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Processing of *Bombyx mori* silk for biomedical applications

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Abstract: This chapter highlights various processing methodologies that may be utilized to create a variety of structural forms from the protein fibroin, which is derived from the *Bombyx mori* silkworm cocoon. Modulation of fibroin material properties will be considered with respect to induced protein secondary structure formation. Additional considerations will be explored for sericin removal and fibroin extraction. A review of recent scaffold processing techniques will be reported, which include materials such as native fibers, electrospun fibers, sponge scaffolds, hydrogels, and microspheres. Finally, a perspective on the future trends relating to fibroin protein based technologies will be discussed.

Key words: silk fibroin, biomaterial, scaffold, tissue engineering, biopolymer.

3.1 Introduction

The use of silk is ubiquitous in society as one of the most useful and oldest natural fibers in the world with an evolutionary history of over 380 million years (Shear *et al.*, 1989). Similar to how humans use concrete, metals, and plastics to build the world around us, arthropods have employed nearly 40,000 different silk proteins to produce varying structures such as webbing, nests, cocoons, and underwater air sacks (Kaplan, 1994). Historically, humans have harnessed the fibers produced by the domesticated *Bombyx mori* silk-worm for uses in textile applications due to the extraordinary mechanical and visually appealing properties of the material (Shao and Vollrath, 2002). Commercial grade silk fibers are derived from the mulberry leaf-fed, domesticated *B. mori* silkworm cocoon, which is formed during the pupae phase of its life cycle (Jin *et al.*, 2002; Valluzzi *et al.*, 2002). The *Bombyx mori* silkworm cocoon is composed primarily of three proteins, which consist of the glue-like glycoprotein sericin and heavy and light chains of the structural fibrous protein fibroin (Kaplan, 1994).

It has been shown that fibroin may be resolubilized into an aqueous solution, and then formed into a number of different geometrical forms...
Table 3.1 Selected structures previously formed from silk fibroin solution with specific material characteristics and associated references

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<tr>
<td>Native fibers</td>
<td>Ropes or fibrous mats providing uniaxial strength</td>
<td>Extracted from cocoon by boiling in sodium carbonate solution then drying</td>
<td>Ligament, tendon, and soft tissue repair</td>
<td>Altman et al., 2002, 2003; Vunjak-Novakovic et al., 2004</td>
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<td>Electrospun fibers</td>
<td>Fibrous mat or tubular scaffolds composed of nano- to micron-sized fiber diameters</td>
<td>Silk solution is ejected onto a flat or tubular surface through the use of an electrostatic charge differential</td>
<td>Wound healing, cardiovascular grafts, soft tissue repair, intervertebral disc formation, peripheral nerve regeneration</td>
<td>Jin et al., 2002, 2004; Kim et al., 2012; Schneider et al., 2009; Soffer et al., 2008; Sukigara et al., 2003; Wharram et al., 2010; Zhang et al., 2012</td>
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<td>Films</td>
<td>Thin sheets of isotropic material with highly controlled length, width, and thickness dimensions</td>
<td>Silk solution is cast upon a molding substrate and water is evaporated to grow a continuous film structure</td>
<td>Cell culture substrates, corneal tissue, optics, sensors, electrical insulator, drug stabilization, nerve regeneration, tube formation</td>
<td>Bray et al., 2012; Horan et al., 2005; Hwang et al., 2012; Lawrence et al., 2008; Minoura et al., 1995; Rogers et al., 2012; Zhang et al., 2012</td>
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<td>Sponge scaffolds</td>
<td>Molded scaffolds with interpenetrating pores</td>
<td>Silk solution is blended with leachable solutes (i.e. salt or beads), cast using a mold, and then leached using the appropriate solvent system</td>
<td>Bone, cartilage, soft tissue repair, <em>in vitro</em> 3D culture systems, adipose</td>
<td>Liu <em>et al.</em>, 2012; Mandal <em>et al.</em>, 2012; Nazarov <em>et al.</em>, 2004; Papenburg <em>et al.</em>, 2009; Swinerd <em>et al.</em>, 2007; Wang <em>et al.</em>, 2008; Yan <em>et al.</em>, 2012</td>
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<td>Hydrogels</td>
<td>Filamentous scaffold interspersed by high amounts of water</td>
<td>Silk solution is subjected to an agitating force (i.e. ultrasonic cavitation or vortex mixing) which induces gelation process</td>
<td>Dermal void filling, cell encapsulation, intervertebral disc</td>
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<td>Microspheres</td>
<td>Nano- to micron-sized sphere structures</td>
<td>Silk solution is dissolved within a solvent system and post processed to produce desired particulate size</td>
<td>Drug delivery, vascular applications, chemical processing</td>
<td>Breslauer 2010; Lammel <em>et al.</em>, 2010; Wang <em>et al.</em>, 2007, 2010; Wenk 2008; Wenk <em>et al.</em>, 2011</td>
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(Jin and Kaplan 2003; Rockwood et al., 2011) (Table 3.1). This silk solution is termed ‘regenerated’ silk to indicate the natural origins of the fiber, which has subsequently been processed to produce a formable biopolymer solution (Altman et al., 2002). It is well documented that regenerated B. mori derived silk is highly biocompatible within the body, and also demonstrates an impressive range of material properties based on a variety of processing protocols (Altman et al., 2003; Minoura et al., 1995; Omenetto and Kaplan, 2010; Vepari and Kaplan, 2007). To that end, over the last two decades numerous investigators have been devoted to further understanding the potential of regenerated silk fibroin solution for use in biomedical applications in relation to tissue engineering, regenerative medicine, and drug delivery applications, which will be the focus of this chapter.

