Part I

Biomimetic principles, production and properties

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Recombinant DNA methods applied to the production of protein-based fibers as biomaterials

F. TEULÉ and R. LEWIS
University of Wyoming, USA

W. MARCOTTE JR and A. ABBOTT
Clemson University, USA

Abstract: The protein compositions of selected high-performance biological materials such as silks, collagen and mussel byssus threads are reviewed. The possible roles of key amino structural motifs present in these fibrous proteins are outlined. Experimental investigation of structure/function relationships in fibrous proteins is discussed. More specifically, the structural characterization of artificial silk proteins, produced through the expression of native silk cDNAs, synthetic silk gene analogs or designer silk genes in different expression systems, is reviewed in detail. Finally, the most popular and most successful unicellular or multicellular expression systems available for the production of such fibrous proteins are described.

Key words: expression of native or synthetic silk gene analogs, structural characterization, recombinant silk proteins, biological materials, expression systems.

1.1 Introduction

Biological organisms produce a wealth of natural fibrous polymers that have evolved to achieve highly specialized roles and to allow organism adaptation and survival. Some of the more interesting fibrous materials such as wool, silks, skin, cartilage and tendons are composed of fibrous proteins.

The literature is replete with details on the compositions, structures, mechanical and other physical and chemical properties of many of these protein-based biomaterials. This richness of information, along with scientific progress in the fields of genetic engineering and materials science, make it feasible to realize the production of biomaterials using recombinant DNA technologies.

It is the intent of this review to touch upon much of the rationale and methods for creation of genetically engineered systems for the production of fibrous protein polymers. We will focus on the utilization of spider silk gene mimics as a demonstration of a successful design, implementation and production scheme.

1.2 Biomimetics and protein-based biomaterials

Protein-based materials or protein fibers were thought for a long time to be a unique characteristic of the animal kingdom. However, such fibrous proteins have since been identified in plants. In wheat, a large, highly elastic storage protein called gluten, important in the expansion or rising of wheat-based doughs, was recently characterized as one of the first plant fibrous proteins (Tatham and Shewry, 2000).

The basic components of these natural fibrous materials are structural fibrous proteins. Such proteins are found in hair, tendons, cartilages, skins, arteries, muscles of mammals or in cuticles and silks of many arthropods. The individual proteins that make up these fibers or fibrous materials are of a specific sequence and often share structural amino acid motifs from one type of protein to another. In addition, many of these fibrous proteins have the ability to self-assemble, in an ordered manner, into a supramolecular network. The resulting supramolecular structure is often insoluble and is maintained, depending on its origin, by a combination of intra- and/or inter-molecular cross-linking, hydrophobic interactions, hydrogen bonding and coulombic interactions. Cross-linking is often necessary to hold the 'structure' together and can be different in nature depending on the amino acid residues involved. The molecular architecture (sequence and composition) of the individual proteins forming the network and the level and types of cross-links involved determine the mechanical and physical properties of the final fibrous materials, and the sequence in which these fibrous materials are assembled may be equally critical.

All these bio-based polymers display exceptional mechanical properties. More importantly, they are biodegradable and, thus, are attractive as a potential source of exploitable, environmentally-friendly materials. However, engineering new bio-based fibers having desired or customized mechanical properties is extremely challenging. To achieve this goal, the molecular architecture of the molecules composing these fibers, as well as their assembly processes, need to be fully understood in order to control assembly in the manufacturing process. Protein-based fibers are very suitable for bioengineering and remarkable efforts are being made in this field to understand the structure/function relationships of protein polymers. Because of the availability of technologies allowing the manipulation of genes encoding proteins, the prospect of designing and manufacturing new, possibly customized, protein-based polymers seems within reach.

Although there are many diverse types of fiber proteins, we will limit our discussion primarily to silk and collagen proteins for the sake of brevity. However many of the rules that govern the structure/function relationships of these specific fibrous materials or fibers are relevant to others as well (e.g. elastins-, lamprin-based materials).

1.3 Characteristics of some natural protein-based materials

1.3.1 Homopolymers

Silks

Many arthropods such as insects (Lepidoptera), and arachnids (Araneidae) produce silks. These highly insoluble proteinacious fibers share several structural motifs and characteristics. The primary structures of the different silk proteins are usually highly repetitive and comprise combinations of crystalline structures (tightly packed β-pleated sheets with more loosely associated β-sheets) and amorphous regions (α-helices, β-spirals or 'spacer' regions) (Xu and Lewis, 1990; Beckwitt and Arcidiacono, 1994; Guerette *et al.,* 1996; Simmons *et al.,* 1996; Hayashi and Lewis, 1998). Although most silk-producing organisms may only produce one type of silk, some arachnids such as Araneidae spiders have a more complex spinning apparatus and are able to produce several types of silks (Peters, 1955; Lucas, 1964). In these spiders, the different silks originate from different silk glands and differ in both amino acid composition and mechanical properties. The wide range of mechanical properties exhibited by these different silks allow for their adapted use in various applications from web construction, prey swathing, safety line or dragline, to cocoon construction (Lewis, 1992).

The cocoon silk of moth larvae such as *Bombyx mori* (Bombycidae, Lepidoptera) is the main source of silk exploited by textiles industries. *B. mori*, like all lepidopteran caterpillars, possesses a pair of modified salivary glands (labial glands) that are responsible for silk production. This silk-secreting system is linked to an outlet by which the silk is pulled out as the worm moves its head from left to right following a figure-eight-shaped trajectory (Sehnal and Akai, 1990).

The fibrous core of *B. mori* cocoon silk is composed of a 350 kDa heavy-chain fibroin (H-fibroin, *Bm-Fhc*) and a 25 kDa light-chain fibroin (L-fibroin, *Bm-Lc*) covalently linked by disulfide bonds. These fibroins are also associated with a small glycoprotein of approximately 27 kDa (P25) but there is no evidence of covalent links (Tanaka *et al.,* 1999a; Tanaka *et al.,* 1999b). P25 may play a role in the transport of the two fibroins from the cells to the gland lumen. The heavy-chain fibroin is the fiber protein responsible for silk formation.

B. mori silk fibroins are characterized by a high glycine content. The fibroin primary sequence is highly repetitive and displays a noticeable $(GA)_n$ motif containing glycine and alanine residues interspaced with serine-containing polyhexapeptide repeats GAGAGS and GAGAGX with X = Y or V (Zhou *et al.,* 2000). The short GAGAGS repeat can form crystalline regions in both morphological states of silk studied: silk I and silk II. Silk I or 'water soluble silk' is obtained from the desiccated gland content without any mechanical disturbance (storage state of the fibroin) (Lotz and Cesari, 1979). The more stable silk II (β-silk

fibroin) is observed in the spun cocoon fiber. Silk I is converted to silk II by mechanical stress or shearing that occurs when the fiber is being pulled out. The crystalline structure of silk II is viewed as extended polypeptide chains arranged in hydrogen-bonded structures. In this structure, the GAGAGS motif adopts an anti-parallel β-sheet so that all the glycine residues are projected on one side of the sheet and all alanine or serine residues are projected on the other side. However, in silk I, these β-sheets are imperfect (Lotz and Keith, 1971; Fossey *et al.,* 1991).

In *B. mori* silk, crystalline regions account for approximately 40 to 50% of the silk structure. Typically, the fibroins consist of twelve large crystalline domains. These crystalline domains are periodically interspaced with short distinct 'spacers' (sequences with bulky amino acid residues like Y, W, E and R) that are more amorphous regions with random orientation. These spacers are important because they may give some flexibility to the fiber (Denny, 1980). The sticky protective coating of *B. mori* silk is composed of a protein called sericin. This 'glue' has a critical role since it provides proper adhesion of the cocoon fibers with one another (Grzelak, 1995).

B. mori silk has a very specific function since it is used by the caterpillar to build the cocoon in which it pupates. As a result, to ensure proper development of the pupae, the silk has to be very strong and resilient; elasticity in this case can be considered a luxury. The mechanical properties of *B. mori* silk are a tensile strength of 0.5–0.74 GPa, an elastic modulus (Young's modulus) of 5–17 GPa and an extensibility of 19–24% (Denny, 1980; Cunniff *et al.*, 1994; Perez-Rigueiro *et al.*, 2000).

The most interesting spider silks are those produced by the orb weavers *Nephila* and *Araneus* (Araneidae). These spiders possess seven types of glands and are able to produce six types of silks (and a glue) with very different mechanical properties (Denny, 1976; Gosline *et al*., 1984; Gosline *et al*., 1999; Gosline *et al*., 2002) and amino acid compositions (Andersen, 1970; Work and Young, 1987; Lombardi and Kaplan, 1990). Each has a determined function and use (i.e. web building silks, cocoon silk, dragline and swathing silk). The most fascinating and thus best-characterized silks are the dragline or major ampullate silk and the flagelliform or viscid silk. Dragline silks are used for construction of the web frame or as a safety line when the orb weaver spider drops from high elevations and flagelliform silks make up the capture spiral of the web. These silks differ in their repeat structures resulting in different tensile strength and elasticity.

Studies on several major ampullate dragline silks revealed that this fiber is composed of two proteins called spidroins (spider fibroins, MaSp 1 and MaSp 2) that contain glycine-rich and polyalanine repeats (Xu and Lewis 1990; Hinman and Lewis, 1992). X-ray diffraction and NMR spectroscopy studies demonstrated that the polyalanine regions are organized in anti-parallel β-sheets alternating with the glycine-rich amorphous regions (Hirijida *et al*., 1996; Simmons *et al.,* 1996). The polyalanine domains can form non-covalent cross-links or hydrogen bonds between the individual proteins and alternate either with a glycine-rich tripeptide

domain [$(GGX)_n$, MaSp 1] or with a glycine-rich, proline-containing pentapeptide [$(GPGXX)_n$ = GPGGY or GPGQQ, MaSp 2]. The GPGXX motif is predicted to be responsible for the fiber's elasticity (Guerette *et al.,* 1996; Hayashi and Lewis, 1998) and the GGX motif, which can form a small 3_{10} helix or more likely a glycine II (gly II) helix, is predicted to link the highly crystalline and more amorphous regions (Hayashi *et al.,* 1999). The structure of this GPGXX pentapeptide motif resembles the elastin pentapeptide VPGVG and thus may form β-turns. Tandem repetitions of these structures would yield an elastic β-spiral and act as a spring (Hayashi *et al.,* 1999).

Flagelliform silk is rich in glycine and proline (Andersen, 1970) and characterized by the presence of long elastic domains containing a GPGGX elastic motif similar to that found in dragline silk proteins, interspaced with GGX tripeptides. The GGX tripeptides always precede non-repetitive 'spacer' regions that are thought to be involved in cross-linking of the silk fiber (Hayashi and Lewis, 1998; Hayashi and Lewis, 2000). In contrast to dragline silk proteins, flagelliform silks do not contain polyalanine repeat motifs.

Both dragline and flagelliform silks behave like viscoelastic materials. Spider dragline silks (major ampullate silks) are very tough fibers (160 MJ m^{-3}) due to a combination of high tensile strength (1.1–4 GPa), high stiffness or elastic modulus (10 GPa), and high extensibility (35%) (Denny, 1976; Gosline *et al*., 1984; Gosline *et al*., 1999; Gosline *et al*., 2002). Like dragline silk, flagelliform silks are also very tough fibers (150 MJ.m^{-3}), but they are far more elastic than dragline silks (200–270%) and, thus, have a lower stiffness (3 MPa) (Denny 1976; Gosline *et al*., 1984; Stauffer *et al*., 1994). Their extreme elasticity seems to be the result of the marked presence of these long regions containing repeats of several elastic motifs (Guerette *et al*., 1996; Hayashi and Lewis, 1998; Hayashi *et al*., 1999). The mechanical properties of this viscid silk are similar to those of lightly cross-linked rubbers (Gosline *et al*., 1986).

A phenomenon called supercontraction has been observed for some spider silks. Although at room temperature *Araneus diadematus* dragline silk is very stiff, immersion of that silk in water causes a contraction to about 55% of its dry length, a diminution of the elastic modulus and an increase in extensibility (Gosline *et al.,* 1984).

Collagens

Collagens represent one of the most abundant proteins found throughout the entire animal kingdom. Most of the time, these highly insoluble fibers are used to support the framework of many body parts as stress-bearing components. For instance, skins or leathers, cuticles, and connective tissues such as cartilages, teeth, tendons, ligaments, blood vessels and bones contain collagen fibers.

The extracellular protein matrix consists of self-assembled tropocollagen molecules synthesized in specialized cells (fibroblasts). Usually, the individual tropocollagen α-chains contain a GXY tripeptide repeat in which X and Y are

bulky and inflexible proline and hydroxyproline residues, respectively. Each of the individual α-chains adopts a left-handed helix and three helical chains twist together to form the tropocollagen right-handed triple helix. Intermolecular hydrogen-bonding stabilizing the triple helix may occur between glycine and the carbonyl of the X residue present at the same level.

1.3.2 Copolymers: marine mussel byssus threads

Marine mussels (*Mytilus edulis*) have the unique ability to generate protein-based tendons or byssus threads to bond to solid surfaces in wet, saline, and turbulent environments (Pujol, 1970; Waite, 1992). Bonding is rapid and it only takes a mussel five minutes to generate a 2–4 cm thread by injection molding (Waite, 1992). This type of bonding is also permanent and extremely versatile. Such byssus threads or 'beards' are extra-corporeal bundles of tiny tendons which are attached distally to a foreign substrate such as a rock and proximally by insertion of the stem root into the byssus thread retractor muscles.

Unlike regular tendons, byssus threads have a non-periodic microstructure. Two collagen-like polypeptides called Col-P and Col-D, both homotrimers (50 and 60 kDa), exist in complementary gradients along the length of the byssus thread. Col-D dominates at the distal end of the thread or close to the adhesive plaque and Col-P dominates at the proximal end or close to the shell. Both precursors of Col-D and Col-P, called preCol-D and preCol-P, were identified in mussel foot extracts (Coynes *et al*., 1997; Qin *et al*., 1997). A third protein, preCol-NG, may mediate the graded progression of preCol-P and preCol-D between proximal and distal ends (Qin and Waite, 1998).

The preCol-P protein was the first described natural block copolymer containing a hard collagenous domain flanked on either side by soft elastin-like domains. It contains a central region of 435 residues corresponding to the collagenous domain or 146 (GXY) repeats (X = proline or *trans*-hydroxyproline; Y = *trans*-4-hydroxyproline). The (GXY) repeat is interrupted after the eleventh repeat GST by a single missing glycine (G) causing a kink in the molecule and the bending of the triple helix (Coynes *et al*., 1997). An acidic patch of 15 amino acids rich in glutamic acid (E) and aspartic acid (D) is located after the collagen sequence. Elastic domains flank the collagen domain on both sides and could provide solubility to the protein. These domains, dominated by glycine, proline (P) and bulky hydrophobic residues are 'proline-containing pentapeptide repeats' (XXXPX). This pentapeptide resembles the elastin VGVPG pentapeptide in which glycine, proline and the hydrophobic amino acids of elastin are critical for the entropically driven elastic recoil (Coynes *et al*., 1997).

The primary structure of the predicted preCol-D protein consists of four domains symmetrically arranged around a 45.5 kDa collagenous domain (= 175 GXY repeats) that is identified as a type-III collagen because of the high level of glycines. These repeats more closely resemble a GGX repeat rather than a GXY

repeat. This GGX tripeptide destabilizes the triple helix and thus may give more flexibility to the chain in the affected regions (Qin *et al.*, 1997). There are also differences from regular collagens such as glycine substitutions and residue deletions or the presence of unusual amino acids such as tyrosine (Y) and cysteine (C). Apparently, these breaks in the collagen continuity form stable bends or kinks in the triple helical structure since they are resistant to high pepsinization. As for preCol-P, there is a stretch of 20 hydrophobic and acidic amino acids at the end of the collagenous domain and there is also a proline-rich hinge region of 44 residues. Flanking the central collagenous domain of preCol-D are silk fibroin-like domains (dragline silk spidroin 1-like) containing several polyalanine motifs of 11–14 amino acids (2 and 5 repeats in the N- and C-termini, respectively) and GGX tripeptide repeats. Under shear, these polyalanine segments may form β-sheets with expanded sheet spacing because of various amino acid substitutions and the presence of bulky amino acids (R, Q).

Like preCol-D and -P, preCol-NG contains a central collagenous domain, flanking domains, an acidic patch and histidine-rich termini. Its flanking domains resemble the glycine-rich proteins of plant cell walls with tandem $(XG)_n$ repeats (X = A, L, or N but not P). PreCol-NG also has sequences similar to the (GA) and polyalanine runs found in arthropod silks. This protein seems to act as a mediator between preCol-D/-P molecules during assembly (Qin and Waite, 1998). The byssus thread fibers are highly cross-linked. N- and C-termini of preCol-P, -D, and -NG contain histidine-rich regions which may be involved in cross-linking through metal (possibly zinc) complexing (Coynes *et al.*, 1997; Qin *et al.*, 1997). Dityrosine bonding or aryl grouping involving tyrosine (Y) residues in preCol-P and preCol-D also stabilize end-to-end adhesion (Waite *et al.*, 2002).

Each byssus thread is a stiff strong tether at one end and a shock absorber at the other end owing to the graded distribution of tensile molecular elements. Collagen domains may confer self-assembly to all parts of the structure while histidine-rich regions provide cross-linking sites resulting in metal-induced stickiness holding the structure together. The graded distribution of both proteins resulting in the graded axial distribution of elastic elastin-like and stiffer silk-like flanking domains seems critical to moderate the stress concentration of this highly composite structure (Waite *et al.*, 2002). These threads, which are five times tougher than Achilles tendons, can shrink and have a melting temperature exceeding 90 ºC. The presence of the elastin blocks in the Col-P protein may improve the extensibility of the collagen and its tensile stress. As a result, the material exhibits a breaking strength lower than that of tendons (35–75 MPa) but is much tougher than tendons (35–45 MJ. m^{-3}) because it is more extensible (109–200%; Bell and Gosline, 1996).

1.4 Experimental characterization of model fibrous proteins

The characterization of natural fibrous proteins has shown that many are highly

repetitive and contain similar short amino acid structural motifs. The sequence and order of these structural motifs (primary sequence and repeat pattern) impact folding of the protein chain as well as further interaction of the protein with itself and/or others. Therefore, in a macromolecular polymeric material formed by the assembly of similar structural protein chains, those amino acid repeats may be responsible for the physical and mechanical properties of the polymer. It is currently possible to produce proteins of particular specific amino acid sequence and length and there is a great interest to use these technologies to produce sufficient fibrous protein materials for research because of the huge potential for the development of 'bio-inspired or biomimetic materials'.

1.4.1 Production of fibrous protein analogs

Overall, biophysical and chemical data on spider silk (x-ray diffraction, FTIR, NMR and CD) provide a limited understanding of the mechanical properties of spider silks (Hirijida *et al*., 1996; Simmons *et al*., 1996; Parkhe *et al*., 1997; Dicko *et al*., 2004). The ideal situation would allow the use of full-length, native fibrous proteins for direct assessment of structure/function relationships and assembly mechanisms. However, because of the highly repetitive nature and frequent large size of these polypeptides, this has not been possible. As a result, recombinant DNA technologies have been exploited as a way to produce significant amounts of native or tailored fibrous proteins to further investigate these relationships and gain a better understanding of how these molecules perform in the natural protein polymers, including mechanisms of protein self-assembly. In addition, analogs of structural proteins may yield materials with novel properties. Most protein polymers engineered so far have been modeled on natural structural proteins such as elastin, silks or collagen. To be concise, we will focus on the studies relating the genetic engineering of silk-like analogs.

Expression of native silk cDNAs

Several partial dragline silk cDNA sequences have been cloned and expressed to produce native silk analogs. A partial MaSp 1 cDNA from *Nephila clavipes* (1.5 kbp) was expressed in *Escherichia coli* (Arcidiacono *et al.,* 1998) and the 43 kDa His-tagged (polyhistidine-tagged) recombinant protein was produced *in vivo* at low levels (~4 mg of purified protein L^{-2}). However, the presence of truncated proteins was evident on Western-blot analysis suggesting some level of gene recombination in *E. coli*. Other cDNA sequences encoding dragline silk proteins from two spider species (*N. clavipes* MaSp 1 and MaSp 2; *Araneus diadematus* ADF-3) were cloned and expressed in two mammalian cell lines, bovine mammary epithelial alveolar cells/MAC-T or baby hamster kidney cells/BHK (Lazaris *et al.,* 2002). Multimers of the ADF-3 cDNA sequences were also engineered. The secreted silk proteins, ranging from 60 to 140 kDa, were soluble in phosphate

saline buffer, possibly owing to the presence of the native hydrophilic C-terminus, and easily purified. The larger the protein, however, the lower the protein yields. Protein production levels reached 25–50 mg/L^{-1} in BHK cells for the ADF-3 monomer gene and synthetic fibers were obtained by wet spinning (Lazaris *et al.,* 2002). A more recent study reports the expression of small flagelliform and MaSp 1 cDNAs (*N. clavata*) into BmN insect cells (*B. mori*-derived cells; Miao *et al.*, 2006; Zhang *et al*., 2007). This MaSp 1 cDNA was also expressed in transgenic silkworm larvae (Zhang *et al*., 2007). The enhanced green fluorescent protein (EGFP)-MaSp 1 protein (70 kDa) was highly insoluble and had a tendency to aggregate. The recombinant silk protein yields were only 5% of the total proteins in cell culture and 6 mg per transgenic larvae (Zhang *et al*., 2007).

Expression of synthetic silk analogs

Many laboratories also copied consensus repeat sequences of native dragline silk proteins (*N. clavipes* MaSp 1 and MaSp 2) to engineer synthetic silk-like gene replicates for the production of spider silk-like proteins in different expression systems. Synthetic MaSp 2 genes (*N. clavipes*) containing 8–32 repeats of a monomer unit (105 bp) encoding a PGGYGPGQQGPGGYGPCQQGPSGPGS $(A)_8G$ consensus sequence were cloned in *E. coli* (Lewis *et al.,* 1996). The recombinant MaSp 2 protein (16 repeats) was produced at level of 2–10 mg/g^{-1} of wet cells. The pure lyophilized silk protein was dissolved in formic acid (2–4 mg/ml) and extrusion of the protein solution into methanol yielded a solid fiber thread (Lewis *et al.,* 1996). Native MaSp 1 or MaSp 2 sequences (*N. clavipes*) were also used to construct larger inserts using bigger synthetic DNA monomer sequences (303 bp MaSp 1 and 357 bp MaSp 2 monomers) (Fahnestock and Irwin, 1997). Three MaSp 1 synthetic variants (each with native deletion patterns) were constructed. The synthetic MaSp 2 sequences constructed, about 4.8–5.6 kbp (up to 16 monomer tandem copies), were cloned in *E. coli* (Fahnestock and Irwin, 1997). Additional synthetic MaSp 1 genes containing 8–16 repeats of the 303 bp monomer sequences were constructed and cloned in yeast (*Pichia pastoris*) (Fahnestock and Bedzyk, 1997). Using the recombinant genes described above, silk proteins of different sizes were produced. Although longer genes encoding proteins containing up to 3000 residues could be expressed in *P. pastoris,* the protein yields dropped drastically. In contrast to *E. coli,* yeast stable integrants did not show any evidence of truncated synthesis or gene recombination (Fahnestock and Bedzyk, 1997).

Synthetic silk-like genes were also expressed in transgenic tobacco and potato (Scheller *et al.,* 2001). The synthetic spidroin 1-like sequence, engineered by ligation of multiple sequence cassettes flanked by appropriate restriction sites, thus taking into account that the MaSp 1 repeats have slight differences, replicated almost exactly the native MaSp 1 sequence. The 1.8 kbp synthetic MaSp 1 construct, placed under the control of the CaMV 35S viral promoter lacked the

native 3' non-repetitive terminus but contained an N-terminal LeB (legumin) signal sequence to direct the protein into the secretory pathway, a C-terminal ER retention signal (KDEL) and a c-myc tag for immunological detection. MaSp 1 proteins (12.9–99.8 kDa) were produced at a level of 2% of the total soluble protein in tobacco leaves or potato tubers (Scheller *et al.,* 2001).

1.4.2 Production of designer fibrous protein analogs

Several laboratories engineered synthetic spider silk-like gene constructs to specifically investigate the mechanisms of interaction of the individual silk molecules as well as the structural conformations taken by identified key silk amino acid structural motifs. In some cases, the gene engineering, which usually targets protein self-assembly, includes self-assembly trigger sequences in order to better manage protein aggregation.

Homoblock protein polymers made of silkworm silk-like crystalline and flexible blocks were produced in *E. coli* (Capello *et al.,* 1990). DNA sequences encoding the short repeat motifs corresponding to the crystalline region [poly(GAGAGS)] of the *B. mori* silk fibroin alone or containing a non-crystalline region from silk (GAAGY) were first cloned in *E. coli* as monomer units (Capello *et al.,* 1990). Silk-like protein polymers (SLP) were produced and purified from bacteria using a highly concentrated lithium bromide solution (LiBr). Another group designed an additional copolymer, 'SLPF', containing the silk-like repeat GAGAGS juxtaposed to a fibronectin sequence GAAVTGRGDSPASAAGY which was produced in *E. coli* (Anderson *et al*., 1994). The SLPF protein $[(GAGAGS)_9$ GAAVTGRGDSPASAAGY$]_{12}$, had the physical stability of silk and the cell attachment bioactivity of human fibronectin given by the (RGD) triplet. The structures of lyophilized SLP and SLPF proteins were analyzed by wide angle x-ray diffraction scattering (WAXS) and transmission electron microscopy (TEM). WAXS data supported the theoretical x-ray diffraction pattern indicating that dry SLP and SLPF proteins accommodate the same anti-parallel β-sheet crystal structure characteristic of native silks due to their silk component. The overall crystallinity of the SLP polymers is affected when adding non-crystallizing blocks (i.e. GAAGY) to their sequence. The SLPF polymer is semi-crystalline. TEM studies showed that the fundamental unit of the morphology of SLPF is a whisker crystal that can form sheaves (Anderson *et al.,* 1994). The SLP and SLPF polymers were solubilized at concentrations up to 400 mg/ml in LiBr. These synthetic polymers solutions were also spun into fibers.

Another synthetic multiblock ('polymer 1') containing sequences inspired from dragline silk protein sequences was produced in *E. coli* (Qu *et al.,* 2000). The 'polymer 1' produced $((AEAEAKAK)_2AG(GPGQQ)_6GS]_9(AEAEAKAK)_2AG$ (GPGQQ) could spontaneously form a self-supporting macroscopic film as a result of conformational transition (α-helices to β-strands) of segments within the polypeptide. FTIR, CD spectroscopy and ^{13}C NMR showed that the alanine-

containing segments formed α-helices in solution and β-sheets in films (Qu *et al.,* 2000).

Winkler *et al.* (1999, 2000) expressed small genetically engineered spidroin variants (25 kDa) containing methionines (M) or RGYS*L trigger sequences recognized by a cyclic AMP-dependent kinase (cAPK) flanking a penta-alanine motif in *E. coli*. Once oxidized, the methionine residues acted as sterical triggers to prevent β-sheet assembly, thus allowing control over the protein assembly process. The RGYS*L trigger present in the same type of protein served to study the effect of phosphorylation/dephosphorylation on the recombinant protein β-sheet self-assembly capacity (Winkler *et al.,* 2000). Phosphorylation of these analogs on their trigger sequences increased their solubility by disrupting the β-sheets. These approaches demonstrate clever ways to control protein self-assembly chemically or biologically, thus preventing the premature aggregation of genetically engineered silk-like proteins.

Other studies focused on investigation of the structure adopted by the spider silk-like (GGX) motifs. Synthetic MaSp 1 homopolymers or MaSp 1/MaSp 2 block copolymers of variable sizes were expressed in *E. coli* and the His-tagged proteins ranging from 14.7 to 41.3 kDa were purified by IMAC (immobilized metal affinity chromatography) followed by reverse-phase HPLC. CD data on the recombinant silk proteins indicated that two-thirds of the modular proteins adopted β-sheet-structures and the remaining one-third was present as β-turn structures (Prince *et al.,* 1995). These results suggested that the GGX motifs from the MaSp 1 sequences adopted β-sheet structures (Prince *et al.,* 1995). In a later study, a sequence encoding a SCAP 1 polypetide (GLGGQGGGAGQGGYG) was engineered and cloned in *E. coli* (Fukushima, 1998). The SCAP 1 sequence was then condensed to form the SCAP multimers containing 4 to 13 tandem repeats of SCAP 1. All the SCAP sequences were expressed in *E. coli* and purified by IMAC. Typically, protein yields in fermenters decreased with increased molecular weight of the produced proteins (from 5.2 to 1.2 mg/L). Study of lyophilized SCAP 13 (13 repeats) suggested that the glycine-rich region of the protein adopted random coils whereas as in a film cast from formic acid, it adopted β-sheet structures. These results also indicated that the glycine-rich sequence present in MaSp 1 (GGX) adopted β-sheet structures (Fukushima, 1998).

