alanine [85]. Flag proteins from *N. clavipes* spiders comprising blocks of $(GGX)_n$ assumed to form 3_{10}-helices; blocks of GPGXX leading to β-turn spirals imparting elasticity/flexibility to the proteins [102–104]; and a highly conserved non-silk-like spacer sequence (such as TIIEDLDITIDGADGPITISEELTI) the function of which is uncertain, however, its polar hydrophilic nature may be important for both cross-linking and hydration of the fiber [112].

1.2.2.4. Pyriform silk. The **pyriform** silk gland produces a sophisticated protein glue (Pyriform silk) that is used to securely attach the MA scaffold to a substrate (such as trees, walls, etc.), and subsequently Flag silk to the MA scaffold [113–115]. Pyriform silk proteins from *A. diadematus* spiders contain relatively low amounts of small non-polar amino acids, significant quantities of polar and charged amino acids (important for cross-linking), and are assumed to be randomly coiled before and after secretion facilitating the maximum number of cross-links [85].

1.2.2.5. Aggregate silk. The **aggregate** silk gland produces a mixture of sticky glycoproteins and small highly hygroscopic peptides that are used to coat the capture threads of orb webs [105,116,117]. Aggregate silk proteins from *Latrodectus hesperus* spiders [117] contain relatively low amounts of small non-polar amino acids, significant quantities of proline, and both polar and charged amino acids that are important for cross-linking and hydration of the fiber [85].

1.2.2.6. Cylindriform/tubuliform silk. Silk fibers made of proteins produced in the **cylindriform/tubuliform** silk glands (cylindriform/tubuliform silk) are used to produce tough egg case silk [118–126]. Cylindriform proteins of *A. diadematus* spiders are composed of blocks of A_n and $(GGX)_n$ (X = A, L, Q, or Y) yielding protein structures rich in β-sheet stacks (similar to major ampullate silk proteins produced by *N. clavipes* spiders) [127].

1.2.2.7. Aciniform silk. Silk fibers made of proteins produced in the **aciniform** silk gland (aciniform silk) serve numerous purposes: one type is used as a soft lining inside the egg case used for a spider's offspring, whereas another type is used to reinforce the pyriform silk cement matrix [128–131]. Studies of the amino acid contents of aciniform silk from *Argiope trifasciata* [128] and *L. hesperus* [130] spiders indicate the presence of GGX domains in the proteins, but that they are both notably different from other types of silk proteins [85].

2. Silk protein processing

2.1. Protein solubility

Proteins in aqueous solution tend to be covered by a highly ordered layer of water (known as the hydration layer), with 10% greater density and 15% greater heat capacity than that of bulk water [132–136]. The solubility of a protein in water is affected by the presence of other solutes such as ions, organic solvents, polymers or surfactants. Low concentrations of salt tend to improve the solubility of proteins (known as 'salting in'), due to the formation of ion-rich hydration layers in the vicinity of charged and polar amino acid residues (as described by the Debye–Hückel theory). High concentrations of salt tend to have the opposite effect, causing the protein to precipitate (known as 'salting out'). The magnitude of this effect is dependent upon the particular ions, and usually follows the Hofmeister series (see Fig. 4) [132,137–150].

Originally it was thought that an ion's influence on the properties of macromolecules was primarily due to its effects upon bulk water structure; kosmotropic ions are highly hydrated and were thought to be 'water structure makers' that stabilized protein structure and salted-out proteins, whereas chaotropic ions were thought to be 'water structure breakers' destabilizing folded proteins and giving rise to salting-in behavior. More recent studies demonstrate that although ions interact strongly with water, this is

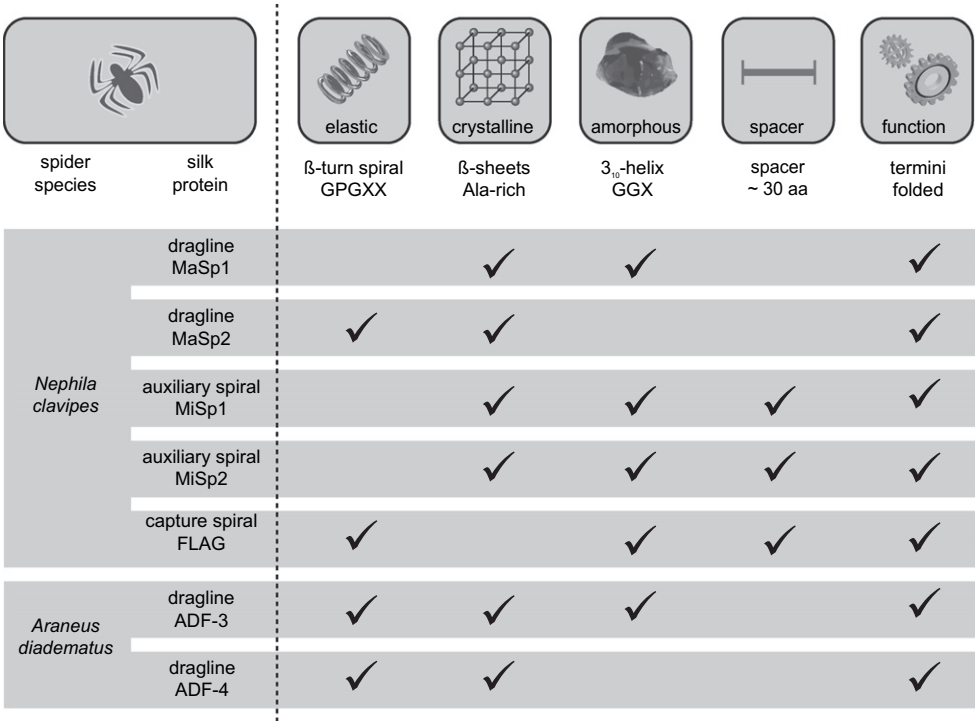

Fig. 3. Structural motifs occurring within the primary structure of spider silk proteins determine their mechanical properties. X indicates a residue that may vary within or between proteins. The spacer represents non-repetitive but conserved regions that disrupt the glycine-rich repeats. More details on the motifs can be found in the text.

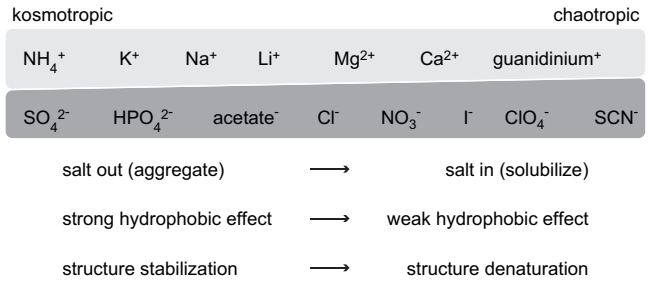

Fig. 4. The Hofmeister series.

a local effect, and hence does not affect the bulk structure of water [134,135,140,141,144,145,147,149–154].

As noted above, the presence of both kosmotropic and chaotropic ions reduces the hydration of hydrophobic residues causing the protein to precipitate or 'salt out'. Furthermore, kosmotropic anions polarize water molecules that are hydrogen bonded to amide moieties, thereby reducing hydration of the amide (favoring 'salting out'), and finally, chaotropic anions interact directly with amides (favoring 'salting in'). This allows the explanation of the Hofmeister effect by direct ion–macromolecule interactions and interactions of the ions with water molecules in the first hydration shell of the macromolecule [132,140,141,145,146,149,150].

The pH of the solution also plays a critical role in protein solubility. The isoelectric point (p*I*) of a protein is the pH at which the number of positively charged residues is equal to the number of negatively charged residues. Therefore, if the pH is below the p*I* the protein has a net positive charge, if the pH is equal to the p*I* it has no net charge, and if the pH is above the p*I* it has a net negative charge. The greater the net charge a protein carries, the greater the charge repulsion between its similarly charged residues and the less attracted it is to other similarly charged proteins. In cases where the protein is uncharged it is more prone to aggregation. Also noteworthy is that most proteins unfold at a certain pH value, which encourages aggregation due to exposure of hydrophobic residues [138,148,155–157].

Uncharged solutes also interact with proteins, affecting both their folding and solubility. Urea is a well-known chaotropic agent, which at molar concentration interferes with the hydrogen bonding motifs that stabilize the secondary structure of proteins, and therefore causes the unfolding of proteins into a random coil (with protein-dependent effects on solubility) [158,159]. Polymers such as poly(ethylene glycol) may aid protein crystallization due to the fact that they restrict the motion of the protein due to volume exclusion, and that they reduce the hydration of hydrophobic residues favoring 'salting out' [160,161].