3.2 Modulation of silk biomaterial properties

Regenerated silk fibroin solution is produced by dissolving silk cocoons into water through the use of chaotropic agents, such as heavy salts, to disrupt the high degree of hydrogen bonding that exists between the individual protein molecules (Jin and Kaplan, 2003; Keten et al., 2010). The regenerated silk solution can then be reformed into a variety of three-dimensional geometries (Rockwood et al., 2011). Silk fibroin has previously been described as an engineering grade biopolymer due to the degree of control over its protein secondary structure formation (Lawrence et al., 2008b). Fibroin offers great potential for use in medically related applications due to the high degree of biocompatibility and non-inflammatory properties when implanted within the body (Meinel et al., 2005; Panilaitis et al., 2003; Wang et al., 2008b). This chapter will explore various aspects of the material properties of silk fibroin and the multitude of applications this protein has found in biomedical related applications.

Recent work has shown that constructs formed from silk solution are biocompatible in vivo, and have proven to be both non-inflammatory and non-immunogenic upon implantation within the body (Etienne et al., 2009; Fini et al., 2005; Huang et al., 2012; Mandal et al., 2012; Meinel et al., 2005; Pra et al., 2005). Current animal trials are underway to assess the use of silk solution in the creation of scaffolds for bone, ligament, and nervous tissue (Mandal et al., 2012; Wang et al., 2006; Yang et al., 2011). In addition, a number of scaffolds are being developed as in vitro tissue analogs for corneal, intervertebral disc, cardiac, breast, skin, and articular cartilage (Altman et al., 2010; Etienne et al., 2009; Harkin et al., 2011; Lawrence et al., 2009; Park et al., 2012; Patra et al., 2012; Yan et al., 2012). As with traditional tissue engineering approaches, the silk scaffolds are typically seeded in vitro with a specific cell type, and then cultured over time to produce tissue analogs (Kim et al., 2005). It has been shown
that the silk fibroin protein can be degraded through a number of naturally occurring proteolytic enzymes (Horan et al., 2005; Li et al., 2003). The hydrolyzed silk protein is believed to be cleared from the tissue through cellular phagocytic pathways (Desjardins, 2005). Silk fibroin protein is primarily composed of glycine and alanine amino acids that can be reused for new protein synthesis post-degradation (Kaplan, 1994). As a result, silk degradation products do not collect in the local environment to cause an induced inflammatory response, which is commonly associated with other synthetic biomaterials like poly-(lactic-co-glycolic acid) (PLGA) (Onuki and Bhardwaj, 2008).