These results, though in agreement with previous data collected by TEM on spider dragline silk fibers (Thiel *et al.,* 1994) are in total contradiction with NMR (Kümmerlen *et al.,* 1996), FTIR (Dong *et al.,* 1991) and REDOR-NMR studies (rotational echo double resonance) (Jelinski, 1998) that all suggested that these GGX segments could not form β-sheets and instead assumed compact helix-like structures. Others also suggested that these GGX segments most likely took on helix-like structures with three amino acids per turn (3_{10}) (Hayashi *et al.,* 1999).

A different MaSp 1/MaSp 2 hybrid sequence was engineered and expressed in *E. coli* (Ouroudjev *et al.,* 2002). This sequence comprised four MaSp 1-like sequences fused to one MaSp 2-like domain [pS(4+1)]. The individual MaSp 1

sequence (38 amino acids) encoded a 16 amino acid hydrophobic segment (polyalanine/(GA)), flanked on each side by 22 amino acid glycine-rich regions $(GGX)_{22}$. The MaSp 2 sequence encoded the $(GPGGX)_2$ polypeptide. Atomic force microscopy (AFM) studies on the synthetic pS(4+1) proteins revealed that the synthetic soluble recombinant proteins spontaneously formed nanofibers characterized by a segmented substructure (Ouroudjev *et al.,* 2002). The model proposed in order to explain the morphology of these nanofibers suggested that the protein molecule folded on itself via arrangement of the four MaSp 1 hydrophobic polyalanine/(GA) segments into anti-parallel β-sheets (crystals) stabilized by hydrogen bonding. The more hydrophilic $(GGX)_n$ segments of MaSp 1 assumed random coils or 3_{10} helices that alternated with the hydrophobic regions. The MaSp 2 sequences formed loops allowing the molecule to fold back onto itself. Thus, in this model, each folded molecule resembles a slab composed of alternating hydrophobic crystalline regions with more hydrophilic glycine-rich regions. Approximately 30 folded slab-like molecules form a 'stack' or 'nanofiber segment' that piles up through hydrophobic interactions of their alanine-containing segments (Ouroudjev *et al.,* 2002). When stretched or drawn, as in the natural silk spinning process, the $(GGX)_n$ segments composing the semi-amorphous matrix of the fiber may undergo a conformational change to more extended structures (3_1 helix or β-sheet) stabilized by newly formed intra- and inter-hydrogen bonds (Ouroudjev *et al.,* 2002).

Additional studies focused on the role and structure of the flagelliform silk-like $(GPGGX)_n$ motif (with X = A, V, Y, or S) that differs slightly from dragline silk MaSp 2 $(GPGX_1X_2)_n$ motif (with X_1X_2 = QQ/GY). The first study produced the recombinant 'polypetide 1' $[(GPGGSGPGGY)_2GPGGK]_{11}$ in *E. coli* (Zhou *et al*., 2001). Structural data of the purified polypeptide 1 analyzed by CD, FTIR and NMR techniques indicated that the GPGGX motif formed small amounts of β-turns in solution and higher amounts in films (dehydrated polypeptide). In a more recent study, two synthetic silk proteins variants ($A1S8_{20}$ and $Y1S8_{20}$, each 60 kDa) were produced in *E. coli* to study the impact of a single amino acid substitution (**X**) in the $(GPGG\mathbf{X})_2$ motif on protein structure and self-assembly (Teulé *et al*., 2007). The $A1S8_{20}$ and $Y1S8_{20}$ proteins contained 20 repetitions of sequences composed of the flagelliform-like motif ($[GPGG\mathbf{X_1}\ GPGG\mathbf{X_2}]_2$, ($\mathbf{X_1}/\mathbf{X_2}$ = A/A or Y/S for the 'A1' or 'Y1' elastic versions, respectively) linked to an MaSp 2-like 'S8' motif {= $[GGPSGPGS(A)_8]$}. Both $A1S8_{20}$ and $Y1S8_{20}$ were heat-stable. CD data indicated that, in aqueous buffers, both proteins went through a heat-inducible β-sheet transition (attributed to the S8 hydrophobic crystalline-forming segment) that was more prominent and only irreversible for $Y1S8_{20}$. The more hydrophobic A1 motif $(GPGG\mathbf{A})_4$ present in the $A1S8_{20}$ protein seemed to favor a more random coil conformation with few 'loose' β-sheets in solution until the reversible structural transition of the S8 motif was induced by heat. The more hydrophilic Y1 motif $(GPGG\mathbf{Y}\ GPGG\mathbf{S})_2$ favored a higher initial amount of stable β-sheet that increased during heating, albeit irreversibly. The A1 and Y1 motifs seemed to adopt different

conformations in solution (random coil and possibly β-turns, respectively). Self-assembly of the pure proteins in aqueous environments was spontaneous for $Y1S8_{20}$ and shear-induced for $A1S8_{20}$ but, in both cases, the surface liquid films formed could be used to generate artificial silk fibers of different properties (Teulé *et al.*, 2007).

1.5 Expression systems available for recombinant fibrous protein production

With the advent of molecular biotechnologies, recombinant protein production is now possible in a variety of prokaryotic and eukaryotic systems. The choice of the host system depends on several factors such as the nature of the protein to be produced (requiring significant *vs.* little post-translational modifications), the purpose of its production (structural characterization *vs.* large-scale production), the mode of production (intracellular *vs.* secreted), and the method of purification. In all cases, a chimeric gene, containing sequences encoding for the desired protein, is introduced in the multicloning site of a plasmid expression vector that is introduced in the host system by transformation (chemical, electroporation, bacterial or biolistically mediated). The vector features required for proper gene expression depend on the host expression system but the simplest ones generally contain (1) a multicloning site, (2) promoter sequences that most of the time are chemically inducible or viral in origin (strong promoters), (3) appropriate 5'UTR/3'UTR flanking the sequence of interest, (4) a selectable trait (usually antibiotic resistance gene) allowing the selection of the transformants, and (5) an appropriate origin of replication for replication of the vector if the gene is not integrated in the host genome.

1.5.1 Prokaryotes

The simplest of all systems is bacteria, in particular *E. coli.* The advantages of bacterial systems include relative ease of handling, rapid rate of reproduction in simple liquid media, and tolerance to harsh treatment conditions. In addition, there are a large variety of inducible expression vectors, specialized bacterial strains that accommodate rare codons leading to enhanced expression levels, and multiple options for affinity purification of recombinant proteins (Makrides, 1996; Hannig and Makrides, 1998; Baneyx, 1999; Hunt, 2005). Moreover, recombinant bacteria grown in bioreactors allow mass production of the desired proteins.

For example, in this type of system, the coding region for a specific protein can be cloned into a plasmid vector under the control of a promoter juxtaposed to a lac operator (part of the lactose operon). In the absence of lactose, a repressor is bound to the operator preventing binding of RNA polymerase to the promoter region thus negatively regulating transcription. Addition of the lactose analogue IPTG (isopropyl-β-D-thiogalactopyranoside) to the culture media causes release of the

repressor and thus transcription of the gene and protein production. Addition of sequences encoding a removable histidine-tag located at the amino or carboxy terminus of the chimeric protein facilitates purification of the proteins by IMAC (Hochuli *et al*., 1987; Hochuli, 1988; Hochuli, 1990).

However, *E. coli* cells have been reported to have difficulties handling repetitive DNA sequences (recombination and deletion). In some cases, truncated products were observed even when expressing relatively short silk-like polypeptides (Arcidiacono *et al.,* 1998). The *E. coli* system was also depicted as limited because of the observed premature termination errors of protein synthesis when attempting to produce proteins containing more than 1000 amino acids (Fahnestock and Irwin, 1997). In other cases, genetic instability of larger silk-like inserts resulted in internal deletion or duplication resulting in the generation of a ladder of protein products (Fahnestock and Irwin, 1997). Further, some studies showed that even though the codon used to engineer synthetic silk genes was optimized to maximize expression levels in *E. coli,* recombinant protein yields were inversely proportional to the length of the multimer sequence (Prince *et al.,* 1995).

As prokaryotes, bacteria are also limited in the type of post-translational modifications that can be performed. While disulfide cross-links can be made, bacteria are unable to perform typical eukaryotic post-translational modifications (secretion signal processing, O- and N-glycosylations, or lipid additions). As a result, in certain cases the protein formed is often improperly folded.

1.5.2 Eukaryotes

Unicellular systems: yeast

Yeast is a simple single-celled eukaryotic organism, which, like bacteria, has a rapid rate of reproduction, can be propagated in simple liquid media in bioreactors, and is suitable for large-scale protein production. The simplicity of the techniques used to genetically manipulate yeast makes this system extremely tractable for eukaryotic expression of designer proteins. Expression vectors are available for both *Saccharomyces cerevisiae* (baker's yeast) and *P. pastoris* (methylotrophic yeast). Moreover, the characterization of secretion signals in *S. cerevisiae* such as the 87 amino acid MAT α-prepro signal peptide (Lin and Cregg, 2001) and its engineering in yeast expression vectors allow the secretion of the produced recombinant protein outside the cell. All secretion signals are cleaved upon targeting of the protein to the secretion pathway and thus do not interfere with the native secondary structure of the protein. Protein secretion is also an option with *P. pastoris*, although secretion of native proteins occurs at lower levels compared with *S. cerevisiae* (Cereghino *et al*., 2002).

Despite the differences in secretion efficiency, the *P. pastoris* system has the advantage of permitting very high culture densities in bioreactors. Designed vectors for the *P. pastoris* system (e.g. Invitrogen) are shuttle/expression vectors

for easy gene engineering in *E. coli* with subsequent transfer to yeast for protein production (intracellular or extracellular). Upon transformation, the linearized recombinant plasmid becomes inserted in the yeast genome by homologous recombination, usually by a single cross-over event, at the alcohol oxidase 1 (AOX1) gene locus.

The sequence of interest is thereby placed under the control of the AOX1 gene promoter and its expression in yeast is induced by the addition of methanol to the culture media (Cregg and Madden, 1988). The native AOX enzyme, required for methanol metabolism, accumulates intracellularly in the peroxisome, where it catalyzes the formation of formaldehyde using methanol as a substrate. Some of the formaldehyde leaves the peroxisome and is further oxidized to generate energy for the growth of this organism while the remaining is integrated in a cyclic pathway to generate cell constituents (Cereghino and Cregg, 2000). The levels of accumulation of AOX can reach 30% of the soluble protein fraction in cells grown in fermenters supplied with methanol. Thus, the *P. pastoris* system produces more significant amounts of protein intracellularly and extracellularly than other yeasts.

Many eukaryotic post-translational modifications are also possible in yeast. For instance, yeast produces at least two kinds of amine oxidases differing in heat stability and substrate specificity, methylamine oxidase and benzylamine oxidase. A *P. pastoris* benzylamine oxidase was characterized and, although this 120 kDa enzyme displayed a wide range of substrates, there was a clear preference for peptidyl lysine. This enzyme was named PPLO for *P. pastoris* lysyl oxidase (Kuchar and Dooley, 2001). Thus, *P. pastoris* is able to cross-link proteins through lysyl residues. Other enzymes such as protein disulfide isomerases (PDI) are also produced in *P. pastoris* (Warsame *et al*., 2001) and, thus, protein cross-linking through disulfide bonds is possible. *P. pastoris* is also able to perform post-translational modifications such as glycosylation. Indeed, evidence for the presence of O- and N-linked carbohydrates to several recombinant proteins was observed (Cereghino and Cregg, 2000). However, the glycosylation mechanisms are not well known and some foreign proteins that are not extensively glycosylated in their native host sometimes end up hyperglycosylated in *P. pastoris.*

This system also seems to be more tolerant in handling repetitive DNA sequences (e.g. silk-like sequence) compared with *E. coli*. In contrast to *E. coli,* stable yeast integrants do not show any evidence of truncated synthesis or silk-like gene recombination (Fahnestock and Bedzyk, 1997). However, although longer silk-like synthetic genes encoding proteins containing up to 3000 residues could be expressed in *P. pastoris,* the protein yields dropped dramatically (Fahnestock and Bedzyk, 1997), similar to that observed in *E. coli* (Prince *et al.,* 1995).

Multicellular systems

Multicellular eukaryotic organisms such as transgenic insects, mammals and plants are capable of expressing engineered protein constitutively or in a tissue

specific manner. Tissue-specific expression allows the accumulation of the recombinant protein in a selected compartment (targeting signals tagged on the proteins), or storage organ like a gland facilitating purification of the protein. Moreover, these systems may provide appropriate post-translational modifications and thus enable more appropriate folding of the most complex proteins.

Insects

Baculovirus-mediated insect cell protein expression systems have been developed for many years and are now commonly used for the expression of foreign proteins in different insect cell lines (O'Reilly *et al.,* 1992; see Hunt, 2005 for review). The availability of a strong polyhedrin gene promoter allows a high level of recombinant protein production in the baculovirus expression system. In addition, this system is capable of mediating a variety of common eukaryotic post-translational modifications (O'Reilly *et al.,* 1992). Though the most traditional and commonly used insect cell lines for exogenous protein production are the Sf9 and Sf21 cell lines, which are derived from *Spodoptera frugiperda* pupal ovarian tissue (Vaughn *et al.*, 1977), a *B. mori*-derived cell line (BmN insect cells) is also available. A recent study reports the use of a newly developed Bac-to-Bac/BmNPV (nuclear polyhedrosis virus) system (Motohashi *et al.,* 2005) to express a small MaSp 1 cDNA (*N. clavata*) into BmN insect cells and transfected silkworm larvaes (Zhang *et al*., 2007; see subsection entitled 'Expression of native silk cDNAs' above). Unfortunately, the 70 kDa recombinant EGFP-MaSp 1 protein was highly insoluble and its yields were extremely low both in insect cells and in transfected larvae. The primary cause for this low yield was attributed to premature aggregation of the silk proteins (Zhang *et al*., 2007).

The engineering of transposon-based gene vectors has allowed the production of several transgenic insects (for review see: Handler, 2001; Atkinson *et al*., 2001; Handler, 2002; Atkinson, 2002; Robinson *et al*., 2004). In Lepidoptera, the *piggyBac* transposable element was instrumental in the development of insect vector systems that were used to generate stable germ-line transgenic *B. mori* silkworms (Tamura *et al*., 2000). Very soon after, the production of fibrous proteins was achieved in insect systems. For instance, a fusion cDNA coding partly for the *B. mori* fibroin L-chain and a partial human type III collagen (helical domain) was successfully expressed in transfected silkworms (Tomita *et al*., 2003). The use of a fibroin L-chain specific promoter to control the fibroin–collagen sequence allowed targeted transgene expression into the silk glands of transgenic silkworms. Unfortunately, this fibroin–collagen fusion protein only represented 3.67% of the total protein extracted from cocoons (Tomita *et al*., 2003).

Mammals

Production of recombinant proteins in mammalian cell cultures is feasible and this system has been extensively used for the production of most currently commer-

cialized monoclonal antibodies (Pollock *et al*., 1999). The use of mammary gland cell cultures allows secretion of the recombinant proteins. All types of mammalian cell culture systems require more delicate handling than simple organisms like bacteria and yeast. In addition, although they can be grown using steel tank bioreactors, large-scale bioreactor production is rather expensive. For instance, at a 10 000 L fermentation scale, the cost of purified antibodies is between 1000 and 2000 US$ per gram (Werner, 1998). Thus, other systems such as transgenic animal and plant systems are being investigated.

Because most cell culture systems are onerous, the use of transgenic animals, more specifically dairy animals (mammary gland expression system), to produce specific proteins, may be more adaptable for low-cost, large-scale production. In this system, the gene encoding the protein of interest is fused to milk specific gene regulatory elements (whey acid proteins, lactalbumin or casein genes) and the transgene is introduced by pronuclear microinjection into fertilized ovules collected from a female donor. The embryos are implanted in the uterus of a recipient female and are carried to term. The resulting offspring are screened and the animals containing the transgene are identified. Once mature, the transgenic animal can be hormonally induced to lactate for collection of the recombinant proteins or bred to generate a herd of transgenic animals for large-scale production (reviewed in Pollock *et al*., 1999). Caprine mammals such as dairy goats have a gestation period far shorter than cattle (5 months versus 9 months) and, thus, are attractive for such large-scale production. Indeed, a doe has an average milk production of 600–800 L per 300 days of lactation. Human antibody expression levels of 14 mg/ml were observed for a female caprine founder versus 5 mg mL^{-1} for its offspring (Pollock *et al*., 1999).

As with any system, there are problems associated with expression of recombinant proteins in transgenic animals, not the least of which being the ethical arguments against this approach. However, there are also technical hurdles in that protein extraction can be difficult, particularly from a high-protein, high-fat environment such as milk.

Plants

Of all the expression systems, plants require little material input and can produce significant levels of multimeric and fully processed recombinant proteins (Hondred *et al*., 1999; Perrin *et al*., 2000;). Transformation systems are available and the use of strong viral promoters such as the cauliflower mosaic virus CaMV 35S promoter leads to the constitutive expression of the transgene in essentially all plant tissues. Moreover, targeting of foreign protein accumulation to special organelles within the cell, such as chloroplasts or endoplasmic reticulum (ER), is possible by adding appropriate signals to the sequence of interest. As an example, plants such as tobacco that are relatively easy to transform and regenerate, were extensively and successfully used for the production of foreign proteins such as human collagen (Ruggiero *et al*., 2000), or growth hormone (Staub *et al*., 2000).

Plants have also been used for the production of antibodies or 'plantibodies'. These plant bioreactors are expected to generate 10 kg of antibodies per acre in tobacco, maize, soybean and alfafa (Larrick and Thomas, 2001). Some reports also show the expression of transgenic proteins in plant seeds of corn (Russell, 1999), soybean (Zeitlin *et al*., 1998), tobacco (Fiedler and Conrad, 1995), and barley (Horvath *et al*., 2000).

Chloroplast transformation is also an option in plant transgenics. As chloroplasts are not normally transmitted through pollen (Daniell, 2002), plastid transformation significantly reduces the risk of gene escape into neighboring wild populations. However, plastid transformation may not be the method of choice for highly repetitive sequences like silks. Since the introduction of transgenes relies on homologous recombination, genetic instability like that seen in bacterial systems may be problematic.

1.6 Artificial material production, properties and performance

We encourage the reader to explore other chapters in this book that are devoted to these subjects.

1.7 Conclusions

Through the continued exploration of the nature, process and production of protein-based biomaterials, a greater understanding of the structure/function relationships of structural protein materials is certain. Through the integration of this knowledge with bio-processing systems and recombinant DNA technologies, there is great promise for future production of bio-inspired materials with novel and desired properties for industrial and medical applications. We have made significant inroads into the understanding of the primary structure of these natural protein polymers and extensive studies have revealed much about their secondary and tertiary assemblies as well. With current recombinant engineering and cellular production systems, large quantities of pre-polymer subunits can be produced and purified. Coupling these living production systems with material spinning technologies will complete the process needed to prove the concept and provide the pipeline for large-scale production of bio-inspired materials. The next chapters in this book address these latter issues and demonstrate that the exciting future of bio-inspired materials is indeed on the horizon.

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2

Purification of protein solutions

S. HARCUM
Clemson University, USA

Abstract: Purification and separation methods currently used for proteins are discussed, with a special emphasis on the application of these techniques to biologically inspired textile proteins. Many natural proteins found in a cell are enzymes that only function when soluble; in contrast, recombinant DNA proteins destined to be textiles will be required to function as insoluble or solid materials. The methods, which have to separate the target protein and purify it without severe losses of material, include filtration and centrifugation for insoluble removal, ultrasonic shock for mechanical cell disruption, chromatographic methods for soluble separations, and drying and crystallization for finishing.

Key words: protein purification, chromatography, recombinant DNA, textile proteins./

2.1 Introduction

Synthetic proteins, with a design founded in biological inspiration, can find applications in textile materials. These 'biologically inspired textile proteins', derived by recombinant DNA techniques, have the potential for precisely tailored characteristics, such as strength, elasticity, solubility, hydrophobicity, size and charge. The variety of characteristics available to biologically inspired textile proteins are due to the ability to determine the amino acid sequence within a protein at the DNA level, and then produce the biologically inspired textile protein in a rapidly growing organism under controlled environmental conditions. Once produced, however, the biologically inspired textile protein will require processing steps to separate it from the proteins the host organism cellular components and purify it before spinning or other fiber formation steps.

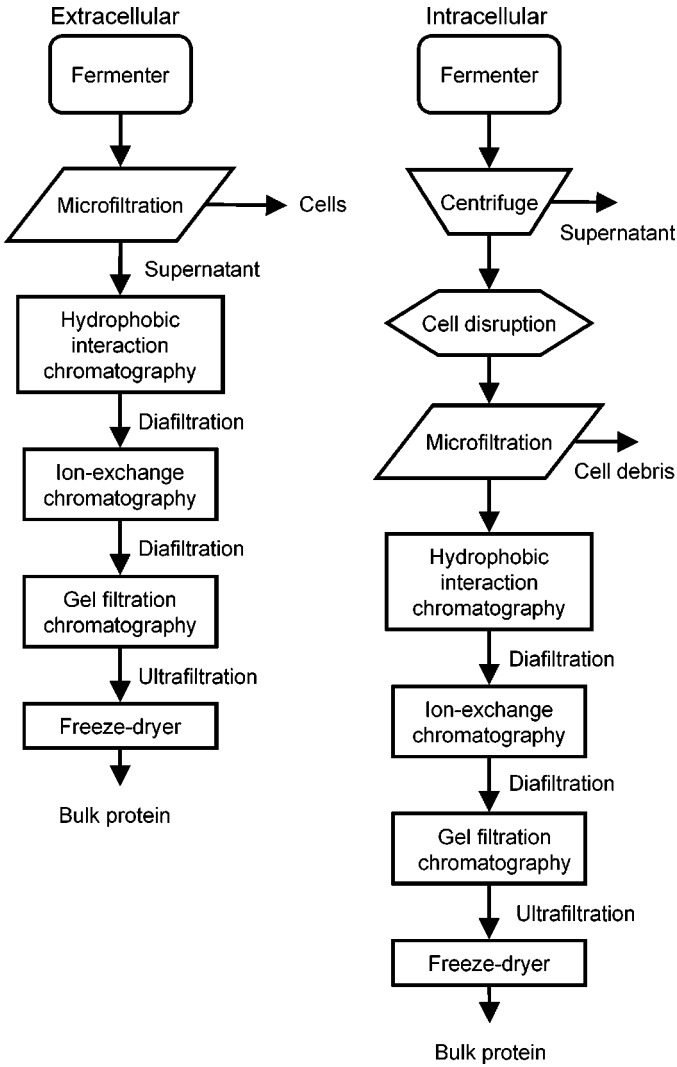
Many natural proteins found in a cell are enzymes that only function when soluble; in contrast, recombinant proteins destined to be textiles will be required to function as insoluble or solid materials. Yet, all proteins begin as soluble proteins inside a cell. Therefore, one of the greatest challenges for biologically inspired textile proteins will be the separation and purification of the target protein without severe losses of material, i.e. low yields, due to premature aggregation.

Therapeutic recombinant proteins, such as recombinant human insulin, are very expensive compared to textiles, on the order of $10 000 per kilogram for

'inexpensive' therapeutic proteins. Fortunately for the healthcare consumer, the amount of recombinant protein needed (a dose) is very small (milligram amounts). Additionally, people are willing to pay significant sums of money for drugs to sustain and improve quality of life. Since biologically inspired textile proteins are also produced by recombinant DNA techniques, the tools needed to separate and purify these proteins will need to rely on the methods developed and refined by the biotechnology industry, where the focus of the traditional biotechnology industry is to obtain a highly purified protein with less regard to costs and yields than will be required or desired for biologically inspired textile proteins.

Recombinant proteins are expressed by the host organism and can remain inside the cell or be secreted by the host, termed intracellular and extracellular, respectively. From a protein purification perspective, the fate of the recombinant protein, intracellular or extracellular, shapes the initial separation and purification steps. Proteins expressed extracellularly are often much easier and cheaper to purify, since steps are not required to separate the desired protein from most of the host cellular components, including host proteins. A host organism can contain up to 1000 different protein species, DNA, RNA, lipids, and polysaccharides. Intracellular proteins are the source for most therapeutic proteins due to the rapid growth characteristics of the bacteria *Escherichia coli*. This rapid growth rate allows for large amounts of protein to be manufactured in a short time; however, the overall protein yield for a typical intracellular therapeutic protein is 5 to 20%, compared with over 50% for extracellular proteins. Additionally, for most therapeutic proteins and many industrial enzymes (including non-recombinant) the separation and purification steps account for over 50% of the total manufacturing costs.

Protein separation and purification methods are loosely grouped by the principle of separation, i.e. size, solubility, or charge. A series of separation and purification methods are used that rely on different principles of separation (termed orthogonal methods) in order to obtain a purified target protein. In general, protein separation methods have one of four basic functions: (1) removal of insoluble material; (2) primary isolation and/or concentration; (3) removal of major contaminants; and (4) final product preparation. Since water is the major contaminant that dilutes the product, process economics usually position the water removal steps early in the purification process. Many of the equipment units used for isolation and contaminant removal are the same, thus the distinction between these steps is often not made. Figure 2.1 shows two examples of flow diagrams outlining the major steps used to purify extracellular and intracellular proteins for therapeutic proteins, which are often injectable drugs with very stringent purity standards. The order of the chromatography steps depends on the characteristics of the target protein and contaminants. For biologically inspired textile proteins, there may be a limit on water removal that is much lower than that allowed for therapeutic proteins, owing to the propensity of textile proteins to precipitate (become a solid) and the difficulties one encounters with re-solubilization, as has been observed with



2.1 Simplified diagram of the major separation and purification steps for extracellular and intracellular recombinant proteins.

natural silk proteins. Additionally, it is anticipated that the number of steps will be lower for biologically inspired textile proteins compared with therapeutic proteins.

2.2 Insoluble removal

Independent of the location of the desired protein (extracellular versus intracellular), the first step after the fermentation step is separation of the cells and culture broth, an isolation step. If the protein is extracellular, the cells are discarded.

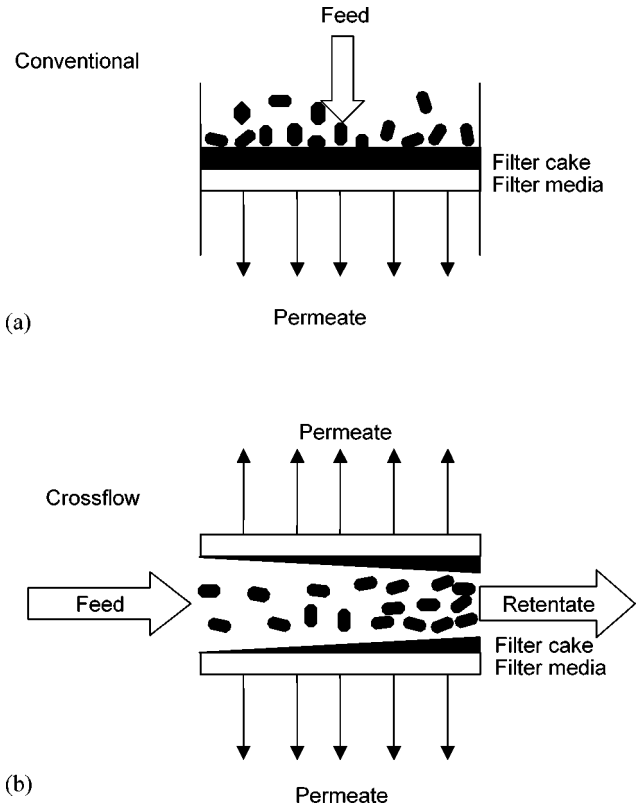
Conversely, if the protein is intracellular, the culture broth is discarded. There are two well-established methods to separate soluble and insoluble materials. One method is based on size differences (filtration) and the second method is based on density differences (centrifugation). A third method, coagulation and/or flocculation, relies on the addition of an agent that causes the cells or other soluble materials to aggregate or precipitate, which then is followed by a size- or density-based separation step. These three methods will be described in more detail below with information regarding the application of the method to biologically inspired textile proteins.