Organic solvents also affect the solubility of proteins in water [162], primarily because charged and ionic residues tend to be less well solvated by solvents with lower dielectric constants, and also due to the fact that they interfere with the non-covalent interactions that determine the protein's secondary structure. Alcohols are known to both promote and disrupt secondary structure elements of proteins, but this effect is heavily dependent upon the protein under investigation: for example trifluoroethanol generally stabilizes α-helices at the expense of β-sheets due to its interaction with carbonyl oxygen atoms and hydrophobic residues (although certain investigations with model peptides indicate weak interactions with hydrophobic residues) [163–166]. In contrast, ethanol and methanol promote β-sheet formation at the expense of α-helices due to dehydration of the α-helices [162]. In certain cases it is possible to dissolve proteins in non-aqueous solvents, although the fact that the physical properties (dielectric constant, dipole moment, hydrophobicity, etc.) of such solvents are different from water may significantly affect the folding of the protein due to poor solvation of the charged and polar regions, favorable solvation of the hydrophobic regions of the protein and disruption of the hydrogen bonding patterns [162].

Temperature is another important factor governing the solubility of proteins. Increasing the temperature has two important effects: first, decreasing the order in the structure of water, thereby decreasing the solvation of the hydrophobic regions of proteins, and encouraging aggregation through hydrophobic interactions; and second, unfolding the protein, thus exposing more hydrophobic regions to water, which also encourages aggregation through hydrophobic interactions [140,141,145,146,149].

2.2. Biological processing of silk proteins – fiber spinning

The previous section highlighted the environmental conditions that are important for protein solubility and folding. In this section, we shall briefly review the folding and fiber assembly processes of major ampullate silks produced from the spidroins of *N. clavipes* (MA spidroins 1 and 2) and *A. diadematus* (ADF-3 and ADF-4) spiders; which is assumed to be similar to the spinning process for silkworms (see Fig. 5) [167].

The requisite proteins of individual spider silks are synthesized in specific cells and excreted into the lumen of specific glands, as described above, for storage (i.e. MA spidroins 1 and 2 are synthesized in different cells and subsequently excreted into the lumen of the MA silk gland). MA spidroins are amphiphilic proteins, composed of repetitive hydrophilic and hydrophobic blocks with highly conserved non-repetitive polypeptide sequences at the N- and C-termini. These proteins are stored at remarkably high concentrations (up to 50% w/v) without the occurrence of undesirable aggregation [35].

The flow of the proteins through a narrow duct to the spinning warts is controlled by a valve located at the exit of the duct. As the protein solution passes through the duct, the proteins are salted-out and assemble, finally yielding silk fibers with remarkable mechanical properties.

The origin of the proteins' solubility and anti-aggregation behavior in the lumen of the glands has been the subject of some debate; storage of the proteins in a liquid crystalline state was a particularly attractive hypothesis that would explain the low propensity to aggregation, the low viscosity of the solution, a low critical shear rate for inducing crystallization, and the low draw ratio necessary for the production of uniaxially aligned fibrous structures [168]. However, NMR studies carried out by Jelinski and co-workers indicate that the proteins are stored in the lumen as 'molten globules'. In solution, ^{13}C chemical shifts of the alanines indicate the presence of helices and random coils, and solid state studies of the lyophilized protein show evidence of both 3_{10}-helices and α-helices, suggesting that folding into the β-sheet conformation occurs during the spinning process [35].

In 2003, the Kaplan Group presented an alternative hypothesis suggesting that the stability of *B. mori* silk proteins in the lumen of the gland is due to the formation of micellar structures by the amphiphilic proteins [169]. It was also suggested that the stability of silk proteins in solution is due to the periodic hydrophobicity inherent in their primary structure [170], as demonstrated in theoretical models [171]. Our investigations using microfluidic devices also support the latter model [172].

As the protein solution passes along the duct, a variety of chemical and physical stimuli drive the assembly of the silk proteins into fibers [173]. For example, the protein solution is stored in the lumen at pH 7.2, and as the protein solution passes along the duct the pH drops to 6.3 encouraging aggregation of the proteins (silk proteins tend to contain more acidic than basic residues, hence their p*I* is typically in the region of 4–7) [174–176]. Ion exchange

also occurs in the duct, and the more chaotropic sodium and chloride ions present in the lumen are exchanged for the more kosmotropic potassium and phosphate during passage along the duct, encouraging protein self-assembly [170,177].

In addition to a decrease in pH and ion exchange, water is extracted during flow along the duct. Water extraction occurs via a cuticle with the structure of a hollow fiber dialysis membrane that lines the duct, concentrating the proteins inside the spinning dope, and promoting aggregation [178,179].

Additionally, as the proteins flow from the lumen along the duct, they undergo shear-induced elongation and alignment, encouraging hydrophobic and hydrogen bonding interactions. As the flow rate increases, so does the alignment of the proteins in solution, resulting in more regular stacking of β-sheet-rich regions, and therefore stiffer, stronger, but less extensible fibers [172,180–182].

Temperature has also been observed to affect the fiber formation process; experiments carried out by forcibly silking immobilized live *Nephila edulis* spiders (non-anaesthetized) at a variety of temperatures between 5 and 40 °C show that as the temperature increases, so does the breaking strain, and it was postulated that the temperature dependence of the viscosity of the solution during spinning was the cause of this effect [168,183].

We have used microfluidic devices in order to study the assembly process of engineered spidroins (*e*ADF-3 and *e*ADF-4) that are based upon the consensus sequences of one of the major ampullate silks (ADF-3 and ADF-4) of *A. diadematus* spiders. We demonstrated that fiber formation for *e*ADF-3 requires a combination of salting out, pH drop and elongational flow. Increasing the phosphate concentration to 500 mM (salting out) induces the assembly of *e*ADF-3 into spherical aggregates. The pH drop (to pH 6) must be accompanied by elongational flow to induce the aggregation of the *e*ADF-3 spheres into β-sheet-rich fibers, i.e. fiber formation does not occur in the absence of either a pH drop or elongational flow. In contrast, *e*ADF-4 assembles into spheres under all conditions tested, and only formed fibers in the presence of *e*ADF-3 [172].

2.3. Technical processing of silk proteins – fibers

2.3.1. Non-naturally spun fibers (μm scale diameters)

As described above, silkworms and spiders have both evolved highly efficient methods of spinning high performance silk fibers with μm scale diameters, and there have been a number of attempts to mimic their processing techniques to enable the production of fibers from both natural and recombinant protein sources. Fibers with μm scale diameters can be prepared by wet spinning (extrusion of a solution of protein into a non-solvent/coagulant), hand-drawing (literally drawing a fiber from solution by hand) and microfluidic devices; some examples of the different techniques and solvents used are highlighted below.

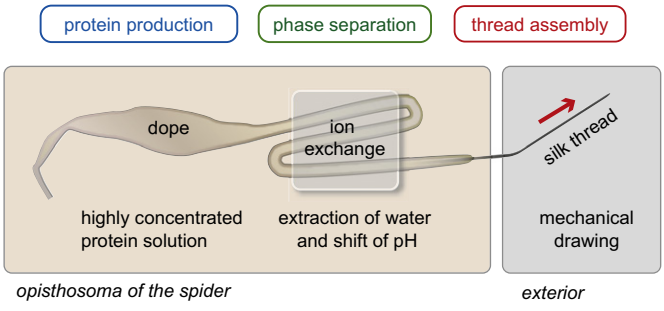

Fig. 5. Biological processing of spider silk proteins – fiber spinning.

2.3.1.1. Aqueous solvents. Fibers have been prepared via hand-drawing from a 40 wt% solution of a 76 kDa engineered protein (containing extensive repetitions of the hexapeptide GAGAGS derived from the crystalline region of *B. mori* fibroin) in 9 M LiBr into an acetic acid–acetone coagulation bath. The fibers were cured in the coagulation bath under slight tension for 30 min, and subsequently washed with water and air dried at 80 °C. The resulting fibers had non-uniform diameter (10–20 μm) and poor mechanical properties [184].

Fibers with diameters of ca. 9 μm were prepared by hand-drawing from approximately 0.1 wt% aqueous solution of *N. edulis* major ampullate spidroins (solutions were prepared by forcible reeling of the silk from *N. edulis* spiders, dissolution of the fibers in 8 M guanidinium hydrochloride, concentration by centrifugation, and removal of the guanidinium hydrochloride by gel filtration) and β-sheet formation was induced by stretching the fibers [185]. The extensibility of the resultant fibers was similar to natural spider silk, yet the other mechanical properties were poorer. Similarly, fibers with diameters of 7–21 μm were hand-drawn from aqueous solutions of engineered spidroins (containing repeat sequences occurring in the flagelliform (GPGGX) and major ampullate (A_8) proteins of *N. clavipes*) [112]. The extensibility of the fibers was comparable to native MaSpII spider silk, but the mechanical strength was much lower. During our investigations of the fiber spinning process, we also demonstrated the ability to prepare β-sheet-rich fibers (with diameters of ca. 5 μm) via hand-drawing from concentrated aqueous solutions (>15% w/v) of *e*ADF-3 [170].