The degradation rate and by-product formation of silk fibroin is directly related to the secondary structure content of the protein (Chen et al., 2001; Hu et al., 2006, 2011; Mo et al., 2006). By increasing or decreasing the presence of these structures the silk degradation rate can be adjusted from minutes to years (Kim et al., 2010; Wang et al., 2008b). This significant range of material processing is a key attribute of fibroin protein and is one of the important reasons for the potentially broad application to biomedical applications. The ability to control silk material properties offers a number of advantages over other biopolymer systems like collagen, chitosan, and alginate. The formation of silk structures begins with fibroin proteins aggregating into protein globules in solution (Jin and Kaplan, 2003; Keten et al., 2010; König and Kilbinger, 2007). The fibroin globules then aggregate to form larger bulk macromolecular structures that can then be modified through a variety of processing methods (Jin and Kaplan, 2003). The silk material properties can then be controlled through inducing protein secondary structure formations, such as alpha-helices and beta-sheets, through a variety of post-processing techniques (Chen et al., 2001; Hu et al., 2006, 2011; Mo et al., 2006) (Fig. 3.1).

The formation and organization of these structures modulates the total hydrogen and hydrostatic bonding within the bulk structure of the material, which affects the material properties at the macroscale. A variety of silk processing methods have been developed to produce a multitude of structures, and range from the use of physical factors, such as mechanical stress and heat, to the use of chemicals, from water to organic solvents, in order to induce controlled secondary structure formation (Agarwal et al., 1997; Gupta et al., 2007; Jin et al., 2005; Lu et al., 2010; Mandal et al., 2012). As a result, fibroin material properties such as degradation rate, hydrophobicity/hydrophilicity, transparency, mechanical strength, porosity, oxygen permeability, and thermal stability may be altered (Agarwal et al., 1997; Horan et al., 2005; Jin et al., 2004b; Kim et al., 2005; Motta et al., 2002; Tretinnikov and Tamada, 2001). In this regard, silk proteins can be considered as an engineering class of biopolymers in which the material properties can be defined for a given application.

Water plays an important role in processing regenerated silk fibroin materials. The control of regenerated silk fibroin material properties is primarily achieved through modulation of hydration state (Agarwal et al., 1997; Hu
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Water acts as a lubricant, which allows for protein chain movement and secondary structure formation (Hu *et al*., 2007, 2008, 2011). As a result, processing of regenerated silk materials can be achieved through aqueous based processes, however, organic solvents can be utilized as well (Chen *et al*., 2001; Hu *et al*., 2011; Mandal *et al*., 2012). The use of water vapor to control protein secondary structure formation is commonly referred to as water-annealing (Hu *et al*., 2011; Jin *et al*., 2005). The extent of secondary structure modification is controlled through enhancing water vapor content available for interaction with the fibroin protein chains (Hu *et al*., 2007; Lawrence *et al*., 2008b; Mo *et al*., 2006). With more extensive secondary structure formation there is an increase in internal hydrogen bonding which modulates the apparent material properties, which may be specified for a given application (Hu *et al*., 2008).

Previous research indicates that beta-sheet content can be modulated between 10% and 60% of total protein secondary structure through various water-annealing processes (Lawrence *et al*., 2008b). For example, placing newly cast silk films in a chamber with water placed in the basin to increase chamber humidity will cause the formation of up to 24% beta-sheet content, however if this is used in conjunction with high pressure and temperature steam, the beta-sheet formation will increase to 60% (Lawrence *et al*., 2008b).
Further work has demonstrated that exposing the silk material to increasing water vapor content will drop the glass transition temperature \( T_g \) from 180 to 40°C (Agarwal et al., 1997; Hu et al., 2006, 2011). Practical applications of this drop in \( T_g \) have been utilized to produce rapid surface imprinting methods and also for modifying silk scaffold degradation \textit{in vitro} and \textit{in vivo} (Amsden et al., 2010; Arai et al., 2004; Wang et al., 2008b). These specific examples provide compelling evidence for the large range of material processing windows attainable for controlling fibroin protein secondary structure formation.