2.2.1 Filtration

The purpose of standard filtration is to remove particles from a solution, where the principle of separation is the size differences between the particles and the fluid. Standard filtration is routinely used for extracellular fermentation products, including antibiotics and industrial reagents, such as citric acid. For recombinant protein fermentations, standard filtration is only used for extracellular proteins. In standard filtration, two fluid flow patterns are commonly used: conventional and crossflow, as shown in Fig. 2.2. For conventional filtration, the flow of the feed, which can be the harvested broth from the ferementer, is perpendicular to the filter media. The permeate stream is the fluid that passes through the filter. In conventional filtration, filter aids are often added to the feed to prevent cake compression. A common filter aid is diatomaceous earth. The addition of a filter aid usually precludes the further use of the cells. For crossflow filtration the flow of the feed is tangential to the filter media, the cells exit the system in the retentate as a concentrated cell slurry, and the permeate exits as clarified culture broth. Since it can be difficult to maintain sterility of the cells deposited on a filter media, if it is desired to save the cells, tangential flow is more desirable. Depending on the amount of material to be processed, continuous filtration versus batch filtration devices can be used for both conventional and crossflow filtration. For biologically inspired textile proteins, an extracellular product is more desirable, as a filtration step to remove cells is relatively inexpensive and results in fewer required purification steps.

2.2.2 Centrifugation

Centrifuges separate solids from liquids based on the density difference between the solid particles and fluid. The solid particles are typically whole cells or cell debris. Centrifuges can be operated in batch or continuous modes. The cells or culture broth can be easily saved for further purification steps. Owing to the small difference in density between cells (<1.1 g cm^{-3}) and the culture broth (1.0 g cm^{-3}), relatively high rotational speeds are used to decrease the equipment operation times, i.e. for a centrifuge spinning at 1000 rpm that requires 5 h to completely



2.2 Schematic diagram of (a) conventional and (b) crossflow filtration.

pellet the cells, the same centrifuge spun at 5000 rpm would only require 12 min. The g-force of a centrifuge depends on the rotational speed and the radius of the centrifuge, where the g-force is proportional to both parameters. Cell debris requires significantly high rotational speeds, often approaching 10 000 *g*. Centrifuges are more expensive to purchase than filtration devices, while operational costs are usually lower. Since centrifuges are closed, retaining the cell pellet or culture broth for further processing is possible. Thus, for intracellular biologically inspired textile proteins, centrifugation or crossflow filtration may be appropriate.

2.2.3 Coagulation/flocculation

Coagulation and flocculation are used in conjunction with filtration and centrifugation to improve the efficiency of these other processes. For example, if the cells to be removed from a culture broth are very small, it is possible that the filter media can become fouled (or clogged) owing to the cells filling the void space in the filter media. However, if the cells can be made to aggregate into larger more rigid particles,

filtration becomes more efficient because of the lower pressure drops required across the filter media. The same principle can be applied to centrifugation, an agent can be added to the culture broth that causes the cells to aggregate with this agent, such that the cell aggregate has a higher density. Centrifugation efficiencies increase proportionally based on the density difference. Coagulation and flocculation agents are normally relatively inexpensive; however, the fate of the cells may preclude some agents. Typically, diatomaceous earth and calcium chloride can be used as flocculation agents. High-molecular-weight, water-soluble organic compounds can also be used; however, waste disposal costs may be higher. Thus, for extracellular biologically inspired textile proteins, coagulation and flocculation may be appropriate alternatives, since the cells are normally discarded.

2.3 Cell disruption

The purpose of cell disruption is to release the product from the cells for intracellular products, such as a recombinant protein. *E. coli* does not secrete recombinant proteins, so recombinant proteins made in this host cell will require a cell disruption step to release the protein into the soluble fraction, such as a biologically inspired textile protein. Yeast strains can be engineered to either secrete or retain a recombinant protein, thus a cell disruption step may be needed. Mammalian cells usually secrete the recombinant protein, such that the cells are usually removed, and not disrupted. Additionally, mammalian cells do not have rigid cell walls like yeast and bacteria, and thus can be disrupted by far gentler treatments, if only intracellular expression can be obtained.

Cell disruption causes nearly all the contents of a cell to enter the soluble fraction along with the desired protein. The isolation and purification steps then must remove host cell proteins, DNA, RNA, lipids, and polysaccharides. Cell disruption methods can be divided into two types: chemical and mechanical. Industrially, mechanical cell disruption methods are preferred because of the lower operational costs and because there is no need to add chemicals or materials to the process that need to be removed at a later stage.

2.3.1 Mechanical

Mechanical cell disruption methods rely on pressure differences between the outside and the inside of the cells to break open the cells by physically moving the fluid around the cells or by moving the cell relative to itself (shearing the cell). The pressure differential can be generated by cavitation (bubble burst), high pressure on the fluid, and grinding (high pressure on cell). High physical pressure on the fluid or cells is more commonly used industrially owing to costs.

Ultrasonic shock

Ultrasonic shock or sonication is a commonly used laboratory-scale method to

disrupt cells. This method uses high frequency sound waves to cause cavitations within the fluid. When a cavitation bubble bursts near a cell, a pressure gradient is generated that disrupts cell. Sonication is very expensive to use because of its low efficiency to generate cavitation, and the subsequent amount of heat generated due to this inefficiency. For heat-susceptible proteins, high levels of cooling are required, which further increases costs. At the laboratory scale (0.5 to 1.5 mL), sonication provides a rapid method of cell disruption. This method is fairly harsh and difficult to scale-up due to the heat removal requirements.

Pressure difference

Pressure difference methods use high pressures (20 to 40 kpsi) to break cells. For laboratory-scale devices (i.e. French presses) the cell slurry is pressurized, then forced through a small orifice with atmospheric pressure on the other side, where the sudden change in pressure causes the cells to burst. Industrial-scale devices pressurize the orifice chamber then the cell slurry is pumped into the chamber and forced out through the orifice to atmospheric pressure. Continous flow devices have a more even pressure drop across the entire run compared with batch devices, since the flow chamber is pressurized to a constant value. The cost of these devices is moderate and the process is fairly harsh. Cooling is required to counterbalance the friction effects of the culture broth moving through the small orifice; however, this cooling load per liter of fluid is significantly lower than sonication. Pressure difference cell disruption may be an appropriate method for intracellular biologically inspired textile proteins.

Grinding

There a number of grinding cell disruption methods, including ball mills, homogenizers, and grinders. All of these grinding methods use a high-speed impeller, glass beads, steel balls, or even flat plates to smash the cells and create shear stresses across the cell. Grinding units vary from laboratory- to industrial-scale. If a bead or ball system is used, the beads and balls must be separated from the disrupted cell debris slurry or cheap enough to discard with the cell debris after a second removal of insolubles step. These grinding methods can be batch or continuous and tend to be moderately harsh. Also, these methods are typically less expensive than pressure difference methods. Grinding methods for cell disruption may be appropriate for intracellular biologically inspired textile proteins.

2.3.2 Chemical (non-mechanical)

Osmotic shock

Osmotic shock relies on the chemical balance that cells maintain with their

environment. Specifically, the osmotic balance cells have with respect to salts and sugars. Normally, cells have higher concentrations of salts and sugars inside the cell than in the culture broth, which causes an osmotic imbalance that is counterbalanced by the cell wall strength. Cells expend energy to bring many small molecules into the cell to maintain their osmotic balance. Also, as part of this osmotic balance, water freely diffuses across the membrane from the high water concentration outside the cell to the lower water concentration inside the cells. Thus, when cells are placed in a solution that has a lower concentration than normal, the net flow of water increases towards the inside of the cell. Eventually, the volume of a cell exceeds the cell wall strength capacity or cell membrane and the cells ruptures. This cell disruption method is inexpensive and gentle. For some very robust cells, osmotic shock is not sufficient to release the cell contents in a timely manner, so combining osmotic shock with freezing, enzyme degradation, or pressure disruption can enhance recoveries. For intracellular biologically inspired textile proteins produced in yeast, osmotic shock alone would not be sufficient to break open the cells.

Enzymes

Enzymes, such as lysozyme, can be used to digest the cell wall of most bacteria. Once the cell wall is digested, the cells burst owing to a weakened cell wall that cannot counterbalance the normal osmotic pressure of the cell. Other enzymes with or without lysozyme can be used to disrupt some bacterial species and yeast in order to enhance yield and/or decrease process time. Freezing and thawing cells treated with enzymes has also been used to improve efficiencies. Two major drawbacks for this method include high costs and the need to remove any enzymes added in later purification steps. For biologically inspired textile proteins, the addition of an enzyme may not be as problematic as it is for therapeutic proteins; however, the effect of the enzyme on the final material properties needs to be assessed to determine if enzymes are appropriate.

Solvent and detergents

Solvent and detergents are more often used with yeast and mammalian cells. Mammalian cells do not have cell wall, but have bilipid cell membrane, where solvents, such as toluene, can be used to dissolve the cell membrane, thus completely disrupting the cell. Acetone is a solvent that has been used to successfully disrupt yeast cells. Detergents that target cell membranes are mainly used for mammalian cell disruption. With solvent methods, the solvent is most often removed by evaporation. Removal of the detergent from the solution can be achieved by most chromatography steps. Solvent and detergent methods are less expensive than enzyme methods; however, they can increase the duration or number of later process steps. The effect of the solvent or detergent addition and

need for removal needs to be assessed with respect to the final material properties of the biologically inspired textile protein.

2.4 Soluble protein separations

Once the majority of the insoluble particles have been removed, the target protein needs to be separated from other soluble host cell materials, such as host DNA, RNA, lipids, polysaccharides, and proteins. The degree of purification is determined by the end-use of the protein. For example, therapeutic proteins have extremely stringent purity standards, whereas industrial enzymes used in laundry detergent have much lower purity requirements. Biologically inspired textile proteins are more likely to have purity standards similar to industrial enzymes; however, material properties may be affected by contaminants necessitating a higher level of purity.

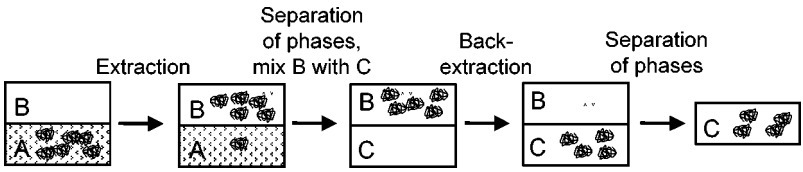
Separation of DNA and RNA from a target protein is usually the easiest separation owing to the highly negative nature of DNA and RNA under physiological conditions. However, this separation can be more challenging if the target protein is also highly negatively charged. Lipids are highly polar species, such that extraction and some chromatography steps can be used to remove this contaminant. Polysaccharides are most often removed by a de-pyrogen chromatography step. The remainder of this section will highlight the principles of the most commonly used separation and purification methods, starting with the least expensive method, extraction, and progressing to the most expensive method, chromatography.

2.4.1 Liquid–liquid extraction

Liquid–liquid extraction is a method of separating molecules based on the solubility of the species present in two immiscible phases, normally both liquids, one usually an organic solvent. The principle of separation relies on the target molecule having different solubility in the two phases, and different solubilities than the contaminants. Thus, the target molecule will be preferential transferred from one solvent to the other solvent with different affinities than the contaminants. Extraction is an equilibrium process, where the partitioning between the phases is defined as K, the partition coefficient, for each species in the system, as shown in Equation 2.1.

$$K = \frac{y}{x} \qquad [2.1]$$

where y and x represent the concentration of the molecule (solute) in the extraction solvent and waste solvent (raffinate), respectively. K is highly dependent on pH, temperature, solvents, and concentration of salts in the solvent phases. The sensitivity of K to process parameters has allowed for 'tuning' of the extraction



2.3 Schematic diagram of an extraction process. Solution B is contacted with Solution A, which contains all the target protein under conditions where the target protein has a greater affinity for Solution B. Solution A is removed and Solution B is contacted with Solution C, which may be the same solvent as was in Solution A. If so, the conditions are different from the first step (i.e. different pH, temperature, or salt concentration). The target protein transfers to Solution C and Solution B is removed.

process to favor the target molecule relative to the contaminant molecules. For example, penicillin was discovered to be highly soluble in an organic solvent at low pH and then almost completely insoluble in the same solvent at high pH. This allowed for the development of an inexpensive penicillin process, where penicillin is first extracted into the solvent at low pH, and then back-extracted into water at high pH. Figure 2.3 shows the basic steps in the extraction process, including the back-extraction step.

2.4.2 Aqueous two-phase extraction

Many proteins are irreversibly denatured by organic solvents, such that traditional liquid–liquid extraction using organic solvent was limited. A second-generation extraction system was developed where the solvents are aqueous, termed aqueous two-phase extraction. This newer extraction method relies on two immiscible solvents that are both aqueous, where one or both of the solvents contain a polymer that alters the solvent's density and solubility characteristics. Poly(ethylene)glycol (PEG)–dextran systems are commonly used to separate such proteins from host nucleic acids (DNA and RNA species) and polysaccharides. By changing the ratio of PEG to dextran, the K value for a particular target molecule can be varied to optimize the separation. The steps shown in Fig. 2.3 also represent the steps used for aqueous two-phase extraction. Owing to the aqueous environment, this method has been used to isolate and purify proteins with little denaturing of the protein. The concentration of the protein in the phases is still relatively low, so this method might be well-suited to biologically inspired textile protein to prevent self-assembly induced by concentration.

2.4.3 Precipitation

Precipitation or 'salting-out' is a relatively inexpensive way to concentrate and purify molecules, including proteins. A concentrated salt solution is slowly added

to a solution of the target solute, the precipitates being removed periodically by filtration or centrifugation. Usually ammonium sulfate is used as the salt. Proteins assume structures in aqueous solutions that minimize the contact of the hydrophobic amino acid residues with the solvent (water), and maximize the contact between the polar amino acid residues and the solvent. Many protein characteristics, such as size, shape, and charge together determine the salt concentration necessary to precipitate a protein. Additionally, environmental factors, such as solvent and temperature, can be used to alter the salt concentration necessary for precipitation. The 'salting-out' concentration for any one protein needs to be determined experimentally. For proteins that are difficult to solublize or re-solubilize, such as many biologically inspired textile proteins, any precipitation should be avoided until contaminants have been removed to sufficient levels, or only used to remove the contaminants by precipitation.

2.4.4 Adsorption

Adsorption, like chromatography, relies on the different affinities solutes have for a solid surface. This is an equilibrium process (Equation 2.2) and can be described by an equilibrium constant (K_{eq}) that is dependent on the concentration of the solute [C], the concentration of empty adsorption sites [S], and the concentration of occupied adsorption sites [CS], as shown in Equation 2.3.

$$\mathrm{C} + \mathrm{S} \xleftrightarrow{K_{eq}} \mathrm{CS} \qquad [2.2]$$

$$K_{eq} = \frac{[\mathrm{CS}]}{[\mathrm{C}][\mathrm{S}]} \qquad [2.3]$$

Since [S] can not be readily measured during the process, various mechanisms have been proposed to describe the process mathematically in terms of measurable parameters. The most commonly used method to describe adsorption for proteins is the Langmuir isotherm method (Equation 2.4). An alternative description is the Freundlich isotherm, which has been used successfully to describe steroids, antibiotics, and hormones adsorption. In the Langmuir isotherm, the total number of adsorption sites S_{total} and the solute concentration determine the amount of solute that can be adsorbed (i.e. recovered).

$$[\mathrm{CS}] = \frac{K_{eq} S_{total} [\mathrm{C}]}{1 + K_{eq} [\mathrm{C}]} \qquad [2.4]$$

Each species in the system has an equilibrium constant. Additionally, species may even have a different number of total sites due to size constraints. The Langmuir equation predicts a saturation value of $K_{eq}S_{total}[\mathrm{C}]$ for [CS], if K_{eq} is small relative to 1. In contrast, the Langmuir equation predicts that all the sites will be occupied if K_{eq} is large for a particular species. Since adsorption is relatively inexpensive, this may be an appropriate separation and purification method for biologically

inspired textile proteins. However, as the high concentration of the biologically inspired textile protein at the surface sites may be a problem, this method may prove to be better for removal of contaminants, rather than the adsorption of the biologically inspired textile protein.

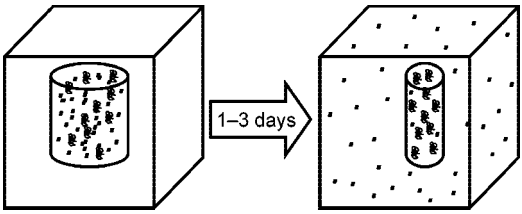
2.4.5 Microfiltration and ultrafiltration

Microfiltration and ultrafiltration operate under the same principle of separation as standard filtration (size); however, the operational characteristics are different due to the very small pore sizes of the membranes used as the filter media. Microfiltration is defined to separate in the range 0.5 to 5 μm, which includes viruses, bacteria, and paint pigments. Ultrafiltration is defined to separate in the range 0.001 to 0.2 μm. Normally, ultrafiltration membranes are specified by the molecular weight that does not pass through the membrane, also called the cut-off molecular weight (i.e. a 10 000 cut-off means that most molecules smaller than 10 kDa will pass through the membrane and most molecules larger than 10 kDa will be retained by the membrane. Thus, ultrafiltration can be use to concentrate protein solutions in a continuous system using a tangential (crossflow) flow configuration.

In ultrafiltration processes a phenomenon called gelling can occur at the membrane due to the high concentration of protein that can develop in the boundary layer just above the membrane. Tangential flow and mixing can help reduce this phenomenon; however, a boundary layer always develops above the membrane. With regard to biologically inspired textile proteins, operation characteristics must minimize this phenomenon in order to prevent self-assembly under the local high concentration at the membrane.

2.4.6 Dialysis

Dialysis is a commonly used laboratory-scale process to remove salt, or reduce the salt concentration, from a solution. A semi-permeable membrane is used to contain the target protein. The target protein in solution is placed into the dialysis tubing and the dialysis tubing is sealed and placed into a large container of water, as shown in Fig. 2.4. The salt in the dialysis tubing moves from high concentration to low concentration via passive diffusion. The final salt concentration in the protein solution is the weighted average of the salt concentration based on the volumes of the starting protein solution and the water. As the volume of the solution in the dialysis tubing decreases, the protein concentration increases. Continuous dialysis is called diafiltration, and the protein solution is diluted with large amounts of water and then passed through an ultrafiltration device to remove both the salts and water. Both diafiltration and dialysis methods can be repeated by adding or contacting, respectively, the target solution with more water.



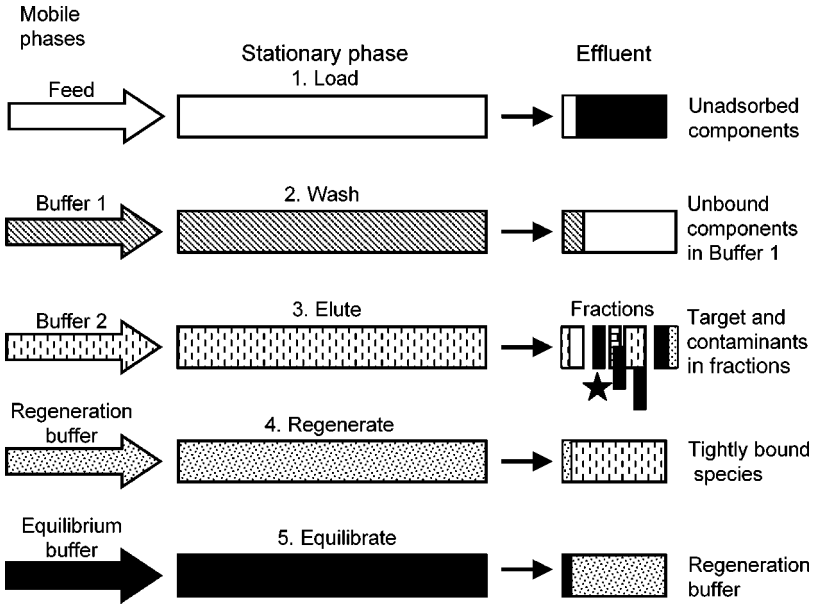
2.4 Schematic diagram for a dialysis system. The proteins (target and other large molecules) are represented by the globular shapes and the smaller molecular species are shown as dots. Initially, all the salt is in the dialysis tubing. After 1–3 days the salt and water exchange through the semi-permeable membrane reaching equilibrium. The salt concentration inside the dialysis tubing and outside the dialysis tubing are equal.

2.4.7 Chromatography

Chromatography is an adsorption process where the solid phase is stationary and packed into a column. The solutes are in the mobile phase and are passed through the column under pressure. The pressure drop in the column due to the frictional effects of flow through a packed bed require that the resins used are sufficiently stabilized to withstand these pressures without collapsing. Differences in affinity for the solid phase of the target protein and contaminants enable separation and purification of the target from other molecules. There are many types of solid surfaces that can be used as the stationary phase including silica, dextran, polyacrylamide, and agarose. Figure 2.5 shows the basic steps of chromatography. The target protein is collected in a fraction during the elute step. Depending on the protein and contaminants, there may be more than one wash, regeneration, and/or equilibrium steps required. Elution chromatography uses buffers with gradient properties in order to improve separations. Example elution profiles for three species are shown in Fig. 2.6.

Adsorption

Adsorption chromatography only differs from adsorption in how the process operates, not the principle of separation. For adsorption chromatography, the adsorbent is used as the stationary phase. The solute binds to the adsorbent via van der Waal forces and steric interactions. Since the adsorption sites are typical only on the outer surface of the stationary phase, fairly small particles are used as the stationary phase. Smaller-particle stationary-phase materials have higher frictional effects and, thus, larger pressure drops during operation. Thus, the stationary phase for adsorption chromatography needs to be able to handle the pressure drop necessary for the mobile phase to flow through the packed-bed column. Typical adsorbents include alumina- or silica-based resins, which are very rigid.



2.5 Schematic of the basic steps of chromatography.

Liquid–liquid partitioning

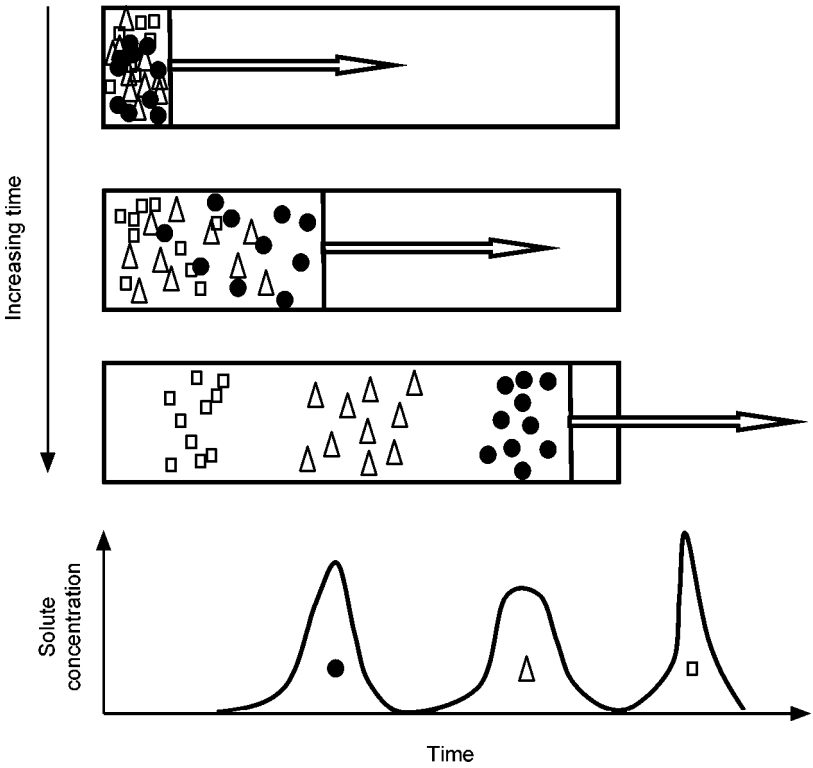
Liquid–liquid partitioning chromatography relies on differences in solubility of solutes in a liquid phase that is adsorbed onto the stationary phase. Most often the adsorbed liquid phase is a non-polar solvent, where the mobile phase is aqueous.

Ion exchange

Ion-exchange chromatography relies on the charge differences of the solutes for separation. The stationary-phase resins have a charged species associated with them that is displaced (or exchanged with) by the solute species. Figure 2.7 shows a schematic of the ion-exchange process for an anion-exchange resin. The negatively charged protein displaces the chlorine anions because of its greater negative charge at the pH of the mobile phase. By changing the mobile phase pH, the charge on the solutes can be manipulated to displace the ion, or so that the solute is displaced by the ion. Because of the sensitivity of a protein to pH, and a processing goal to minimize salt additions to a process, buffers that can adequately buffer at less than 0.05M are commonly used. Example buffers used for ion-exchange chromatography include: Tris, phosphate, acetic acid, and triethanolamine.

Gel filtration (size exclusion)

Gel filtration is a chromatography method that separates molecules by size on the

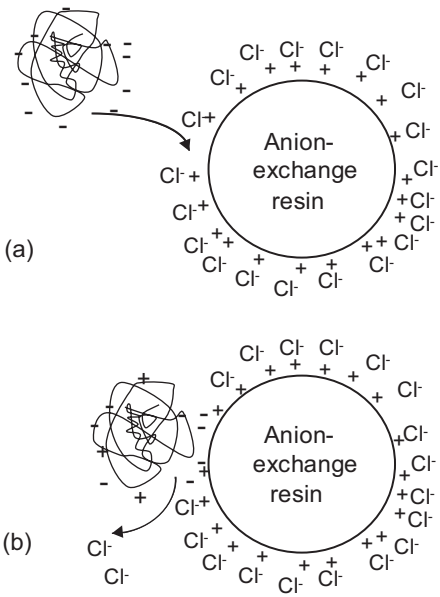


2.6 Schematic of elution chromatography. The three solutes exit the column at different times corresponding to the elution buffer concentration, where the equilibrium favors the fluid over the stationary phase.

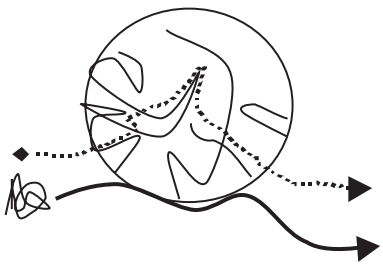
same size-scale as ultrafiltration. Unlike ultrafiltration, the molecules are not retained by a filter media or membrane, but pass through the column packed with a stationary phase resin that is a soft spherical gel particle. The rate at which a molecule travels through the packed bed (stationary phase) is inversely dependent on size, i.e. large molecules exit first. The size exclusion resins are porous, such that small molecules have longer path lengths and, thus, take longer to go through the column. Figure 2.8 shows a diagram of how this system operates at the resin-scale for a small molecule and a protein. For therapeutic proteins, gel filtration is one of the few chromatography methods that does not require an additional step to remove the separating agent from the product stream.

Hydrophobic

Just as all proteins have charge, all proteins have hydrophilic and hydrophobic tendencies. It is because of these hydrophobic characteristics of a protein that the



2.7 Schematic of the ion-exchange process for an anion-exchange resin: (a) negatively charged protein displaces the chlorine anions due to its greater negative charge at the pH of the mobile phase. (b) The elution buffer pH decreases, which causes the protein to become more positively charged so that it is displaced by the chloride ion.



2.8 Schematic of a gel filtration resin with path length for two molecules of different sizes.

target protein can be separated from contaminant proteins. The proteins are adsorbed on to the stationary phase under very high salt concentration. The adsorbed proteins are desorbed as the salt concentration is decreased in order of decreasing hydrophobicity. Salt removal from the target solution is required after this step.

Affinity

Affinity chromatography relies on the specific interactions of a solute with a ligand. The ligand is attached to a support resin. The specific interaction between the solute and ligand is strong, similar to substrate–enzyme interactions. The affinity may be based on molecular size and shape. The forces controlling the interaction can include ionic, covalent, and/or hydrogen bonds. Most commonly, an antibody is used as the ligand, which recognizes the target protein with 'lock-and-key' precision. Affinity chromatography is the most expensive chromatographic method, since often a highly purified protein (the antibody) must also be manufactured before the target protein. Owing to the highly specific binding affinities, affinity chromatography can be used to give highly purified proteins in a single step, if the feed material is relatively free of cellular proteins, such as can be obtained from mammalian cell cultures grown in protein-free media. For biologically inspired textile protein, this method is too expensive to be considered practical or economically viable.

2.5 Finishing steps

Finishing steps are very dependent on the end-use of the protein and the protein characteristics of the final product. For example, some proteins denature under mild heat, so drying has limited use. Other proteins will not form crystal structures due to the amorphous nature of the protein, thus limiting crystallization. Finishing steps do not purify the target protein, however, but often significantly concentrate the protein.

2.5.1 Drying

Drying or precipitating concentrates a solution and transforms the solution into a solid by applying heat to evaporate the solvent, usually water. The solids formed normally have poorly defined morphology and small particles. This method is relatively inexpensive. Since many proteins are susceptible to heat, may have only limited application to protein solutions.