In another approach, fibers were prepared via wet spinning 20 wt% solutions of recombinant major ampullate spidroin 2 (from *N. clavipes*) into a coagulant bath of either aqueous alcohol (methanol or ethanol) or ammonium sulfate solution [186]. The fibers were drawn (improving their mechanical properties by alignment of the β-sheet crystallites), washed with water and dried in air (below 100 °C). The resulting fibers had uniform diameter of ca. 70 μm and mechanical properties sufficient for certain tissue engineering applications (although poorer than naturally spun spider silks). In a similar approach, fibers with diameters of 10–60 μm were wet-spun via extrusion of aqueous solutions of 25 wt% engineered spidroins (43 or 55 kDa proteins containing consensus sequences from major ampullate spidroins 1 and 2 from *N. clavipes*) into an aqueous-methanol coagulation bath. As expected, drawing of the fibers aligned the β-sheet crystallites, however, the poor mechanical properties of the fibers and a lack of pure material prevented acquisition of mechanical data on these proteins [187]. This spinning process was further improved by post-spin double drawing, yielding fibers with diameters of 10–40 μm [188] with mechanical properties similar those reported for wet-spun regenerated spider silk [189], yet still poorer than naturally spun spider silks.

We have demonstrated that it is possible to prepare fibers from aqueous solutions of *e*ADF-3 (and blends with *e*ADF-4) at a concentration of 20 mg/ml using a microfluidic device. A combination of salting out, pH drop and elongational flow within the microfluidic device yields β-sheet-rich fibers with diameters of ca. 1–5 μm. Mechanical tests of fibers produced in this fashion could be performed with atomic force microscopy or optical tweezers, enabling detailed investigation of structure–function relationships of the proteins [172].

2.3.1.2. Non-aqueous solvents. Silk proteins have been shown to be soluble in certain non-aqueous solvents, and there have been a number of reports of fiber spinning from non-aqueous solvents. The first of these described the wet spinning of fibers from 5 to 25 wt% solutions of *B. mori* fibroin in hexafluoroisopropanol (HFIP) into a methanol coagulation bath. Naturally spun *B. mori* fibroin fibers are insoluble in HFIP, hence HFIP solutions were prepared by dissolving degummed *B. mori* fibroin in 12.5 M lithium thiocyanate,

followed by dialysis to remove the lithium thiocyanate, leaving an aqueous solution of denatured fibroin. The solvent was allowed to evaporate, yielding a film of fibroin that was found to be HFIP soluble [190].

The wet-spun fibers were left in the bath overnight to allow diffusion of the HFIP from the fiber, then hand-drawn to the desired draw ratio whilst still wet with methanol, and finally immobilized to prevent recoil before drying overnight. The treatment with methanol induced β-sheet formation, rendering the fibers insoluble in HFIP. Their mechanical strength (initial moduli and tenacity) exceeded that of natural silkworm fibers. Investigation of this wet-spinning process in greater detail showed that *B. mori* fibroin adopts a predominantly α-helical structure in HFIP solution, and extrusion of this solution into methanol produced β-sheet-rich fibers with diameter of ca. 160 μm. The β-sheet crystallites in undrawn fibers are randomly oriented, and post-spin drawing reduced the diameter of the fibers to ca. 90 μm, increased the β-sheet content of the fiber and aligned these crystallites with the axis of the fiber [191]. In order to improve this technique, a wet-spinning apparatus was designed for the preparation of fibers (with diameters from 10 to 100 μm) from small quantities (ca. 10 mg) of *B. mori* fibroin, which would be useful for recombinant proteins produced in small quantities [192].

Naturally spun major ampullate silk from *N. clavipes* is soluble to a certain extent in HFIP, and initial attempts to wet-spin 2.5 wt% solutions of *N. clavipes* spidroin in HFIP into an acetone coagulation bath resulted in fibers with diameters of ca. 40 μm containing surprisingly high levels of residual HFIP even after storage under vacuum at 60 °C. Solvation of the fiber by residual HFIP is reflected in the high α-helix content and low β-sheet content relative to that in naturally spun silk fibers and explains the poor mechanical properties of these fibers. Soaking the fibers in water removed residual HFIP, and encouraged further β-sheet formation, although the fibers still had poor mechanical properties [193]. This process was significantly improved by drawing the fibers whilst they were wet with acetone, soaking in water, and a second drawing whilst wet with water. The mechanical properties of the resultant fibers (with diameters of ca. 30 μm) were comparable to (yet poorer than) naturally spun spider silks (strength 320 MPa vs. 875 MPa in naturally spun silk; and modulus 8.0 GPa vs. 10.9 GPa in naturally spun silk) [189].

HFIP is not the only organic solvent that has been used to process silk proteins, especially since certain silk proteins are insoluble or poorly soluble in HFIP as described above. Hexafluoroacetone hydrate (HFA-hydrate) was demonstrated to be a superior solvent to HFIP as it can dissolve naturally spun *B. mori* and *Samia cynthia ricini* fibroin fibers (which are insoluble in HFIP). It was also demonstrated that it was possible to prepare fibers by wet-spinning 10 wt% solutions of *B. mori* fibroin in HFA-hydrate into a methanol coagulation bath (although this process did not work for *S. cynthia ricini* fibroin). Washing and drawing in water and subsequent steam annealing yielded fibers with aligned β-sheet crystallites [194].

Ionic liquids such as 1-ethyl-3-methylimidazolium chloride and 1-butyl-3-methylimidazolium chloride have been demonstrated to be good solvents for naturally spun *B. mori* fibroin fibers, producing β-sheet free solutions [195]. It was also demonstrated that it was possible to prepare fibers by wet-spinning 10 wt% solutions of *B. mori* fibroin in 1-ethyl-3-methylimidazolium chloride into a methanol coagulation bath inducing β-sheet formation. The fibers were left in the coagulation bath overnight to remove the residual 1-ethyl-3-methylimidazolium chloride, yielding clear solid fibers of 250 μm diameter. Post-spin drawing of the fibers to twice their length reduced their diameters to 150 μm, and aligned the β-sheet crystallites. Unfortunately, in both cases the fibers were brittle when dried [196].

In contrast to halogenated [197–200] or ionic liquid solvents [201–206], *N*-methyl morpholine *N*-oxide (NMMO) is a cheap environmentally friendly organic solvent. Fibers have been prepared by wet-spinning 10–25 wt% solutions of *B. mori* fibroin in NMMO monohydrate into methanol or ethanol coagulation baths, collected by reeling, washed in either methanol or water, drawn whilst still wet and left to dry in air. The diameter of the finest fibers was 19 μm, yet the mechanical properties of these fibers were poorer than naturally spun fibers [207–210].

2.3.2. Electrospun fibers (nm scale diameters)

Electrospinning is a popular technique for the preparation of fibers with nanoscale diameters. The typical experimental setup involves pumping a solution of polymer through a needle, forming a droplet at the tip. Application of a voltage to the tip causes the droplet to stretch into a structure known as a Taylor cone. If the solution viscosity is too low, droplets are electrosprayed, whereas if the solution is sufficiently viscous an electrified jet is formed. The jet is elongated and stretched by electrostatic repulsion resulting in the preparation of fibers with uniform nanometer scale diameters.

2.3.2.1. Aqueous solvents. Early attempts to electrospin *B. mori* fibroin from relatively dilute aqueous solutions were unsuccessful due to the surface tension and viscosity of the solution being too low to maintain a stable drop at the end of the needle tip. The addition of up to 1.8 wt% PEO to 2–7 wt% *B. mori* fibroin solutions altered the surface tension and viscosity of the solution enough to allow smooth fibers with diameters of 590–910 nm to be successfully electrospun from aqueous solutions [211,212]. Treatment of these fibers with 90/10 (v/v) methanol/water induced a conformational change from random coil to predominantly β-sheet, and the surface of the fibers became rough due to phase separation of fibroin and PEO, and potentially extraction of some of the PEO. It was later found that using more concentrated solutions of *B. mori* fibroin (ca. 28 wt%) increased the surface tension and viscosity of the stock solution enough to enable the successful electrospinning of fibers with an average diameter of 700 nm and some β-sheet content [213,214]. It is also possible to electrospin fibers with a core/shell morphology by a two-fluid electrospinning technique and water as the common solvent [215]. The two-fluid electrospinning process (one fluid containing *B. mori* fibroin and the other PEO) is the key to produce a core filament of unblended fibroin and allowed a variety of core/shell diameters to be prepared.

2.3.2.2. Non-aqueous solvents. Fibers can also be electrospun from solutions of silk proteins in suitable non-aqueous solvents. It has been demonstrated that it is possible to electrospin fibers from solutions of 0.2–1.5 wt% *B. mori* fibroin and *N. clavipes* major ampullate silk in HFIP. The fibers had diameters that were tunable between 2 and 2000 nm. The as-electrospun fibers were α-helix-rich, and β-sheet formation was induced by high temperature annealing (205–245 °C for *B. mori* fibroin or 210–225 °C for *N. clavipes* major ampullate silk) [216]. It has also been demonstrated that it is possible to electrospin fibers from HFA-hydrate solutions of both *B. mori* fibroin (3 wt%) and *S. cynthia ricini* fibroin (10 wt%) (previous attempts to wet-spin *S. cynthia ricini* fibroin were unsuccessful). Soaking the fibers in methanol overnight, followed by storage under vacuum for two days removed the residual HFA-hydrate from the fibers. The fibers were β-sheet-rich and had diameters tunable between 100 and 1000 nm. Interestingly, in the case of the *S. cynthia ricini* fibroin fibers, the as-spun fibers were β-sheet-rich, and post-spin treatment with methanol only served to remove the residual HFA-hydrate from the fibers [217].

