CHAPTER 5

Inspiration from Natural Silks and Their Proteins

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Contents

1. INTRODUCTION

Silk, a kind of fine, strong, and lustrous fiber, consists of a series of natural polymers spun by any of various caterpillars and spiders. The source of commercial silk is mainly from the cocoons produced by the domestic silkworm larvae, *Bombyx mori*. In textile and fashion industry, natural silk is unfailingly popular for its smooth feeling and shimmering luster. Nowadays, however, silk and its component protein have received much attention as a kind of reproducible natural biomaterial. The outstanding mechanical properties and specific structure of silk provide a good model for researching and gaining better

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understanding of the relationship between molecular compositions, the associated structure forming capacity, and the ultimate property of final biomaterials. Moreover, silk and its protein demonstrated to have great potential in the biomedical field, for its biocompatibility, biodegradability, and excellent mechanical properties.

From the viewpoint of zootaxa, the silkworm and the spider belong to insect and arachnid of arthropod, respectively. Their silk proteins (fibroin for silkworm silk and spidroin for spider major ampullate silk) do not have any genetic heritage in common and their amino acids sequence compositions are different too. However, the silkworm and spider employ a similar spinning process to produce silk. Furthermore, the silkworm silk and the major ampullate silk have a number of similar structural characteristics, both at the level of the secondary protein structure and the condensed silk morphology. Therefore, for the sake of convenience, they are discussed together in some parts of this text.

First, a review of the properties and the structure of silk is given followed by a discussion on the relationship between molecular composition, assembled protein structure, and mechanical properties. Second, artificial spinning of silk proteins and their bioapplications are emphasized. Finally, the potential role of silk proteins in biomineralization is introduced and discussed.

2. STRUCTURE AND PROPERTIES OF PROTEINS AND SILKS

The *B. mori* silk fiber is made up of two kinds of protein. One is called sericin, a water-soluble protein responsible for the gum-like, sticky coating covering the fiber, and the other is referred to as fibroin, the core filament of silk. The inner part of silk fiber is composed of two monofilaments called brins (Figure 1a) (Poza et al., 2002; Shao and Vollrath, 2002).

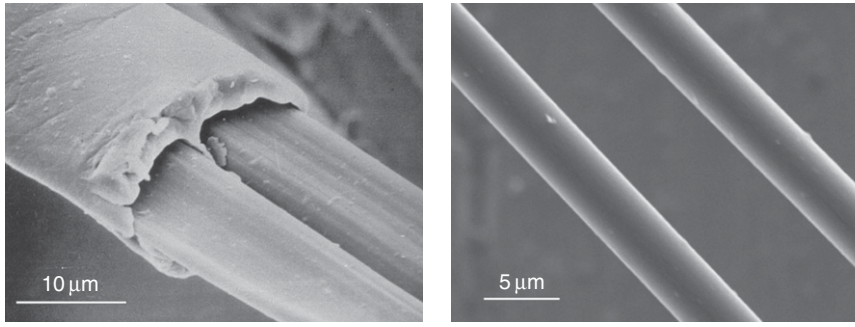

Figure 1 (a) the sericin (outer layer) and fibroin filaments of *Bombyx mori* silkworm; (b) the major ampullate silk of *Nephila edulis* spider.

Sericin which constitutes 25–30% of the weight of fiber can glue the fibers together to form a cocoon. In addition, it has several extraordinary properties aimed at protecting the silk, cocoon, and pupa inside, such as oxidation resistance, antibacterial, and regulating the moisture content (Zhang, 2002). Sericin is also responsible for sensitization and has some surprising properties in bioapplications as well (Zhaorigetu, 2001). However, most research focusses on the fibroin and therefore the outer sericin is removed by washing the cocoon in weakly alkaline solution or hot water (degumming).

The core filament of *B. mori* silk, fibroin, is composed of a heavy chain fibroin (H-fibroin, 391 kDa) and a light chain fibroin (L-fibroin, 28 kDa), as well as P25 protein (25 kDa). These three constituents assemble into the secretory units in the ratio of 6:6:1 (H:L:P25) (Inoue et al., 2000; Shimura et al., 1976). H-fibroin and L-fibroin link together via a disulfide bond and the P25 is thought to act as a kind of chaperon to assist the transport and secretion of the insoluble H-fibroin (Sehnal and Zurovec, 2004; Tanaka et al., 1999; Zhou et al., 2000). H-fibroin has a much higher molecular weight and takes up 90% weight of the core filament. The properties of the core filament are mainly attributed to H-fibroin, which is often referred to as fibroin.

In comparison to the silkworm which produces silk fibroin from a single silk gland, spiders have a set of highly differentiated glands that can produce several kinds of silk. Generally, an orb web spider has at least seven separate and morphologically distinct protein glands that can produce six different kinds of silk, as well as a glue substance. The different kinds of silk and glue substance have distinct amino acid compositions, structures, and properties that serve various biological functions (Lewis, 2006; Hu et al., 2006b; Vollrath and Porter, 2006). The dragline silk produced by the major ampullate gland has drawn the majority of research interests for the gland is relatively big and easy to study, while the fiber has outstanding mechanical properties. Therefore, further reference to spider silk and spidroin implies dragline silk and corresponding spidroin (Figure 1b).

Spider silk only has one protein monofilament, and the core-skin structure has been observed in some of them (Frische et al., 1998; Poza et al., 2002). It is thought that both the skin and the core are mainly composed of spidroins, which are differed from the primary structure (spidroin 1 and spidroin 2, >350 kDa calculated from mRNA) (Hinman and Lewis, 1992; Sponner et al., 2005a, b; Xu and Lewis, 1990).

2.1. Structure of the proteins

To some extent, the properties of the protein are mainly determined by its primary structure (i.e., the amino acid sequence). The two kinds of structural protein, fibroin and spidroin have a distinct and highly repetitive primary structure, which results in specific secondary and tertiary

Table 1 The structure elements in fibroin and spidroin (Hakimi et al., 2007).

	Fibroin (*Bombyx mori*) (ExPASy http://www.expasy.ch/)	Spidroin (*Nephila clavipe*) (Shao et al., 2003)
Main amino acids composition (%)	Gly 43.5 Ala 28.0 Ser 12.3 Val 2.3 Tyr 5.0	Gly 40.3 Ala 28.4 Glu/Gln 10.1 Pro 9.4 Arg 2.3
Repeating motifs	GAGAGS, GAGAGY (Asakura et al., 2004; Sehnal and Zurovec, 2004)	GPGGX/GPGQQ, $(A)_n/(GA)_n$, GGX (Hayashi et al., 1999; Hinman et al., 2000)
β-sheet content	40–50%	35%
Size of crystallite	$2 \times 5 \times 7$ nm (Hakimi et al., 2007)	$2 \times 5 \times 6$ nm (Grubb and Jelinski, 1997)
Molecular weight	391 kDa (Shimura et al., 1976)	>350 kDa (Hinman and Lewis, 1992; Xu and Lewis, 1990)

structures. These structural regularities provide fibroin and spidroin with outstanding properties.

Table 1 shows key structural elements in fibroin. The amino acid repeat unit in *B. mori* silk is mostly Gly-X (Glycine, G) [65% of X is Ala (L-alanine, A), 23% Ser (L-Serine, S), and 9% Tyr (L-Tyrosine, Y)], representing 94% of the amino acids in the total sequence (Zhou et al., 2001). The repeating motif GAGAGS – the first-order repeat among three kinds of repeats at different structural levels – is responsible for the crystalline region in silk. Strings of varying numbers of GAGAGS followed by a terminating repeat (usually GAAS) form the second-order repeat. Some Ala or Ser are replaced by Tyr within the second-order repeat, which is thought to disturb the periodicity to a certain extent. About 2–6 second-order repeats and an amorphous sequence of 43 amino acid residues compose the third-order repeat. The amorphous sequences are rich in charged amino acid residues and function as spacers breaking the repetitive region. Fibroin contains 12 third-order repeats with N- and C-termini. Both of the N- and C-termini contain a high proportion of hydrophilic residues (Sehnal and Zurovec, 2004; Zhou et al., 2001). The repetitive structure of fibroin is pictured schematically in Figure 2a. The charged residues in the amorphous sequence and the termini greatly influence the solubility and the assembly behavior of fibroin (Jin and Kaplan, 2003; Shulha et al., 2006). They also represent sites of fibroin that can interact with

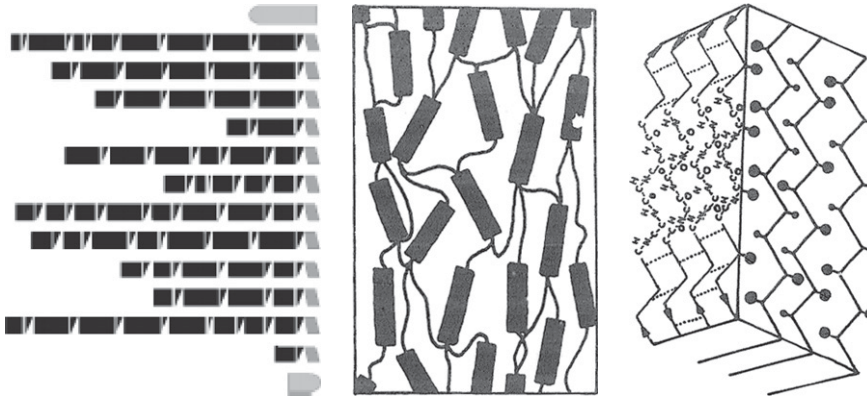

Figure 2 Schematic representation of the repetitive structure of (a) fibroin (Sehnal and Zurovec, 2004), (b) the crystalline and amorphous regions in silk, and (c) the structure of the parallel β-sheet crystals (Zhou et al., 2001). The bullnose symbols in (a) depict the nonrepetitive termini. Each pair of rectangle and triangle represents a second-order repeat unit. Each line on the symbols is the unit of third-order repeat. Crystalline regions are shown in black (Gosline et al., 1986; Heslot, 1998).

ions (Hossain et al., 2003; Peng et al., 2005; Zhou et al., 2003, 2005a, b, 2006b, 2007; Zong, 2004). The gray parts in Figure 2a stand for the hydrophilic regions in fibroin. The hydrophilic and hydrophobic blocks are located alternately along the molecule chain (Jin and Kaplan, 2003; Shulha et al., 2006).