2.5.2 Freeze drying

Freeze drying or lyophilization is basically a drying operation, except the solvent, usually water, is removed by sublimination of the solvent from the solid to the vapor phase. Often a vacuum chamber is used to increase the speed of this process and allows for lower temperatures to be used, a gentler process for heat-susceptible proteins. The resulting solid has a relatively low density and re-dissolves readily back into the solvent. This method has been successfully used on enzymes and bacterial suspensions, where biological activity was recovered once the enzyme or

bacterial were re-hydrated. Freeze drying may be appropriate for biologically inspired textile proteins as a means to concentrate the protein and then re-dissolve it in a solvent appropriate for the fiber formation steps.

2.5.3 Crystallization

Crystallization is a method for transforming a solution into a solid, where a supersaturated solution nucleates the solute by a chemical equilibrium controlled process. Uniform particles with well-defined morphology are formed, and these readily re-dissolve. Crystals tend to be brittle. Amorphous materials are more difficult to crystallize than highly ordered structures. Protein crystallization is more of an art than a science, but does allows researchers to determine the structure of a protein based on x-ray diffraction patterns. Due to the amorphous nature of many of the biologically inspired textile proteins, such as silk, crystallization may have only limited use.

2.6 Conclusions and sources of further information and advice

This chapter briefly covers the field of bioseparations as related to biologically inspired textile proteins. For economic considerations and slightly more in-depth coverage of the various unit operations, see *Bioseparations Science and Engineering* by Harrison *et al.* (2003). *Bioprocess Engineering* by Shuler and Kargi (2002) provides some background biochemistry and cell physiology, as well as detailed descriptions of the various separation and purification methods. An advanced-level, comprehensive coverage of bioseparations can be found in Ladisch's *Bioseparations Engineering* (Ladisch, 2001).

2.7 References

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3

Spinning of fibers from protein solutions

F. TEULÉ
University of Wyoming, USA

Abstract: The development of spinning technologies adapted for the processing of pure silk, collagen or elastin fibrous protein solutions made from natural and artificial fibrous protein sources is outlined. The natural silk-spinning mechanisms of spiders and silkworms are described to stress the physical and physiological requirements paramount to mimic the proper processing and spinning of pure fibrous protein solutions. Examples of the artificial production of collagen, elastin and silk fibers using wet-spinning or electrospinning technologies of pure protein solutions in different solvents as well as the properties of the materials produced are reviewed.

Key words: spinning technology, protein-based fiber, fibrous proteins, spider silk, silkworm.

3.1 Introduction

Fibrous materials are extremely prevalent in nature and their main components are usually polysaccharides or proteins. The industrial production of cellulose-based fibers is already a source of 'natural' polysaccharide-based fibers. Fibrous proteins, however, represent another interesting facet of 'natural' materials that deserves further exploration. The *in vivo* formation of some of these protein-based fibrous materials often involves 'spinning'. As a first step toward 'mimicking' natural protein-based fiber production, a thorough understanding of the native mechanisms of such fibrous protein processing is required. Therefore, the first part of this chapter is devoted to natural spinning mechanisms, where, after citing a few examples of 'spun' and 'non-spun' protein fibrous biomaterials, the *in vivo* silk-spinning mechanisms of orb-weaver spiders and silkworms are reviewed. The second part deals with the possible requirements to realize such artificial spinning of fibers from protein solutions outlining the main obstacles to overcome. Third, the pioneer work and current studies focused on the development of such spinning technologies are examined considering only the spinning from pure protein solutions. Finally, before concluding this chapter, the possible applications of synthetic protein-based fibers and possible future trends in this type of artificial spinning are addressed.

3.2 *In vivo* or natural spinning of protein-based fibers

In this chapter, the word 'spinning' refers to the specific process, performed at will, allowing the transformation of soluble fibrous proteins into an organized fiber or biofilament requiring the use of specialized apparatus. However, in nature, the production of such protein-based fibrous materials may not always require 'spinning'. Yet, biomaterials with exceptional properties are produced and, thus, their protein components are worth investigating as they too represent potential candidates for artificial spinning technologies.

3.3 Protein-based fibrous materials

3.3.1 'Non-spun' materials

Elastin and collagen are particularly versatile and may be the most prominent natural fiber proteins. Both elastin and collagen are found in vertebrates and collagen is also present in invertebrates. For instance, elastins are key components of elastic ligaments, skins or leathers, and blood vessels, whereas collagens are found in bones, cuticles, connective tissues, tendons, teeth, and ligaments. In vertebrates, both elastin and collagen proteins are components of the heavily crosslinked insoluble supramolecular structure known as the extracellular matrix where they assume specific roles (Indik *et al*., 1987; Debelle and Alix, 1999; Wallace and Thompson, 1983; Ushiki, 2002). In the extracellular matrix, the collagen fibrillar system sustains the scaffold of cells and tissues while the microfibrillar elastin system homogeneously disperses stress to preserve the resilience adapted to local tissue requirement (Ushiki, 2002). Interestingly, these protein matrices are formed immediately following synthesis and secretion of the fiber proteins outside specialized cells displaying their ability to spontaneously self-assemble when reaching the extracellular compartment. This aggregation process occurs at several levels. First, nanofibrils form and self-organize into microfibrils leading to fibril formation which eventually yield a higher order structure. Several enzymes participate in this 'self-assembly process' and help stabilize the matrix by crosslinking the molecules and fibrils (Debelle and Alix, 1999; Wilson *et al*., 1998; Edens *et al*., 2001). In the invertebrate group of jawless fish, a different type of self-aggregating fibrous protein can be found in the extracellular matrix. For example, in sea lampreys, some of the cartilaginous structures are composed of collagenous matrices, while others are made of non-collagenous and non-elastin like small proteins called 'lamprins' (Robson *et al.,* 1993). In some insects such as locusts and dragonflies, another kind of elastic fibrous protein called 'resilin' is a component of specialized regions of the cuticle as well as joints and tendons (Weis-Fogh, 1960; Weis-Fogh, 1961). Resilin contains structural motifs resembling those found in elastins and is also crosslinked

through covalent bonds (Andersen, 1964). Its role is to provide long range elasticity to some tissues and, during insect flight, resilin allows energy storage and acts as a damper of vibrations.

Vertebrate muscles also contain numerous fibrous proteins. The giant titin protein, found in sarcomeric filament systems in striated muscles of higher vertebrates, is involved in muscle assembly and provides tissue elasticity (Tskhovrebova and Trinick, 2003).

Finally, keratin proteins also yield heavily crosslinked fibrous materials and are another interesting example of biomaterials. Here again, no obvious spinning mechanism is used but rather a controlled self-aggregation process that, in one case, yields true fibers such as hair or wools while in others forms more compact fibrous structures like nails, horns or quills (Chapman, 1969; Feughelman, 2000).

3.3.2 'Spun' materials

Very few organisms seem to have the ability to deliberately spin fibrous proteins into fibers using specialized spinning apparatus but many arthropods do possess such skills. This spinning process allows for the accumulation and storage of the self-aggregating fibrous proteins in a soluble form until their assembly is initiated to form the final water-insoluble fiber.

Marine mussels for instance attach themselves to rocks and to each other in order to survive in tidal waters using protein-based fiber anchors. These byssal threads or 'beards' are external collagen-based tendon-like structures. These tough holdfasts are 'spun' and glued to a hard surface, underwater by the foot of the mussel (Pujol, 1970; Waite, 1992). Although the nature and the repartition of the proteins making up the byssus are known (Qin *et al.*, 1995; Qin *et al*., 1997; Coyne *et al*., 1997; Qin and Waite, 1998; Waite *et al*., 1998; Coyne and Waite, 2000), the actual spinning process used to generate these byssal threads is not yet fully understood but seems to resemble injection molding (Waite, 1992).

Insects and arachnids also fabricate protein-based fibers at will using spinning apparatus. Prominent examples are silkworms (insects, Lepidoptera) and spiders (arachnids, Araneidae) which are able to spin water-insoluble dry silk filaments from a liquid silk protein solution (or 'silk dope'). These fibers have diameters from 2 to 5 μm for spiders' silks and 10 to 20 μm for silkworms' silks, and are used by these organisms as external gear in their daily life. The self-assembly of silk proteins seems to require some still uncharacterized processing and the use of sophisticated and tightly regulated spinning apparatus to create fibers. Silks are not simply extruded or squeezed outside the organism's body but rather are drawn out by the spider's legs (Foelix, 1996) or by the silkworm's figure-eight-shaped head movements (Akai, 1983). Silks are of great interest because of the wide range of their mechanical properties due to the widespread variety of their uses (Craig, 1997).

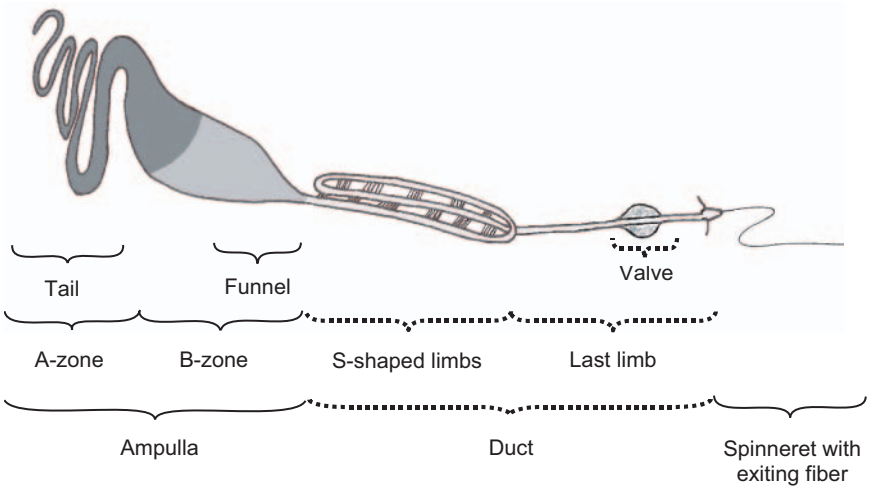
The sequences of the proteins constituting the silks of silkworms (Kusuda *et al*., 1986; Kikuchi *et al*., 1992; Mita *et al.*, 1994; Zhou *et al*., 2000; Datta *et al*., 2001)

and many spiders (Xu and Lewis, 1990; Guerette *et al*., 1996; Hayashi and Lewis, 1998; Colgin and Lewis, 1998; Hayashi and Lewis, 2000; Gatesy *et al*., 2001; Tian and Lewis, 2004) are available. The data suggest that these proteins are highly repetitive, usually contain high amounts of glycine, serine and alanine, and share several structural motifs (hydrophilic amorphous motifs and hydrophobic crystalline forming motifs) that are believed to play specific roles in mechanical properties (Gosline *et al*., 1999; Hayashi *et al*., 1999). For example, dragline and flagelliform silks, two components of orb-weaver spider webs (frame and radius, and catching spiral, respectively), are made of large silk proteins with distinct primary structures (MaSp 1 and MaSp 2, and Flag, respectively) that share similar structural motifs but in different combinations and amounts (Xu and Lewis, 1990; Hinman and Lewis, 1992; Hayashi and Lewis, 1998; Hayashi *et al*., 1999; Hayashi and Lewis, 2000). As a result, dragline and flagelliform silks, both extremely tough fibers (160 and 150 $MJ{\cdot}m^{-3}$, respectively), exhibit different tensile strengths (σ_{max} = 4 and 0.5 GPa, respectively) and elasticities (ε_{max} = 35 and 200%, respectively) specifically adapted to their roles (Gosline *et al*., 1984; Gosline *et al*., 1999; Denny, 1976; Stauffer *et al.*, 1994). In comparison, *Bombyx mori* silkworm silk combines a tensile strength of 0.61–0.74 GPa and an elasticity of 15–24% (Denny, 1980; Perez-Rigueiro *et al*., 2000).

Interestingly, while elastin-, collagen-, and keratin-based fibrous materials are stabilized through a combination of covalent and non-covalent interactions, some silks are only stabilized through non-covalent interactions. Moreover, it is worth mentioning that silks proteins in general are significantly larger proteins than other self-assembling fibrous proteins. The sizes of both dragline protein monomers (MaSp 1 and MaSp 2), estimated by gel filtration, are 300–350 kDa (Sponner *et al*., 2005). The heavy-chain fibroin from silkworm silk (*Bm-Fhc*) is also roughly 350 kDa. However, in silkworm silk, the heavy-chain fibroin is crosslinked through disulfide bonds to the 25 kDa light-chain fibroin (*Bm-FLc*) protein and the *Fhc/Flc* fibroin complex is non-covalently associated to the 27 kDa P25 glycoprotein (Tanaka *et al.,* 1999a; Tanaka *et al.,* 1999b). In comparison, the sizes of collagen protein monomers are usually 30–100 kDa (Kaddler *et al*., 1989; Cox, 1992; Kramer, 1994; Ray *et al*., 1996; VanderEycken *et al*., 1994; Wang *et al*., 1998).

3.4 Silk production in spiders and insects: a natural spinning process

Silk production and spinning processes in both silkworms and spiders are initiated in specialized internal organs or silk glands that are linked to external spinneret structures through essentially what are short or long tapering ducts. The spinnerets are covered by one or more openings functioning as spigots through which the solidified fiber exits. Thus, silk production is achieved under physiological conditions in a regulated aqueous environment, at ambient temperature as well as



3.1 Major ampullate gland spinning system in orb-weaver spiders (*Nephila edulis*). After Vollrath *et al.* (1998) and Vollrath and Knight (1999 and 2001).

low hydrostatic pressure and with relatively slow extrusion/drawing rates. While displaying evident anatomical differences, the silk-producing systems of spiders and silkworms seem to share similarities in their design and mode of operation.

3.4.1 Orb-weaver spiders

Orb-weaver spiders: spinning apparatus

The silk glands are located in the spider's abdomen and the spinnerets are found externally on the ventral side of its lower abdomen (Lucas, 1964; Foelix, 1996). The spinning systems of several spiders have been investigated (Kovoor, 1977; Kovoor, 1986; Kovoor, 1990; Vollrath and Knight, 1999). The most recent reports thoroughly describe the major ampullate spinning system of the *Nephila edulis* (Araneae) orb-weaver responsible for the production of the major ampullate silk used as dragline and web bearing frame (Vollrath *et al.*, 1998; Vollrath and Knight 1999).

In this system, the ampulla is divided in two zones (A and B) lined with secretory epithelium (Fig. 3.1). The A-zone (proximal) is composed of a long tail and the first part of the sac while the B-zone (distal) comprises the widest part of the ampulla up to the funnel (constriction). Previous histochemical data suggested that the nature of the secretions in the two zones differed (Kovoor, 1986) and it was initially thought that most protein secretions in the A-zone constituted the core of the silk fiber. In this zone, tiny spherical viscous droplets are visible which gradually increase in size, most likely coalescing with one another. As they move forward in the ampulla towards the duct region, the growing droplets seem to be

stretched by the elongational flow (Knight and Vollrath, 1999). In the B-zone, the secreted protein mixture seemed to coat the fiber (Vollrath and Knight, 1999). Recent immunological data determined that MaSp 1 and MaSp 2, both dragline silk proteins, are produced and secreted throughout the A- and B-zones of the gland (Sponner *et al*., 2005). Thus, both proteins accumulate in the gland lumen and constitute the core of the fiber while a third protein coats the nascent fiber. In fact, a glycoprotein, detected in both gland (Kovoor, 1977) and thread (Weiskopf *et al*., 1996), may constitute the coating (Vollrath and Knight, 2001) and plasticize the thread by retaining moisture (Vollrath and Tillinghast, 1991). An increasing gradient of peroxidase from the proximal B-zone to the region just before the funnel was also detected though the role of this enzyme remains obscure since silks do not contain any di- and tri-tyrosine linkages. The silk material exits the gland through a funnel and flows into a long narrow S-shaped (first limbs of the duct) tapering duct ending with a valve located a few millimeters before the spinneret (Fig. 3.1). Surfactants like lipids are added in the last stages (last limb of the duct before the valve) of the spinning process. The S-shaped duct is characteristic of major and minor ampullate glands spinning systems while such a valve is only found in the duct of major ampullate glands (Vollrath *et al*., 1998). Owing to the nature of its cuticle, the duct functions like a dialysis tubing, thus silk dope's pH as well as salt and surfactants concentrations allowing water removal, are regulated at different positions along the spinning duct system during native spinning (Knight and Vollrath, 2001; Vollrath and Knight, 2001). Indeed, staining techniques followed by scanning electron microscopy–energy dispersive spectroscopy (SEM-EDAX) demonstrated a decrease in pH from the ampulla tail region to the end of the duct just right before the spigot (7.2 to 6.3). In addition, the presence of a proton pump in the third limb of the duct showed evidence of a final acidic treatment of the material (Vollrath *et al*., 1998). Finally, changes in ion concentrations along the duct are such that there is a decrease in sodium ions (Na^+) concurrent with an increase in the more chaotropic potassium ions (K^+).

Orb-weaver spiders: spinning mechanism

Data from magic angle spinning nuclear magnetic resonance (MAS NMR) indicated that the silks proteins within the sac of the major ampullate gland of *Nephila edulis* [concentration of 30–40% (w/v)] constitute an isotropic phase (Hronska *et al*., 2004). However, at concentrations greater than those existing in the gland, the silk dope behaving as a lyotropic liquid crystal enters a nematic phase (Kerkam *et al*., 1991; Willcox *et al*., 1996; Viney, 1997; Knight and Vollrath 1999; Vollrath and Knight, 2001). During spinning, this lyotropic liquid crystal goes through a gel-like state that is then converted into a dry solid fiber. This liquid crystalline phase plays a rheological role in the spinning process and therefore impacts the mechanical properties of the spun fiber (Viney, 1994; Viney, 1997; Vollrath and Knight, 2001).

This state seems to arise partially because silk proteins are amphiphilic molecules alternating hydrophobic and hydrophilic motifs (Vollrath and Knight 2001; Kerkam *et al*., 1991). Thus, in the aqueous environment of the gland and duct, and in response to appropriate changes in solvent conditions (acidification, and increase in K^+ concurrent with decrease in Na^+), the silk proteins which were in a soluble storage conformation, supposedly a mix of random coils and helices, start to unfold exposing hydrophobic residues. These 'surfacing' hydrophobic residues thermodynamically trigger the coalescence of the silk proteins through a structural transition to crystalline β-sheets resulting in exclusion of water, and thus phase separation (Knight and Vollrath, 2001; Chen *et al*., 2002; Braun and Viney, 2003; Dicko *et al.*, 2004a; Dicko *et al*., 2004b). Coincident with these chemical changes in the dope, stress-induced forces orienting the protein chains are also critical in bringing about this transition leading to phase separation (Vollrath *et al*., 1998; Knight *et al*., 2000; Knight and Vollrath, 2001). Studies relying on Congo red staining determined that the origin of these stress-induced forces was mostly due to elongational flow in the gland and duct rather than shearing from wall friction (Knight *et al*., 2000). Such elongational flow is a direct consequence of the geometry of the gland and duct which combines a funnel giving into a slow long narrowing duct that functions as a hyperbolic die with rapid internal draw-down taper occurring in the third limb of the duct before the valve. Phase separation occurs at the beginning of the draw-down taper where β-sheet formation seems to be initiated, thus it is at this position that a solid fiber thread surrounded by an aqueous phase becomes visible (Knight *et al*., 2000). Moreover, lipids added in the final part of the duct where the draw-down occurs, may also favor protein unfolding in solution acting as plasticizers (Vollrath and Knight, 2001). Evaporation may lead to additional water loss when the spider finally draws the fiber into the air (Vollrath and Knight, 2001; Willcox *et al*., 1996).

3.4.2 Silkworms

Silkworms: spinning apparatus

The domesticated *B. mori* silkworms (Lepidoptera, Bombycidae) possess one pair of silk glands (modified salivary glands) arranged symmetrically on the ventrolateral sides of the mid intestine of the insect (Akai, 1983). These glands are each connected to a short tapering duct. Both ducts eventually unite (Y-junction) into a single large spigot constituting the spinneret mounted on the base of the labium just posterior to the mouth of the insect (Tnau, 2003). A single silk gland contains distinct sections assuming different functions: posterior, median (three regions), and anterior. The posterior region of the gland secretes the silk fibroin dope (heavy- and light-chain fibroin proteins) while the three distinct middle parts

secrete the protein coating the fiber (sericin) and also serve as a reservoir for the storage of the silk fibroin proteins (Kikuchi *et al*., 1992; Mita *et al*., 1994; Grzelak, 1995; Zhou *et al*., 2000; Datta *et al*., 2001). The silk dope moves from the posterior region towards the anterior region of the gland before flowing into the tapering duct. Since both ducts coming from each gland are joined before reaching the single press placed right before the spigot, the silk fiber drawn out of the silkworm's mouth is formed of two individual coated filaments (Magoshi *et al*., 1985a, 1985b; Sehnal and Akai, 1990). The press may act as a restriction die able to regulate diameter (Asakura *et al*., 2007). The pH decreases from the proximal part of the gland towards the end of the duct (from 6.9 to 4.8).

Silkworms: spinning mechanism

Although extensively investigated (Akai, 1983; Magoshi *et al*., 1985a, 1985b; Asakura *et al*., 2007), the spinning process in silkworms is not completely understood. Like spider silk processing mechanisms, studies of *B. mori* native silk dope also suggest the existence of a lyotropic liquid crystalline phase (Magoshi *et al*., 1985a; Nakamae *et al*., 1989; Kerkam *et al*., 1991), as well as shear forces, elongational flow, and existence of a draw-down taper that are able to promote phase separation of the silk dope (Asakura *et al*., 2007). In parallel with these stress-induced forces, additional factors such as changes in protein composition and concentration, pH regulation and changes in ion concentrations (K^+, Na^+ and apparently mainly an increase in Ca^{2+}) (Zhou *et al*., 2000; Zhou *et al*., 2003; Wong Po Foo *et al*., 2006a; Asakura *et al*., 2007) that affect protein conformational transitions and water content also play a decisive role in phase separation leading to fiber formation (Asakura *et al*., 2007). Here again, the lyotropic liquid crystalline silk dope enters a nematic phase in the region of the draw-down taper right before the silk press (Asakura *et al*., 2007). Indeed, the birefringence increases dramatically in the internal draw-down taper suggesting an increase in molecular orientation (Asakura *et al*., 2007). In conclusion, a change in silk protein conformation from soluble silk (silk I) to insoluble silk (β-sheet or silk II) seems to be initiated in the spinning duct before the press at the drawn-down taper site (Asakura *et al*., 2007).

3.5 Elements to consider for the *in vitro* or 'artificial' spinning of protein-based fibers

After reviewing known natural spinning mechanisms involved in silk production, several key factors seem to be critical in promoting fibrous proteins self-assembly and thus should be considered when developing artificial spinning technologies. In this section, the details of the critical factors required in natural spinning are explored and their possible management in the development of artificial spinning technologies is discussed.

3.6 Factors involved in native self-assembly processes

Important factors involved in fibrous proteins self-assembly seem to be protein primary structure, solvent conditions, and, in the case of spun fibers, the design of the spinning apparatus. As mentioned earlier, these soluble fibrous protein monomers possess a self-organization capability, which, under appropriate aqueous solvent conditions and processing, allows for the formation of the final, sometimes crosslinked, insoluble supramolecular structure. Additionally, all of these factors have a noticeable impact on the mechanical and physical properties of the materials formed.

3.6.1 Protein primary structure and choice of solvent

The fact that the primary sequences of these diverse natural fiber proteins rely on a set of specific structural motifs stresses the importance of the role of polypeptide primary sequence. In fact, sequence similarities between many natural fibrous proteins helped to assign structure–function relationships. Hypotheses regarding the secondary structures of certain amino acid motifs comprising some of these fibrous proteins as well as their possible role in the mechanical properties of the final supramolecular matrices or fibers were formulated (Urry *et al*., 1984; Hayashi *et al*., 1999; Tatham and Shewry, 2002). For example, proline-containing pentapeptides found in elastins and some orb-weaver spider silks or poly(A)/poly(GA) [poly(alanine)/poly(glycine–alanine)] motifs identified in silks adopt distinct secondary structures (β-turns/β-spirals or β-sheets, respectively) inherent to their primary structures.

Such types of structural motifs in fiber proteins are thought to grant elasticity or strength, respectively, to the final fiber or matrix produced (Gosline 1978; Urry *et al*., 1986; Gosline, 1987; Urry, 1988; Hayashi *et al*., 1999). Furthermore, as described earlier in the native silk spinning process, the secondary structures adopted by the motifs present in these fibrous proteins are flexible thus may change under different chemical and physical conditions stressing the importance of the solvent and its intricate relationship with the proteins. Indeed, structural transitions resulting in protein conformational changes (refolding) in response to an evolving surrounding environment subjected to various extrinsic factors can be such that a soluble protein may suddenly become insoluble under specific conditions. In the case of spider dragline and silkworm silks, this type of change in protein conformation resulting from the structural transition of poly(A) or poly(GA) motifs from random coil and/or helical to crystalline β-sheets seems necessary to initiate the coalescence of the silk proteins. However, this functional role in self-assembly is not just restricted to alanine-containing motifs.

Recent studies on several elastin structural motifs suggested that specific glycine-rich sequences devoid of proline may behave in an amyloidogenic manner

thus promoting aggregation through structural transition involving β-sheet formation (Rausher *et al*., 2006). In fact, such types of glycine-rich sequences are found in many self-aggregating proteins which may or may not contain any poly(A) and/or poly(GA) sequences. More specifically, some of these glycine-rich sequences found in elastins are also present as $(GGX)_n$ tripeptides in several silks (Colgin and Lewis, 1998; Xu and Lewis, 1990; Hayashi and Lewis, 1998), or as $(GGLGY)_n$ pentapeptides in lamprin proteins (Robson *et al*., 1993; Bocchochio *et al*., 2001). As a consequence, the artificial spinning of fibrous proteins appears to be more challenging than the spinning of a simple uniform chemical polymer because of the complexity and heterogeneity of the polypeptide primary structure and also its behavior in different solvent conditions, creating complex yet critical intramolecular and intermolecular interactions between amino acids of the chains.

3.6.2 Spinning technologies

As mentioned in the case of natural spinning, besides protein sequence and chemical modification of the dope, the design of the spinning device is also fundamental to promote fiber formation. Indeed, additional stress-induced forces caused by elongational flow and shearing help in phase transition and separation in native silk processing (Vollrath *et al*., 1998; Knight *et al*., 2000; Knight and Vollrath, 2001). Thus, applying conventional spinning methods to spin synthetic fibers from aqueous solutions is more challenging. Besides the fact that these spinning techniques were developed for industrial large-scale production of synthetic chemical polymer fibers and thus are not particularly suited for small-scale recombinant production, these systems are somewhat 'closed', consequently, they do not offer the option of progressive chemical modification of the spinning dope to help in structural transitions.

It is also important to keep in mind that in *in vivo* spinning processes, elongational flow and spinning occur simultaneously and are important in promoting molecular orientation. However, in an artificial spinning system, depending on the technique used, elongational flow may or may not occur during spinning consequently extra post-spinning treatments are required to improve the mechanical properties of the 'as-spun' fibers.

3.7 'Mimicking nature'

To generate protein fibers, the starting protein materials used are 'native' if extracted from natural materials (animal tissues, silks) or 'synthetic' if produced as recombinant protein analogs. With recombinant protein production, the fibrous protein gene may be cloned as cDNA or as 'true synthetic' gene (mimetic). Sometimes, the molecular weights of native and recombinant proteins may be different. In the case of silks, the synthetic analogs will most likely be smaller than the native versions and the repeat structure of a true synthetic will be stricter than

that found in natural silks. Synthetic fibers made from smaller analogs may have limited overlaps of molecular chains, thus, fewer possible stabilization interactions and perhaps smaller crystals than those found in native silks. As a result, the mechanical properties will be affected. It is critical to investigate the nature of these interactions in order to design analogs capable of promoting optimized molecular interactions. Usually, native or synthetic materials are available as lyophilized proteins and the first step in artificial spinning comprises solubilizing these in appropriate solvents that will prevent their premature aggregation. Once again, the choice of the solvent may vary depending on the nature and condition of the material.