### 3.3 Silk fibroin materials and their use in biomedical applications

The silk fibroin protein is a structuring molecule that has the ability to be processed into numerous forms through a variety of techniques. Due to the material’s inherent biocompatibility, the protein has been recently utilized as a scaffold for developing a number of biomedical product ideas for use in clinical applications. A few examples of such clinical applications are explored below.

#### 3.3.1 Sericin extraction and fibroin solubilization

Silk fibers were traditionally used as medical sutures before the development of biodegradable synthetic polymers (Altman et al., 2003). However, silk sutures would in many cases need to be removed due to their slow biodegradation rate and the potential for inflammatory reactions to their presence (Altman et al., 2003). This inflammation was largely found to be a reaction to the presence of the glycoprotein sericin fiber coating, which could be removed through cleaning the fibers with detergents (Barker, 1975; Santin et al., 1999). Once the sericin was removed the regenerated structural silk fibroin protein was well tolerated by the body (Meinel et al., 2005; Panilaitis et al., 2003; Santin et al., 1999; Wang et al., 2008b). As a result both native silk fibers free of sericin and regenerated fibroin protein solution offer desirable material properties for designing silk-based biomedical devices.

Silk fibroin solubilization has been documented since the beginning of the twentieth century (Matthews, 1913), but only recently has the use of regenerated silk solution for biomedical applications been studied (Altman et al., 2002; Minoura et al., 1995). The most ubiquitous method to produce regenerated silk fibroin solution is through the use of NaCO₃ and heavy salts like LiBr (Lawrence et al., 2012b; Rockwood et al., 2011) (Fig. 3.2). In addition, mixtures with urea and ionic liquid solutions have been used to produce silk solution (Gupta et al., 2007; Shaw, 1964); these processes will not be described here, however, for brevity.

Silk fibers may be cleaned of contaminating sericin proteins by boiling cocoons or fibers in 0.02M NaCO₃ in less than 1 h. The extracted fibers should
be thoroughly rinsed with pure water, and then allowed to dry using clean convective air. The dried fibers can then be dissolved using a 9.3M LiBr solution to disrupt hydrogen bonding between the fibroin protein chains (Lawrence et al., 2012b). The solution should then be allowed to thoroughly dissolve for up to 4 h at 60°C to ensure complete dissolution, while making sure the reaction container is covered to prevent water evaporation. The heavy salts, in this case LiBr, can then be removed from the silk solution through dialysis against pure water over a period of 48 h. Typically the molecular weight cut-off for the dialysis membrane is 3500 Da, which is permeable enough to allow for the LiBr salts and water to travel freely while retaining the 25 and 390 kDa fibroin light and heavy protein chains, respectively (Kaplan, 1994). Final silk solution concentrations range from 6% to 10% wt./vol. content, however dialysis against high molecular weight water-soluble polymers, like polyethylene glycol (PEG), allow for concentrations of the silk solution of over 20% wt./vol. (Kim et al., 2005; Mandal et al., 2012; Nazarov et al., 2004).

3. Rinse and dry fibroin fiber extract

Structures formed from silk fibroin solution:
- Films
- Tubes
- Scaffolds
- Hydrogels
- Microspheres
- Electrospun fibers
- Microfluidic devices

4. Dissolve fibers into LiBr solution

5. Dialyze silk solution for 48 hrs

6. Centrifuge silk solution at 10 000 g

7. Use silk solution or store at 4°C

3.2 Schematic diagram detailing the key steps for preparing silk fibroin solution from Bombyx mori silkworm cocoons.
The silk solution can then be stored in a 4°C refrigerated environment, where the material has shown stability for up to two months post production (Le et al., 2008; Matsumoto and Lindsay, 2008).

3.3.2 Native fibers

Native silk fibers derived from the *B. mori* silkworm cocoon provide the raw material that is used to produce regenerated silk solution. Silk fibers have found wide utility in the medical world as sutures over the past millennia (Altman et al., 2003). As mentioned earlier these fibers are composed of the fibroin protein, which provides robust mechanical strength in the native silkworm cocoon. Recently, the utility of ‘extracting’ the native fiber from the sericin coating proteins has been invaluable for the use of this protein in medical applications (Altman et al., 2003). The extracted silk fibers have relatively high tensile strength recorded between 500 and 740 MPa, a modulus between 5 and 17 GPa, and an elongation to break ranging from 4% to 20% (Altman et al., 2003). This is in the order of 5–10 times higher when compared to other synthetic and natural polymers. Native silk fibers can then be further used for the design of medical devices.