2.4. Technical processing of silk proteins – other morphologies

In addition to fibers, silk proteins can be processed into a diverse set of morphologies, and the following section of the review is devoted to their preparation.

2.4.1. Gels

Gels are solid-like materials, comprising a liquid that is immobilized by another component, where the other component is more commonly known as the gelator. Gel phase materials have a variety of applications including cosmetics and drug delivery.

2.4.1.1. Aqueous solvents. Spontaneous hydrogelation occurs in a matter of hours in sufficiently concentrated aqueous solutions of *B. mori* fibroin (>23 wt%) due to inter- and intramolecular interactions of the proteins in solution. Inter- and intramolecular β-sheet formation lowers the solubility of fibroin in water, encouraging the formation of physical cross-links between the proteins that eventually leads to hydrogelation. Hydrogelation at lower fibroin concentrations also occurs, although the timescale of this process is highly concentration-dependent (at concentrations below 4 wt% this process takes many days under comparable conditions). A pH near the isoelectric point of fibroin (pI = 3.8–3.9) [218,219] accelerates gelation, as the proteins are more prone to aggregation due to hydrophobic interactions. The presence of K^+ ions was found to have no effect upon the kinetics of gelation between 2.5 and 20 mM, whereas the presence of Ca^{2+} ions in the same concentration range is found to decrease the gelation time.

SEM images of freeze-dried gels show that in the presence of K^+ ions the fibroin had a flat 'leaf-like' morphology; by comparison, the presence of Ca^{2+} ions causes the fibroin to self-assemble into highly cross-linked sponge-like morphologies potentially due to Ca^{2+} mediated inter- and intramolecular interactions. Gelation time is also dramatically reduced (from days to hours) as the temperature increased from room temperature to 60 °C. There are two explanations for this, firstly, that hydrophobic regions of proteins are less well solvated at higher temperatures, encouraging aggregation through hydrophobic interactions; and secondly, that proteins unfold at higher temperatures, exposing more hydrophobic regions to water thereby encouraging aggregation [220,221]. Gelation time could also be reduced to a matter of hours by ultrasonication, as this dehydrates the hydrophobic segments, hence promoting aggregation [222]. Similarly, engineered fibroins based upon the consensus sequences of *B. mori* fibroin have been demonstrated to form hydrogels with various biomedical applications [223–231].

Hydrogel formation is also possible with spider silk proteins. The major ampullate silk proteins (of *A. diadematus* spiders) are stored in the lumen at approximately pH 7.4 without the onset of undesirable aggregation. Lowering the pH to 5.5 (protonation of carboxylate anions) was observed to trigger gelation, as the proteins were more prone to aggregation due to hydrophobic interactions. Interestingly, returning the pH to 7 (deprotonation of the carboxylates) reverses the assembly process [174].

We have demonstrated that certain engineered spidroins (*e*ADF-4 proteins) spontaneously form hydrogels (at concentrations of ca. 2 wt%) composed of nanofibrillar assemblies [232–234]. The self-assembled hydrogels could be disrupted by agitation or shearing, and chemical cross-linking of the hydrogels (10 mg/ml protein) with ammonium peroxodisulfate and tris(2,2′-bipyridyl)dichlororuthenium(II) (a light-inducible cross-linker) yielded elastic hydrogels [233] (Fig. 6).

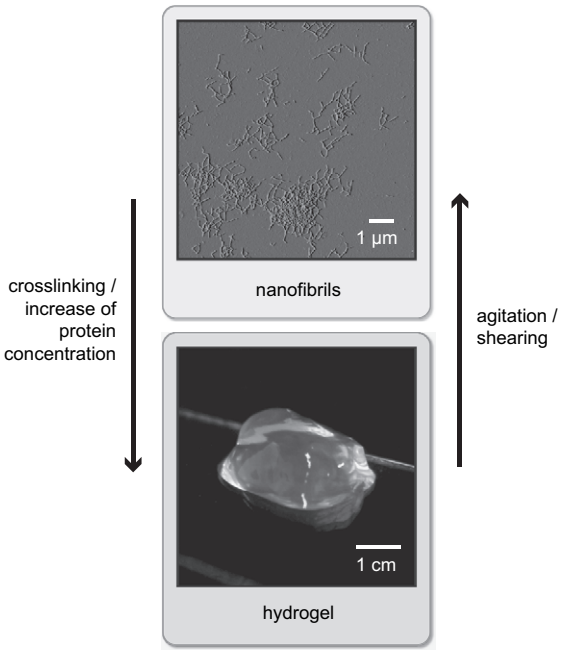

Fig. 6. Top: an AFM deflection image of air dried self-assembled silk nanofibrils and bottom: an SEM image of a hydrogel prepared from recombinant spider silk protein.

2.4.2. Foams

Foams can be prepared by a variety of techniques allowing fine control of their three-dimensional structure and mechanical properties, which ultimately dictates what they may be used for.

2.4.2.1. Aqueous solvents. Foams can be formed by bubbling gases through aqueous solutions of *B. mori* fibroin due to its surfactant-like properties [235], and foams prepared from slightly acidic solutions were observed to be β-sheet-rich [236–242]. Foams can also be prepared via freeze-drying hydrogels formed with *B. mori* fibroin [243]. Early studies demonstrated that the pore structure of the foams could be controlled via freezing temperature, solution pH or the addition of methanol.

Kaplan and co-workers reported the preparation of mechanically stable foams via freeze-drying *B. mori* fibroin hydrogels with pore sizes in the region of 10–70 μM, porosity up to 99% and a maximum compressibility of 30 kPa [244]. They also prepared mechanically stable *B. mori* fibroin foams by casting an aqueous solution of fibroin onto a salt template, and subsequently washing away the salt (a process commonly known as salt-leaching). This procedure generated foams with pore sizes between 470 and 940 μM, porosities of >90%, and compressive strength between 300 and 3800 kPa depending upon the processing conditions [245].

2.4.2.2. Non-aqueous solvents. Mechanically stable *B. mori* fibroin foams can additionally be prepared by pouring a solution of fibroin in HFIP over ammonium bicarbonate granules. Evaporation of HFIP followed by treatment with methanol induces β-sheet formation, and finally treatment with hot water causes sublimation of the ammonium bicarbonate template and expansion of the foam. This process yields foams with pore sizes between 40 and 260 μM, porosity up to 97% and a maximum compressibility of 280 kPa [244]. Alternatively, following a similar procedure using a sodium chloride template yielded foams with pore sizes between 100 and 300 μM, porosities of 84–98%, and compressive strength up to 175 kPa [244]. An adaptation of this process allowed the preparation of foams with a gradient of pore sizes (by packing differently sized salt particles into containers) resulting in foams with structures analogous to bone [246].

2.4.3. Films

Films are generally prepared by casting solutions of silk proteins onto a substrate and allowing the evaporation of the solvent. Once the solvent has evaporated, the films can be peeled off for further use, modified chemically (e.g. cross-linked), or modified structurally via treatment with another solvent.

2.4.3.1. Aqueous solvents. As-cast films made from aqueous solutions of *B. mori* fibroin are mechanically weak, and typically unstructured or α-helix-rich. β-sheet formation can be induced by treatment with methanol [247–249], annealing at high temperature [247], stretching [250], storage [251], or UV radiation [251]. Such conformational conversion renders the films insoluble in water and improves their mechanical properties. Interestingly, exposure to γ-radiation (from a Co^{57} γ-ray source) induces decrystallization of the β-sheets [250,252]. Mechanically stable films may be prepared by casting films from aqueous solutions of *B. mori* fibroin with a suitable cross-linking agent (such as polyethylene glycol diglycidyl ether) and drying at 40 °C for 4 h [253].

It is possible to prepare porous films by casting aqueous solutions of *B. mori* fibroin and a suitable porogen such as polyethylene oxide. Treatment of the films with aqueous methanol induces β-sheet formation, and the polyethylene oxide could subsequently be washed away with water yielding porous films. The topography of the films is tunable via the ratio of fibroin to polyethylene oxide [254].

2.4.3.2. Non-aqueous solvents. As with the preparation of fibers, gels and foams, films can also be made from solutions of silk proteins in non-aqueous solvents. Films have been formed via casting or dip-coating solutions of engineered *B. mori* fibroins in formic acid [255,256]. Scanning probe microscopy carried out on these films allowed the investigation of periodic features and the length-scale dependence of the surface roughness, which determine if cells can adhere to the films, and is therefore important for biomedical implants.

It has also been demonstrated that it is possible to prepare films by casting ca. 10 wt% solutions of *B. mori* fibroin in 1-butyl-3-methylimidazolium chloride onto a substrate. The 1-butyl-3-methylimidazolium chloride was washed away with methanol (inducing β-sheet formation) yielding clear solid films with a rough surface [195]. Films prepared via a similar procedure using a patterned template as a substrate, followed by removal of the template, gave patterned *B. mori* fibroin silk films that were suitable for use as scaffolds to support cell proliferation and growth [257].