In developing artificial spinning technologies, it is fundamental to understand how to control the protein unfolding and refolding processes necessary to create the proper protein structural transitions leading to self-assembly. Sequences known to promote 'self-assembly', like crystalline-forming motifs, should be considered when designing synthetic fibrous protein genes. However, the lyotropic nature of these types of molecules renders the handling of the protein solution more difficult due to the tendency of the peptides to aggregate at a specific concentration threshold. Studies showed that addition of chemically or enzymically controlled trigger sequences allowed control over self-assembly of silk analogs (Winkler *et al*., 1999; Winkler *et al*., 2000; Wong Po Foo *et al*., 2006b). The recombinant silk proteins contained polyalanine motifs flanked by methionine (M) redox or phosphate (RGYS*L) trigger sequences. Oxidation of the methionines or the phosphorylation of the RGYS*L sequences increased the solubility of the silk analogs by sterically disrupting β-sheets. Self-assembly was initiated when reversing the state of the triggers (reduction or dephosphorylation). Many studies on silks demonstrated that other chemical factors involved in natural silk spinning could be used *in vitro* to promote self-assembly. Indeed, acidification (Dicko *et al*., 2004c) and increase in concentrations of different cations (Dicko *et al*., 2004c; Chen *et al*., 2002) affected β-sheet transitions in silk aqueous dopes. In addition, temperatures (up to 85 °C) also promote several critical structural transitions in native (Dicko *et al.*, 2004a) and synthetic silk proteins (Teulé *et al*., 2007) required for protein self-assembly in aqueous solutions. For spinning, temperature-induced structural changes, when applicable, would be easier and cheaper to implement than chemical or enzymic processes.

The main available spinning technologies used to spin fibrous protein materials initially relied on 'wet-spinning' (or 'dry-jet' wet-spinning) and more recently expanded to 'electrospinning'. Wet-spinning usually produces single fibers with rather large diameters (tens to hundreds of micrometers) whereas electrospinning, which is cheaper, generates nanometer-scale fibers as non-woven meshes. Thus, depending on the future application of the fibers, one technique may be chosen over the other. It is important to outline that elongational flow fields occur during electrospinning, while no elongational flow forces are present during wet-spinning. Therefore, the nature and extent of the post-spinning treatments necessary to

improve mechanical properties may not be the same in both cases. These post-spinning treatments are essential to improve the overall assembly and internal structure of the 'as-spun' fibers usually by increasing crystallization (dehydration) and crystal orientation (drawing) thus maximizing molecular interactions. Once again, the nature and condition of the material along with the mode of spinning will dictate the types of appropriate post-spinning processes. Other spinning factors such as extrusion rates, choices of dope solvent and coagulation solvents are also important.

Therefore, from one study to another, spinning 'dope' preparations, spinning techniques and conditions, as well as post-spinning processes may differ to suit a particular fibrous protein. Consequently, the mechanical performances of the artificially spun and processed fibers may be highly variable.

3.8 Examples of protein-based fibers produced through artificial spinning technologies

Today, while films and hydrogels are generated from native or recombinant collagens, elastins and silks, spinning fibers from these fibrous proteins is still an area of active research. This section will therefore only focus on the studies reporting protein fiber production from pure protein materials.

3.9 Wet-spinning of fibrous proteins

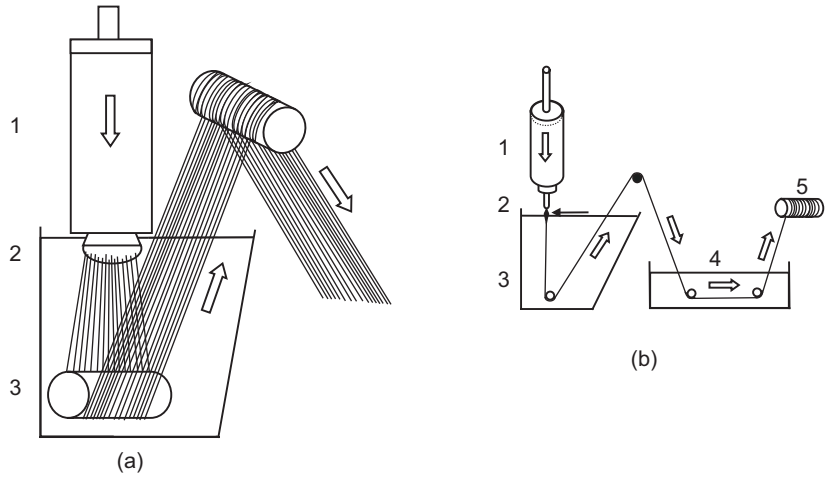
3.9.1 What is wet-spinning?

Wet-spinning is a technology based on extrusion processes and is widely used in industry to produce synthetic and regenerated cellulose fibers. The manufacturing of synthetic fibers based on long-chain synthetic polymers such as acrylic, aramid or spandex, or natural polysaccharide polymers such as rayon (http://www.fibersource.com/f-tutor/techpag.htm) rely on wet-spinning techniques. In this mode of fiber production, the polymer solubilized in a solvent forms a viscous liquid substance which is pushed through the tiny holes of a spinneret in a process referred to as extrusion. The spinneret is immersed into a chemical bath to coagulate the emerging polymer filaments and form solid fibers (Fig. 3.2).

3.9.2 Wet-spinning applied to the production of protein-based fibers

Wet-spinning of collagen fibers

While natural collagen extracts have been used as biomaterials for a long time, only a few reports of successful trials regarding the wet-spinning of collagen fibers may be found in the literature. These studies rely exclusively on the use of



3.2 Wet-spinning techniques. (a) Example of industrial-scale wet-spinning set-up; (1) extruder containing the spinning dope; (2) shower-head type spinneret with multiple openings; (3) chemical coagulation bath. The solidified fibers are taken up by a bobbin. (b) Example of small-scale wet-spinning set-up for artificial production of protein fibers; (1) extruder containing the fibrous protein dope (i.e. syringe type extruder with mechanically or manually controlled plunger); (2) single-spigot spinneret (i.e. hypodermic needle or small peek tubing); (3) coagulation bath with single pully taking up the solid fiber; (4) example of post-spinning drawing treatment performed in solvent. Two godets rotate at different speeds to stretch the fiber (filled arrow); (5) the processed fiber is collected on a winder.
In (a) and (b), the spinning dope is pumped through the spinneret and solidifies upon contact with the coagulation bath (empty arrows). The solid fibers (thin lines) are directed towards the rest of the spinning line [not represented in (a)], where post-spinning processes may be applied (b). The technique used may be 'dry-jet' wet-spinning (b) when the spinneret is not immersed in the coagulation bath (2, black arrow showing air gap). For 'gel spinning', the dope is modified (1) so that its state is no longer a solution but not yet a solid (i.e. gel-like).

extracted natural animal collagen materials or gelatins (hydrolyzed collagen), which can be effectively processed into powders. In a patented aqueous wet-spinning process, collagen aqueous solutions of very low pH (pH <3) were extruded into a coagulation bath containing an inorganic salt (NaCl, $MgCl_2$, $Na_2(SO)_4$, $(NH_4)_2SO_4$, or $Al_2(SO_4)_3 16H_2O$) (Furukawa *et al*., 1994). The dry fiber was consecutively crosslinked by treatment with formaldehyde or glutaraldehyde (Furukawa *et al*., 1994). More recently, another patented procedure described the extrusion of a 0.05 wt% collagen–acetic acid solution into a heated (35 ºC) alginic acid/boric acid bath (pH 8–10) (Fofonoff and Bell, 1999). In this particular case, the neutralization of the acidic dope by the alkaline bath initiated the self-assembly of the collagen fibrils into a fiber. The fibers which were successively dehydrated

by treatments with acetone and ethanol had diameters in excess of 100 μm. Other studies showed that 20 wt% gelatin aqueous solutions could also produce fibers which were dried by heat-treatment (at 150 ºC for 3 h) (Nagura *et al*., 2002). The dry gelatin fibers achieved tensile strengths and initial modulus (σ_{max} = 130 MPa, $E_{initial}$ = 7 GPa) (Nagura *et al*., 2002) comparable to that of some mammalian collagen tendons (σ_{max} = 120 MPa, $E_{initial}$ = 1.2 GPa) (Pollock and Shadwick, 1994).

More recently, dimethyl sulfoxide (DMSO) was investigated as an alternative solvent for gelatin (Fukae *et al*., 2005). In this study, a 10 wt% gelatin–DMSO dope maintained at 60 ºC was extruded into a methanol bath held at –20 ºC in a gel-spinning process. The fibers were drawn immediately at different lengths and consecutively immersed in methanol for one week to remove the solvent before mechanical testing. Unprocessed fibers had low tensile strength and stiffness (σ_{max} = 28 MPa, $\sigma_{initial}$ = 0.7 GPa). While drawn fibers (having a draw ratio, or DR, of 4) had improved mechanical properties (σ_{max} = 81 MPa, $E_{initial}$ = 1.9 GPa), additional crosslinking with gluteraldehyde had little effect on their performance (σ_{max} = 112 MPa, $E_{initial}$ = 1.9 GPa). However, extensive post-spin drawing of the gelatin fibers (DR = 8) drastically improved both their tensile strength and stiffness (σ_{max} = 180 MPa, $E_{initial}$ = 3.4 GPa). X-ray diffraction data showed that drawing induced orientation of pseudo-crystallites present in the fiber (Fukae *et al*., 2005).

Wet-spinning of silk fibers

The availability of degummed silkworm silk materials (silk fibroins) facilitated the regeneration of silks using wet-spinning. In 1993, the first process using wet-spinning to regenerate silk fibers from *B. mori* silks was patented (Lock, 1993). The method relies on the use of silk fibroins resolubilized in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) as a spinning dope, which is extruded through a small needle into a methanol bath. The method was later used to regenerate silkworm silk fibers from 15 wt% silk fibroin-HFIP dopes to study the structural changes occurring during spinning and consecutive post-spinning drawing (Trabbic and Yager, 1998). The fibers were drawn into the air while still wet with methanol (DR = 1–3.5) and dried at constrained lengths (Trabbic and Yager, 1998). Circular dichroism and Raman spectroscopy data indicated that the fibroins which predominantly adopt distorted α-helix conformation in HFIP, mainly transitioned to β-sheet structures in the 'as-spun' fiber due to the methanol treatment. X-ray data showed that while the 'as-spun' fibers were more than 50% crystalline with little crystallite orientation, post-spinning drawing (DR ≥ 2.5) was critical in promoting crystallite orientation close to that observed for the native silk (Trabbic and Yager, 1998). Almost simultaneously, a miniature spinneret allowing the wet-spinning of meters of fibers from solutions containing as low as 10 mg of proteins was constructed using silicon technology (opening = 80–160 μm) (Liivak *et al.,* 1998). This microspinneret was used to spin native silkworm silk and later *Nephila clavipes* dragline spider silk. For silkworm silk, a 2.5 wt% silk-HFIP dope was extruded

into methanol (Liivak *et al.,* 1998) following previous methods (Lock, 1993; Fahnestock, 1994). The fibers, drawn while wet, were successively soaked in methanol overnight before drying and annealing (at 40 ºC for 1 h). The mechanical properties of the regenerated fibers were close to those of the native silk (Liivak *et al.,* 1998). NMR studies indicated that a decrease in aperture size with an increased draw ratio caused an increase in the β-sheet fraction constituted by the alanine domains. Additionally, maximum stresses were higher when both β-sheet fraction and crystal orientation were increased (Liivak *et al.,* 1998). Conformational transition to β-sheet structures and successive molecular orientation of the formed crystallites occurred as a result of these spinning/post-spinning processes (Jelinski *et al*., 1999). For spider silk though, the 2.5 wt% silk-HFIP dope had to be extruded into acetone to regenerate fibers (Seidel *et al*., 1998; Seidel *et al*., 2000). Under SEM, the brittle regenerated fiber appeared spongier than the native spider silk. NMR data of the dry 'as-spun' and water-treated fiber indicated that subsequent water-treatment increased the fraction of alanine residues adopting β-sheet structures (Seidel *et al*., 1998).

Later, the same team used a two-step post-spinning procedure during which the fibers were first drawn into air while wet with acetone, dried at constrained length, and then soaked in water before being stretched again while still wet (Seidel *et al*., 2000). Mechanically, these double-drawn fibers outperformed the single-drawn and as-spun fibers (σ_{max} = 320 MPa, $E_{initial}$ = 8.0 GPa) (Seidel *et al*., 2000). These values, closer to those of native dragline silk, were far superior to the single air-drawn fibers made with synthetic spider silk analogs (Fahnestock, 1994). These experiments established the importance of the role of water in the post-spinning processes. Silkworm silks were also successfully spun using 10 wt% silk fibroin–hexafluoroacetone hydrate (HFA) dopes and methanol as a coagulant (Yao *et al*., 2002). The drawn (DR = 3) and steam-annealed (at 125 °C for 30 min) fibers displayed an initial modulus and a tensile strength comparable with and half that of native silk, respectively (Yao *et al*., 2002).

Although these initial studies using organic solvents are encouraging, their high price and toxicity are problematic for any eventual large-scale fiber production, therefore alternative solvents have been investigated. Recently, silk fibroins were extruded from formic acid (FA) solutions using 13–19% (wt/v) dopes (Um *et al*., 2004; Ha *et al*., 2005), a solvent used to artificially generate a fiber from a recombinant MaSp 2 protein (Lewis *et al*., 1996). The regenerated silkworm silks (drawn while wet) had interesting properties ($\sigma_{max} \approx 1$ GPa, ε_{max} = 30%), yet again demonstrating the impact of fiber processing on mechanical performances (Um *et al*., 2004; Ha *et al*., 2005).

N-Methylmorpholine *N*-oxide (NMMO) monohydrate, a recyclable non-toxic solvent, was used to regenerate silkworm silks with improved mechanical properties (Marsano *et al*., 2005). The 13 wt% silk fibroin–NMMO solution was extruded into ethanol. The data showed that an increase in draw ratios (DR_1/fiber take up = 1–15 and DR_2 = 1–2.7) resulted in a decrease in fiber diameter (130 to 19 μm),

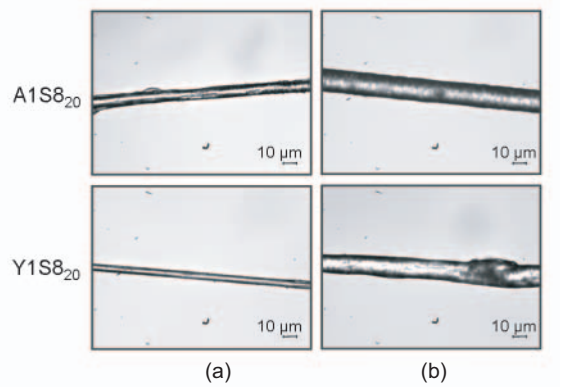
concomitant with increases in birefringence, initial modulus ($E_{initial}$ = 2.6–7.2 GPa), tensile strength (σ_{max} = 43–120 MPa) and elasticity (ε_{max} = 2–35%) (Marsano *et al*., 2005). The regenerated fibers still exhibited initial modulus and tensile strength lower than those of native silkworm silk ($E_{initial}$ = 15–17 GPa, σ_{max} = 610–690 MPa) (Perez-Rigueiro *et al*., 2000) and their birefringence index was half that of native silk (Marsano *et al*., 2005). However, the best processed fibers were more elastic (ε_{max} = 35%) (Marsano *et al*., 2005) than the native silk (ε_{max} = 15%) (Perez-Rigueiro *et al*., 2000).

Finally, a 10 wt% silk fibroin dope prepared with ionic liquids (1-ethyl-3-methylimidazolium chloride) was extruded into methanol. Wide-angle x-ray scattering (WAXS) data showed that the drawn fibers (DR = 2) had more oriented crystallites (Phillips *et al*., 2005).

A patented process describes the first wet-spinning of fibers using recombinant spider silk proteins (Fahnestock, 1994). These silks had lower tensile strength and initial modulus (σ_{max} = 140 MPa, $E_{initial}$ = 4.6 MPa) than those reported for native or regenerated dragline silk (Seidel *et al.*, 2000).

A spider dragline silk cDNAs (ADF-3, or *Araneus diadematus* MaSp 2 protein) expressed in mammalian cells generated enough recombinant ADF-3 spider-silk proteins to spin fibers (Lazaris *et al*., 2002). The 28% (w/v) spinning dope of the 60 kDa ADF-3 rc protein prepared in an aqueous buffer (160 mM urea, 10 mM Na_2HPO_4, 10 mM glycine, 1 mM tris pH 5) was extruded into 70–80% methanol. The double-drawn fibers (drawn in methanol and then water) had the best mechanical properties, though still not as strong or as tough as native dragline silk (Lazaris *et al*., 2002).

Finally, a recent study reports the production, self-assembly characteristics, and wet-spinning of two synthetic spider silk-like analogs produced in *E. coli* (Teulé *et al*., 2007). The basic repeats of these two proteins (60 kDa) contained a Flag-like elastic motif $[(GPGGX_1\ GPGGX_2)]_2$, with X_1/X_2 equal to A/A or Y/S for the 'A1' or 'Y1' elastic versions respectively) adjacent to a MaSp 2-like strength motif {[linker-$(poly(A)_8$] called 'S8'}. The '$A1S8_{20}$' and '$Y1S8_{20}$' proteins were purified by nickel affinity chromatography after heat-treatment (at 80 ºC for 10 min) of the total protein extracts. Circular dichroism (CD) data of melting (0–85 ºC) and successive annealing (85–0 ºC) for both proteins in aqueous buffers (5 mM Tris-HCl pH 8 or 0.1X PBS) showed a heat-inducible β-sheet transition irreversible only for $Y1S8_{20}$. Self-assembly of the pure proteins in aqueous environments was spontaneous for $Y1S8_{20}$ and shear-induced for $A1S8_{20}$. Moreover, 'dipping' forceps into the top layer of the pure protein fractions and pulling away resulted in fiber formation ('hand-pulled') in both cases (Fig. 3.3). Additionally, 25–30% (w/v) $A1S8_{20}$-HFIP and $Y1S8_{20}$-HFIP dopes extruded into 90% isopropyl alcohol generated fibers (Fig. 3.3). The fibers formed in aqueous environments had far better mechanical properties than those made from HFIP suggesting a better fiber internal organization. Within the 'pulled' fibers, the $Y1S8_{20}$ fibers were tougher (10.6 MJ.m^{-3}) and had an elasticity similar to that of dragline silk (ε_{max} = 34%)



3.3 Synthetic spider silk artificial fibre. Light microscope observations of the A1S8$_{20}$ and Y1S8$_{20}$ artificial fibers (Teulé *et al.*, 2007): (a) fibers made from aqueous solutions ('pulled') and (b) fibers made from organic solvents ('as-spun'). Scale as indicated.

while displaying much lower maximum strength ($\sigma_{max} = 50$ MPa) (Teulé *et al.*, 2007) than dragline or flagelliform silks ($\sigma_{max} = 4$ GPa or 500 MPa) (Gosline *et al.*, 1999; Denny, 1976). However, within the HFIP-spun fibers, the A1S8$_{20}$ 'as-spun' fibers performed better than the Y1S8$_{20}$ ones therefore emphasizing the importance of fibrous protein primary structure and fiber formation conditions (Teulé *et al.*, 2007).

3.10 Electrospinning of fibrous proteins

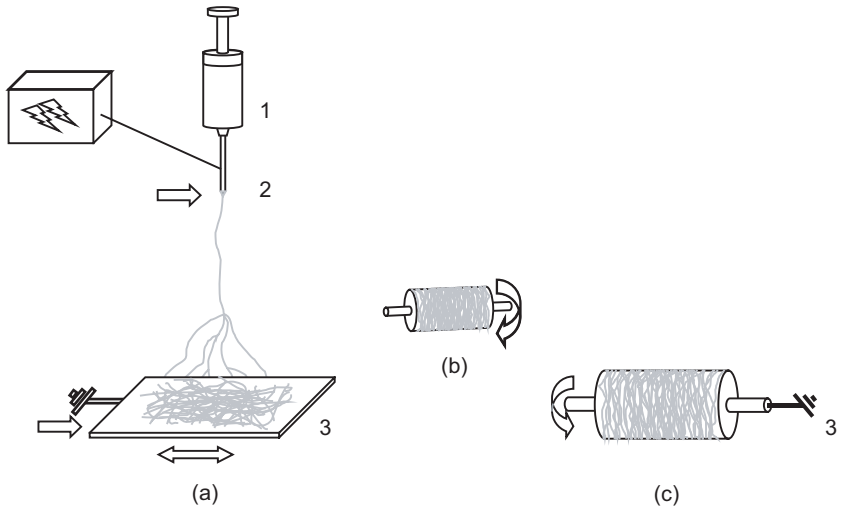
3.10.1 What is 'electrospinning'?

In electrospinning, the polymer solution, supplied through a thin needle positioned opposite a collecting plate or target, is subjected to a high voltage (Fig. 3.4). Once the applied electric field overcomes the surface tension of the solution droplet, a jet forms and travels toward a grounded collecting plate. During this process, as a result of solvent evaporation, the jet thins down into nanoscale fibers that are deposited on the target plate as non-woven, highly porous, meshes (Bowlin, 2002). Using a rotating collector between the needle and the target plates (Xu *et al.*, 2004; Deitzel *et al.*, 2001) or a grounded mandrel (Matthews *et al.*, 2002) allows the collection of aligned fibers.

3.10.2 Electrospinning applied to the production of protein-based fibers

Electrospinning of collagen and elastin fibers

Initial reports suggested that the electrospinning of collagen from very acidic



3.4 Electrospinning techniques: (a) example of a classic electrospinning set up. The spinning dope is pumped through a syringe (1) and exits through a capillary (2), which is connected to an adjustable high-voltage supply (box with lightning arrows). The formed polymer jet thins down, becomes unstable (splitting), and generates a mat of nanofibers collected on a grounded target/collection plate (3). For 'mixing electrospinning', the collection plate moves back and forth (double arrow) between two different polymer jets (second jet not represented) (Kidokaki *et al.*, 2005). (b) A rotating collector may be placed between the tip of the capillary and the target plate in (a) (Deitzel *et al.*, 2001; Xu *et al.*, 2004). (c) Alternatively, a rotating grounded mandrel may serve as a target in (a) (Matthews *et al.*, 2002). Both (b) and (c) allow the collection of more aligned fibers.

solutions such as 1–2 wt% type I collagen from rat tail tendons in HCl (pH 2) was not feasible (Huang *et al.*, 2001). However, increasing the viscosity of this solution with poly(ethylene oxide) (PEO) (such that collagen/PEO = 1:1 or 1:2) and its conductivity with NaCl (34 mM) allowed the non-toxic production of non-woven fiber networks by electrospinning (Huang *et al.*, 2001). The best conditions were when spinning a 2 wt% collagen/PEO (1:1) containing 34 mM NaCl at a flow rate of 100 μm min^{-1} under 18 kV (distance tip/target = 15 cm). Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) pictures showed uniform and perfectly blended fibers with diameters of 100–150 nm thus demonstrating a good PEO/collagen interface. The dry fibers displayed poor mechanical properties (σ_{max} = 0.37 MPa, $E_{initial}$ = 12 MPa) (Huang *et al.*, 2001). A later study also reported the electrospinning of type I collagen from calf skin using the same method (Buttafoco *et al.*, 2006). The dope composed of 5% (w/v) of PEO/2% (w/v) collagen (PEO/collagen = 1:1)/42.4 mM NaCl was spun at a flow rate of 69 μl min^{-1} under 10–25 kV (20–30 cm) producing uniform collagen/PEO fibers

with average diameters of 400 nm. The fibers became insoluble in water once crosslinked by treatment with 1-ethyl-3-dimethyl aminopropyl) carboiimide (EDC) in the presence of *N*-hydroxysuccinimide (NHS) in aqueous 70% ethanol. During this process, the PEO and NaCl totally leached out without disrupting the scaffold (Buttafoco *et al*., 2006).

Organic solvents like HFIP or 2,2,2-trifluoroethanol (TFE) were also investigated. A first study reports the spinning of collagen isotypes from different tissue origins and the collection of aligned fibers using a rotating mandrel (Mathews *et al*., 2002). The best conditions to generate calf skin type I collagen fibers were spinning a 8.3% (w/v) collagen–HFIP dope delivered at 83.4 μl/min in an electric field of 2 k•V cm^{-1} (25 kV). Aligned fibrils 100 nm in diameter were deposited on the surface of the mandrel moving at 1.4 m•s^{-1}. Sheets of spun material exhibited poor mechanical properties (σ_{max} = 1.3–1.7 MPa, $E_{initial}$ = 47.1–57.5 MPa). Under identical parameters, type I collagen from human placenta produced aligned filaments of slightly different structures with diameters of 100–730 nm. However, under these conditions, electrospinning human type III collagen required the use of 4% (w/v) collagen–HFIP dopes. Apparently, the source and collagen isotype and, thus, protein sequence, affect the polymer's structural properties (Mathews *et al*., 2002). A different research group also spun calf skin type I collagen from a 10% (w/v) collagen–HFIP dope delivered at least at 83.34 μl min^{-1} and under 10 kV (15 cm) (Li *et al.*, 2005). In addition, an 8% (w/v) gelatin (bovine)-HFIP was spun using the same parameters. Both produced uniform collagenous mats with fibers of 200–500 nm. The gelatin and collagen fibers had tensile strengths of 8 and 12 MPa, respectively, and elasticities of 8 and 10%, respectively. The collagen fibers were much stiffer than the gelatin fibers ($E_{initial}$ = 262 versus 42.6 MPa) (Li *et al*., 2005) and had improved mechanical properties compared to those published earlier (Matthews *et al*., 2002).

A last study reports the electrospinning of type I collagen (bovine skin) and styrenated (ST) gelatin from HFIP using multilayering and mixing techniques (Kidokaki *et al*., 2005). A 10 wt% ST–gelatin–HFIP dope was spun at a flow rate of 50 μl/min and using 25 kV (15 cm) whereas type I collagen was spun at the same flow rate from a 5 wt% dope and using 15 kV (15 cm). Both non-woven collagenous meshes contained fibers with diameters of 0.2–2 μm. To get insoluble fibers, the collagen fibers were crosslinked by UV light whereas the gelatin fibers were photopolymerized by visible light using camphorquinone. No mechanical data were reported (Kidokaki *et al*., 2005). TFE was used to spin 5–12% (w/v) gelatin (porcine skin) solutions delivered at 13.4 μl/min under 10–12.5 kV (Huang *et al*., 2004). The fiber mats collected contained fibers with average diameters of 100–340 nm. The best tensile strength (σ_{max} = 4.75 MPa) was achieved by fibers from mats spun using a 7.5% (w/v) gelatin–TFE dope. These fibers were also fairly elastic (ε_{max} = 6%) (Huang *et al*., 2004).

The first example of successful electrospinning of elastin was achieved using 5–20 wt% solutions of an 81 kDa recombinant elastin solubilized in deionized water

(Huang *et al*., 2000). Filaments of 0.2–3 μm composed the non-woven meshes which had interesting mechanical properties (σ_{max} = 35 MPa, $E_{initial}$ = 1.8 GPa) (Huang *et al*., 2000).

Later, elastin was also electrospun using soluble bovine α-elastin and a 64 kDa recombinant human tropoelastin (Li *et al*., 2005). Twenty per cent (w/v) α-elastin-HFIP or tropoelastin-HFIP (Martin *et al*., 1995) dopes were spun at a flow rate of 16.7–50 μl/min using 10 kV (15 cm) (Li *et al*., 2005). The fibers were wider and thicker (several micrometers) than those made from collagen or gelatin. The α-elastin fibers were the most brittle ($E_{initial}$ = 184 MPa, σ_{max} = 1.6 MPa, ε_{max} = 1%). The tropeoelastin fibers were as strong (σ_{max} = 13 MPa) as the collagen or gelatin fibers, more elastic (ε_{max} = 15%) than both collagenous fibers and as stiff ($E_{initial}$ = 289 MPa) as the collagen fibers (Li *et al*., 2005).

Soluble bovine elastin was also spun from aqueous solutions composed of 5 wt% elastin/1% (w/v) PEO (elastin/PEO = 1:5)/42.5 mM NaCl delivered at 50 μl/min under 10 kV (25 cm) (Buttafoco *et al*., 2006). The fibers had a rough surface and were 5–10 nm wide with a thickness of 0.5 μm. They had characteristics similar to those of native elastin. The elastin fibers became insoluble in water upon crosslinking and were then PEO- and NaCl-free (Buttafoco *et al*., 2006). The same group also electrospun mixtures of collagen and elastin using PEO and NaCl. Collagen/elastin mixtures of 1–5% (w/w ratios of 2:1, 1:1, 1:2, or 1:3) containing 0.5 wt% PEO and 42.5 mM NaCl were spun at a flow rate of 30 μl/min under 10–30 kV (20–30 cm). The network was formed of perfectly blended and indistinguishable elastin and collagen fibers. The crosslinked insoluble collagen/elastin scaffolds supported the growth of smooth muscle cells (SMC) (Buttafoco *et al*., 2006).