The highly repetitive primary structure leads to the two-phase semicrystalline structure of fibroin (Figure 2b). X-ray diffraction measurements reveal the presence of secondary, β-sheet (Sehnal and Zurovec, 2004) structures (crystal parts) resulting from the repeating motif GAGAGS. In the β-sheet structures, Gly side chains protrude on one side and Ala side chains on the other side of the pleated sheet layer (Figure 2c) (Marsh et al., 1955). The β-sheet structure interlocks with adjacent chains via hydrogen bonds to stack into small 3D crystallites. The size of the crystallites is about $2 \times 5 \times 7$ nm, which is thought to contribute to the strength of silk (Hakimi et al., 2007). As a result, fibroin is traditionally described as rigid with inextensible crystallites embedded in a rubbery matrix (Figure 2b). The matrix is formed by the amorphous parts of the molecules, which have relatively poor orientation and exist as random coils (Mita et al., 1994; Shen et al. 1998). In some cases, they are recognized as 3_1-helices rich in Gly. These helices are somehow oriented parallel to the fiber (van Beek et al., 2002; Kummerlen et al., 1996). The helical region is thought to be related with the extensibility of the silk.

The tertiary structure of fibroin is stabilized by a combination of hydrogen bonds, a high level of crystallinity, and hydrophobic interactions. Such a

combination makes the fibroin insoluble to most aqueous solutions including dilute acids and bases. However, several methods have been developed to prepare fibroin solutions. Dissolving silk after degumming in a high-concentrate aqueous lithium salt solution or a $CaCl_2$/ethanol/water solution is widely used (Yamada et al., 2001). Stable fibroin solutions can be prepared with 1,1,1,3,3,3-hexafluoro-2-propanol or hexafluoroacetone (Yao et al., 2002). Recently, preparation of a fibroin solution using an ionic liquid has been reported allowing production of fibroin materials with different morphologies (Gupta et al., 2007; Jiang et al., 2007; Phillips et al., 2004, 2005, 2006). Other strategies allow for high concentration and high-molecular-weight fibroin solutions (Chen et al., 2004; Zhou et al., 2003).

Table 1 shows differences between spidroin and fibroin at the various structure levels. It is noted that spidroin contains high percentages of Ala and Gly, just as fibroin. Spidroin however, contains also Glu (L-Glutamic acid, E), Gln (L-Glutamine, Q), Pro (L-Proline, P), and Arg (L-Argenine, R). Pro mainly exists in spidroin 2 to form repeating motifs, that is, GPGGX/GPGQQ, besides the motifs of $(A)_n$,$(GA)_n$, GGX (Hayashi et al., 1999; Hinman et al., 2000). Amino acids that do not belong to repeating motifs are called "spacers." The $(A)_n/(GA)_n$ module can thus assemble into a β-sheet structure (Hinman et al., 2000). The interactions between the β-sheets are schematically shown in Figure 3. The poly(Gly–Ala) regions have a lower binding energy than poly(Ala) (Hayashi et al., 1999). The GPGGX/GPGQQ repeating units have been suggested to be responsible for β-turn spirals, which account for the elasticity of silk (Liu et al., 2008) and are highly involved in flagelliform silk, an other kind of spider silk with much higher elasticity. The GGX repeating unit is proposed to form helical conformations, 3_{10}-helix (Kummerlen et al., 1996), that could serve as a transition or link between β-sheets and less-rigid protein structures. These motifs are suggested to be structural modules (Figure 3a), and the spacers accordingly contain more charged amino acid residues and separate the repeating units into clusters.

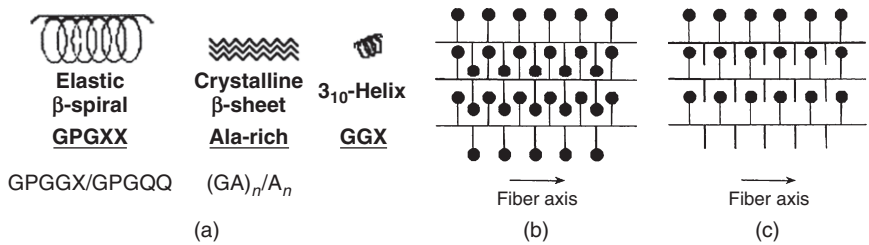

Figure 3 The structural modules as a result from certain amino acid motifs (a) in spidroin (Hayashi et al., 1999; Hinman et al., 2000). The interaction of two different β-sheet forms: poly(Ala) (b) and poly(Gly–Ala) (c) (Hayashi et al., 1999). Poly(Ala) has a tighter structure.

C- and N-terminal regions of spidroin are highly conserved among the spider silk proteins and play an important role in the assembly of spidroin (Huemmerich et al., 2004; Motriuk-Smith et al., 2005).

In addition, one hypothesis for the secondary structure in spidroin suggests that there are amorphous phases, highly oriented crystals, and oriented noncrystalline phases coexisting (Grubb et al., 1997). This structure model has been used to explain the super-contraction of dragline (Liu et al., 2005b).

2.2. Mechanical properties of silk materials

Although the amino acid sequence as well as the secondary structure of fibroin differs from those of spidroin, the fibers spun from these proteins, that is, silkworm silk and spider silk have comparable mechanical properties. These may be attributed to the structural characteristics, both at the molecular and filament level. The superior mechanical properties of silk-based materials, such as films, coatings, scaffolds, and fibers produced using reconstituted or recombinant silk proteins, are determined by their condensed structures.

In view of the various level of structural organization, it is worthwhile to draw a comparison between natural silk fibers (silkworm silk and spider silk) and man-made silk-based materials.

2.2.1. Mechanical properties of natural silk fibers

The significant research attention and the broad number of applications of silk is mainly driven by the prominent mechanical properties. From the mechanical point of view, spider silk (mainly dragline) is regarded as biosteel. The dragline perfectly combines attractive tensile strength with toughness. This composite property is superior to the strongest synthetic fiber such as Kevlar® (Poly-aramide, i.e., poly-paraphenylene-terephtalamide) or highly oriented ultra high-molecular-weight polyethylene. Silks have a better tensile strength than steel considering their much lighter weight. Table 2 compares the mechanical properties of several animal silks and some other natural and man-made fibers (Hinman et al., 2000).

Orb web spiders can produce several kinds of silk. These silks are used for different purposes depending on the different properties of each silk. For example, flagelliform silk is used to capture prey because of its high elasticity. Dragline silk is used for frame and radius building of the web as well as life-saving thread due to its high strength and toughness (Hu et al., 2006b). Interestingly, the dragline also has shape-memory, which prevents an abseiling spider from swinging (Emile et al., 2006).

The properties of natural silk are affected by numerous factors, such as nutrition, temperature, hydration state, extension rate, reeling speed (Knight et al., 2000; Madsen et al., 1999; Riekel et al., 1999; Vollrath and Knight, 1999; Vollrath et al., 2001), and spinning medium during the manufacture (Chen et al.,

Table 2 The mechanical properties of animal silks in comparisons to other man-made and natural materials

Material	Strength (N/m^2)	Elasticity (%)	Energy to break (J/kg)
Silkworm cocoon silk	0.4×10^9	15	6×10^4
Major ampullate silk	1.2×10^9	35	1×10^5
Flagelliform silk	1×10^9	>200	1×10^5
Minor ampullate silk	1×10^9	40–50	3×10^4
Kevlar	4×10^9	5	3×10^4
Rubber	1×10^6	600	8×10^4
Tendon	1×10^9	5	5×10^3
Nylon (type 6)	7×10^7	200	6×10^4
High tensile steel	2×10^9	<1	8×10^4
Collagen	$0.9–7.4 \times 10^6$	24–68	–
Polylactic acid	$2.8–5.0 \times 10^7$	2–6	–
Bone	1.6×10^8	3	5×10^3

Modified from literature (Engelberg and Kohn, 1991; Gosline et al., 1999; Hinman et al., 2000; Lewis, 2006; Pins et al., 1997; Vollrath and Porter, 2006; Vepari and Kaplan, 2007).