We have cast α-helix-rich films from solutions of recombinant spidroins in HFIP and β-sheet formation was induced by exposure of the films to methanol or potassium phosphate rendering the films insoluble in water. We have also cast β-sheet-rich films from solutions of recombinant spidroins in formic acid. The surface of such films could be chemically modified via carbodiimide-mediated coupling of active enzymes through the carboxylic acid groups displayed on the backbone of the spidroin [258–261].

2.4.4. Spheres and capsules

Due to their highly tunable structures, spheres and capsules are very popular morphologies in applications such as the encapsulation and delivery of active ingredients including drugs, dyes, flavors or perfumes.

2.4.4.1. Aqueous solvents. As described above, spontaneous hydrogelation occurs in a matter of hours in sufficiently concentrated aqueous solutions of *B. mori* fibroin (>23 wt%), whereas at concentrations below 4 wt% this process takes many days under comparable conditions. SEM images of freeze-dried 2 wt% solutions of *B. mori* fibroin show smooth sponge-like morphologies. The addition of methanol (10% v/v) to these solutions induced β-sheet formation and made the morphology of the sponges rough; high magnification images of the sponges revealed that they were composed of aggregated particles. It was demonstrated that the particles formed when using fibroin with a molecular weight between 40 and 100 kDa were large and irregularly shaped, whereas use of fibroin with a molecular weight between 20 and 40 kDa resulted in smooth spheres of ca. 1 μm [262].

Microspheres may also be prepared via spray drying aqueous solutions of *B. mori* fibroin followed by storage in a desiccator to assure that the particles were dry [263]. The as-sprayed microspheres were unstructured with diameters of approximately 5 μm. The particles could be stored for up to a month with no change in the secondary structure of the fibroin, whereas storage at a relative humidity of 89% for 24 h allowed crystallization [264,265] of the β-sheet forming segments of the protein, rendering the microspheres insoluble in water.

Lipids such as 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) self-assemble into uni- and multilamellar vesicles that may be used for drug delivery [266–271]. Large multilamellar vesicles are commonly transformed into small unilamellar vesicles simply via freezing and thawing solutions of multilamellar vesicles [272,273]. Kaplan and co-workers have used large multilamellar DOPC vesicles for the encapsulation of *B. mori* fibroin and protein drugs, and subsequently generated small unilamellar vesicles by freezing and thawing steps, lyophilization of which yielded a free-flowing powder. Treatment of the powder with either methanol or aqueous sodium chloride induced β-sheet formation in the fibroin (entrapping the protein drugs) and removed the DOPC, yielding water-insoluble microspheres (ca. 2 μm) with a mixture of unilamellar and multilamellar structures [274].

We have prepared microspheres using one of the aforementioned engineered spidroins *e*ADF-4 (see Fig. 7). The addition of potassium phosphate at concentrations >400 mM to solutions of *e*ADF-4 spidroin leads to the formation of smooth solid β-sheet-rich microspheres, through the liquid–liquid phase separation of a protein-rich phase in a protein-poor supernatant due to the salting out of the protein-rich phase. Sphere size can be controlled with two simple parameters, protein concentration and mixing intensity; increasing the protein concentration resulted in larger microspheres, whereas increasing the mixing intensity resulted in smaller microspheres [275,276].

We have also demonstrated that *e*ADF-4 spidroin spontaneously assembles at a toluene/water interface in water-in-toluene emulsions. This assembly process results in β-sheet-rich capsules that are mechanically and chemically stable (see Fig. 7). The microcapsules can be transferred into aqueous solution either via centrifugation into an aqueous sub-layer, or dilution of the toluene by the addition of an excess of ethanol and water. Adjusting the emulsion droplet diameter allows tuning of the microcapsule diameter between 1 and 30 μm [277,278].

3. From science towards applications

3.1. Textiles

B. mori silk fibers have been used for the production of textile goods for centuries due to their characteristic luster, moisture absorbance and strength. Early research was largely focused on dyeing methodology and dealing with its shortcomings (photo-yellowing [279–281], rub resistance [282] and wrinkle recovery [283]).

Attractively colored *B. mori* silk fibers can be prepared by various dyeing processes, and fiber dyeing has been achieved with acidic [284–287], basic [288], azo [289], disperse [290–294], mordant [295,296], natural [297,298] and reactive dyes [299,300]. There is a vast quantity of literature detailing dye synthesis and fiber dyeing processes due to their economic importance.

The variety of reactive amino acid residues displayed on the backbone of silk proteins has provided synthetic chemists with plenty of scope for the modification of their properties. Modification of *B. mori* fibroin fibers with various acid anhydrides improved their crease recovery properties and protected them from photo-yellowing [279,282,301–303], primarily due to chemical

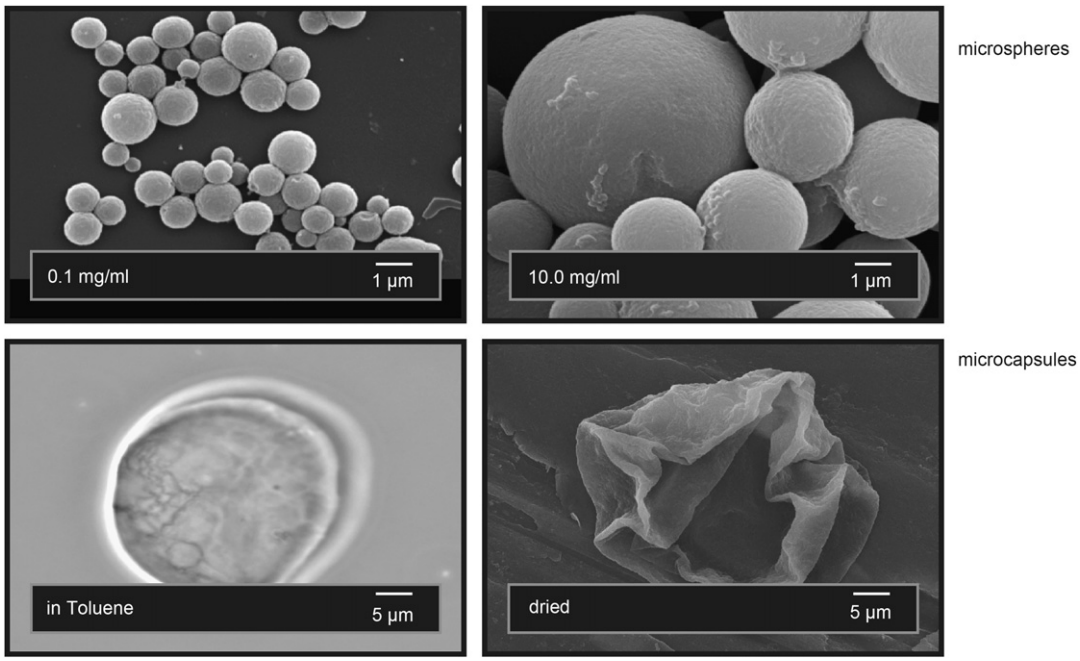

Fig. 7. SEM and optical microscope images of microspheres and microcapsules of *e*ADF-4.

modification of the tyrosine residues, and fibers modified with dibasic acid anhydrides markedly improved the cationic dye affinity [304].

Another method of modifying the properties of silk proteins is the preparation of graft copolymers (imparting some of the properties of the grafted polymer). Fibroin-*graft*-(methyl methacrylate) [305] and fibroin-*graft*-vinyltrimethoxysilane [306] showed improved wrinkle recovery; fibroin-*graft*-(hydroxy-ethyl methacrylate)-*co*-(methacrylamide) [307] displayed an increased moisture content; and fibroin-*graft*-styrene [308,309] showed higher affinity for disperse dyes and can consequently be applied in heat transfer printing.

Water-repellent silk fibers have been prepared via reaction of *B. mori* fibroin fibers with alkyl acid anhydrides [310,311], or coating naturally spun major ampullate silk fibers produced by *N. edulis* spiders with hydrophobic gold nanoparticles [312], at no detriment to their mechanical properties.

We envisage that, in the future, biotechnologically produced silk fibers will allow the preparation of a new generation of high performance fibers with even more built-in properties for a variety of textiles related applications.

3.2. Sutures for wounds

Silk fibers have been used as sutures for wounds for centuries due to their strength, biocompatibility and low immunogenicity, yet only recently have detailed biological studies been undertaken [313–315]. The mechanical properties of sutures determine if they are successful in their application, therefore the investigation of the properties of fibers and yarns is of great importance [316]. Braided yarns could be used as stiff sutures for wounds where small diameter yarns are preferable, and tissue in-growth is unnecessary [317,318].

3.3. Tissue scaffolds

Tissue engineering is the development of substitutes for biological tissues that replace or restore tissue function. Tissue scaffolds are the extracellular matrices designed to support the cells that constitute the tissue to be replaced or restored. Silk-based polymeric materials are interesting tissue scaffolds due to their biocompatibility and their highly tunable morphologies.