Electrospinning of silk fibers

In 2000, electrospinning allowed the regeneration of spider dragline (*N. clavipes*) and silkworm (*B. mori*) silk fibers with diameters of 100–200 nm and 6.5–100 nm, respectively (Reneker *et al*., 2000). The patented process describes the spinning of 0.23–1.2 wt% dragline silk–HFIP and 0.74 wt% silk fibroin–HFIP solutions at 24–30 kV (15 cm). The fibers were annealed at different temperatures (Reneker *et al*., 2000; Zarkoob *et al*., 2004). TEM and wide-angle x-ray diffraction (WAXD) data indicated that crystal orientation only visible in the annealed fibers occurred at 230–280 ºC and 205–240 ºC for dragline and silkworm silks respectively. However, no mechanical data were reported (Zarkoob *et al*., 2004). Another study reports the regeneration of silks from domesticated (*B. mori*) and wild (*Samia cynthia ricini*) silkworms through electrospinning using HFA-hydrate as a solvent (Ohgo *et al*., 2003). These silks greatly differ in primary sequences and so far the wet-spinning of only *B. mori* fibroins using HFA-hydrate was successful (Yao *et al*., 2002). The fibroin–HFA dopes prepared from *B. mori* and *S. c. ricini* silkworm silks (Ohgo *et al*., 2003) were spun using an electric field of 1 kV/cm and

concentrations of 3 and 10 wt%, respectively. Once dried, the fibers were immersed in methanol to initiate crystallization. During this process and after successive drying of the fibers under vacuum, the HFA-hydrate was completely removed. SEM data revealed that both types of regenerated fibers had diameters of 200–300 nm (Ohgo *et al*., 2003). The ^{13}C crosspolarization (CP)/MAS NMR data showed a structural transition of the alanines from random coil (silk I) in the *B. mori* 'as-spun' fibers to anti-parallel β-sheet (silk II) caused by the methanol treatment. However, the data for the *S. c. ricini* fibers suggested that these structures formed upon drying the fibers, i.e. before methanol treatment. The mechanical properties of the regenerated *B. mori* (σ_{max} = 15 MPa, ε_{max} = 40%) and *S. c. ricini* (σ_{max} = 20 MPa, ε_{max} = 20%) fibers were different (Ohgo *et al*., 2003).

Other research groups investigated the use of formic acid (98–100%) as a solvent to electrospin *B. mori* silk fibers using voltages of 10–50 kV (Sukigara *et al*., 2003; Sukigara *et al*., 2004; Ayutsede *et al*., 2005; Min *et al*., 2004). Initial experimental data showed that using 12–15 wt% silk fibroin–FA dopes and electric fields of 3–4 k/cm regenerated uniform fibers with diameters smaller than 100 nm (Sukigara *et al*., 2003). Later, when a response surface methodology (RSM) approach was applied to the experimental data, a model predicted that electrospinning 8–10 wt% silk fibroin–FA dopes with electric fields of 4–5 kV/cm should produce fibers of diameters smaller than 40 nm (Sukigara *et al*., 2004). Electrospinning 9–15 wt% silk fibroin–FA dopes at electric fields of 2–4 kV/cm (10–50 kV) generated fibers with circular diameters smaller than 100 nm (Ayutsede *et al*., 2005). FTIR, Raman spectroscopy and WAXD data indicated that the regenerated fibers were more crystalline (48%) than native silks (39%). The authors observed that the dissolution of fibroins in FA enhanced β-sheet crystallization and may facilitate β-sheet formation in the electrospun fiber. However, according to WAXD data, the lack of crystallite orientation in the 'as-spun' fiber was ultimately responsible for the poor mechanical properties of the fiber mat produced ($E_{initial}$ = 515 MPa, σ_{max} = 7.25 MPa, ε_{max} = 3.2%) (Ayutsede *et al*., 2005) compared with that of the native fiber ($E_{initial}$ = 15–17 GPa, σ_{max} = 610–690 MPa, ε_{max} = 15%) (Perez-Rigueiro *et al*., 2000). Note that no post-spinning processing was done on these fibers (Ayutsede *et al*., 2005). A last study reports the electrospinning of 3–5 wt% silk–fibroin–FA dopes at 15 kV (7 cm) to regenerate silkworm silk fibers (Min *et al*., 2004). The fibers were successively treated with 50% methanol. SEM data showed circular and smooth fibers with an average diameter of 80 nm, and a wide range of pore sizes desirable for cell attachment. Though no mechanical performance was reported, the fiber mats promoted cell adhesion and spreading of type I collagen (Min *et al*., 2004).

Finally, a different study provides an alternative protocol to electrospin nanometer-scale protein fibers from aqueous silk fibroin dopes using PEO as an additive (Jin *et al*., 2002). Silk fibroin/PEO dopes of concentrations from 4.8–8.8 wt% spun at flow rates of 20–50 μl/ml using electric fields between 0.5–0.6 kV/cm (10–12 kV) generated fibers with diameters averaging 800 nm. FTIR data showed

that silk I was the predominant structure in the 'as-spun' fibers and that successive immersion of the fiber mats in aqueous 90% methanol for 10 min induced β-sheet formation (Jin *et al*., 2002).

Very few studies report the electrospinning of recombinant silk-like proteins to produce fibers. Previous work described the production of the genetically engineered SLPF (silk-like polymer with fibronectin) hybrid protein characterized by the crystalline $(GAGAGS)_9$ segment from *B. mori* silk adjacent to a fibronectin sequence containing the RGD tripeptide promoting cell attachment (Anderson *et al*., 1994). This SFPL polymer was dissolved in 96% FA to prepare 0.8–16.2 wt% dopes that were spun using electric fields of 2–8 kV/cm (Buchko *et al*., 1999). Using concentrations equal or superior to 12.1 wt% resulted in the formation of non-beaded filamentous coatings. Only the SLPF films were later tested and mechanical data showed that they were relatively brittle ($\varepsilon_{max} = 3\%$) (Buchko *et al*., 2000). Later, in a different study, a recombinant silkworm silk protein composed of adjacent *B. mori* crystalline and *S. c. ricini* glycine-rich segments was used to prepare a chimeric silkworm silk–HFA dope that was successfully electrospun (Ohgo *et al*., 2003). SEM observations showed homogenous chimeric silkworm silk fibers with diameters of 100 nm though no mechanical data were obtained.

3.11 Applications

Such synthetic protein fibers with customized mechanical properties represent important potential alternatives as more natural materials for several medical, civil and military applications. In the medical field, provided they are biocompatible, these protein-based fibers could be used as strong suture threads, or artificial ligaments. Additionally, the architecture of the produced electrospun meshes which naturally resemble that found in most extracellular matrices make these materials suited for use as protein-based scaffolds for tissue and bone regeneration. Ropes, fabrics or filtering systems are some examples of possible civilian uses while antiballistic gear, strong light-weight gear, parachutes and harnesses constitute possible military applications.

3.12 Future trends and conclusions

Studies show that the spinning of native or synthetic fibrous protein materials is feasible and may soon generate a new source of natural materials on the market. However, to achieve this goal, the spinning technologies adapted to the creation of customized high-performance protein fibers need to be further developed. This may require a more complete knowledge of the behavior of the individual protein materials used in solutions and deeper investigations of the necessary protein structural transitions or refolding processes that are crucial to controlling proper self-assembly. Cleverly designed synthetic protein analogs can be produced to help determine the molecular mechanisms that confer strength and/or elasticity.

However, further investigation is needed to optimize the primary structures of model fibrous proteins as well as the processing of the produced synthetic analogs. Moreover, since these protein-based fibers are designed to be 'more natural' materials, the ultimate goal is to produce synthetic fibers using innocuous processes. Therefore, research efforts in fiber production may shift mostly toward the use of aqueous non-toxic and non-polluting solvents instead of harsh organic solvents. Non-toxic recyclable organic solvents such as NMMO still seem to be appropriate for the production of synthetic protein fibers as demonstrated in the case of regenerated silk fibers (Marsano *et al*., 2005).

The use of non-toxic, ultimately removable, chemical polymers such as PEO in blends with fibrous proteins has proven to be a way to produce fibers in aqueous conditions (Jin *et al*., 2002; Buttafoco *et al*., 2006). Although synthetic protein fibers have been generated, their mechanical performances need to be improved to become viable products. An alternative way to modify the mechanical and chemical properties of synthetic protein fibers is to blend fibrous protein materials with other known natural non-protein fibrous materials such as polysaccharides. Examples of such biofiber blends are the production of synthetic 'natural' fiber blends of silk fibroin/chitin (Park *et al*., 2006) or fibroin/cellulose and fibroin/chitin/cellulose (Hirano *et al*., 2002). In another case, the blending of collagen with chitosan allowed the production of fibers with improved blood biocompatibility thus allowing their possible use in wound dressing treatments (Hirano *et al*., 2000). Recently, a novel chimeric fusion protein containing silk and silica-forming domains was used to create new silk–silica films and nanoscale protein fibers in aqueous conditions thus opening the door to a new kind of interesting biomaterial (Wong Po Foo *et al*., 2006b). All of these aspects of natural protein fiber production are still currently under active research. This ever-expanding field offers exciting and promising possibilities for the creation and exploitation of more natural yet smarter biological materials.

3.13 References

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4

Biomimetic principles of spider silk for high-performance fibres

C. HOLLAND and F. VOLLRATH
University of Oxford, UK

Abstract: The desirable properties of silk and the relationships between structure and composition are explored. Fibres spun from feedstocks with identical rheology have differing mechanical characteristics. However, supercontraction has linked the role of an individual amino acid, proline, to the mechanical properties of dragline silks. The practical difficulties in matching feedstock chemistry with the appropriate extrusion/spinning conditions for copying native silks, spider and silkworm, are discussed. Both processing conditions and protein composition are important. Producing commercial silk the spider's way would give excellent fibres through an ecologically compatible process.

Key words: silk, spider, silkworm, supercontraction, rheology.

4.1 Introduction

In order to mimic or copy silk we must first understand it. Understanding means not only knowing the relevant protein motifs but also knowing their function and, importantly, their structure–property relationships. This is where the gap is in our present knowledge. Silk proteins have been patented by many research groups and companies and been expressed in bacteria, plants and animals (Arcidiacono *et al.*, 2002; Fahnestock and Bedzyk, 1997; Scheller and Conrad, 2005; Menassa *et al.*, 2004; Lazaris *et al.*, 2002; Karatzas *et al.*, 2005; Huemmerich *et al.*, 2004). But no one, to our knowledge, has succeeded in successfully configuring, i.e. spinning, those proteins into anything resembling the natural fibre, either in its microstructure (which is rather complex) or in its mechanical properties (which are outstanding) (Matsumoto *et al.*, 1996; Shao *et al.*, 2003; Madsen *et al.*, 1999; Marsano *et al.*, 2005; Lazaris *et al.*, 2002; Xie *et al.*, 2006; Liivak *et al.*, 1998; Yao *et al.*, 2002; Zuo *et al.*, 2006; Seidel *et al.*, 2000). The difficulty in creating fibres lies as much in the correct processing conditions, as in the correct protein composition, as we shall discuss in this chapter.

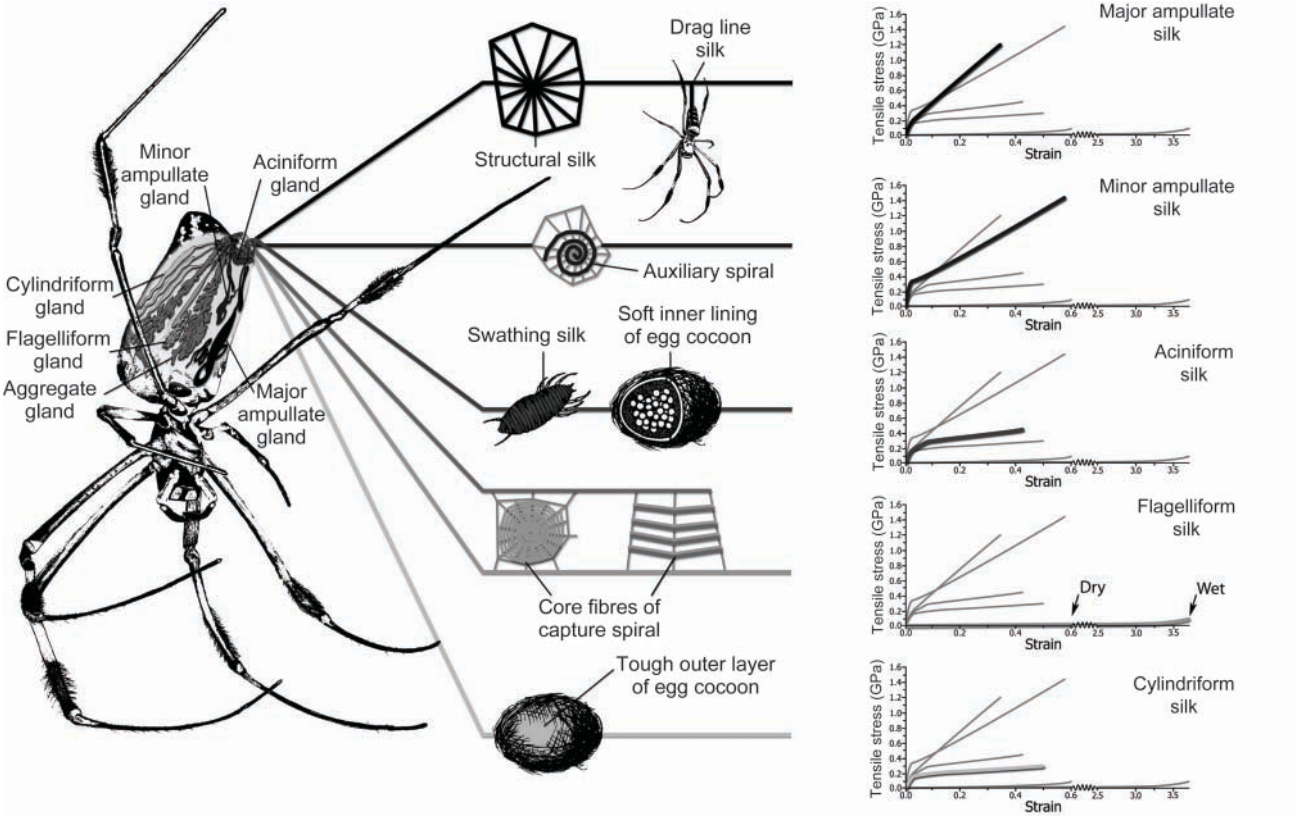
Silks have evolved over hundreds of millions of years and are adaptations to very particular selection pressures (Craig, 1997). Thus, briefly examining the natural function of a particular silk will reveal interesting features about its internal structure which is, after all, what we are trying to copy biotechnologically. Spider silks have

excellent mechanical properties, which overall tend to be better than those of any insect silk known (Vollrath and Knight, 2001). For spiders, the use of silk to catch prey has developed in a vigorous 'arms race' with insects during their 400 million years or so of co-evolution (Foelix, 1996). Therefore, both the architecture of the web-trap structure and the building materials, the spider silks, have experienced aeons of very strong optimising selection pressure for toughness (Vollrath and Knight, 2001). On the other hand, insect silks, specifically silk moth silks, are under very different selection pressures, i.e. to build a cocoon in which to pupate from the caterpillar into the moth or butterfly imago (Fedic *et al.*, 2002). For such an 'eggshell', the integration of the fibres into a strong composite is more important than individual fibre toughness itself and, indeed, the structure–function properties of silkworm silks are well matched to this specific task (Zhao *et al.*, 2005).

Micro-morphological studies on the silk production system of the spider reveal that it differs slightly from the Lepidoptera (Kitagawa *et al.*, 2001; Frische *et al.*, 1997; Thiel *et al.*, 1994; Poza *et al.*, 2002). Unlike Lepidopteron insects, which tend to spin a single fibre issuing through the mouth from a merged pair of huge glands, spiders tend to have a whole battery of silks, produced by a multitude of glands, between which they can 'choose' (Foelix, 1996). Every one of these silks has evolved for a particular task, or set of tasks (see Fig. 4.1). This manifests itself in the wide variation of material properties exhibited by different silks, thus serving as excellent indicators of structure–function relationships (Vollrath and Knight, 2001).

Although there are many different types of spider webs and silks, we shall focus our examination on the dragline of a few species (but mainly the golden orb-weaving spiders of the genus *Nephila* sp.), and the comparison of that silk with the cocoon silk of the common (and commercial) silkmoth *Bombyx mori* (hereafter referred to as the silkworm). Other spider silks that are interesting structurally but will have to be ignored here encompass the super-elastomeric (because fully hydrated), micro-windlass capture silks of the ecribellate orb weavers (Vollrath and Edmonds, 1989) or the hackled, nanofilamentous and electrostatic capture silks of the cribellate spiders (Hawthorn and Opell, 2002) as well as the cocoon silks of any spider or the cement silks used to stick down and anchor other silk threads (Foelix, 1996).

The dragline silk produced by the major ampullate glands is not only the principal silk of the 'advanced' spiders, used for both safety and for the guy/anchor threads that suspend the web in mid-air, but it is also the silk that is most easily drawn from the animal under controlled conditions and in enough quantity for comprehensive study (Madsen *et al.*, 1999). Cocoon silk produced by *Bombyx mori* has been artificially selected for over 6000 years, and is produced on an industrial scale equivalent to other high-performance polymers. Therefore, this silk presents an excellent comparative material to that of (mechanically superior) spider silk as it is easy to obtain, available in large quantities and very well characterised.



4.1 Glandular origin, function and tensile mechanical properties of silks produced by *Nephila* spiders. Lines are differing shades of grey to match gland of origin and stress–strain plots.

4.2 Unravelling structure–function relationships

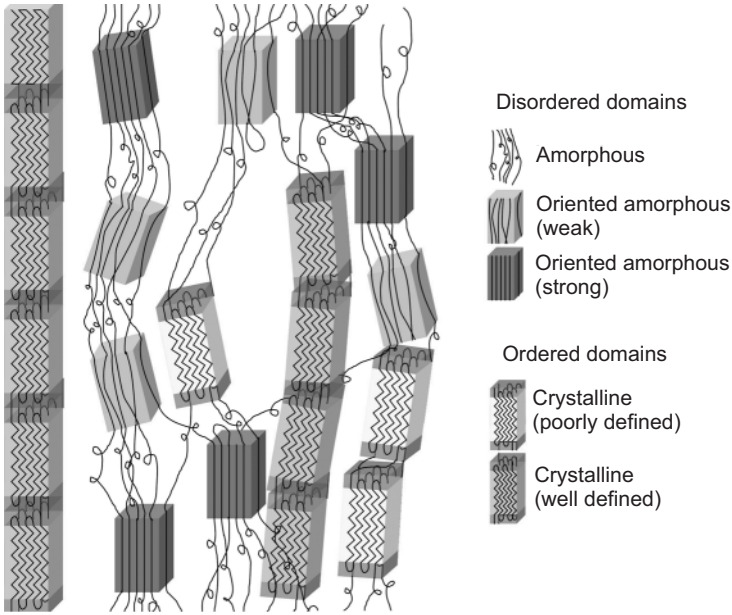
Probing structure–function relationships in these materials, requires, initially an understanding of their composition. Silk in particular has leant itself very well to structural analysis as it is one of a few biological materials which is highly concentrated, orientated and designed to perform outside the body, thus techniques originally developed to study man-made fibres can be translated rapidly, and successfully, to the study of silk.

4.2.1 Structure from composition

Spider dragline silk fibres consist of single protein monofilaments (Vollrath and Knight, 2001) whereas the silk of the silkworm consists of a pair of protein monofilaments (brins) coated by a glycoprotein (sericin) used as a matrix to glue fibres together forming the cocoon composite (Poza *et al.*, 2002). Studies on the core of these filaments reveal remarkable similarities between spider and silkworm silks. Both contain nano-fibrillar structures oriented (primarily) along the long axis of the fibre which can form larger structures (microfibrils) thus leading to a hierarchical structure (Frische *et al.*, 1998; Oroudjev *et al.*, 2002; Sapede *et al.*, 2005; Eby *et al.*, 1999).

The core of spider dragline also contains elongated and filled canaliculi 'cavities' oriented parallel to the silk fibre axis and in appearance very similar to the 'elongate vacuolar droplets' of the silk of some moth larva (Akai *et al.*, 1993; Frische *et al.*, 1998). This overall skin-core structure is the result of a rheological pattern originating in the two secreting regions recognised in *Nephila* silk glands with the canaliculi indicating flow inclusions (Knight and Vollrath, 1999c). Stressing a fibre until fracture can lead to cracks forming between canaliculi (Shao *et al.*, 1999a), and it may be that this potentially damaging mechanical energy is diverted on a microscopic level, thus contributing to the exceptional tensile strength and toughness of the fibre.

It has been recently hypothesised that the hierarchical order of structures in silk causes mechanical energy dissipation at not just the microscopic level, as seen in the canaliculi, but by the very nanostructure itself (Porter and Vollrath, 2007). Evidence beginning with the earliest x-ray crystallographic studies in the 1950s (Pauling and Corey, 1951; Lucas *et al.*, 1955; Riekel and Vollrath, 2001) up to today's NMR (Van Beek *et al.*, 2002) and neutron scattering work (Sapede *et al.*, 2005), has revealed silk's molecular architecture to be composed of ordered (crystalline) and disordered (amorphous) domains. This has been refined over the years to a general model of ordered regions, which are composed of β-sheet protein structures with a high degree of internal bonding, and disordered regions, possessing a more heterogeneous distribution of protein structures and bonding (Termonia, 1994; Beek *et al.*, 2002; Porter *et al.*, 2005; Vollrath and Porter, 2006a) (see Fig. 4.2). This combination of, and energetic communication between, ordered



4.2 Diagrammatic representation of the nanostructure of dragline silk. Molecular chains are oriented parallel to the long axis of the fibre and whilst only displayed in two dimensions the network extends into the third. Disordered domains dissipate mechanical energy and are composed of amorphous chains, oriented amorphous chains disrupted by weak polar solvents (such as alcohols), and chains disrupted only by strong polar solvents (such as water). Ordered domains can only be disrupted by strong chaotropic agents and are responsible for the strength of the fibre. These domains consist of β-sheet crystallites whose orientation is either poorly or well defined. Chains of crystallites arrange together to form nanofibrils (far left).

and disordered structures on the nano-scale, enables the dissipation of mechanical energy and reduces local stress concentrations which might precipitate fracture, making silk tough even on the macro-scale (Porter *et al.*, 2005; Porter and Vollrath, 2007).

4.2.2 Structure from processing conditions

Information from static structural studies of silk can be enhanced through the act of controlled perturbation. By applying physical and chemical forces to silk, we can assess how structure *determines* function. For example, the stress–strain characteristics of *Nephila* dragline silk change significantly with processing conditions. Reeling speed (the rate at which silk is experimentally drawn from the spinneret onto a bobbin) as well as body temperature both affect fibre diameter as well as all conceivable mechanical properties (Vollrath *et al.*, 2001), something

also observed in silkworm silk (Shao and Vollrath, 2002). Online micro x-ray diffraction (Riekel *et al.*, 2000; Riekel and Vollrath, 2001) or Raman spectroscopy (Young *et al.*, 1998), of single fibres as they are spun, shows corresponding changes in structural parameters such as the degree and orientation of the highly ordered, crystalline domains.

A wide range of solvents are able to modify the material properties of many silks (Shao and Vollrath, 1999; Shao and Vollrath, 1997; Perez-Rigueiro *et al.*, 2001; Perez-Rigueiro *et al.*, 2000). A solvent of particular use in assessing structure–function relationships in dragline silk is water. Dragline silk, unlike other spider silks, undergoes supercontraction when wetted, shrinking to up to half its original length and double its diameter (Work and Morosoff, 1982). This interesting feature has been correlated with a recoverable disruption to partially oriented structures by water in the amorphous domains of the silk (Grubb and Ji, 1999; Simmons *et al.*, 1996; Fornes *et al.*, 1983; Eles and Michal, 2004). The degree to which a dragline silk contracts depends, in part, on the processing conditions, with silks spun at higher rates contracting more due to a higher degree of partially oriented amorphous region for the water to plasticize (Liu *et al.*, 2005). Thus, a fibre's capacity to shrink has therefore been proposed as an indicator of molecular chain orientation; as once supercontracted it can be considered to be in a reference state only slightly affected by processing conditions (Perez-Rigueiro *et al.*, 2003; Liu *et al.*, 2005). Liu *et al.* have argued that supercontraction is actually an evolutionary constraint upon dragline silk, if high strength and toughness are to be achieved then shrinkage when wet has to be accepted (Liu *et al.*, 2005). This is an important factor to note when considering dragline-based biomaterials for use in wet environments.

Previous attempts to link amino acid sequences or protein compositions to mechanical properties have been inconclusive (Zax *et al.*, 2004; Brooks *et al.*, 2005; Brooks *et al.*, 2007; Swanson *et al.*, 2006). However, supercontraction has linked, for the first time, the role of an individual amino acid, proline, to dragline silks' mechanical properties (Liu *et al.*, 2007). By comparing the supercontraction properties of dragline silks differing in amino acid composition, but reeled to have similar breaking strains, Liu *et al.* demonstrated that, whilst processing conditions can alter the capacity of a silk to shrink within a certain range, it is proline content that determines the range in which the capacity to shrink can be adjusted.

It would seem that solvents with different polarities affect different regions of spider silk's composite microstructure (Vollrath *et al.*, 1996), probably by modifying the conformation of the different molecular chains. A comparison of Raman spectra shows clear pre-solvent and solvent-induced differences in a wide range of material properties in the major ampullate dragline silks in four spiders representing different families *Araneus diadematus* (Araneid orb-weavers), *Nephila edulis* (Tetragnathid orb-weavers), *Latrodectus mactans* (Theridiid tangle web-weavers) and *Euprosthenops* sp. (Pisaurid nursery web-weavers) allowing further assignation of conformationally sensitive regions (see Fig 4.2) (Shao *et al.*, 1999b).

Although silkworm silk does not supercontract, water does have a plasticising effect. In order to unravel silk from the cocoon, the sericin matrix embedding the fibres must be removed by 'degumming'. The most common methods of degumming are made possible because of the high content of serine and other hydrophilic amino acids in the sericin proteins (Komatsu, 1975; Gamo *et al.*, 1977) making them soluble in hot alkaline water. Complete removal of sericin has proven to represent a technical trade-off, as prolonged degumming degrades silk's mechanical properties (Perez-Rigueiro *et al.*, 2002), but the slightest presence of sericin can cause problems for the biomedical application of silk-based materials (Panilaitis *et al.*, 2003). Once degummed, immersing silk in water reduces its strength, whilst dehydrating agents, such as acetone and alcohols, increase its stiffness (Perez-Rigueiro *et al.*, 2000) and boiling solutions of salts shrink and can damage the fibre (Tsukada *et al.*, 1994).

The ultimate perturbation of the molecular structure of silk occurs when a solvent interacts with the ordered domains (β-sheet rich), disrupting the amide–amide hydrogen bonds, resulting in a partial/complete breakdown of the fibre. This can only be achieved using highly chaotropic agents such as concentrated solutions of lithium salts (Yamada *et al.*, 2001; Sponner *et al.*, 2005) or halogenated alcohols (Seidel *et al.*, 1998; Fahnestock, 1994). This process is popularly known as regeneration, or perhaps more correctly reconstitution, and is the typical approach to produce a spinning feedstock for artificial silk fibre production.

4.2.3 Structure from modelling

Studies such as those outlined above, aiming to integrate mechanical data with structural information, are important in order to prize apart structure–function relationships. The real value of such studies will become apparent when we are able to integrate information on protein and peptide sequences into this data set. As previously mentioned, we already know that the proline content of a silk correlates well with both the overall silk mechanics as well as supercontraction (Liu *et al.*, 2007), a relationship originally hypothesised in our models (Vollrath and Porter, 2006b). Furthermore, the roles of other silk amino acids (such as glycine) are beginning to be elucidated (Dicko *et al.*, 2007), permitting us to extend our hypotheses to the impact on mechanical behaviour of the different compositions of silk proteins (Vollrath and Porter, 2006b). Clearly, for such insights, modelling is a key tool (Termonia, 1994; Van Nimmen *et al.*, 2005), and recent models are surprisingly good at leading the way towards a comprehensive understanding of silk structure–function relationships (Du *et al.*, 2006; Vollrath and Porter, 2006a; Vollrath and Porter, 2006b; Porter *et al.*, 2005) and even down to the core of protein instability and denaturing (Porter and Vollrath, 2008).

4.3 Spider and worm spinning *in vivo*

So far we have demonstrated that the processing conditions are as important as the material itself, to the extent that even silks with different amino acid compositions can be tuned to have almost identical material properties (Liu *et al.*, 2007). Therefore, successful (i.e. controlled) biomimetic production of these materials will only be possible by understanding not just the material itself but also how the fibres are formed naturally.