2002a, b, 2006b; Liu et al., 2005a). Dragline silk is very sensitive to water, which will affect its mechanical properties. The phenomenon is defined as super-contraction when dragline silk shrinks to a certain degree (varies with the species of spider) along its long axis once it contacts with water (Work, 1977). It is conjectured that spiders take advantage of super-contraction to form slack in webs and restore web shape when the silk contacts moist air (Guinea et al., 2003; Savage et al., 2004). It is thought that super-contraction of spider silk results from hydrogen bond breaking by water (or other solvents) and local molecular chains deorientation. The shrinkage depends on the various structure of silk, for example, content of Pro, crystallization of spidroin, orientation of well-defined regular and ill-defined regions, and the ability of solvents to interact with the organization (Eles et al., 2004; Liu et al., 2005b, 2008b). After contraction, silk will increase extensibility and decrease stiffness (Shao and Vollrath, 1999; Shao et al., 1999b, c); however, it can recover its mechanical properties as it can restore the major molecular order (van Beek et al., 1999). The mechanical properties (stress–strain curve) of silk can be determined by its super-contraction ability (Liu et al., 2005b).

Super-contraction, the chaperonage of the special structure of spidroin, is indeed an obstacle to the use of native spider dragline silk, especially in bioapplication (usually wet condition). Recently, it was found that the intrinsic properties of silk fibroin are much better than the data listed in Table 2. The inferior properties are generated by the spinning habit of

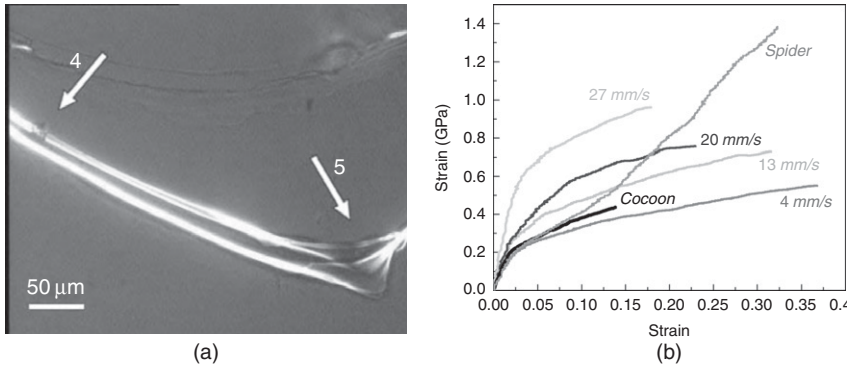

Figure 4 The defects (arrows pointed) on the natural spun silkworm silk (a), and a comparison of stress–strain curves of silks drawn at different speeds from the silkworm *Bombyx mori* (b).

silkworm, which causes defect points in the structure (Figure 4a). The artificial reeling of silk from immobilized silkworms under steady and controlled conditions can produce fibers that are superior to naturally spun ones and approach mechanical properties of *Nephila* spider dragline silk. The silkworm under controlled conditions produces stronger, but more brittle fibers at faster spinning speeds, whereas slower speeds lead to weaker, more extensible fibers (Figure 4b) (Shao and Vollrath, 2002). Consequently, high-performance silkworm silk whose properties are comparable to dragline silk can be produced and used in wet conditions.

The draglines of *N. edulis* have a good (even better) performance at low temperature. This "abnormal" property not only indicates the possibility of such silk to be used as "super-fiber" under severe conditions, but also supplies a model for researchers to investigate the contribution of inter- or intramolecular hydrogen bonds to the physical properties of protein materials (Yang et al., 2005).

2.2.2. Man-made silk materials

The attractive properties of silk fibers as a natural, sustainable product have inspired researchers to look for options to fabricate such fibers without the use of worms or spiders. Furthermore, these natural polymers, silk proteins (both fibroin and spidroin), allow for adjustable mechanical properties, thermal resistance (Drummy et al., 2005; Motta et al., 2002), as well as biomedical compatibility (Vepari and Kaplan, 2007).

The research endeavor to use silk proteins has been a lengthy one. It has generated a lot of insight in the hierarchical organization of proteins into secondary, tertiary, and quaternary structure and is starting to develop industrially viable applications as reflected in multiple spin off companies.

Beyond the well-known textile applications, silk proteins are being proposed for usage especially in the biomedical area (Vepari and Kaplan, 2007). Films, nonwoven mats, sponges, and micro/nanospheres prepared from silk proteins (mainly fibroin) are finding new areas of application. For example, thin films of silk protein are interesting for biomedical (Altman et al., 2003) sutures and optical applications (Putthanarat et al., 2004). A number of approaches aim to synthetically produce silk-like materials using block copolymers or explore recombinant silk proteins by genetic engineering. The mechanical properties of some man-made silk materials are listed in Table 3.

Several methods have been developed to produce silk-based materials with different morphologies, such as wet spinning (fiber) (Zhou et al., 2006a), electrospinning (nonwoven mat) (Jin et al., 2002), casting (film) (Mo et al., 2006), lyophilization (sponge) (Vepari and Kaplan, 2007), and mixing solution (sphere particle) (Cao et al., 2007; Zhang et al., 2007). Of particular interest are wet spinning and electrospinning. However, the complexity of the process requires careful control of multiple parameters such as the composition of solution, the coagulation, and post-treatment of the films or fibers.

Substantially more work is done on the elucidation of properties of regenerated silk, compared to film materials in view of the difficulty to fabricate uniform films. From Table 3, it can be concluded that the mechanical properties of man-made silk materials are inferior to the natural ones. This can be attributed to the fact that the final properties are greatly affected by the structural hierarchies. Typically, the "artificial" materials do not contain the controllable microstructure and supramolecular structure that natural ones possess.

2.2.3. Properties result from structure

The structural differences between natural and man-made silk materials determine the property differences. Even minor changes in the chemical structures give rise to a wide range of variability in mechanical properties (Porter et al., 2005). How this happens is both a challenge to understand and to control when fabricating silk artificially.

For one, the crystalline (or regular) regions mainly define the strength while the amorphous regions allow for the good elasticity in natural silks. Furthermore, the crystallites and most of the amorphous regions are highly aligned along the fiber's long axis. There are, however, some subtle difference between silkworm silk and spider silks.

In *B. mori* silk, the main repeating motif GAGAGS forms the β-sheets, with Gly facing one side and Ala the other side, as depicted in Figure 3c. The small 3D crystallites are stacks of β-sheets. This stacking combined with the hydrophobic interaction between the β-sheets locks the molecules in place. These small crystallites are regarded as the physical cross-links that

Table 3 The mechanical properties of some man-made silk materials

Morphology	Tensile strength (MPa)	Extensibility (%)	References
Film	100	0.5–3.0	Jiang et al. (2007)
Film	9.2	4.0	Li et al. (2003)
Film	58.8	2.1	Jin et al. (2004)
Film (wet)	28.5	51.3	Lv et al. (2005b)
Film (wet)	29.8	59.6	Lv et al. (2005a)
Film (wet)	4.5	10	Kweon et al. (2001)
Film	21	0.7	Freddi et al. (1999)
Fiber	80	11	Matsumoto et al. (1996)
Fiber	very weak	1.5	Ha et al. (2003)
Fiber	630	29.3	Ha et al. (2005)
Fiber	160	17	Um et al. (2004a)
Fiber	190	18	Zhao et al. (2003)
Fiber	180	16	Yao et al. (2002)
Fiber	500	20	Lin et al. (2007)
Fiber	127	12.7	Corsini et al. (2007)
Fiber	0.82 (cN/dtex)	25	Zuo et al. (2007)
Fiber	1.44 (gf/d)	8.4	Ki et al. (2007b)
Fiber	2.0 (gf/d)	20	Ki et al. (2007c)
Fiber	120	35	Marsano et al. (2005)
Fiber (spidroin)	320	4–8	Seidel et al. (2000)
Fiber (spidroin)	110–140	10–27	Shao et al. (2003)
Fiber (recombinant DP-1B)	140	8–10	O'Brien et al. (1998)
Fiber (recombinant ADF-3)	170	43.4	Lazaris et al. (2002)
Fiber (chemical synthesis)	13	22.9	Rathore and Sogah (2001)
Nonwoven mat	2.5	2	Jin et al. (2004)
Nonwoven mat	7.25	3.2	Ayutsede et al. (2005)
Nonwoven mat	1.5	1.6	Chen et al. (2006a)

provide the strength of silk. The amorphous sequences of 43 residues are rich in charged amino acid sequences. They rarely interact with the motifs and function as flexible links between the crystalline regions. Accordingly, a model of crystallites embedded into an amorphous matrix (Figure 2b)

explains the high tensile strength and good fiber flexibility (Sehnal and Zurovec, 2004; Termonia, 1994).

Some designing details are conserved in the fibroin to prevent excessive crystallization. GAGAGS occupy most of the repeat length, but the extent of crystallization is about 40–50% (Table 1). If all the GAGAGS formed β-sheets and crystallites, the silk fiber would become a rigid rod. NMR studies on the conformation of the synthetic peptide $(GA)_nG$ found that β-sheets are preferred when n is larger than 2 and that some β-turns appear when n is larger than 9 (Asakura and Yao, 2002). This indicates that long strings of GAGAGS may limit the degree of crystallization. The presences of quantities of Ser and Tyr in the repeating sequence can disturb the homogeneous motif catenations. Tyr partly incorporates into the β-sheets which causes local disorder in the crystal and increases the distance between β-sheets. Such defects are believed to split up the β-sheets under a smaller force, allowing some increase of the extensibility (Asakura and Yao, 2002; Asakura et al., 2002). Sehnal and Zurovec (2004) also suggested that the irregular length at the third-level repeats separated by spacer is important for defining the precise nature of the secondary protein structure and hence for defining properties.