Silk fibers that have been spun into yarns and subsequently textured via permanent deformation may be useful as non-load-bearing spacers in tissue grafts where tissue in-growth is desirable. Cabled yarns have highly tunable mechanical properties and therefore potential in load-bearing tissue engineering applications [317].

Fibers electrospun from aqueous solutions of *B. mori* fibroin and PEO were used as a scaffold for human aortic endothelial cells (HAECs) and human coronary artery smooth muscle cells (HCASMCs); in both cases *in vitro* culture in endothelial growth medium led to the formation of vascular tissues within a week [211].

Stem cells can differentiate into a diverse range of specialized cell types and their use in implanted tissue scaffolds has been proposed [319]. Human mesenchymal stem cells (hMSCs) can be isolated from bone marrow aspirates, and can form bone, cartilage, fat and other connective tissues, and therefore have significant potential in the regeneration of diseased or injured tissues. Fibers electrospun from aqueous solutions of *B. mori* fibroin, PEO and bone morphogenetic protein-2 (BMP-2) were used as a scaffold for hMSCs; *in vitro* culture in osteogenic media led to the formation of bone-like tissue. Addition of hydroxyapatite nanoparticles to the fibroin solution prior to electrospinning produced fibers with the nanoparticles embedded inside and was found to improve bone formation [320]. *In vivo* implantation of electrospun *B. mori* fibroin fibers in calvarial defects in mice facilitated the complete healing of the defect with new bone within 12 weeks [321].

Manually silked major ampullate silk fibers (from adult female spiders of the genus *Nephila*) have been used as a scaffold for nerve cell growth *in vitro*. Schwann cells were observed to adhere quickly, and proliferate and survive successfully, and the cells completely ensheathed the fibers within a week of cultivation [322]. Recent *in vivo* studies in rats have demonstrated successful nerve regeneration with regrowth of regularly aligned axons [323].

B. mori fibroin hydrogels have been used as a scaffold for hMSC growth *in vitro*. The cells were able to grow, proliferate and survive for weeks in static culture conditions [222]. Similarly, *B. mori* fibroin

hydrogels have been used as scaffolds for bone tissue growth both *in vitro* and *in vivo* in rabbits without inflammatory effects [324–326].

As a further alternative silk morphology, foams prepared from *B. mori* fibroin have been used as scaffolds for the attachment and proliferation of fibroblasts *in vitro*. It was observed that cell colonies were located preferentially at the surface of the foam, potentially due to the cell-seeding process, and/or lack of nutrients inside the foam [327]. Foams seeded with hMSCs in chondrogenic medium formed cartilage-like tissue, whereas those in osteogenic medium formed bone-like tissue [246,328]. Importantly, foams seeded with adipose derived stem cells formed adipose-like tissue *in vivo* in rats [329].

It has proven possible to prepare artificial blood vessels by coating steel wires with porous films of *B. mori* fibroin, and subsequently removing the wire template. Porous films were prepared by casting aqueous solutions of *B. mori* fibroin and PEO (a fibroin immiscible porogen). Treatment of the films with aqueous methanol induces β-sheet formation and the polyethylene oxide could subsequently be washed away with water yielding porous films [254]. The level of porosity was controlled by varying the ratio of fibroin to PEO, and the diameter of the fibroin-based vessels was determined by that of the wire template. Low porosity microtubes demonstrated superior mechanical properties in terms of higher burst pressures, but displayed poor protein and cell permeability; whereas higher porosity tubes had lower burst strengths but increased permeability and enhanced protein and cell permeability [330].

3.4. Biocompatible coatings

Coatings are commonly applied to the surface of materials to improve their surface properties. The biocompatibility and non-immunogenicity of silk proteins should allow their application as coatings for biomedical implants, potentially as anticoagulants, and either promoters or inhibitors of cell adhesion.

Water soluble anticoagulants (such as the highly sulfated polysaccharide heparin) are commonly used for blood treatment during dialysis and surgery, and water-insoluble anticoagulants are useful coatings for implants such as stents. Sulfation of water soluble *B. mori* fibroin (modifying the amine and hydroxyl containing residues) was found to impart anticoagulant activity to the fibroin [331–333], and more recently *B. mori* silk fabrics were sulfated for application as water-insoluble anticoagulants [334,335].

B. mori fibroin films cast from aqueous or HFIP solution and subsequently treated with methanol (inducing β-sheet formation) are suitable for osteoblast-like cell adhesion, and it has been demonstrated that cell adhesion can be significantly improved using films cast from fibroin that has been chemically modified with the RGD integrin recognition sequence that is well-known for its cell-adhesive properties [336]. Peptides derived from the repetitive motif found in collagen [GERGDLGPQGIAGQRGVV-$(GER)_3$GAS]$_8$GPPGPCCGGG and fibronectin [TGRGDSPAS]$_8$ were demonstrated to be cell-adhesion sites in *in vitro* studies. Genetically engineered chimeric proteins based upon *B. mori* fibroin and the peptides above should be capable of both self-assembly and cell-adhesion. Films cast from solutions of these genetically engineered chimeric proteins were demonstrated to have much improved cell-adhesion properties over those cast from *B. mori* fibroin alone; additionally, the cost of these materials would be a fraction of the cost of collagen or fibronectin [337].

Many sugar residues in lipids and proteins are involved in the recognition of lectins present on cell surfaces. Films cast from aqueous solutions of *B. mori* fibroin chemically modified with a sugar (lactose bearing the β-galactose residue) were treated with aqueous methanol solution to induce β-sheet formation, and render the films insoluble in water. Levels of hepatocyte attachment to the films cast from lactose conjugated fibroin was demonstrated to be ca. eight times higher than films cast from unmodified fibroin [338].

2-Methacryloyloxyethyl phosphorylcholine (MPC) inhibits blood cell–platelet, monocyte and macrophage adhesion. The graft polymerization of MPC onto *B. mori* silk fabrics yielded fabrics with the desirable mechanical strength of silk fabrics that significantly inhibit blood cell–platelet adhesion to ca. 10% of the level of the untreated fabric, that are expected to have potential as sutures or wound dressings [339,340].

3.5. Biomineralization

The process by which biological organisms produce mineralized tissues such as bones, diatoms, shells and teeth is known as biomineralization. The biomimetic use of biomineralization processes to construct organic–inorganic hybrid materials is a very active area of research. Silk-based materials have been used as organic scaffolds for the biomineralization of hydroxyapatite (found in bones and teeth) and silica (found in diatoms).

Naturally spun major ampullate silk fibers (from daddy longlegs spiders) have been used as a template for the nucleation and growth of oriented hydroxyapatite crystals [341].

B. mori fibroin films with calcium chloride contents of 5 wt% or greater were prepared by casting from aqueous solution, and β-sheet formation was induced by treatment with methanol. Incubation of the films in simulated body fluid led to the growth of hydroxyapatite crystals in a matter of hours [342,343].

Naturally, dentin matrix protein 1 is involved in the nucleation and oriented crystallization of hydroxyapatite. Genetically engineered chimeric proteins based upon major ampullate spidroin (from *N. clavipes*) and dentin matrix protein 1 are capable of both self-assembly and biomineralization. Films cast from HFIP solution were treated with methanol to induce β-sheet formation in the major ampullate domain of the chimeric protein, rendering the film insoluble in water. Incubation of the films in simulated body fluid led to the growth of hydroxyapatite crystals on the surface of the film, whereas films cast from the control protein without the dentin matrix protein 1 domain did not induce biomineralization [344].

Silaffins are low molecular weight proteins involved in silica formation in nature. *In vitro* studies using a peptide derived from the repetitive motif found in silaffin proteins (known as the R5 peptide) demonstrated that this peptide promotes and regulates silica formation at neutral pH. Genetically engineered chimeric proteins based upon major ampullate spidroin (from *N. clavipes*) and R5 peptide are capable of both self-assembly and biomineralization. Fibers electrospun from HFIP solution, and films cast from HFIP solution were treated with methanol to induce β-sheet formation in the major ampullate domain of the chimeric protein, rendering the materials insoluble in water. Incubation of the fibers and films with a water soluble silicon species led to biomineralization [345].

3.6. Drug delivery

For a drug to have its optimal effect it is important that its release profile is both reliable and controlled, particularly important in cases where the drugs have undesirable side effects. Silk proteins may find application in drug delivery as drug carriers owing to their biocompatibility and their highly tunable morphologies.

L-Asparaginase has been used in clinical trials in humans to prevent the progress of L-asparagine-dependent tumors, however, its half-life of activity is limited due to rapid clearance, and it is both antigenic and immunogenic. Conjugation of L-asparaginase to

water soluble *B. mori* fibroin via glutaraldehyde improved its half-life and reduces its antigenicity and immunogenicity *in vitro* [346].