Recently, silk fibroin native fibers have been combined to produce scaffolding structures that have found utility in soft tissue repair, such as ligaments (Altman et al., 2002; Vunjak-Novakovic et al., 2004). Specifically a silk fiber mesh can be twisted together to produce a rope structure that mimics the natural mechanics of the anterior cruciate ligament (ACL) (Altman et al., 2002). The structure can then be implanted *in vivo* to serve as a scaffold to regenerate new ligament tissue (Vunjak-Novakovic et al., 2004). Extending from this work these fibers can then be resolubilized into regenerated silk solution as described above to produce a number of geometrical structures, which are described further below.

3.3.3 Electrospun fibers

Electrospinning silk solution is a favored processing methodology for producing nanometer- to micron-scale fibers that result in a high degree of available surface area for use in creating scaffolds for tissue engineering and regenerative medicine purposes (Jin et al., 2002). In brief, electrospun materials are produced by applying a strong electric field between a polymer solution and a collection device (Soffer et al., 2008). In the case of silk fibroin this has been readily accomplished using both organic solvents and aqueous based processes (Jin et al., 2002; Sukigara et al., 2003). The electrospun silk fibers have found utility in producing scaffolds for a variety of biological applications such as growing cardiac, bone, nerve, and skin tissue
One area of work has focused on the use of a silk fibroin and poly(ethylene oxide) (PEO) solution blend that reduces material brittleness and enables ready processing and handling of the final material (Jin et al., 2002). Fiber diameters range between 700 and 900 nm for varying silk/PEO blends. Further work using silk/PEO blends was undertaken to form electrospun mats for use in culturing human bone marrow stromal cells (hBM-SCs), which demonstrated excellent cell attachment, spreading, and culture growth over a 14-day period (Jin et al., 2004a). These materials compared favorably with native silk fiber controls. Building from this work silk/PEO electrospun fibers were used to produce tubular scaffolds for use as small-diameter vascular grafts (Soffer et al., 2008). Human endothelial and smooth muscle cells were successfully grown on these scaffolds, and mechanical testing demonstrated the ability of the constructs to withstand arterial pressures and tensile properties comparable to native vessels (Soffer, 2006).

Similar blended silk/PEO electrospun constructs were developed for use in wound healing applications (Schneider et al., 2009; Wharram et al., 2010). Initial material characterization proved that these scaffolds exhibited aqueous absorption, water vapor transmission, oxygen permeation, and enzymatic biodegradation profiles applicable for full thickness wound site regeneration (Wharram et al., 2010). Further studies used a human skin-equivalent wound healing model to demonstrate physiological feasibility (Schneider et al., 2009). The study showed that the silk/PEO electrospun fibers could be loaded with epidermal growth factor (EGF) and release 25% of the molecule content over a one-week period. It was shown that the electrospun mats increased wound closure by a remarkable rate, increased by 90% when compared to controls. Additional work has shown successful implantation of electrospun silk fibroin constructs in vivo using a subdermal rat model (Kim et al., 2012). Results indicated that electrospun constructs demonstrated varying biodegradation profiles based on post-processing with varying blends of ethanol and propanol concentrations, where higher ethanol concentrations showed longer degradation times. In addition, implanted constructs proved highly biocompatible, and supported cell infiltration for materials treated with lower ethanol concentrations. Together these findings suggest promise for such materials in the use of chronic wound healing.