In spider dragline silk, the repeating motif which is responsible for β-sheets is $(A)_n$. Successive Ala residues placed on alternate sides of the backbone (Figure 3b) induce an hydrophobic interaction between molecules that is much stronger than in $(GA)_n$ β-sheets. Also the $(A)_n$ β-sheets have a more compact structure than $(GA)_n$ β-sheets giving rise to $(A)_n$ β-sheet regions with stronger binding energy than $(GA)_n$ region. This structural feature explains why minor ampullate silk [with $(GA)_n$] has a lower tensile strength than major ampullate silk [with $(A)_n$] (Hayashi et al., 1999). This also supports the understanding that tensile strength results from the β-sheet (crystalline) region.

As mentioned above, the Gly-rich domains (GGX) is found to form a helical conformation, 3_{10}-helix (Kummerlen et al., 1996), which serves as a transition between β-sheets and less rigid protein structures. Hydrogen bonds form between these helices and will interlock molecules to some extent, while it also keeps the molecules aligned (Hayashi et al., 1999). Therefore, the GGX sequence may contribute to both the fiber's tensile strength and extensibility.

The repeating motif GPGGX/GPGQQ only exists in major ampullate and flagelliform silk known to have the highest extensibility. This motif has been proposed to conform to a spiral having a similar function as the β-turn spiral (Urry et al., 1975). The Pro residues serve as the points for the retraction after stretching while the side chains of Tyr and Ser stabilize the β-turn spiral through hydrogen bonds. GPGGX/GPGQQ acts as a spring and provides super-elasticity (Figure 5d) (Becker et al., 2003; Hayashi and

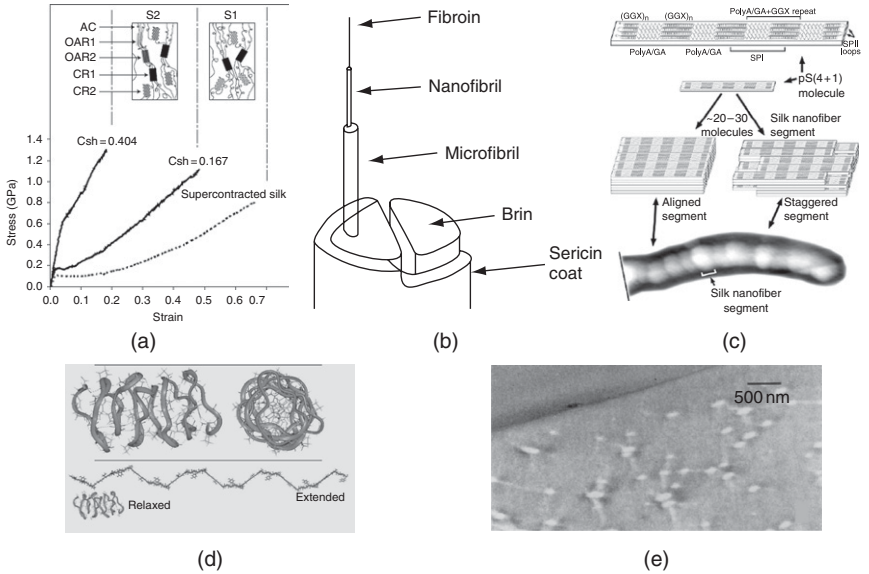

Figure 5 Schematic representation of various molecular structures and supramolecular structure encountered in silk. (a) The two states and the stress–strain curves of their representative spider silks under different degrees of contraction. The supercontraction is induced by loss of alignment with the destruction of hydrogen bonds by a solvent. AC, amorphous molecular chains; OAR1, oriented amorphous region where weak polar solvents such as alcohols can penetrate; OAR2, oriented amorphous region where only strong polar solvents such as water can penetrate; CR1, crystalline region (well defined); CR2, crystalline region (poorly defined) with S1–S2 having increased degree of orientation of this region (Liu et al., 2005b). (b) the schematically hierarchical structure of *B. mori* silk thread (Hakimi et al., 2007). (c) A model for silk nanofibrils organization: well-defined single molecules stacked together to form a nanofibrillar substructure (Oroudjev et al., 2002). (d) Molecular models for relaxed and extended $(GPGGX)_{15}$, which indicates the extensibility of such repeating motif (Becker et al., 2003). (e) the TEM of the silk that was stretched until rupture. Cracks are formed between the light domains. The crack can take up strain energy (Shao et al., 1999a).

Lewis, 1998). The flagelliform silk contains much more GPGGX/GPGQQ than dragline silk, so it is surprisingly elastic (Table 2).

Besides the structural elements mentioned above, mechanical properties of silks are influenced by many other factors. A major one is the aggregation states of the molecular chains (Chen et al., 2006b). Silk properties are strongly influenced by the spinning conditions (Chen et al., 2006b; Liu et al., 2005a). It is theorized that the fabrication conditions have little impact on the crystallites but greatly affect the alignment and orientation of the molecular chains in the amorphous region (Liu et al., 2005b). The combination of these structural elements, alignment of crystallites, and alignment of amorphous region results in the outstanding mechanical

properties of silk. Also the alignment of amorphous regions could explain the different capacities to shrink (super-contraction) of the different fibers (Figure 5a) (Liu et al., 2005b). The observation that the spider silk has different properties under different degree of contraction suggests that the overall morphology of silk is very important to the mechanical properties.

The mechanical superiority of silks is also related to the thread substructure, namely the supramolecular structure (Riekel et al., 2001; Sapede et al., 2005). *B. mori* silk and spider dragline silk are made up of bundles of nanofibrils, about 5 nm in diameter (Poza et al., 2002). The nanofibrils align parallel to the axis of the fiber and have a strong interaction (Figure 5b and 5c) (Miller et al., 1999; Oroudjev et al., 2002). This hierarchical structure, from the molecular organization to the nanofibrillar bundles, demonstrates to have a high resistance to the crack spread under tension in view of the heterogeneous morphology of dragline silk. It is suggested that the elongated canaliculi may contain fluid that acts as shock absorbers (Figure 5e) (Shao et al., 1999a; Sponner et al., 2005b).

In view of the intricate structural features in natural silk, it becomes understandable that man-made silks in order to achieve satisfying mechanical properties will require a significant step-up in fabrication control to manage for example a proper degree of crystallinity and crystal size, as well as molecular chains organization and orientation.

Regenerated fibroin films usually have poor physical properties, mainly because the molecules do not align well, and parts of the protein sequence adopt coil conformations. The poor alignment of molecular chains causes insufficient intermolecular interaction to induce the sliding between the molecules at low tensile stress. Although the β-sheet content in artificial silk materials can be increased by methanol or ethanol treatment and heating, allowing some enhancement of the film strength, the elongation at break remains poor. It should be noted that wet films usually have a large extensibility (Table 3), suggesting that molecular water acts as plasticizer, easing the molecular realignment and disentanglement under stress (Kweon et al., 2001; Li et al., 2003; Lv et al., 2005a, b).

Jiang et al. (2007) have investigated the structure and mechanical properties of ultrathin fibroin films prepared by spin-assisted layer-by-layer (SA-LBL) method. It was found that the tensile strength of such film is relatively high but its elasticity is low (Table 3). The SA-LBL process increased the β-sheet content and enhanced intermolecular interactions. The ultrathin film may confine the molecules into a 2D plane, but the crystalline structure is poorly ordered. The very high β-sheet content and the poor molecular alignment are associated to the high tensile strength and low elasticity.

These insights indicate that for artificial wet spinning of silk, there are a number of important processing parameters to consider for optimizing performance, including composition of spinning dope, spinning speed, temperature, coagulation bath, and post-treatment.

3. ARTIFICIAL SPINNING OF SILK FIBROIN

Although spider dragline silk has superior mechanical properties, most suited spider species are cannibals and not domesticated, making daily silk production difficult and with very low yield. To date, it is clearly not realistic to even consider spiders as an economically viable option for the commercial silk production similar to the silkworm silk. Still research on dragline silk has led to understand the hierarchical structure–property relationships of such nonbioactive proteins. Recently, genetic engineering has been carried out to clone and express recombinant spider silk protein in different host systems, such as transgenic tobacco, yeast, and mammalian cells (Fahnestock and Steinbuchel, 2003; Lazaris et al., 2002). The recombinant proteins were spun into fibers with fair mechanical properties. However, the high production cost and complicated process make it difficult for such a strategy to produce spider protein viable for commercialization and wide usage, at least for the time being (Chen et al., 2006b; Vepari and Kaplan, 2007).

Accordingly, major hurdles will need to be overcome to achieve required performance including overcoming inherent weaknesses of natural silk such as super-contraction and fabrication consistency (Hakimi et al., 2007). To date, film, sponge-like silk, and nonwoven mat materials can artificially be made from silk solution, as mentioned in previous section (Vepari and Kaplan, 2007).

As to fibers, it was reported that the inferior mechanical properties of silk from cocoons compared to spider silk result from the silkworm spinning process. If silkworm silk is processed at a constant pulling speed rather than constant force pulling, it possesses excellent properties, approaching the spider dragline silk (Shao and Vollrath, 2002). This suggests that the silkworm silk has the potential to produce better fibers, and the regenerated fibroin, which is easy to harvest, has the possibility to be fabricated into a reconstituted super-fiber.

More than 80 years ago, artificial spinning was carried out using regenerated spidroin and fibroin as spinning dope. Although the properties of regenerated silk then and now are still not good enough compared to those of natural silk, much progress has been made on the fabrication methodology.