Hydrogels formed from *B. mori* fibroin were demonstrated to control the release of model drugs with molecular weights ranging from ca. 350 Da to 4.5 kDa, and of buprenorphine (a morphine-like drug used in the treatment of acute pain) *in vitro*. Tuning of the gels' mechanical properties and rate of drug release was achieved simply by varying the concentration of fibroin [347]. Hydrogels formed from engineered proteins based upon *B. mori* fibroin were demonstrated to similarly control the release of model drugs with molecular weights ranging from ca. 400 Da to 500 kDa, and of Pantarin (a mitotoxic protein) *in vivo* in guinea pigs, with no clinical signs of tissue reaction due to allergy, irritancy or toxicity after 28 days [348].

Foams prepared via freeze-drying aqueous solutions of *B. mori* fibroin and aspirin were demonstrated to be capable of controlled release of the aspirin trapped in the scaffold. Preliminary *in vitro* kinetic studies showed a burst release profile for the aspirin, with a significant quantity of aspirin released in the first 2 h, followed by an almost constant rate of release thereafter [243].

Films of *B. mori* fibroin and carbodiimide cross-linked *B. mori* fibroin were deposited on tablets of theophylline (a drug used in therapy for respiratory diseases such as asthma) via dip-coating. The carbodiimide cross-linked films were more mechanically stable and allowed controlled release to be achieved for significantly longer periods of time with zero order release kinetics *in vitro*; moreover, multiple coatings allowed the fine tuning of the rate constants [349,350]. Films of *B. mori* fibroin and various macromolecular drug models (FITC-labelled dextrans, horseradish peroxidase and lysozyme) were shown to be capable of controlled release of the macromolecular model. β-Sheet-rich films showed a strongly molecular weight dependent retardation of the release of all of the macromolecular drug models *in vitro* [351]. Multilayered *B. mori* fibroin films have been used as drug-eluting coatings for stents, incorporating clopidogrel, heparin, and paclitaxel. Clopidogrel and paclitaxel were observed to retard human aortic endothelial cell (HAEC) proliferation and inhibit human coronary artery smooth muscle cell (HCASMC) proliferation. Heparin loaded silk films inhibited HCASMC proliferation and promoted HAEC proliferation, which is ideal for the prevention of restenosis. Moreover, preliminary studies show that these films are also functional *in vivo* in porcine aorta [352].

Alginate and PLGA microspheres containing model enzyme drugs were coated with a *B. mori* fibroin film. The as-cast films retarded the rate of release of the drugs when compared to uncoated controls *in vitro*, and could be further retarded by induction of β-sheet formation by treatment of the films with methanol [353]. *B. mori* fibroin microspheres prepared using lipid vesicle templates have been used for the efficient encapsulation and controlled release of an active model protein drug (horseradish peroxidase) *in vitro* [274].

We have demonstrated that microspheres prepared using one of our engineered spidroins (*e*ADF-4) are capable of the encapsulation of poorly water soluble substances such as β-carotene, and that these microspheres were undigested in artificial gastric fluid and completely digested in artificial intestinal fluid at 37 °C. This sort of controlled release gives them potential use as delivery vehicles for hydrophobic compounds (such as drugs or food ingredients) that remain intact in the stomach and release the compounds in the small intestine [354].

We have also prepared β-sheet-rich microcapsules using *e*ADF-4 via a process of spontaneous assembly at a toluene/water interface in water-in-toluene emulsions. The mechanically and chemically stable microcapsules can be transferred into aqueous solution either via centrifugation into an aqueous sub-layer, or dilution of the toluene by addition of an excess of ethanol and water (see Fig. 8) [277,278].

We have demonstrated that these microcapsules are capable of encapsulation of model macromolecular drugs (such as FITC-labelled dextran) whilst allowing small molecules (such as fluorescein) to diffuse freely across the membrane. The capsules can be degraded upon exposure to Proteinase K in a matter of minutes (see Fig. 9), moreover, this degradation can be prevented by chemical cross-linking of the microcapsules via photo-initiated oxidation with ammonium peroxodisulfate and tris(2,2′-bipyridyl)dichlororuthenium(II) [277,278].

3.7. Solid supports for catalysts

Silk protein-based materials have found application as solid supports for potentially expensive enzyme and organometallic catalysts.

Silk proteins are capable of forming functional complexes with metal ions. One potential application of the metal complexes is in the immobilization of catalysts, the first example of which was a *B. mori* fibroin–palladium catalyst capable of asymmetric hydrogenations. These heterogeneous catalysts were used to prepare

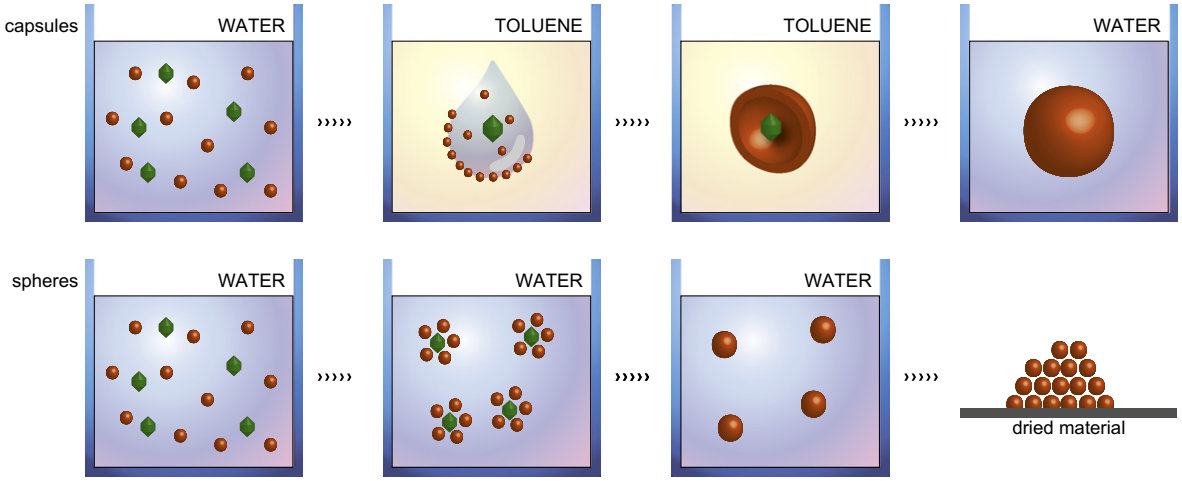

Fig. 8. Schematic of microcapsule and microsphere formation. Top: an aqueous solution of *e*ADF-4 (red) and drug (green) is emulsified in toluene leading to spontaneous assembly of the protein at the toluene/water interface, yielding microcapsules (containing the drug) that can subsequently be transferred into aqueous solution. Bottom: an aqueous solution of *e*ADF-4 (red) and drug (green) is exposed to high concentrations of potassium phosphate leading to liquid–liquid phase separation of a protein-rich phase in a protein-poor supernatant (due to the salting out of the protein-rich phase), yielding drug containing microspheres.

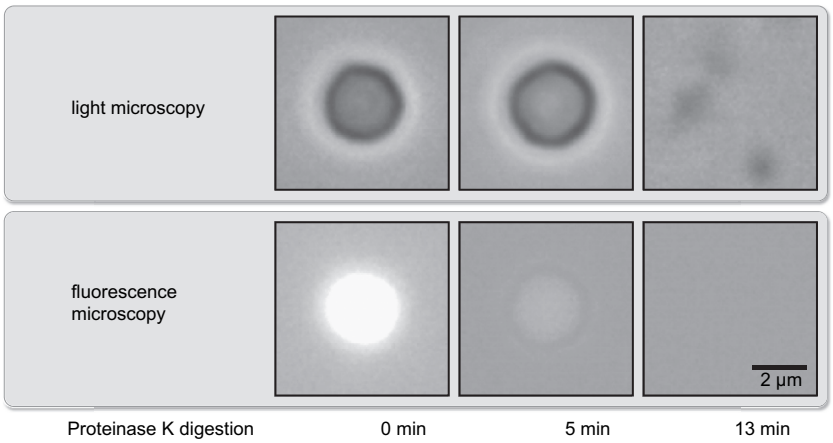

Fig. 9. Proteinase K digestion of spider silk microcapsules encapsulating FITC-labelled dextran, indicating the release of the encapsulated FITC-labelled dextran.

optically active amines and amino acids such as diphenylethylenediamine, glutamic acid and phenylalanine. The preparation, properties, selectivity, stability and structure of these palladium based catalysts were subsequently investigated in more detail [355–357], as were the platinum [358,359] and rhodium analogues [360].

More recently it has also been demonstrated that *B. mori* fibroin–palladium catalysts are capable of highly chemoselective hydrogenation of acetylenes, olefins and azides in the presence of other groups that are readily hydrogenated using Pd/C or Pd/C(en) as a catalyst (such as aromatic aldehydes and ketones, halides, and benzyl ester and N-Cbz protecting groups) [361–364]. *B. mori* fibroin–iron catalysts are capable of hydroxylation of phenol to catechol and hydroquinone. The catalyst had a slight selectivity for catechol over hydroquinone, and could be recovered and reused several times without a significant decrease in its activity [365]. *B. mori* fibroin–metallophthalocyanine catalysts are capable of the elimination of the malodors of hydrogen sulfide and methane thiol via catalytic oxidation, and may find application in air purification [366,367].