3.3.4 Silk fibroin films

Silk films offer an elegant and straightforward biomaterial of choice for medical device design. Due to the inherently less complex nature of films these materials offer rapid characterization and design with regards to scaffold development. The dynamic protein secondary structure of the fibroin beta-
sheet and alpha-helical structures has been largely determined from studies utilizing silk film samples (Hu et al., 2008; Motta et al., 2002; Tretinnikov and Tamada, 2001), and they possess the longest history of scientific experimentation and use (Matthews, 1913; Minoura et al., 1990). As a result, silk films offer the most immediate potential utility for a variety of biomedical applications (Kaplan and Omenetto, 2012b; Omenetto and Kaplan, 2008). As a biomaterial of choice they offer a number of advantages for in vitro characterization due their ease of production and consistent material properties (Lawrence et al., 2012b; Rockwood et al., 2011). Silk film material properties like biodegradation, mechanical properties, chemistry, and optical properties may be readily modulated for a desired application (Arai et al., 2004; Horan et al., 2005; Karageorgiou and Meinel, 2004; Lawrence et al., 2008a; Li et al., 2003; Motta et al., 2002). In addition, fibroin films have been shown to support a multitude of cell types including a variety of cell lines from epithelium, endothelium, and fibroblasts, which allows for the adaptation to a variety of tissue systems (Arai et al., 2004; Meinel et al., 2005; Panilaitis et al., 2003). In vitro results can be quickly translated to in vivo models due to the fibroin film possessing a high level of biocompatibility and material consistency (Arai et al., 2004; Meinel et al., 2005; Panilaitis et al., 2003).

A recent interest has been the use of patterned silk films to develop a multitude of silk scaffolds and devices (Gil et al., 2010; Kaplan and Omenetto, 2012a; Lawrence et al., 2008a, 2009; Omenetto and Kaplan, 2008, 2010; Rogers et al., 2012; Tsioris et al., 2011). A nanopatterned fibroin film surface may be produced by casting silk solution upon a molding surface, then allowing the water to evaporate to form a film, following which the dried silk film is air-lifted from the molding surface (Lawrence et al., 2008a; Omenetto and Kaplan, 2008; Perry et al., 2008). In addition, the water-annealing technique may be employed post-casting to produce water insoluble films through the induction of alpha-helical and beta-sheet secondary structure formations for use in biological applications (Lawrence et al., 2008a; Rockwood et al., 2011).

This technique has been recently used to produce culture surfaces for investigating corneal cell response to silk film surface topography (Gil et al., 2010; Lawrence et al., 2009, 2012a). Results indicated that silk film surface topography could be used to direct corneal epithelial and fibroblast alignment (Gil et al., 2010; Lawrence et al., 2009), and that the edge surface of the patterned topography significantly influenced localization of focal adhesion formations (Lawrence et al., 2012a). One immediate biomedical application for silk films is for use in repairing the cornea after injury due to their transparent nature and high degree of biocompatibility (Bray et al., 2011; Chirila et al., 2008; Liu et al., 2012a).

In addition to the surface patterning capabilities of silk fibroin, the material can also be employed for immobilizing bioactive compounds for
later use without loss of activity or function (Demura and Asakura, 1989; Lawrence et al., 2008a; Liu et al., 1996; Numata and Kaplan, 2010; Tsioris et al., 2011; Zhang et al., 2012a). Recent work has demonstrated that the antibiotic tetracycline and the measles, mumps, and rubella vaccine can be successfully stored within a dried silk fibroin film matrix for six months at 60°C (Zhang et al., 2012a). Such capability eliminates the need for cold storage and may revolutionize the way many labile molecules are stored and utilized throughout the world (Zhang et al., 2012a). In addition, the molecule immobilization work has been combined with the unique surface patterning properties of silk to produce a microneedle delivery system: high aspect ratio topographic features were produced on a silk film surface that were strong and sharp enough to pierce the skin (Tsioris et al., 2011). The fibroin protein secondary structure is then modified to release a therapeutic molecule in a continual release profile after the microneedles are embedded within the hydrated dermis.