It is therefore of interest to review the mechanism of natural silk processing first. In addition, some promising artificial spinning methodologies of silk-like materials are discussed, including wet solution spinning and electrospinning. It is believed that understanding the mechanism and the factors involved in the process will help researchers to produce high-performance artificial silk successfully via a biomimetic method.

3.1. Natural silk fabrication

Silkworms and spiders have developed a set of complicated but efficient spinning systems. They can produce silks with different properties under mild, ambient conditions in an aqueous solution. Considering the supreme properties, they really employ an efficient procedure with minimum energy consumption. Only the conformation transitions happen and no active enzymes work in the solidifying process. As a result, there is a great deal of interest in understanding the precise details of how silk forms from silk proteins – whether in vivo or in artificial circumstances (Fahnestock and Steinbuchel, 2003; Vollrath and Knight, 2001).

Here the production of dragline silk of *Nephila* is considered as an example. The dragline silk is produced in the major ampullate gland which mainly contains four parts, namely tail, lumen (sac), spinning duct, and spinneret (valve and spigot). The schematic structure of the gland is shown in Figure 6a (Lewis, 2006). The tail is the major part of the "spinning dope," serving as a reservoir for the aqueous solution of silk proteins. The sac is responsible for the storage of such spinning dope. The duct has three loops (limbs) and has a tapered shape. Spiders exude the protein solution in a duct where much of the fiber formation occurs. The duct terminates in the valve which has a muscular function that controls the flow rate and the fiber diameter. The final silk thread exits at the spigot (Vollrath and Knight, 2001). The special morphology of duct (three loops, decreasing in diameter with length) favors the formation of the silk. When the silk solution is pulled down a narrow duct, shear and elongational forces can be generated, which cause the alignment of the molecules. The dehydration of the solution also happens in the duct. There is a so-called internal drawdown in the third limb of the duct, in which the forming thread is suddenly stretched. The high elongational stress generated during this drawdown brings the molecules into a more extended conformation, so that they are able to join together forming intermolecular hydrogen bonds. The flexible lips of the spigot can fit tightly around the silk, which is suggested to assist in the water retention (Vollrath and Knight, 1999). Also the pulling force exerted by the spider legs in combination with the holding force by the muscular valve induce an external drawdown on the silk (Vollrath and Knight, 2001).

Vollrath and Knight (2001) have studied and reviewed the spinning process of spiders in detail. The spinning process is believed to employ a liquid crystal strategy. The state of the protein in the sac and in the first and second loop of the duct is a liquid crystal (Knight and Vollrath, 1999). The liquid crystal is considered to be in the nematic phase. The liquid crystal order (as well as the ordered structure observed in the solid silk) seems to form progressively in the duct because silk becomes increasingly birefringent as it passes along the duct while the solution in the sac is not birefringent (Work, 1977). This is attributed to the shear and elongational

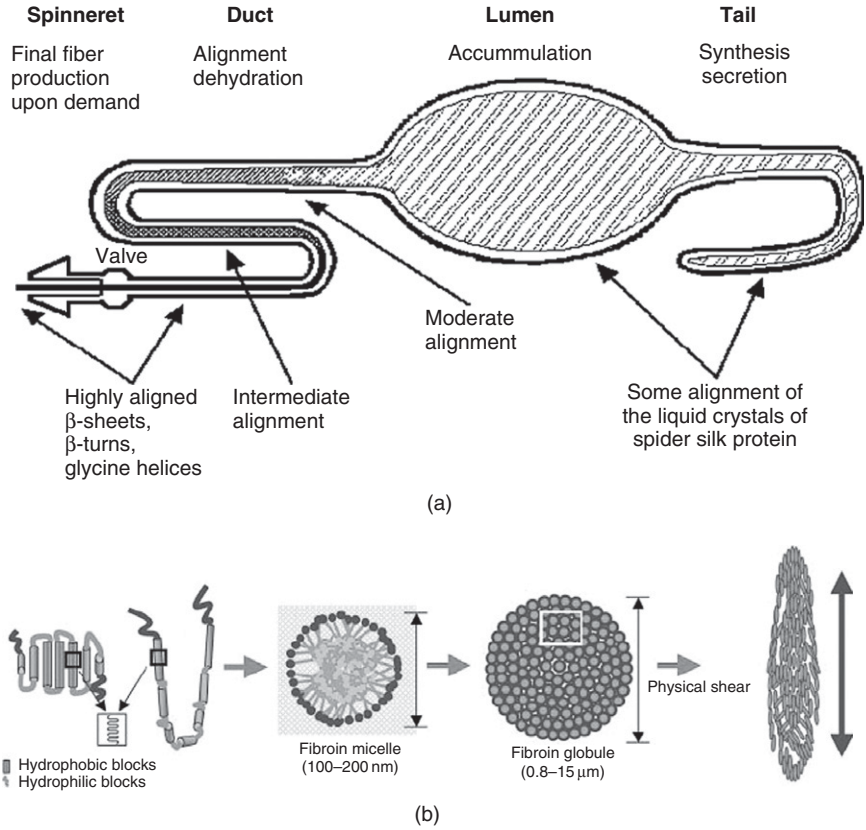

Figure 6 Schematic representation of major ampullate gland (a). The various parts of the gland and their function, as well as the structure of silk under each part, are shown (Lewis, 2006). Also shown is a model of chain folding, micelle formation, globule formation and curing, and shear processing of silk proteins. This model is motivated by experiments in vitro (Jin and Kaplan, 2003) (b).

forces aligning the protein molecules and altering their secondary structure (Lewis, 2006). It is thought that there are several advantages for using the liquid crystal strategy. First, it will prevent fiber formation in the sac. Second, it will form a compact conformation that allows the silk to be processed at high concentrations without aggregation. Third, it allows the viscous solution to flow easily allowing the spider to draw silk using minimal forces. Fourth, the prealignment will prevent defects from forming in the final fiber. Finally, molecular chains can still reorient with the plasticization of water.

Besides the shear force and internal or external drawdown, that is, physical boundary conditions, the chemical environment for fiber formation,

that is, presence of metallic ions and pH value of the solutions, defines the conformational transition and final properties of the fiber (Chen et al., 2002a, b, c; Dicko et al., 2004a, b; Knight and Vollrath, 2001; Zhou et al., 2005b; Zong et al., 2004). For example, there is a gradual decreasing pH from 7.2 in tail part to 6.3 in the third limb of the duct (Dicko et al., 2004b). This acidification of the spinning dope aids the conformational transition of the proteins from the random coil and/or helix into the final conformation of a mix of α-helix, β-spiral, and β-sheet in the final fiber (Chen et al., 2002b, 2006b). Metallic ions, such as sodium (Na^+), whose concentration decreases, and potassium (K^+), whose concentration increases along the duct, induce the formation of β-sheet and nanofibrils (Chen et al., 2002b, c).

The combination of physical and chemical boundary conditions defines how the silk protein solution turns into a solid silk thread consisting of highly oriented molecules and hierarchically organized structures.

Further insight into early steps in fiber production and the phase separation of the fibroin was provided by Kaplan and his coworkers describing the behavior of fibroin and polyethyleneoxide (PEO) blends (Jin and Kaplan, 2003). They proposed a model of molecular folding, micelle and globule formation, and thread development under shearing (Figure 6b). Gradually increasing the amounts of high-molecular-weight PEO to the fibroin aqueous solution induces competition for water between PEO and fibroin. It simulates the state change of fibroin into forming micelles and globules when water becomes less available inducing an assembly process (Shulha et al., 2006). These authors also suggested that the role of sericin is to adsorb water and increase the fibroin concentration.

These insights are forming the basis for advancing artificial spinning through mimicking of the natural process.

3.2. Solution spinning

The overall performance difference between the artificial fibroin silk and natural silk is induced by many factors. Composition of the spinning dope is critical but not the only factor. Important to understand is that the spinning process which determines the condensed structure of silk is crucial. It suggest that knowing the spinning process details it should be feasible to produce high-performance silk artificially and "design" silk.

The natural spinning by silkworm and spider is a "dry-wet spinning." It implies that an aqueous silk protein solution with high concentration is spun into a solid thread through without a coagulation bath. Current artificial spinning of silk protein are nearly all operating using the wet spinning process which needs a coagulation bath.

Regenerated spidroin and fibroin dissolved in various solvents are used as spinning dope while the coagulation baths are mainly alcohol (Table 4). In addition certain fiber post-treatments, such as drawing, are used as well.