Silk is produced and processed by specialist glands in the spider and silkworm in a remarkably similar manner. Silk proteins are released into solution by epithelial cells and stored, potentially for extended periods of time, in a part of the gland serving as a reservoir, known as the ampulla (spiders) or middle division (silkworm) (Knight and Vollrath, 1999a; Akai, 1986). The situation in silkworms is complicated further by the fact that the silk is co-extruded with multiple sericin proteins, produced by a part of the gland further downstream (Gamo *et al.*, 1977). Upon spinning, this protein solution travels down an elongated tapering tubular duct, during which the silk undergoes active chemical and mechanical modification, to convert it from stored gel to final solid fibre as it exits through the spinneret (Vollrath and Knight, 2001; Asakura *et al.*, 2007).

Silk glands can be divided in several zones, each thought responsible for the production of a different combination of silk proteins. In spider dragline silk, this is usually a combination of spidroins (MaSp1 and MaSp2) (Hinman and Lewis, 1992) and, in the silkworm, fibroins (H-fibroin and L-fibroin) and P25 (Shimura *et al.*, 1976; Gamo *et al.*, 1977). Both spidroins and fibroins show a degree of evolutionary convergence in their peptide composition, both possessing high amounts of glycine and alanine in repetitive motifs necessary to propagate the β-sheet structures seen in the fibre (Gatesy *et al.*, 2001; Bini *et al.*, 2004).

Birefringence patterns and the shape changes in the silk droplet inclusions (canaliculi) provided the initial evidence that elongational flow and shear are important in providing the energy for this transition from stored gel to final solid fibre (Knight *et al.*, 2000; Magoshi *et al.*, 1985; Akai, 1986; Akai *et al.*, 1987). There is evidence that this flow is non-Newtonian, resembling nematic liquid-crystalline flow (Knight and Vollrath, 1999a; Knight and Vollrath, 1999b; Asakura *et al.*, 2007) if perhaps not being liquid crystal *sensu strictu* (Holland *et al.*, 2006).

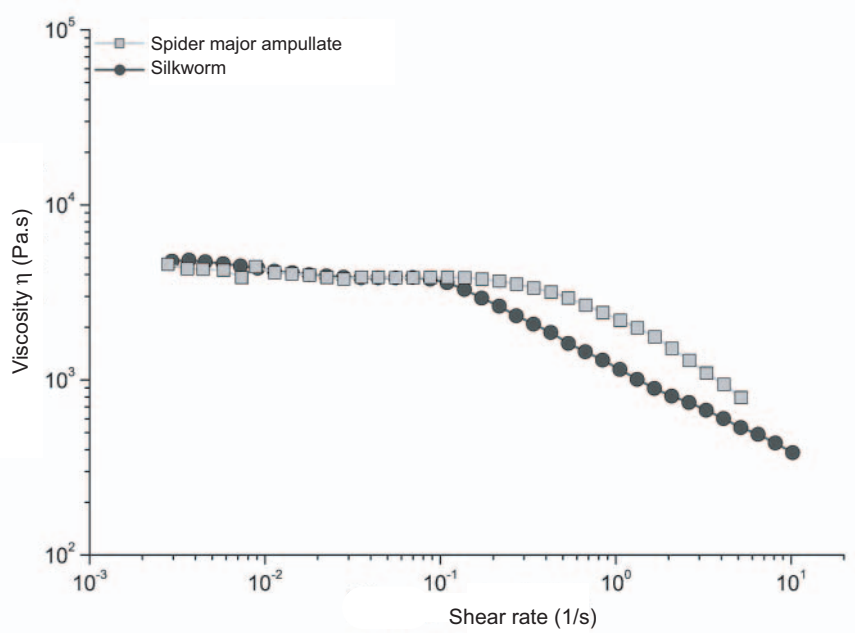
The duct of the silk gland consists of a hollow fibre dialysis membrane that is able to 'handle' the rapid removal of water as well as to precisely control ionic and pH gradients (specifically acidification) during spinning (Foo *et al.*, 2006). This serves to reduce the amount of energy required to undergo a stress-induced phase transition and helps propagate and stabilise the different conformations and arrangements of silk proteins in order to bring about the development of a hierarchical structure (Zong *et al.*, 2004; Magoshi *et al.*, 1997; Dicko *et al.*, 2004a; Dicko *et al.*, 2004b; Dicko *et al.*, 2004c; Vollrath *et al.*, 1998; Knight and Vollrath, 2001; Meyer and Jeannerat, 1939).

The silk gland duct in a spider terminates in a structure [originally termed 'valve' (Wilson, 1962; Wilson, 1969)] because it was thought to work much like the 'press' in the silkworm, i.e. to squeeze the silk and provide shear stress. Whilst this is no longer thought to be used as a 'press' in the spider, it is now seen as a 'ratchet', used to advance broken threads internally (Knight *et al.*, 2000; Knight and Vollrath, 1999b). The 'press' in the silkworm is thought to allow for slight modifications to the degree of post draw applied to the silk because of its relatively narrow range of spinning rates (Magoshi *et al.*, 1985; Iizuka, 1966), whereas the spider can achieve this by altering the rate at which it draws silk with either its legs (if used) or movement of the body (Vollrath *et al.*, 2001). Finally, the thread is stripped of its bathing and coating liquids (save for the sericin in the silkworm) before exiting at a tight spinneret.

Therefore, the process of spinning occurs when silk proteins are pulled through the spinning duct, resulting in a stress-induced phase transition and hierarchical structure development. Other factors, such as the chemical changes to the silk's environment, dehydration and the 'ratchet' or 'press', serve to facilitate this transition or tune the properties of the final fibre. By characterising exactly how these materials respond to shear deformation (mechanical energy input), we can determine how this transition from stored gel to final solid fibre occurs. If we want to spin these materials biomimetically, then an understanding of the stress-induced phase transition will be THE key to controlled processing in order to optimise material properties. By employing rheology, the study of flow and deformation of matter, we are able to examine the spinning forces applied to the feedstock in the duct to samples *in vitro*, providing us with a window into the silk production process.

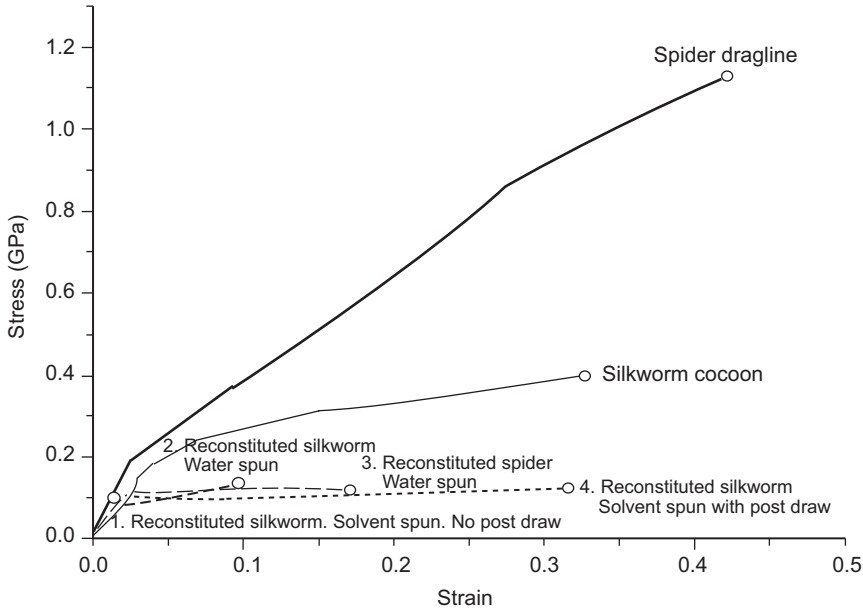
The first rheological investigations into silk began in the 1960s, but technical constraints meant that the experiments required large sample volumes. This spawned two approaches; either dilution of the native spinning feedstock or using a reconstituted silk, each one moving further away from the natural system (Iizuka, 1966; Ochi *et al.*, 2002; Hossain *et al.*, 2003; Chen *et al.*, 2002). The introduction of more sensitive machines made it possible to start using samples at *in vivo* concentrations (20–30% dry weight) (Terry *et al.*, 2004; Kojic *et al.*, 2006; Holland *et al.*, 2006). However, the ability to test a silk feedstock 'straight from the gland' did bring its own set of challenges, specifically concerning repeatability. When previously working with dilute or reconstituted samples, shear history was inconsequential. However, as silk has evolved to be stored at the precipice of a stress-induced phase transition (to make spinning as fast and energetically efficient as possible) any mechanical energy accidentally introduced during an experiment, by mishandling the samples, could cause up to two orders of magnitude variation between experiments (Holland *et al.*, 2006; Terry *et al.*, 2004).

Rheological studies on native concentrations of silkworm feedstock have revealed that the material behaves like a non-Newtonian high molecular weight polymer acting as a weak gel (Terry *et al.*, 2004, Holland *et al.*, 2006). Unspun silk



4.3 Viscosity shear rate profiles of spider (squares) and silkworm (circles) native spinning feedstocks. Note the similarity between the profiles in their response to increasing shear.

behaves as a liquid over long timescales (ideal for storage) and a solid over short timescales (suitable for energy absorption for phase transition). In addition, exposing a silk feedstock to a drop in pH results in a rapid gelation of the material, which is synonymous with the acidification observed in the duct (Terry *et al.*, 2004; Vollrath *et al.*, 1998; Magoshi *et al.*, 1994). Later work directly compared spider major ampullate and silkworm silk feedstocks (Holland *et al.*, 2006; Kojic *et al.*, 2006). The findings revealed that these two materials, despite an evolutionary separation of hundreds of millions of years and being made from completely different proteins and making fibres with very different mechanical properties, both share almost identical shear rheologies (see Fig. 4.3) (Holland *et al.*, 2006). This implies that in order to produce a high-performance fibre naturally, these materials have to flow and absorb energy in a similar manner, which might very well be a key evolutionary constraint for silk spinning. In addition, these materials not only behave rheologically like one another, they also behave like molten polymers under flow. This allows us, for the first time, to use tools and techniques originally developed for the polymer industry to study silks, but also serves as a welcome affirmation that we may, one day, be able to adapt traditional polymer spinning technologies to artificially produce silk.



4.4 The tensile properties of artificial silks compared to native spider and silkworm. Labels describing the natural progenitor and spinning conditions are next to the reconstituted stress–strain curves (Madsen *et al.*, 1999; Xie *et al.*, 2006; Shao *et al.*, 2003; Marsano *et al.*, 2005).

4.4 Spinning *in vitro*

Biotechnologically, the spinning pathway of the major ampullate silk in any spider is a highly advanced fibre production system. It is able to control the energetically efficient production of fibres, with a wide variation of mechanical properties, using the same spinning feedstock, by just altering the spinning conditions. These properties, perhaps even above the high performance characteristics, make silk a highly desirable material in order to spin biomimetically.

Many attempts have been made to produce artificial silk, e.g. Matsumoto *et al.*, 1996; Shao *et al.*, 2003; Madsen *et al.*, 1999; Marsano *et al.*, 2005; Lazaris *et al.*, 2002; Xie *et al.*, 2006; Liivak *et al.*, 1998; Yao *et al.*, 2002; Zuo *et al.*, 2006; Seidel *et al.*, 2000, although none can be considered truly biomimetic (i.e. spun in a natural way). Typically such artificial silk fibres are spun from silk that was reconstituted 'back' into its unspun state and then re-processed in a variety of different ways, ranging from classical alcohol baths (Matsumoto *et al.*, 1996) to electrospinning (Zarkoob *et al.*, 2004). However, to date none of these reconstituted fibres have been able to match the mechanical properties of their natural progenitors (see Fig. 4.4).

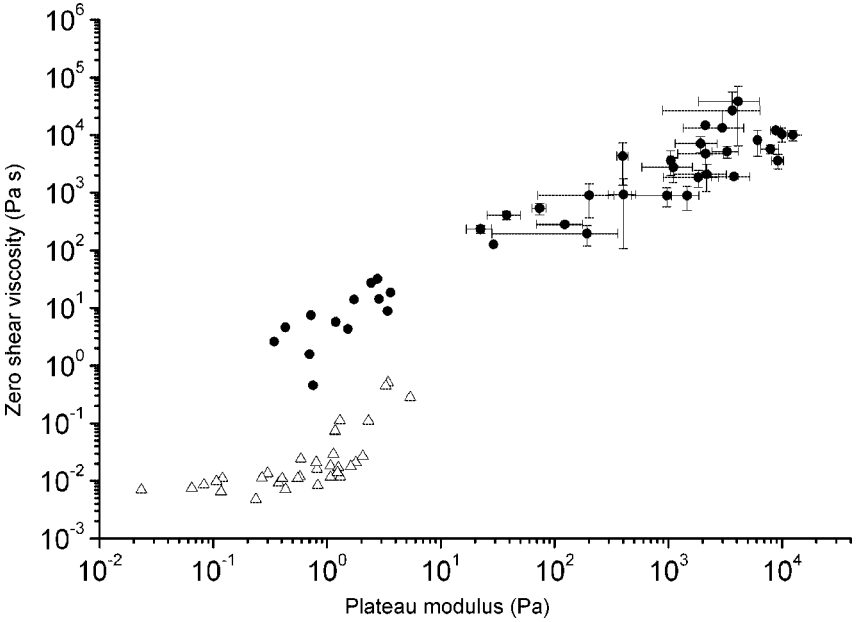
Determining the quality of artificial silks by testing only the mechanical

properties of the final fibre can only provide half the story, as, like the natural fibre, it represents both the quality of the spinning feedstock and the conditions of manufacture. However, from previous work, it has been shown that the rheology of natural silks converges upon a narrow range of rheological parameters, giving us the potential to turn *evolutionary constraints* into *design criteria* (Holland *et al.*, 2007). For biomimetic spinning it will be necessary, so we believe, to first match the rheologies of reconstituted and native feedstocks.

The design criteria are rheological parameters (spinnability indicators) chosen to represent consistent, yet relevant, features seen across the native silk feedstocks so far tested. The first spinnability indicator is *zero shear viscosity*, representing the strength of intermolecular associations (internal friction) between silk proteins in the feedstock. This describes how applied shear energy flows through the material. The second indicator is *plateau modulus,* which reveals how much energy the silk proteins are able to absorb, which is required for a complete transition from stored gel to final solid fibre (Knight *et al.*, 2000). Therefore, by comparing artificial silk feedstocks against these rheological design criteria, we are able to determine the *potential* of a silk to be spun, like a silk, into a silk-like fibre (see Fig. 4.5).

As evidenced from Fig. 4.5 it is clear that native silkworm and artificial reconstituted silkworms spinning feedstocks are significantly different from one another. Clearly, the act of reconstitution seriously degrades not only silk's ability to store energy, required for phase transition, but also degrades or destroys vital associations between silk molecules, required for a hierarchical structure. These flow properties (indicative of *function*) can be related to *structure*. Indeed, the research by Holland and collaborators confirms previous findings about the damaging effects of reconstitution on silk which is seen structurally by a change in conformation and size of the proteins (Yamada *et al.*, 2001; Iridag and Kazanci, 2006; Zuo *et al.*, 2006; Asakura *et al.*, 1985).

Therefore, it is not surprising that industrial methods, such as alcohol baths, extreme chemical modification and temperature are required in order to 'spin' a reconstituted silk fibre (Matsumoto *et al.*, 1996; Shao *et al.*, 2003; Madsen *et al.*, 1999; Marsano *et al.*, 2005; Lazaris *et al.*, 2002; Xie *et al.*, 2006; Liivak *et al.*, 1998; Yao *et al.*, 2002; Zuo *et al.*, 2006; Seidel *et al.*, 2000). These approaches are the only way to introduce sufficient energy into these materials to bring about fibre formation due to the silk protein's severely degraded nature. The massive gap between these two feedstocks may go some way to explaining why it has not been possible to create a reconstituted 'silk' fibre with the mechanical properties (Matsumoto *et al.*, 1996; Shao *et al.*, 2003; Madsen *et al.*, 1999; Marsano *et al.*, 2005; Lazaris *et al.*, 2002; Xie *et al.*, 2006; Liivak *et al.*, 1998; Yao *et al.*, 2002; Zuo *et al.*, 2006), or structural complexity (Eby, 1995; Putthanarat *et al.*, 2000), of a natural silk, let alone process it in the same way. Clearly, artificial silks are inherently different before they are even spun and it is questionable whether, once reconstituted, these materials can even be called silks.



4.5 Spinnability indicators of a similar range of concentrations for native silkworm spinning feedstock (circles), compared with those of artificial, reconstituted silkworm silk feedstock (triangles). Zero shear viscosity indicates the degree of intermolecular associations between silk molecules (for structure development) whilst plateau modulus indicates the amount of energy the molecules can absorb (necessary for phase transition). Error bars for native silkworm spinning feedstock samples >1 Pa, represent standard error based on 3 repeats from the same silkworm gland contents, lower concentration native and reconstituted silkworm spinning silk points represent individual tests due to the increased amount of sample required for accurate characterisation.

4.5 Future trends and applications

As far as we know, none of these enterprises devoted to the commercial production of silk-based materials has managed to make a feedstock that has been spun (or that can be spun) into fibres with properties comparable with those of the role-model, spider dragline silk. We argue that the most logical way forward is to improve the quality of the starting material to match the rheological *design criteria* already provided to us by Nature herself. Future efforts will need to be focused towards a better understanding of what is happening to silk proteins during reconstitution (Holland *et al.*, 2007, Porter and Vollrath, 2008). Through the development of milder, less damaging reconstitution techniques, more benign spinning techniques can be employed and higher performance fibres created (Z. Shao *personal communication*). Another approach is to harness genetic engineering to produce

silk feedstock *ab initio*, thus side-stepping the problems of reconstituting a silk 'back' into its unspun form (Vendrely and Scheibel, 2007). Whilst not without their own set of technical difficulties, current attempts at producing such genetically engineered feedstocks are promising, with recombinant spider silk hydrogels already displaying some of the rheological characteristics of natural silk (Rammensee *et al.*, 2006).

Even when truly biomimetic silk production becomes a reality, it is likely that, at least for the foreseeable future, the costs of such fibres will be high and probably prohibitively high for the mass textile market. The initial market will most likely be in the medical field, both for single threads (suture) and specialist textiles (woven and non-woven) which will make use of silks, excellent mechanical properties, biocompatibility and its ability to be chemically functionalised. However, the potential uses of silk are not strictly limited to a fibrous role. Reconstitution has the unique advantage that it allows silk to be reprocessed into almost any structure, from films to foams, whilst still possessing very good mechanical properties, finding uses in areas of medicine from wound dressings to orthopaedics (Hakimi *et al.*, 2007; Vepari and Kaplan, 2007).

4.6 Conclusions

Spider silks, like silkworm silks, are semicrystalline biopolymers with excellent mechanical properties. Independently, spiders and insects have evolved materials that behave virtually identically rheologically but make fibres with very different mechanical characteristics. This is due partly to differences in chemical composition and partly to differences in the details of the processing conditions. Spider silks, because of their selection in a ferocious arms race with insects, have evolved into materials having a wide range of mechanical properties. This makes them very interesting as models for the design and production of modern artificial synthetic silks. The spider's production system has strong potential to provide us with excellent fibres that are produced along biological principles, i.e. ecologically compatible, therefore it is likely that, in the present economic climate, efforts will be redoubled to produce commercial silks the spider's way.

4.7 Sources of further information and advice

Further information can be found within the references for this article. The first piece of literature before embarking on a study of this topic must be Craig's comprehensive review of the evolution of arthropod silks (Craig, 1997). A good overview of the biology of the spider and the silkworm is given by Foelix (1996) and Fedic *et al.* (2002). For a general introduction to silk processing in spiders we would suggest that by Vollrath and Knight (2001) and for silkworms that by Asakura *et al.* (2007). For introductory papers on nano-scale toughness see Porter and Vollrath (2007), for supercontraction see Liu *et al.* (2007), for the effects of

solvents see Shao and Vollrath (1999), for reconstitution see Shao *et al.* (2003) and Yamada *et al.* (2001), for structure–function modelling see Vollrath and Porter (2006a) and Vollrath and Porter (2006b), and for silk rheology see Holland *et al.* (2006 and 2007) and Ferry (1980). For a review of genetic engineering of spider silks see Vendrely and Scheibel (2007). Of particular importance outside of the field of silks, yet born of its research and with application to all proteins, the authors suggest the work of Porter and Vollrath (2008).

For applications of silkworm silk as a biomedical material the recent review by Vepari and Kaplan (2007) is of note as is that of Hakimi *et al.* (2007), which deals with both spider and silkworm silk.

Websites

Some research groups working within the field of silk

Prof. Fritz Vollrath, Department of Zoology, Oxford University, Oxford, UK. http://www.oxfordsilkgroup.com
Prof. Zhengzhong Shao, Department of Macromolecular Science, Fudan University, Shanghai, China. http://www.polymer.fudan.edu.cn/research/shaozz/English%20version/index.html
Prof. David Kaplan, Department of Biomedical Engineering, Tufts University. Medford, MA, USA. http://ase.tufts.edu/biomedical/faculty-staff/kaplan.asp
Dr Thomas Scheibel, Technische Universität Munich, Munich, Germany. http://www.fiberlab.de/

Companies producing silk-based biomaterials

Oxford Biomaterials. http://www.oxfordbiomaterials.com
Nexia Biotechnologies Ltd. http://www.nexiabiotech.com

4.8 Acknowledgements

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5

A biomimetic approach to the production of sustainable structural composites using plant fibres

C. SANTULLI
University of Reading, UK

Abstract: The production of sustainable structural composites, using cellulosic fibres extracted from plants as a reinforcement, is discussed. Critical factors, including fibre extraction and treatment, impact properties, compatibility with polymer matrices, and selection of plant fibres are explored. Ideally, all of these aspects need to be integrated using a biomimetic approach, i.e. taking into account structural factors linked to the biology of plant fibres, such as the role of the cellular hollows and the presence of hierarchical levels, allowing the fibre to be built from the cell upwards. Fibers studied include celery and jute.

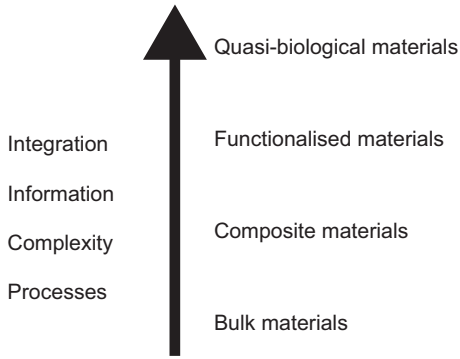
Key words: cellulosic fibres, biomimetics, plant fibres, celery, jute.

5.1 Biomimetic design of composite materials

Biomimetic materials design is a multidisciplinary approach, involving either the conception and realisation of new materials using biological tissues, with high environmental friendliness and a life cycle resulting in a lower resource depletion for the Earth, or tailoring materials for functions, as successfully addressed during evolution.[1]

However, mimicking a natural structure can mean different things in different situations. In other words, the extent of bio-inspiration in materials can vary, from copying the bare geometry of a natural object to reproducing its function or even providing a self-organisation paradigm for the material.[2, 3] The above approaches are in effect complementary, in the sense that the material is 'designed' in nature through self-organisation to perform the required function. Structurally, the inspiration from the natural structure can be obtained at super-molecular, molecular and sub-molecular level. In a more complex and vertical way, it is also possible to envisage that the material acquires higher levels of 'smartness' and subsequent functions, as far as it proceeds from the engineered material, perceived as a continuum, towards the biological material, containing a hierarchical structure, as represented in Fig. 5.1.

The biomimetic approach led in recent years to the conception of innovative fibre-reinforced composites, where at least the reinforcement phase, if not the



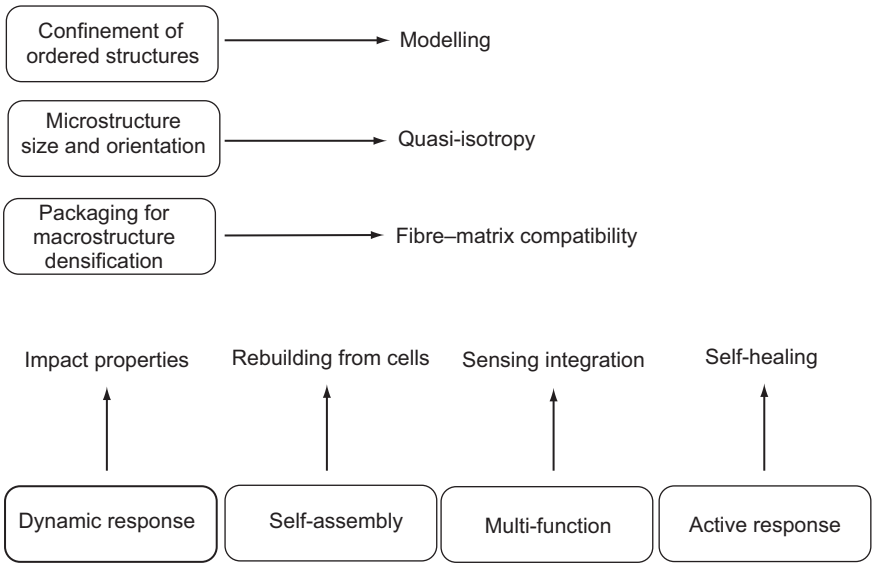
5.1 Vertical levels of complexity in materials (from reference 85).

whole composite, originated from nature. A fibrous morphology can be preferable for a number of reasons, including the possibility of embedding woven or knitted fibre tissues to form fracture-resistant composites: here, the inspiration can be from biological self-assembled tissues, not requiring energy-intensive or environmentally damaging processing steps.[4] Concentrating on the mechanical purpose of manufacturing composite, the matrix allows the re-distribution of concentrated loads and resistance to buckling.[5]

Potential materials for manufacturing innovative composites using a biomimetic approach are those related to cellulose fibres and those based on protein fibres. Five possibilities for reinforcement can be recognised: protein fibres from animals, genetically modified protein fibres, reconstituted animal cellulose (tunicin) fibres, cellulose fibres from agro-waste material, and cellulose or ligno-cellulose fibres extracted from plants. This chapter contains a review of these choices, but concentrates on the alternatives involving the use of cellulose fibres from plants.

General considerations on the possibilities of protein fibres as composites reinforcement are reported below. In particular, beta-keratin is an only partially hydrophilic material, which appears, at least in principle, ideal to be coupled with a hydrophobic matrix and/or with cellulose fibres as a hybrid composite.[6] A number of recent studies using keratin fibres from avian feathers as a reinforcement highlighted that the main issue to obtain a sufficient matrix–fibre compatibility would be having some control over the feather supply and possibly of their melanin content.[7–9]

Another possibility would be silk obtained from silkworm (*Bombyx mori*) and some spiders, such as *Nephila clavipes*. The former, in particular, has been used for centuries as a biomedical material for sutures, biodegradable and chemically suited to the function. Owing to some bio-compatibility problems to be ascribed to sericin contamination, its use as such has been discontinued. In recent years, bio-silk, obtained by genetically modifying silk proteins, has been introduced and this shows strong mechanical properties, making it suitable for use in composites.[10–11] The use of waste silk materials in composites is also reported in literature:[12] this is



5.2 Mechanical design and functional design of a biomimetic composite.

of similar interest as obtaining fibres from agricultural waste, especially in that it may improve the life-cycle analysis (LCA) performance of the final material. Most frequently, agro-waste, such as rice husks, is used as a filler for thermoplastic polymers.[13] In some cases, however, it was also proposed as a material in (randomly oriented) fibrous form for materials reinforcement, although mainly applied to wooden board rather than fibreglass replacement.[14, 15]

5.2 Characteristics of biological materials in biocomposites

Biological materials offer a number of characteristics that are not always available in engineered materials. These characteristics should be available, at least in principle, when synthesising materials using biological sources, such as in composites including natural fibres as a reinforcement for a polymer matrix.