Enzymes can be effectively immobilized via covalently linking the silk protein to the enzyme using well established azide, cyanogen bromide, diazo or glutaraldehyde methodologies. Aspartate aminotransferase, calf intestine alkaline phosphatase, and ribonuclease have been covalently attached to *B. mori* fibroin fibers using such methodologies and were shown to maintain their activity [368–371]. Enzymes may also be effectively immobilized via physical entrapment within silk films. Films are typically cast from

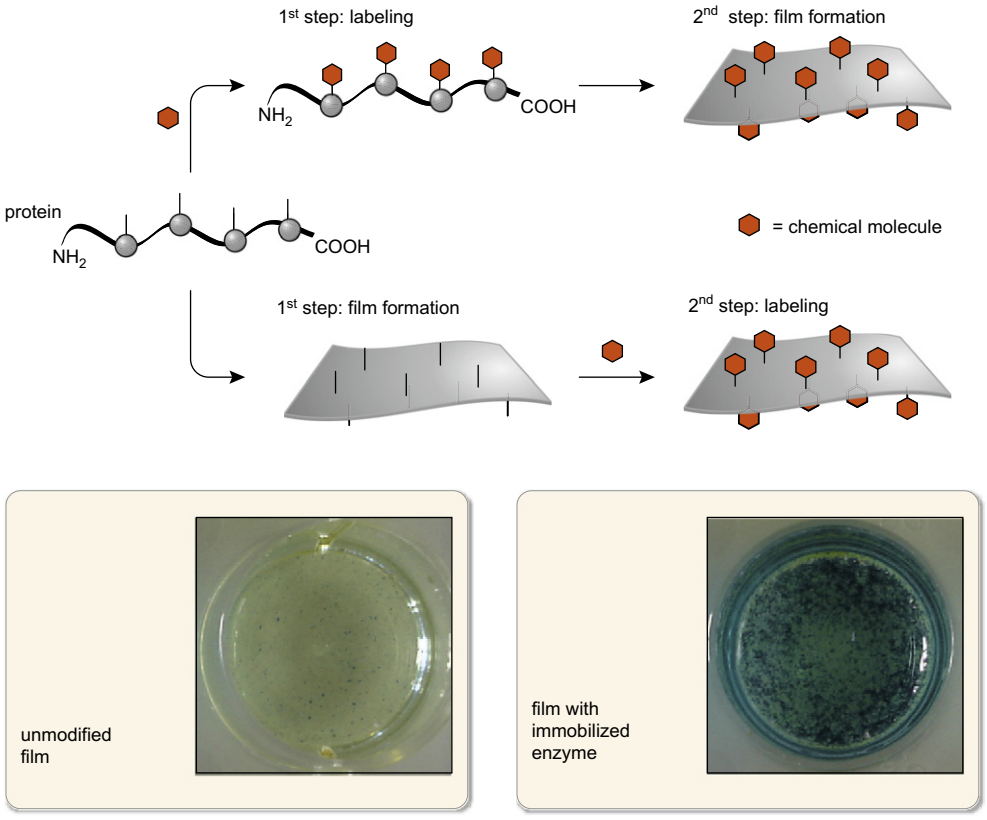

Fig. 10. Schematic of film formation and labeling, and photographs of examples of such films from our lab.

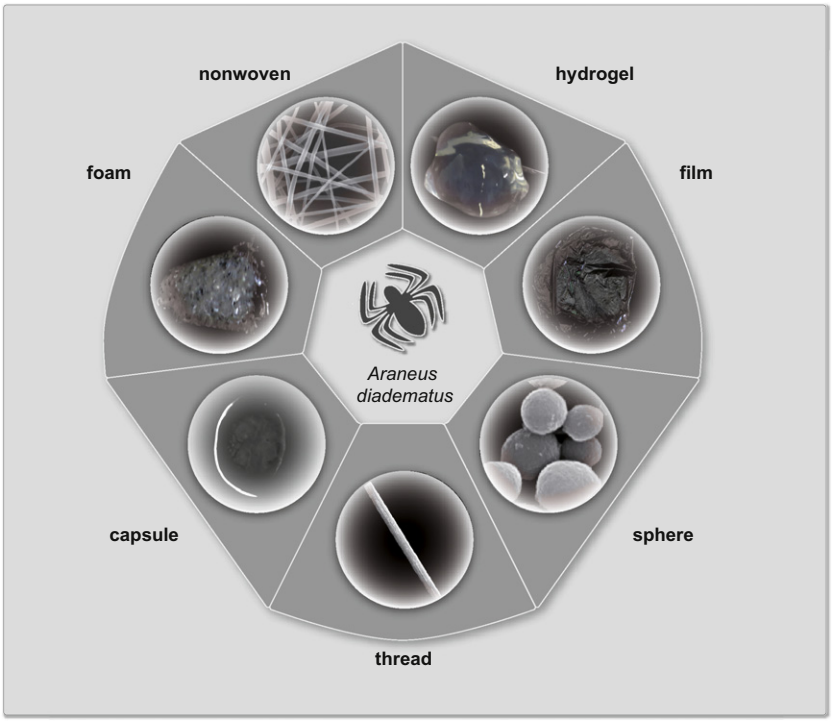

Fig. 11. The various morphologies that can be prepared with a single recombinantly produced engineered spider silk protein *e*ADF-4.

aqueous solutions of *B. mori* fibroin and the desired enzyme, and β-sheet formation is induced in order to immobilize the enzyme. *B. mori* fibroin–glucose oxidase films were demonstrated to be excellent glucose sensors in terms of enzyme leakage, response time and storage stability [250,372,373]. *B. mori* fibroin–horseradish peroxidase films were shown to be excellent biophotosensors for the chemiluminescent detection of hydrogen peroxide by the luminol reaction catalyzed by the immobilized peroxidase [351,374].

We have prepared mechanically stable β-sheet-rich films of *e*ADF-4 displaying active enzymes such as β-galactosidase on their surface, and demonstrated their activity using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as a substrate (see Fig. 10) [258–261].

3.8. Materials with novel electronic, magnetic and optical properties via the encapsulation of colloidal and nanoscale particles

Naturally spun major ampullate silk fibers produced by *N. edulis* spiders can be coated in nanoparticles (via dip-coating in suspensions of nanoparticles and air-drying) with almost no detrimental effect upon the mechanical properties of the fibers. Coating the fibers in superparamagnetic magnetite (Fe_3O_4) allowed the orientation of the fibers in a magnetic field, potentially allowing integration into devices for audio reproduction. It is also possible to form stable coatings of electroconducting polypyrrole, semiconducting cadmium sulfide particles and hydrophobically functionalized gold nanoparticles [312].

Hollow mesoporous silica fibers can be prepared via coating forcibly silked major ampullate silk fibers (produced by *Nephila madagascariensis* spiders) with a silica-surfactant mesophase, and subsequently removing the silk and surfactant via calcinations [375]. Hollow porous silica fibers can be prepared by coating *B. mori* fibroin fibers with tetraethylorthosilicate (a silica precursor) in supercritical CO_2. The silica coating faithfully reproduced the surface features of the *B. mori* silk fibers and it was possible to tune the diameter, wall thickness and porous structure of the silica fibers by varying the processing parameters [376]. Such hollow porous fibers may find application as host materials for a variety of electrically or optically active chemical species [375], as was demonstrated with porous fibers of titania and zirconia containing gold nanoparticles [377].

B. mori fibroin micelles have been used as an active scaffold for growing gold/fibroin core/shell particles via reduction of Au(III) to Au(0) in the presence of fibroin. The cores were 3–20 nm in size and the core/shell particles were stable for months at room temperature, and may be useful in a variety of nanotechnological applications [378]. Spider silk fibers harvested from the webs produced by *Pholcus phalangioides* spiders have been used as an active scaffold upon which to grow gold nanoparticles, via reaction of the silk fiber with aqueous chloroauric acid. Solvent vapor induced structural changes in the fibers, thereby altering their electrical properties, and these materials may find application as vapor sensors [379].

Green fluorescent protein (GFP) is commonly linked to other proteins as a fluorescent tag to determine location of the other protein in microscope-based studies of biological systems. GFP also has interesting nonlinear optical properties, such as levels of two-photon absorption that are high enough to saturate the two-photon transition probability under certain conditions. This creates a population inversion in the chromophore, giving GFP potential application in laser technology [380,381]. Films may be cast from aqueous solutions of *B. mori* fibroin and GFP with a sufficient density of GFP molecules for two-photon absorption and potential application in photonics [382,383].

4. Summary and outlook

Silk proteins can be processed into a diverse set of morphologies with a great potential for various applications. We envisage that in the future, proteins in general will be employed in addition to, and in some cases in place of classical synthetic polymers. Biotechnologically produced silk proteins will allow the preparation of a new generation of protein-based biopolymeric materials with programmed properties for a wide variety of exciting applications (Fig. 11).

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