Table 4 Examples of wet spinning processes using regenerated fibroin, spidroin, and recombinant spidroin, with different solvents and coagulation baths (Zhou et al., 2006a)

Spinning dope	Coagulation bath	References
Spidroin in HFIP	Acetone	Seidel et al. (2000)
Spidroin aqueous solution	Air	Shao et al. (2003)
Recombinant spidroin (DP-1B) in HFIP	Methanol	O'Brien et al. (1998)
Recombinant spidroin (ADF-3) in water	Methanol/water	Lazaris et al. (2002)
Fibroin in $LiBr{\cdot}H_2O$—EtOH—H_2O	Methanol	Matsumoto et al. (1996)
Fibroin aqueous solution (dialysis after dissolving in $Ca(NO_3)_2{\cdot}4H_2O_2MeOH$)	Methanol	Ha et al. (2003)
Fibroin in 95% formic acid and anhydrous TFA[a]	Methanol	Ha et al. (2005)
Fibroin in 98% formic acid[a]	Methanol	Um et al. (2004a)
Fibroin in HFIP[a]	Methanol	Zhao et al. (2003)
Fibroin in HFA[a]	Methanol	Yao et al. (2002)
Fibroin in mixture of formic acid and LiCl	Methanol	Lock (1992)
Fibroin aqueous solution with high concentration	$(NH_4)_2SO_4$	Lin et al. (2007)
Fibroin in NMMO	Air gap and ethanol	Corsini et al. (2007)
Fibroin in HFIP[a]	Ethanol and methanol	Zuo et al. (2007)
Fibroin in 98% formic acid[a]	Methanol	Ki et al. (2007b)
Fibroin in mixture of phosphoric and formic acid	Methanol	Ki et al. (2007c)
Fibroin in NMMO containing *n*-propyl	Ethanol	Marsano et al. (2005)

The properties of some production are listed in Table 3.
[a]The corresponding solutions were prepared by dissolving fibroin films.

The wet spinning of regenerated spidroin was reported in the early 1990s by Jelinski et al. They dissolved spider silk in hexafluoroisopropanol (HFIP) at a concentration of 2.5 wt% to produce an artificial fiber using water, methanol, isopropanol, and acetone as coagulation bath. The reconstituted silk could only be shaped in acetone but the structure

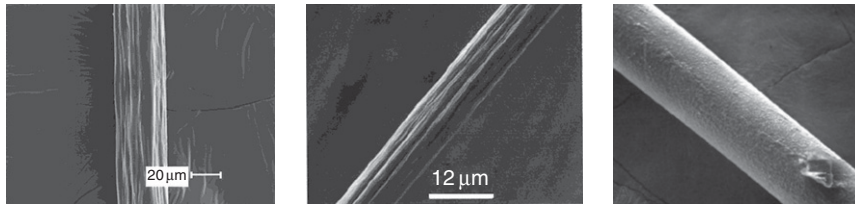

Figure 7 The morphologies of regenerated spidroin silks. (a) The silk formed in acetone and subsequently stretched (Seidel et al., 2000). (b) Silk drawn out of solution into air (Shao et al., 2003). (c) Silk produced from the recombinant ADF-3 (Lazaris et al., 2002).

as well as the properties were of low quality (Figure 7a) (Seidel et al., 1998, 2000). Shao et al. (2003) also used a regenerated spidroin aqueous solution to obtain a thread in air but again the properties and structure of the silk was not satisfactory. Poor structural regularity and lower β-sheet content were considered as causes, as the amino acids sequence was nearly identical with the originating natural silk (Figure 7b).

Recombinant spidroins have also been synthesized and spun into fibers (Hinman and Lewis, 1992; Lazaris et al., 2002; O'Brien et al., 1998; Xu and Lewis, 1990). Among these reports, Lazaris et al. (2002) obtained the best performing artificial spider silk. The spinning dope is the ADF-3 aqueous solution (23 wt%) and the resulting silk has higher elasticity but lower tensile strength compared to dragline silk (Figure 7c).

Considering the source of raw materials, neither regenerated spidroin nor recombinant spidroin is available in large amounts. This is an obstacle for these two production methods when commercial usage or even research is aimed for. Obtaining fibroin from the silkworm silk is well known and widely used as it is easier to obtain fibroin solution as spinning dope. Accordingly, research has focused more on the wet spinning of fibroin solution.

Pioneering work in fibroin wet spinning can be traced back to 1930s. After that, little work has been done until the late 1980s, when more research was done to investigate the spinning dope systems, and structure and properties of the artificial fibroin silk. The composition of the dope is very important to the properties of the final fiber. Several kinds of solvents, such as LiBr—EtOH, $Ca(NO_3)_2$—MeOH, formic acid, HFIP, hexafluoro acetone (HFA), and so on, are used to prepare the spinning dope (Table 4). Very recently, an ionic liquid was used as dope solvent (Phillips et al., 2005).

Hudson et al. used $Ca(NO_3)_2$—MeOH to dissolve silkworm silk and did not manage to obtain good fibers directly. They also casted film from such a solution and then dissolved the film in formic acid and trifluoroacetate acid

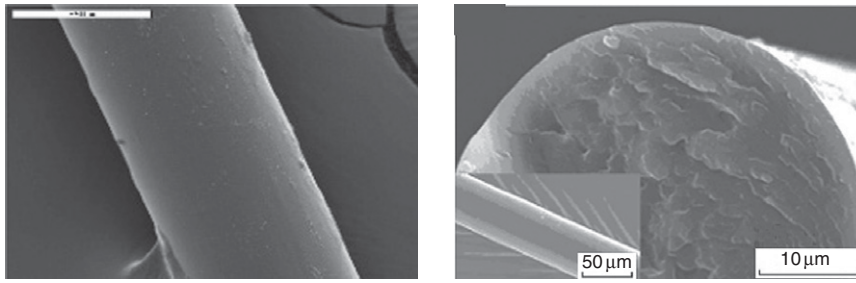

Figure 8 SEM pictures showing the morphology of artificial fibroin fibers from different processings. (a) Drawn fibers from TFA dope solution (Ha et al., 2005). (b) Cross-section and surface (inset) of fiber after post-drawn from aqueous solution (Lin et al., 2007).

(TFA). The reobtained solution could be spun into better fibers (Figure 8a) (Ha et al., 2003, 2005). Park and his coworkers did similar experiments but focused on the formic acid system. They compared the influence of many coagulation solvents and the processing temperature to the morphology of the final fibers. For alcohol systems (ROH), a larger R group will cause nonround fiber cross sections, and increasing the temperature of the coagulation bath will decrease the diameter of fiber (Um et al., 2004a, b). HFIP and HFA are thought to be good solvents for the fibroin wet spinning, as the solvents do not degrade fibroin and the solutions are stable. Although the produced artificial fibroin fibers have very good properties, the applied solvents are very expensive, being prohibitive for further commercial developments (Lock, 1992; Zhao et al., 2003).

Most of the solvent systems mentioned are rather harsh, and some will severely degrade the fibroin. Some will dissolve the fibroin only in relatively low concentration at room temperature. It is also difficult to obtain a spinning dope with a high-molecular-weight fibroin and a high concentration at the same time. Chen et al. (2004), however, have developed a novel method to prepare the fibroin aqueous solution, using high molecular weight and high concentration. Such a solution is good for the conformation transition and for the fabrication. Furthermore, the process of producing the solution is environmental friendly. With this aqueous fibroin solution, Lin et al. (2007) have spun the corresponding fiber with good mechanical properties using $(NH_4)_2SO_4$ as coagulation bath and having a post-treatment, that is, postdrawing and steam annealing.

Overall Table 3 indicates that the properties of artificial silk fibers cannot match those of native fibers (Table 2 and Figure 4).

From a scientific perspective, the artificial silk experiments have provided insight into the morphology of reconstituted silk. In the spinning dope, fibroin molecules adapt a random coil or other less extended conformations.

As the dope is pressed into the coagulation bath, the fibroin precipitates immediately and the random coil state is fixed limiting the intermolecular–protein interactions and producing a brittle fiber. Postspin treatment, such as postdrawn, may extend the molecules and ameliorate some intramolecular interaction into the needed intermolecular ones to the benefit of the orientation of molecules and the formation of β-sheets. These structural changes increase the mechanical properties to a certain degree (Corsini et al., 2007; Ha et al., 2005; Lin et al., 2007). Lin et al. (2007) have obtained an artificial fibroin silk from aqueous dope, whose mechanical properties are close or even better than those of natural silk (cocoon fiber). This encouraging result was obtained from an aqueous fibroin solution with high molecular weight and high concentration (Chen et al., 2004) and from the steam-annealing post-treatment.

Nevertheless, silk spinning remains a very complex process. Spiders and silkworms not only have a set of well-developed spinning glands but also have a set of well-defined and controlled chemical boundary conditions. Besides the composition of the spinning dope, the spinning techniques and the combination of chemical parameters (pH and metallic ions) must be considered and optimized.

3.3. Electrospining

Electrospinning is a direct method to produce nanofibers out of polymer solutions. The formation of fine fibers by electrospinning has been widely explored for applications such as high-performance filters and biomaterial scaffolds for cell growth, vascular grafts, wound dressings, or tissue engineering (Bognitzki et al., 2001; Boland et al., 2006). These applications benefit from the high surface area of the electrospun mat, their good biocompatibility, and fair mechanical properties (Jin et al., 2002). Therefore, electrospinning with fibroin/spidroin solutions is increasingly investigated to broaden the application of silk proteins.

Zarkoob et al. (1998, 2004) were the first to report on the electrospinning of silkworm silk and *Nephila clavipes* dragline protein. They used an HFIP solution of protein as the spinning dope. The resulting fibers had a wide distribution in diameter and the continuity during spinning could be significantly improved.