In addition to mechanical design properties, which suggest embedding fibres into a polymer matrix, composites, to be really biomimetic should be able to perform a number of functions, in what can be defined as functional design. These two levels of composite design are clarified, with reference to plant fibre composite, in Fig. 5.2. In more detail, the functions that biomimetic composites should have are:

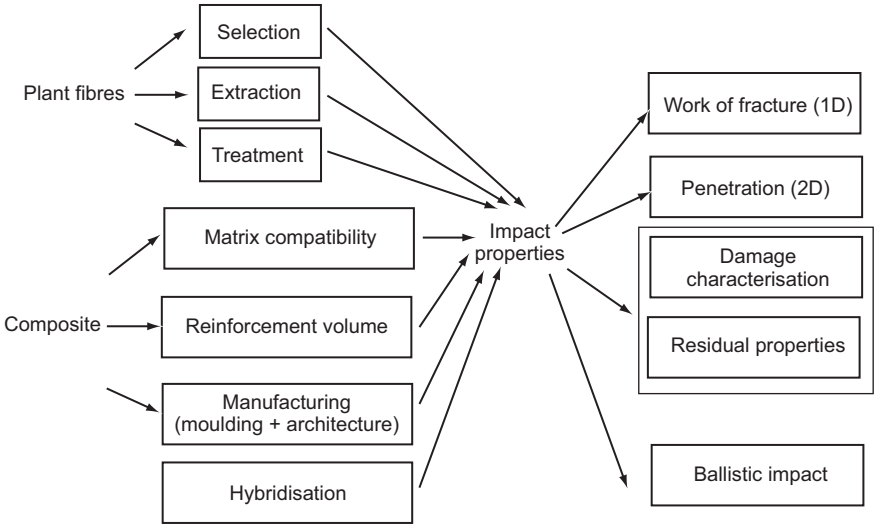
- Building themselves in a hierarchical and optimised way, and, when necessary, coming back to the cell level to 'rebuild' the material (self-assembly). Routes

for self-assembly, which would represent the final stage of an integrated process, including polymer synthesis and mineralisation, have only been proposed in silk replacement materials so far.[16]

- Performing different tasks when required (e.g. allow sensing integration). Here, chemical bio-sensing is, in principle, available in materials embedding cellulosic fibres by incorporation of glucose co-enzyme systems.[17] It appears to be more difficult to include mechanical sensing capabilities in the material. The main reason for this is owing to the reduction of mechanical properties induced by embedding fibre optics systems, usually in carbon fibre composites,[18] and this is unlikely to be compensated for by using cellulosic or lignocellulosic fibres.
- Responding in an active way to damage (e.g. self-healing). Self-healing of composite materials comprises two functions: enhanced damage visualisation and mechanical restoration through healing agent inserted in hollow fibres.[19] Usually borosilicate glass fibres have been used; however, the presence of hollow plant fibres would, at least in principle, allow them to be used for self-healing functions.

As discussed above, the potential of plant fibres in biomimetics depends on the role of the hollow in these fibres. A hollow fibre allows fluids and nutrients to diffuse in the plant. The presence of a hollow, or lumen, in a structure has been for quite some time regarded as solely detrimental for its mechanical resistance. However, it is worth pointing out that having a hollow in the centre of a supposedly cylindrical, loaded structure, means that the neutral axis in the structure is unloaded, a fact which may be beneficial from the point of view of shear tension in composites.[20]

The system of interest leading to the modelling/realisation/validation of plant fibre composites is reported in Fig. 5.3 relative to impact properties, which are important for possible high-tech use of these materials. The three aspects essential for the successful use of plant fibres in composites, i.e. fibre extraction, treatment and matrix coupling, can not be ‘designed’ without considering the presence of the fibre hollow. More generally, the difference between biological artefacts, such as plant fibres, and man-made ones, is particularly reflected in the presence of a hierarchical repeatability, which is different from the engineering macro-scale repeatability of components.[21] For fibre-reinforced composites, which are classically regarded as structures with a low order of hierarchical structure, fibres are embedded in a matrix to form anisotropic laminae that are, in turn, bonded together to form a laminate. Both fibres and matrix are considered to be continuous media in the analysis of the lamina, and the laminae are regarded as continuous in the analysis of the laminate, so that the degree of anisotropy would depend on the stacking sequence of laminae and the orientation of fibres within them.[22]



5.3 System of interest leading to impact-resistant structural plant-fibre composites.

5.3 Fibre extraction, fibre treatment and matrix compatibility in a biomimetic composite

Cellulose forms the structural component of plant fibres. Other components include hemicellulose, lignin and in some cases pectin and waxes, as well as some moisture content. Increasing as much as possible the cellulose percent in the final fibre would require fibre drying and elimination of pectins in the first place. In bast fibres, conventional field or dew retting, based on bacterial action, and followed by decortication, has a number of uncontrollable factors, which may affect the final quality of the fibre. This limitation is particularly significant when structural applications are envisaged for the fibre-reinforced laminates. In addition, it is not at all an environmentally friendly process, whose impact has been discussed in literature.[23, 24] There are also difficulties in applying binders to long fibre bundle lengths, since decortication technology is still not optimised in terms of mechanical reliability, separation efficiency and purity. To avoid extraction problems, an alternative is to leave the fibres unretted with the undesirable result of having more parenchyma, cuticle and epidermal tissue attached to the fibre tissue and therefore a weaker interface between the tissues.[25] For these reasons, on hemp fibres the possibility of green decortication and degumming technology has been explored,[26] whilst, on flax fibres, enzyme retting, involving the use of pectinase and galacturonase for pectins dissolution, is a quite well explored possibility.[27, 28]

Other fibres, which are obtained from fruit, such as coir from coconut, present a comparatively easier extraction, although retting by bacterial complexes is also

commonly applied.[29] In this case, fibre length depends on the size of the de-husked coconut, which is highly variable in different plantations and even more in different regions.[30] A particular situation is that of bamboo, which, in spite of being a traditionally used material, especially in the building industry, was only rarely proposed in fibrous form as a reinforcement for polymeric matrices. In the few works existing on this subject, extraction was nevertheless carried out using a combination of mechanical (e.g. roll mill) and chemical methods.[31]

In general, it can be quite reasonably inferred that, in spite of extensive work on improving fibre extraction, the most common types of plant fibres used in composites have by no means sufficient properties for being used in structural components. As a consequence, it is not possible at this stage to rule out the use of fibre treatments to improve fibre characteristics. A large number of treatments have been proposed and applied, the significance of which has been discussed by Li *et al.*[32] In general, applied treatments tend in most cases to reproduce what has been realised on cotton fibres, in particular to obtain better dye tolerance for the tissue, a characteristic often termed dyeability. This is the case for sodium hydroxide, which proved effective in dissolving hemicellulose, forming microscopic sodium–cellulose local domains, depending on the diffusion of the alkali over the fibre surface.[33] The corresponding macroscopic effect comprises reducing fibre surface asperities and allowing quasi-cylindrical fibre sections to form.[34, 35] However, the improvement of fibre–matrix compatibility by geometrical effect appears to be compensated for by other negative chemical effects: for example mercerisation affects polymerisation, when cellulose fibres are cross-linked with polymer matrices, which can reduce the effectiveness of the treatment.[36]

In other instances, a better fibre–matrix compatibility has been achieved by adopting 'softer' treatments, such as silane, which was demonstrated to be effective, when working on glass fibres.[37–38] When concentrating on the need to have lower water absorption or anti-fungal protection of the fibres, the situation appears to be less defined, so that a number of treatments have been applied with some success. In practice, proposed treatments include acetylation,[39] a particularly flexible treatment, allowing e.g. the transformation of chitosan (i.e. polysaccharide) fibres into regenerated chitin (i.e. protein) fibres, an effective anti-fungal process;[40] benzoylation, particularly effective on cotton fibres in terms of elongation, moisture regain, friction, abrasion resistance and recovery;[41, 42] urea/microwaves;[43] isocyanate;[44] potassium permanganate/photocuring;[45] organic peroxide;[46] corona discharge and ultraviolet rays.[47]

In recent years, a matrix-specific treatment using maleic anhydride grafting on the polypropylene chain (MAPP) has often been applied, e.g. in references 48–50. This is a part of a present trend of employing polypropylene matrices, because they are thermoplastic and more easily recyclable, and as the base for obtaining co-mingled laminates, commonly used in the automotive industry and also proposed with plant fibres reinforcement, for better impact properties.[51]

Some of the above treatments, especially treatment with alkali, are simple and

inexpensive, although their efficacy in improving fibre stiffness and fibre–matrix bond may not result in a similar increase of the static properties of the composite. In particular, it is necessary to optimise the treatment in terms of time, quantity or chemical and possibly temperature of application, verifying microscopically that the required surface modification has been obtained.[52] This optimisation process is by no means obvious and depends on the vegetable species involved and on a number of biological aspects, which will be also dealt with in Section 5.4.

An alternative method appears to be to increase the resistance of fibre/matrix interface by, e.g. allowing penetration of the polymeric resin into plant wall cells, a method demonstrated to be effective on flax fibre/epoxy resin composites.[53] In this regard, the real challenge appears to be improving fibre extraction to avoid the high cost and the not always sure efficacy of chemical treatment and to convert the extracted fibres into appropriate intermediate products as true 2D random mats, bi-directional and unidirectional.

In conclusion, it is still fair to say that no systematic or else optimised approach to fibre treatment is available so far, although a number of comparative studies exist in which different treatments are applied to the fibres, and materials properties obtained as a result are characterised.[54, 55] A recent review, although having a quite optimistic approach to fibre treatment as necessary and potentially able to substantially increase both plant fibres strength and fibre–matrix compatibility, could not neglect the fact that in real terms these issues are far from resolved.[56]

5.4 Approaches to the realisation of plant fibre composites

5.4.1 Possibilities for plant fibre selection in composites

Plant fibres are currently selected in composites, mainly according to geo-political and economical aspects. As a consequence, a number of plant fibres have been proposed in the last decades for reinforcement of polymeric matrices. A list, which is by no means guaranteed exhaustive, is presented in Table 5.1. From an economic point of view, it is significant to notice whether the fibre production represents the main reason for that cultivation, or else it is only a marginal product among a great number of other products. For example, switchgrass can be used also for fibre production, although it is almost exclusively cropped for bio-fuel production, while on the other side there are fibres whose production belongs to a very large and complex industrial sector, such is the case with coir and the coconut industry. Most cases are intermediate between these two indeed.

Of course, the transition to a selection more based on materials *vs.* properties considerations, such as in an Ashby diagram, which exist already in the more general field of natural materials,[57] would require a large number of comparative studies between composites, obtained by the same manufacturing procedure using different plant fibres as a reinforcement. Some of these studies do exist, as reported

Table 5.1 Plant fibres used or proposed for use for materials reinforcement

Plant	Botanic name	Fibres extracted from
Abaca	*Musa* textiles	Leaf
Banana	*Musa sapientum*	Leaf
Bamboo	Various species	Stem
Betelnut	*Araca catechu*[86]	Seed hair
Celery	*Apium graveolens*[87]	Stem
Coir	*Cocos nucifera*	Fruit hair
Date palm	*Phoenix dactylifora, Phoenix sylvestris*	Leaf base (netted structure)
Esparto	*Lygeum spartum, Stipa tenacissima*	Stem
Flax	*Linum usitatissimum*	Stem
Hemp	*Cannabis sativa*	Stem
Henequen	*Agave fourcroydes*	Leaf
Indian grass	*Sorghastrum nutans*[88]	Stem
Jute	*Corchorus* sp.	Stem
Kapok	*Ceiba pentandra, Ceiba occidentalis*	Fruit hair
Kenaf	*Hibiscus cannabinus*	Stem
Lady's fingers	*Abelmoschus esculentus*[88]	Bark
New Zealand flax	*Phormium tenax*[89]	Stem
Oil palm	*Elaeis guineensis*	Fruit hair
Palmyra	*Borassus* sp.[90]	Leaf
Piassava	*Attalea funifera*	Leaf
Pineapple	*Ananas comosus*	Leaf
Ramie	*Boehmeria nivea*	Stem
Roselle	*Hibiscus sabdariffa*	Stem
Royal palm	*Roystonea regia*	Leaf
Sisal	*Agave sisalana*	Leaf
Spanish Broom	*Spartium junceum*[91]	Stem
Sunn hemp	*Crorolaria juncea*	Stem
Switchgrass	*Panicum virgatum* L.[92]	Stem
Talipot	*Corypha umbraculifera*[90]	Leaf
Vetiver	*Vetiveria zizanoides*[93]	Stem

Note: references are given for fibres not typically used in composites

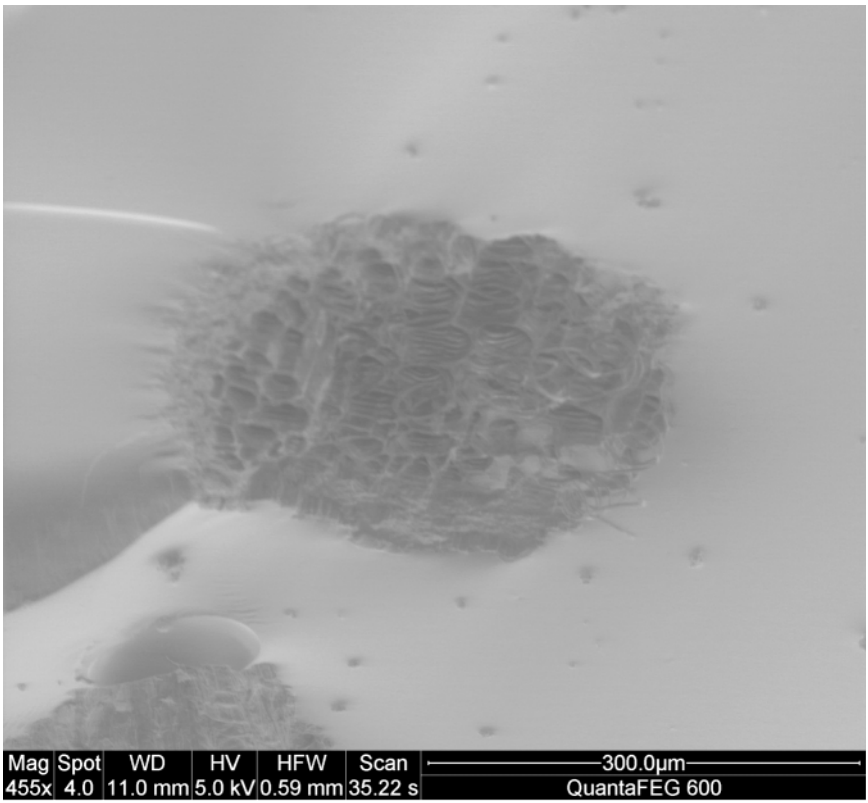
in Section 5.3 for fibre treatment, also on particular aspects of fibre performance, such as impact properties,[58] but a general database is still missing and, in a sense, well overdue.

One reason for this lack of large comparative studies is the complexity of factors involved, which is especially critical when biological factors are included, such as fibres maturity, taxonomic differences, etc., which can largely affect the final properties of the composite produced. In recent years, these aspects tend to be more recognised, so that composite studies tend to be more accurate in declaring the origin of crops from which fibres are extracted: this used to be generally overlooked, leading to the difficulty of tracing back exactly to the non-local species. A good example of this strategy is given by Faria *et al.*,[59] where the study leading to banana fibre/polypropylene composites is more dedicated to the microscopic

characterisation of fibres, reflected macroscopically, e.g. in the degree of crystallinity and the lignin content, than to the crude improvement of fibre strength with an appropriate treatment. Chemical treatment (MAPP in this case) is applied only once a sound knowledge of fibre properties is acquired: this might be a first step towards fibre selection.

A second reason, which appears to be more critical for the possible success of plant fibres as a reinforcement in composites may be deduced. Including biological structures, such as plant fibres, in man-made materials would require changing the engineering perspective to them. In practice, this would mean leaving aside the traditional approach to composites, as formed by fibres, embedded in a matrix with some kind of, possibly strong, interface. This approach requires that the laminate has a recognisable stacking sequence, to which the composite owes its quasi-isotropic behaviour. It has also been proposed as the basis for the production of 'biomimetic composite laminates' reproducing in their stacking structure the one used in biological composites, e.g. insect cuticles and fish scales. The bare reproduction of biological stacking sequences led to interesting results in terms of improvement of mechanical and impact absorption properties in carbon fibre reinforced composites.[60] The application of this philosophy to plant fibre composites would require nonetheless a greater level of complexity to be applied, introducing other factors: in particular, bamboo fibre has been described on a micro scale as a helical, multi-layered hollow cylinder.[61] Accepting these three characteristics of the reinforcement can be sufficient for some general modelling. However, as a consequence of the large dimensional variability of the fibres, starting at the lowest level, from the cell, as shown for example in Fig. 5.4a, representing a celery fibre structure, and in Fig. 5.4b, representing a jute fibre structure, every single laminate would have its personal 'stacking sequence' and hardly predictable properties. In other words, it can be deemed necessary to accept that some form of hierarchical structure is present in the composite, which is incidentally a fundamental observation of studies on natural composite laminates, such as e.g. arthropod cuticles.[62]

Coming back to fibre selection, the above reasons make the traditional approach insufficient for allowing the comparison between different plant fibres as a reinforcement; the different origin of the plant structure (bast, leaves, seed, fruits) would require also the interface and the stacking sequence to be modified to account for these differences. In contrast, the biomimetic approach may allow fibres selection on an objective basis, considering both the presence of hollow and their helical structure, formed by stronger (or crystalline) and weaker (or amorphous) parts. It is worthy to note nonetheless that this modelling can work if reasonably accurate predictions over the influence of defects are obtained. In real plant fibre composites, defects and dimensional variations are easily observable at any level of the hierarchical structure, as shown in Fig. 5.4a (celery fibre embedded in epoxy matrix) and Fig. 5.4b (bundles of jute fibres embedded in polyester matrix). The influence of defects in these materials has also obtained some

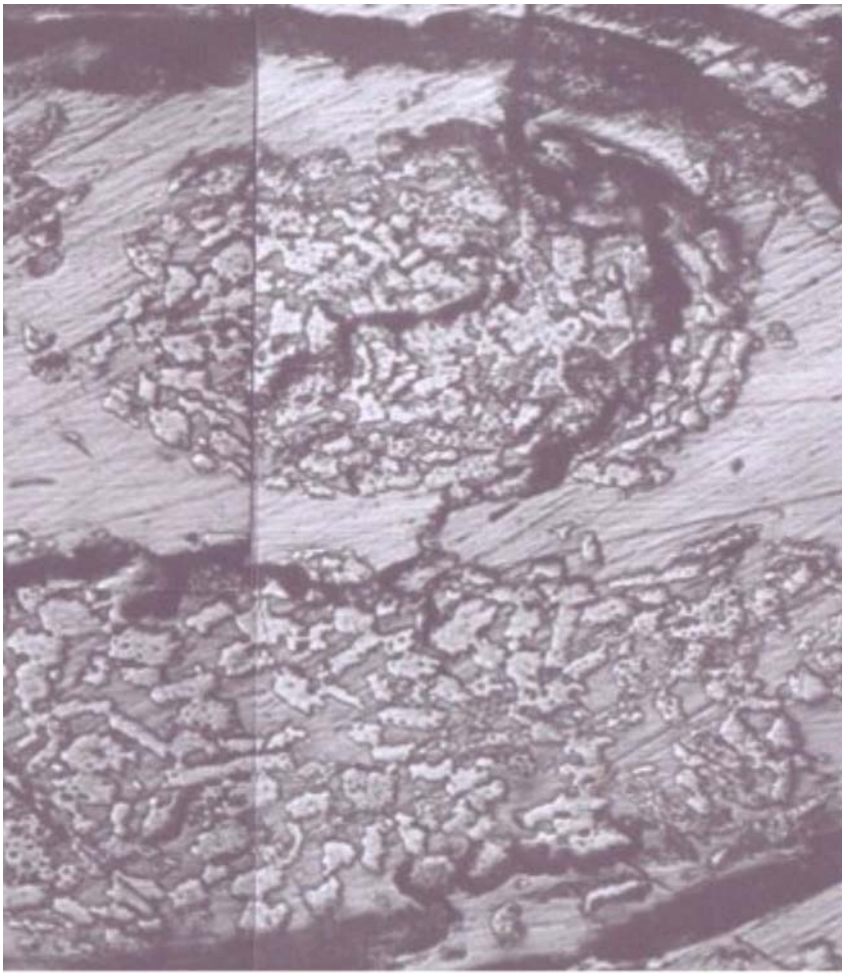


5.4a Structure of a celery fibre.

attention: it is well known e.g. that, in plant fibre composites, the longer the clamping length the lower the tensile strength; this is because of the presence of a larger number of fibre defects, i.e. failure initiators.[63] A similar effect was observed in impact-damaged plant fibre composites, when areas showing poor adhesion, which occur because of geometrical and structural variations of the fibre bundles, act as triggers for impact failure.[64]

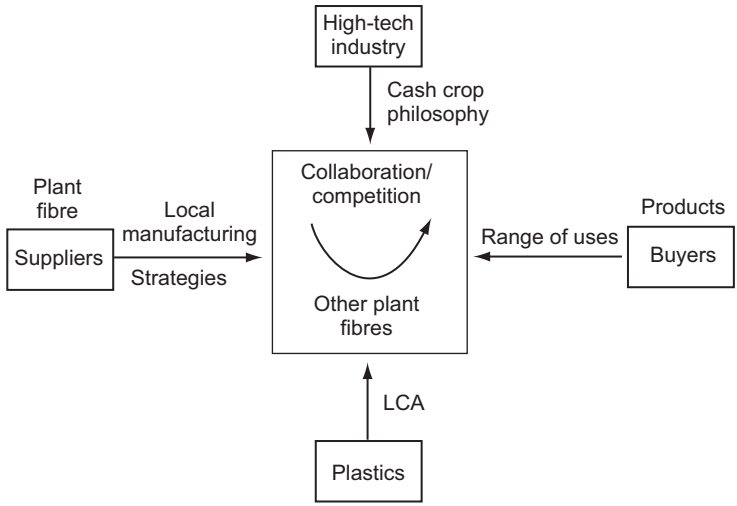
5.4.2 Life-cycle analysis of plant fibre composites and hybridisation as an environmental trade-off

The position of LCA in a typical economic system comprising the use of plant fibres is given in Fig. 5.5. More details on the general environmental significance (and true possibility) of replacing, e.g. glass fibre composites with plant fibre composites, as results from LCA, are given in Joshi *et al.*[65] The main environmental advantages of plant fibre over glass fibre composite appeared to correlate to the lower use of fossil fuels, even considering the use of fertilisers in agriculture, and



5.4b Structure of jute fibre bundles.

the lower level of eutrophication, as recognised also elsewhere.[66] A number of caveats can nonetheless be raised: first, the effect on eutrophication would need an absolute control over all the steps of agricultural operations, such as retting, which does not seem to be the case so far;[67] second, LCA results appear to be strongly influenced by the envisaged application, especially in the sense that the quantity of chemicals applied for treatment is depending on the level of improvement of fibre properties required.[68] Of course, selection of the fibres more suited to the application, albeit not easily obtainable, could assist in reducing the need for chemical treatment.



5.5 General economic system of the production of plant fibre composites.

In fact, in more recent studies LCA comprises three parts: inventory, dynamics, and validation, although traditionally the latter is omitted or disregarded.[69] For LCA studies involving natural fibres, validation cannot be neglected, but it has been hardly investigated so far. In particular, it is essential that the sustainability of the whole manufacturing procedure is assessed, which might involve a number of processes, such as fibre treatment and extraction, far from optimised, even if reasonably environmental friendly when compared with glass fibre sizing. In addition, the selection of the best fibre for a given application may represent a necessary development, which will possibly include modelling the material with analogies appropriate with the use of natural fibres. This 'natural modelling' would particularly require, as mentioned before, accounting for the helical symmetry of stacking sequences, the presence of hollows and the role of water and/or relative humidity in materials processing. Some examples of natural modelling are starting to be considered in composites, as is the case with the development of a particular synthetic fibre, inspired to a biological hollow fibre, referred to as 'technical plant stem'.[70] This can potentially lead to the active incorporattion of defects, for example with probabilistic methods, into modelling of natural fibre composites, in a way not dissimilar to what is done with dislocations in metals.

Meanwhile, the inherent difficulty of validating data obtained from LCA, considering materials with large variability, such as biological fibres, would in some cases require an intermediate approach: hybridisation. It has been recognised that hybrids have often been used in materials science and technology to expand design space, allowing a material to be designed with specific properties.[71] In a specific case, however, a hybrid laminate including two species of fibres, of which

at least one is extracted from a plant, albeit slightly complicated in manufacturing terms, may act environmentally in a kind of trade-off, offering some environmental gain with respect to man-made composites. It could also be 'designed for function', provided of course the assumptions made on it are as close to reality as possible.

In practice, hybridisation, although largely used over the last two decades on E-glass/carbon fibres,[72] kevlar/S-glass[73] and also E-glass/plant fibres[74, 75] with some limited success, has recently been re-introduced in a more global sense in composites reinforced with some plant fibres, in particular the more lignified ones, such as jute and sisal.[76, 77] However, most studies here again are concerned with finding the most suitable stacking sequence (usually plant fibres are used for inner layers and glass fibres for outer layers). This approach can be useful especially for the sake of simplicity, in order to evaluate if a positive hybridisation effect is reached, with reference to the possible validity of the 'rule of mixtures' for static mechanical properties.[78] There is no reason, nonetheless, that hybrids could not be obtained with a more complex structure, with a higher hierarchical order. In practice, as mentioned above, the reduction of mechanical and structural properties obtained with the introduction of some amount of plant fibres can be compensated by some environmental benefits, due to the higher grade of biodegradability of the material. Both the optimal amount of fibres to be introduced and the predicted environmental gain are strongly dependent on the application envisaged for the material. In Table 5.2, a small number of recent studies on hybrid configurations including natural fibres are presented and briefly discussed to give an idea of the issues involved in hybridisation.

Hybrids present advantages that are perfectly suitable in a biomimetic approach: the need, e.g. to account for the non-avoidable presence of water, allow hybrids to be employed in applications for which biological materials are in principle excluded, such as for dielectrics. Hybrids including plant fibres with higher lignin content, such as palm oil fibres embedded in natural rubber matrices as bio-dielectrics, have been proposed to reduce the environmental impact of these materials.[79] It is interesting to note that a trend towards extending the application of hybridisation between fibres exists, for example with hybrids including partially hydrophilic fibres, such as those obtained from avian feathers. Where, however, the need for extensive chemical treatment exists (sanitisation), this represents another source of environmental impact thus modifying the LCA data of the final material.[80, 81]

5.5 Conclusions: plant fibre selection for composites reinforcement

A biomimetic approach to the production of composites including plant fibres represents a possibility for addressing the problem of obtaining a more sustainable material, with a more acceptable LCA profile. In practice, the two combined aspects of accepting the unpredictable presence of defects and seeing the

Table 5.2 Some examples of hybrids including natural fibres

Hybrid fibres	Advantages	Limits
Short glass/empty fruit bunch[94]	Introduced in a composite waste material, reasonably clean, generally improving LCA	Considerable dimensional variations. Difficult compatibility
Jute/glass[76]	Good for industrial applications. Well-known hybrid for decades. Could revive jute market	Quite far from a biomimetic material. Traditional composite modelling (classical laminate). Could require aggressive treatments
Sisal/oil palm[79]	Plant/plant hybrids allow optimising properties of natural fibre composites. Closer to a biomimetic material	Two plant fibres mean double degree of variability in properties
Chicken feathers/ aspen wood[80]	Introduced in a composite waste material, to be treated, ideally improving LCA	Chemical treatment required. Difficult compatibility. Hard quality control of feather supply
Sisal/glass[77]	Ideal for industrial applications. Applications could match the traditional ones for composites (e.g. automotive panels)	Quite far from a biomimetic material. Traditional composite modelling (classical laminate)

opportunities offered by hollow fibres can lead to a new material modelling that includes the development of composite materials different from traditional ones, with some form of hierarchical structure. It is worthy of note that fibre extraction through biological retting can be designed to recognise the degree of hierarchical order present in the plant structure from the cell up to the fibre bundle and therefore contribute to materials design.[82] An essential aspect of this evolution for composite materials is the possibility of constituting woven tissues, which will be more similar to spontaneous tissues formed by plant fibres, according to their respective structure and geometry. In terms of materials selection, this would broaden the possibility of choosing plant fibres for composites to unusual or even infesting crops, such as e.g. nettle.[83]

In environmental terms, it is possibly fair to assume that a biomimetic approach to composites would allow using plant fibres in more sustainable materials, provided that the repeatability of the microstructure (hierarchical nature of the material) would lead to a repeatable result, even if it is not easy to identify it for materials modelling.

However, there are other aspects which need to be clarified, before hierarchi-

cally ordered and environmentally sustainable composites are available; in particular, environmental advantages need not be nullified by the presence of other sensitive issues. This applies particularly to two types of reinforcement recently proposed for composites: bio-silk fibres, where bio-compatibility for envisaged medical use requires sericin removal, obtained by genetic modification,[11] and keratin fibres from avian feathers, where sanitisation, usually by sodium hypochlorite, is required to achieve fibres suitable for use.[84]

An important conclusive consideration is in regard to the use of hybridisation procedures for composite manufacturing: these not only can suggest new applications for composites and cover gaps in existing properties, but also improve the environmental sustainability of existing materials, such as fibreglass with the introduction of plant fibres. Hybridisation, therefore, needs to be considered in the most general possible sense, looking not only at the compatibility between the originating materials, but also at their respective properties, such as in some of the examples discussed in Table 5.2.

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