Since then research on the electrospinning of fibroin has led to improved results (Figure 9a). Most research is focused on improving the electrospinning device and optimizing the processing variables, such as the concentration of the fibroin solution, the strength of electricfield, and distance of collection, all greatly influencing the diameter distribution and the morphology of obtained silk nonwoven mat (Figure 9b). Also, nanofibers of silk protein with different oriented alignment were obtained by changing the shape of the injector electrode defining the Taylor cone geometry (Ayutsede

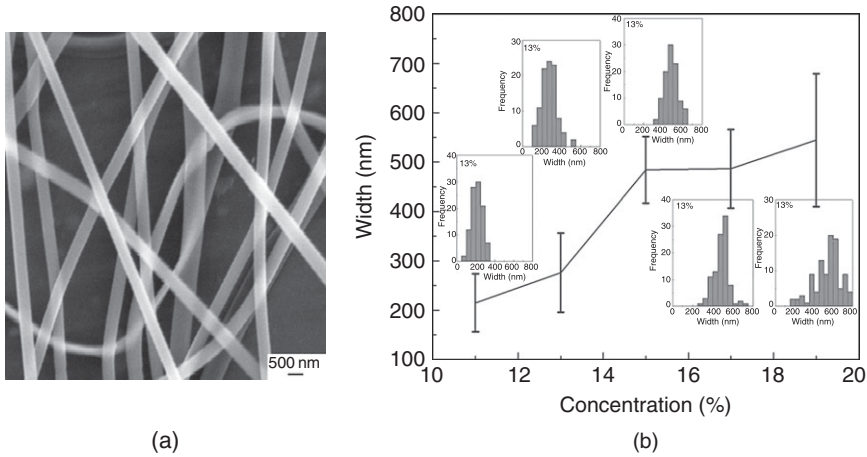

Figure 9 The electrospun fiber of fibroin (a) and the correlation between the concentration of fibroin and the diameter of electrospun fiber (b).

et al., 2006). Similar to the wet spinning process, the spinning dope is important in electrospinning. A number of electrospinning of fibroin experiments are summarized in Table 5.

In order to improve the properties and the spinnability, fibroin sometimes has been electrospun together with other natural or synthetic polymers (Jin et al., 2002; Park et al., 2004, 2006; Wang et al., 2004, 2006). For instance, Jin et al. (2002) developed an aqueous process for silk electrospinning in combination with PEO. More recently, Cao (2008) used PVA/Silk Fibroin (SF), Gelatin/SF, and Hydroxyapatite (HAP)/SF to produce double-layered (core-shell) nanofibers (mats) by coelectrospinning.

Several in vitro experiments have been carried out to examine the biological properties of fibroin nonwoven mats. The results show that the mats are suitable for cell adsorption, differentiation, growth, and propagation. The fibroin nonwoven mats really indicated the great potential for application in wound dressings and tissue engineering (Jin et al., 2004; Kim et al., 2005b; Meechaisue et al., 2007; Min et al., 2004, 2006; Wang et al., 2006).

Most electrospinning of fibroin is performed using formic acid or HFIP as solvent. Considering the biomedical application and environmental issues related to these solvents, electrospinning of fibroin without use of organic solvents is being pursued. All-aqueous processes for regenerated silk fibroin (RSF) electrospinning are reported (Wang et al., 2005, 2006; Zhu et al., 2007), with optimal concentrations of RSF around 30 wt%. In such electrospinning dopes, the RSF can only be considered as "protein fragments" rather than the complete protein in terms of molecular weight. Accordingly, only poor mechanical properties of the prepared nonwoven RSF mat are observed (e.g., breaking stress and strain were 0.82 MPa

Table 5 Some fibroin electrospinning results using different solution system

Solvent	Concentration (%)	Diameter (nm)	References
HFIP	0.23–1.2	6.5–200	Zarkoob et al. (1998, 2004)
Formic acid		30–120	Kim et al. (2003)
HFA	2–10	100–1,000	Ohgo et al. (2003)
Formic acid	5–20	20–400	Sukigara et al. (2003)
Formic acid	3–15	30–120	Min et al. (2004)
Formic acid	10–16.5	<40	Ayutsede et al. (2005); Sukigara (2004)
Formic acid	12	20–150	Ayutsede et al. (2006)
Water	28–37	~1,000	Chen et al. (2006a)
HFIP	5–10	250–550	Min et al. (2006)
Water	17–39	~700	Wang et al. (2005, 2006)
HFIP	7	500–1,200	Jeong et al. (2006)
Formic acid	12–18	100–400	Ki et al. (2007a)
Water	30–38	1,330–2,780	Zhu et al. (2007)

The morphology of the result nano-fibers can be tuned.

and 0.76%, respectively). It should be noted that the mechanism of electrospinning totally differs from that of wet spinning, for determining the final structure and associated performance as previously described.

4. BIOAPPLICATION OF SILK FIBROIN

Fibroin products with different morphologies (such as film, fiber, sponge, and nonwoven mat) have been produced successfully. In view of the overall economics, most applications are in the biomedical field. Silk sutures are used for centuries, and silk fibroin has been applied in clinical repairs and tissue engineering (Vepari and Kaplan, 2007). As a biomaterial, silk fibroin is biocompatible and nonbiotoxic, that is, biologically inert, and possesses some special advantages, namely excellent mechanical properties, controllable proteolytic biodegradability, morphologic flexibility, versatility for sterilization (Karageorgiou et al., 2004; Meinel et al., 2004a; Sugihara et al., 2000), and

options for chemical modification (Gotoh et al., 1998; Hakimi et al., 2007; Vepari and Kaplan, 2006, 2007).

Biodegradability and in particular control over the rate of degradation is critical for tissue regeneration. In an optimized design, the rate of scaffold degradation needs to match the rate of tissue growth (Altman et al., 2003). Although silk itself is defined as a nondegradable biomaterial by the United States Pharmacopeia, it is reported that fibroin is susceptible to proteolytic degradation and can be absorbed slowly after implantation in vivo and after a long-term, indicating that silk materials can provide adequate and robust support over a period of time (Arai et al., 2004; Kim et al., 2005c), in particular useful for slow tissue growth. The degradation of fibroin materials depends on many parameters, such as structure (porosity and pore size), morphology, molecular weight, and biological conditions at different implantation locations. Essential is the content of β-sheet which directly affects the rate of degradation (Huang et al., 2007; Minoura et al., 1990).

Many silk-based materials have been investigated in vitro and in vivo because of the perceived morphologic flexibility and the need for biocompatible materials for use in tissue engineering, bone engineering, and ligament repair. Table 6 lists the silk-based materials with different morphologies in different bioapplications. Some evidence suggests that these materials exhibit better biomedical properties than collagen, another protein fiber and a common biomedical material (Altman et al., 2003; Vepari and Kaplan, 2007). For example, the anterior cruciate ligaments (ACLs) have complex structures and require high mechanical properties. Several

Table 6 A listing of some bioapplication of silk-based materials

Morphology	Application	References
Fiber	Ligament tissue	Altman et al. (2002); Chen et al. (2003); Moreau et al. (2005)
	Tendon tissue	Kardestuncer et al. (2006)
Twisted silk rope	Ligament tissue	Altman et al. (2003)
Film	Wound dressings	Sugihara et al. (2000)
	Bone engineering	Karageorgiou et al. (2004); Kino et al. (2006); Sofia et al. (2001); Tanaka et al. (1999)
	Hepatic tissue	Hu et al. (2006a)
	Antithrombogenesis	Lee et al. (1998)
Nonwoven mat	Bone engineering	Kim et al. (2005b); Li et al. (2006)
	Connective tissue	Dal Pra et al. (2005)
	Endothelial and blood vessel	Fuchs et al. (2006); Unger et al. (2004)

Table 6 (*Continued*)

Morphology	Application	References
Porous sponge	Wound dressings	Yeo et al. (2000)
	Bone engineering	Karageorgiou et al. (2006); Kim et al. (2005a); Marolt et al. (2006); Meinel et al. (2004b, 2005, 2006a, b)
	Cartilage engineering	Aoki et al. (2003)
Hydrogel	Cartilage engineering	Aoki et al. (2003)
	Bone engineering	Fini et al. (2005); Motta et al. (2004)
Spherical particles	Drug carrier and release	Cao et al. (2007); Wang et al. (2007a, b)

Modified from literature (Vepari and Kaplan, 2007).

materials have been tried in ACL, but because of their supreme mechanical properties, silk-based materials seem better candidates in this particular area (Altman et al., 2002, 2003; Ge et al., 2006). Moreover, silk proteins have been probed in bone reconstruction because of their ability to deposit minerals.

As the coating protein of cocoon silk, sericin is proposed for bioapplications, although it has been reported to cause hypersensitivity (Altman et al., 2003). It is suggested that sericin could suppress the development of colon tumor by reducing cell proliferation and nitric acid production (Zhaorigetu, 2001). In addition, it can induce the deposit of bone-like apatite (Takeuchi et al., 2003; Terada et al., 2005; Tsubouchi et al., 2005).

5. BIOMINERALIZATION REGULATED BY SILK PROTEINS

Living organisms are capable of using proteins to deposit several kinds of minerals with specific function such as teeth and bone. This phenomenon is termed biomineralization. Typically, the biominerals formed during such a process have exquisite structure and possess outstanding mechanical and optical properties (Mann, 2001). Among these minerals, calcium carbonate, HAP, and silica have been widely investigated. There are several different morphologies of calcium carbonate in organisms, such as the brick-like structure sandwiched by proteins in nacre (Addadi et al., 2006; Kato et al., 2002), the sponge-like and fenestrated structure in sea urchins (Park and

Meldrum, 2002), the elaborately shaped spicules in ascidians, and finely the sculpted coccolith shells in coccolithophores (Mann, 2001). For silica structure, the diatoms produce the most exquisite siliceous cell wall (Sumper and Brunner, 2006). Also the calcium phosphate in bone shows a highly regulated organization and arrangement. Interestingly, avians have two main biomineralization systems, one produces calcium carbonate for egg shell and the other produces calcium phosphate for bone (Bauerlein, 2000; Mann, 2001).

Research related to biomineralization mainly includes three aspects, investigation of the structure of biominerals and the structure–function relationship (Bauerlein, 2003; Mann, 2001), the mechanism of the formation of minerals (in vivo and in vitro) and the exact control over the morphology of minerals by organism (Xu et al., 2007), and the production of new and high-performance materials under the concept of biomineralization (Slocik and Naik, 2007). In the latter field, new high-performance or functional hybrid-materials that are environmental friendly and low energy consuming could be produced by mimicking the process of biomineralization. Calcium carbonate, for example, is commonly used as model mineral. Synthesizing calcium carbonate-rich hybrid materials with controlled morphology is an important research subject (Colfen, 2003). Generally, the calcium carbonate is synthesized with the collaboration of an insoluble matrix and soluble molecules in the organism (mainly mollusk shell) (Addadi et al., 2006; Wei et al., 2007; Ajikumar et al., 2004; Hosoda et al., 2003; Litvin et al., 1997; Nudelman et al., 2006). In the experiments that investigate mechanism in vitro, synthesis of calcium carbonate in the presence of organic templates and soluble inhibitors is an effective technique, namely the template direction and growth inhibition method. Polyelectrolytes (Sugawara et al., 2003; Wang et al., 2005, 2006), double-hydrophilic block copolymers (DHBCs) (Colfen, 2001; Xu et al., 2007; Yu and Colfen, 2004), and biopolymers (Belcher et al., 1996; Butler et al., 2006; Falini et al., 1996; Feng et al., 2000; Hardikar et al., 2001; Leveque et al., 2004; Li et al., 2002; Raz et al., 2000; Shen et al., 2002; Verraest et al., 1996) including dextran, peptides, and proteins, such as collagen and fibroin, have been employed as soluble additives.

It is well known that well-ordered β-chitin (a polysaccharide) associated with a less ordered protein in the β-sheet conformation is the main component of nacreous organic matrix in shell. The amino acid sequence of such proteins is very similar to those of silk fibroins. Indeed, the amino acid sequence of a major protein from the nacreous shell layer of the pearl oyster resembles that of spidroin (Sudo et al., 1997; Weiner and Traub, 1980). The question of whether silk-like proteins play an important role in shell formation is raised. When Falini et al. (1996) did the experiment with the proteins from the shell, they assembled a substrate in vitro that contained β-chitin and natural silk fibroin and concluded that the silk fibroin may influence ion diffusion or the accessibility to the chitin surface or both. Furthermore, cryo-TEM study of the structure of the *Atrina* shell nacreous organic matrix without dehydration

suggested that the silk-like shell protein was not in the form of a distinguishable ordered layer but in the form of an disordered hydrated gel (Levi-Kalisman et al., 2001). It meant that silk-like proteins acting as an organizing template require the cooperation of other organic materials, for example, β-chitin and other acidic proteins.

The role of the individual silk-like protein played is unclear, and whether the silk-like protein may dominate the crystallization of calcium carbonate or not is still unknown. To provide experimental insights into the interaction of minerals and proteins, a model system containing RSF or spidroin as templates may be used for the crystallization of calcium carbonate.

Li (2005) used regenerated fibroin film as a substrate for $CaCO_3$ crystallization, in cooperation with soluble acidic peptides. It was found that the growth of $CaCO_3$ was aligned along the oriented silk films. It was suggested that fibroin molecules could cause the acid functional groups to be ordered over a large distance by orientation of molecules, which led to the alignment of $CaCO_3$. Moreover, a conformation transition of silk fibroin happened during the crystallization, which implied that the silk-like proteins in mollusk shell play a pivotal role during the formation of aragonite crystals in the nacre sheets through the interaction between crystal and proteins.

The use of fibroin, as a soluble additive rather than an insoluble matrix, can influence the crystallization of $CaCO_3$ (Cheng et al., in press). It was observed that the inherent (self-assembling) aggregation process of silk fibroin molecules affected both the morphology and crystallographic polymorphism of $CaCO_3$. This combination stimulated the growth of a novel, rice-grain-shaped protein/mineral hybrid with a hollow structure with an aragonite polymorphism (Figure 10a). These observations suggest new hypotheses about the role of silk-like protein in the natural biomineralization process. It also may serve to shed light on the formation process of those ersatz hybrids regulated by artificially selected structural proteins.

As fibroin is considered a good biomaterial, a number of experiments have been performed in bone engineering. Experiments in which fibroin or spidroin is used to modulate the crystallization of calcium phosphate have been carried out (Cao and Mao, 2007; Kino et al., 2006; Kong et al., 2004; Yao et al., 2007; Wang et al., 2007; Takeuchi et al., 2005). The fibroin solution can regulate the crystallization of calcium phosphate through the change of the initial structures, which is controlled by some chemical conditions such as metallic ions and pH value (Kong et al., 2004; Yao et al., 2007). Takeuchi et al. (2003) managed to deposit bone-like apatite on natural silk in solutions that mimic physiological conditions. It was found that the deposition benefited from the existence of sericin in natural silk fiber. Cao and Mao (2007) carried out the mineralization experiment

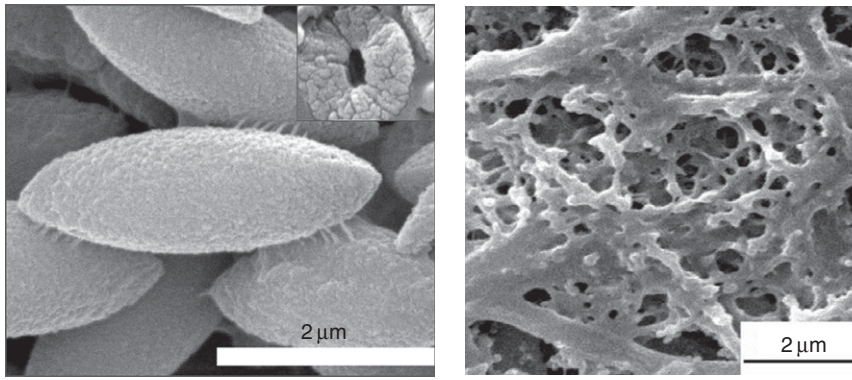

Figure 10 A novel micron-sized hybrid of silk fibroin and $CaCO_3$, with a hollow structure and unusual rice-like shape is synthesized in the presence of regenerated silk fibroin via aggregation-mediated crystallization (a); mineralization of HAP on the electrospun mat of silk fibroin (b).

on the spider dragline silk. As spider dragline silk can be pictured as the oriented organization of protein nanocrystals along the long axis of fiber, the nucleation sites for HAP orient along the fiber, which results in the preferentially c-axis orientation of HAP forming on the surface of silk. The authors suggested that the mineralized silks would combine the good mechanical properties of the spider silks and the biocompatibility of HAP and may be assembled into the ideal biomaterials for bone implants. Furthermore, a preliminary study has indicated that the HAP can mineralize on the surface of electrospun fibroin nanofibers as well as in the interstitial space of the mat (Figure 10b) (Cao, 2008).

Some silk-based materials have been used for bone reconstruction or cell proliferation (Meinel et al., 2004c, 2006b; Sofia et al., 2001; Tanaka et al., 2007; Vepari and Kaplan, 2007; Wang et al., 2007). Both in vivo and in vitro tests show that silk proteins have good affinity to bone cell and good bone conductivity. It is therefore anticipated that silks and their proteins will play more important roles in the field of bone tissue engineering, especially the material blend of silk and HAP or other growth agents (Kirker-Head et al., 2007; Li et al., 2005).

Recently, Foo et al. (2006) produced some novel nanocomposites from spider silk–silica fusion (chimeric) proteins. The composite morphology and structure could be regulated by controlling processing conditions to produce films and fibers. Silk and biomineralization being natural inspiration sources will allow production of numerous new materials in various fields of application.

6. CONCLUSION AND PERSPECTIVE

Silks are a unique group of fibrous proteins with unusually high mechanical strength in fiber form. The impressive mechanical properties result from a well-defined hierarchical structure. The primary amino acid sequence structure and the aggregation state of secondary and tertiary levels of organization of the silk proteins contribute to the fiber performance. Several kinds of silk-based materials such as fibers, films, sponges, and spheres have been produced. Due to their natural origin and good properties, silks and their proteins have been widely investigated in biomedical applications. Different morphologies are used in different tissue engineering applications. The rate of degradability can be adjusted by the β-sheet content and the overall fiber morphology.

Among the man-made silk-based materials, fibroin draws the most interest. Obtaining a "super-fiber" without silkworms or spiders from regenerated fibroin or spidroin is the ultimate goal. To date, artificial fibers still lags behind natural ones in term of mechanical properties. The reason is that the artificial fibers do not have the well-defined structure of natural ones, despite the similar amino acids compositions. Studying the natural spinning process has generated a lot of understanding useful for developing better artificial silk fabrication processes. Key is to optimize the process of artificial spinning by combining the physical (spinning dope, coagulation bath, spinning rate, and post-treatment) and chemical conditions (metallic ions and pH) that allow for good orientation and crystallization of the primary structures.

To extend the application area of silk proteins-based materials, blending the fibroin with other natural macromolecules and synthetic polymers, or even manufacturing composites with silk fibers are a few of the possible strategies.

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