Silk films have also found utility as carrier surfaces for use in biomedical applications. Recently it has been shown that electronic components may be fabricated upon fibroin film surfaces that can later fully integrate or degrade naturally with the surrounding environment (Hwang et al., 2012). Silk fibroin has been shown to be an optimal insulating material of choice in which semiconductor materials, like silicon, can be readily integrated to produce biodegradable and highly functional electronic components (Hwang et al., 2012; Kim et al., 2010; Tao et al., 2012). The fibroin films can be processed to allow for a conformational adhesion to a given surface such as on brain, bone, and food surfaces (Hwang et al., 2012; Kim et al., 2010; Tao et al., 2012). Recent work has demonstrated the feasibility of mapping the feline neural brain surface using sensors that utilize the insulator and carrier abilities of silk fibroin (Kim et al., 2010). Similar efforts have utilized such conformable electronics to adhere to food products to determine spoilage or bacterial contamination (Tao et al., 2012). These studies demonstrate the utility of silk fibroin as a manufacturing material in which continued development will provide a sustainable, biodegradable material platform that can be used for a number of high-tech, medicinal, and environmental applications (Kaplan and Omenetto, 2012b).

3.3.5 Sponge scaffolds

Regenerated silk fibroin solution may also be processed to produce three-dimensional sponge scaffolds for use in tissue engineering (Mandal et al., 2012; Nazarov et al., 2004). Sponge scaffolds provide a framework of interconnected pores with a high amount of surface area within a defined three-dimensional volume, which allows for cell attachment and tissue ingrowth. Initial studies indicate that the porosity and mechanical properties of these
scaffolds can be readily controlled through salt leaching and gas foaming processes (Nazarov et al., 2004). Pores averaged 100 μm for both processing conditions, and reached porosity void volume ranges between 84% and 98%. However, compressive strength measured up to 175 kPa for salt leached scaffolds, and up to 280 kPa for gas foamed structures. Further work identified that silk fibroin concentration was a critical determinant of final porosity formation and mechanical properties (Yan et al., 2012). Specifically, porosity could be dialed in from 80% to 90% by ranging the fibroin concentration between 8% and 16%, respectively. Such scaffolds could find utility in the production of cartilage, bone, or connective tissue engineering applications.

Additional methods to produce silk scaffolds utilize sacrificial template techniques (Swinerd et al., 2007). It has also been shown that silk fibroin solution can be mixed with 500 nm diameter polystyrene beads and then dried to form a solid structure. The dried construct is then treated with methanol to produce the highly stable silk beta-sheet protein secondary structure. The scaffold is then treated with toluene to leach out the polystyrene beads. What is left is a spongy scaffold with 400 nm pores interspersed throughout the scaffold, and could elastically recover from 112 MPa compressive loads while possessing super hydrophobicity properties that may not be desirable for in vivo use (Swinerd et al., 2007).

Follow up in vivo work to assess silk fibroin sponge scaffold biocompatibility was undertaken in rat muscle pockets and subcutaneous animal models (Wang et al., 2008b). Results indicated that the implanted scaffolds were highly biocompatible and encouraged tissue ingrowth to varying extents depending on porosity and solvent processing conditions (i.e., aqueous or organic solvent) (Wang et al., 2008b). It was shown that all aqueous processed scaffolds degraded within a 2–6-month time frame, while scaffolds prepared using the organic solvent hexafluoroisopropanol (HFIP) persisted beyond one year post-implantation (Wang et al., 2008b).

Following on from this work, scaffolds were produced that had high compressive strength characteristics of up to 13 MPa within a hydrated environment, which could allow for functional bone formation (Mandal et al., 2012). These scaffolds utilized a novel processing method in which silk fibers were combined with NaOH pellets in water to produce a sponge-like scaffold construct. Subcutaneous in vivo studies in rats demonstrated a high degree of biocompatibility and neovascularization throughout the scaffold. In addition, the standard sponge scaffolding processing techniques can also be combined with other innovative methods. Silk fibroin was combined with gelatin, dried, and then freeze dried to produce a sponge scaffold matrix upon a molding template surface (Liu et al., 2012b). The template was designed to mimic the liver by producing a scaffold with grooves and substructures patterned on the surface. The scaffolds were then seeded
with primary hepatocyte cultures and, after seeding, the sponge layer was rolled upon itself to form a three-dimensional structure (Liu et al., 2012b; Papenburg et al., 2009). Analysis of the structures revealed the scaffolds were highly biocompatible and encouraged tissue growth. Future processing techniques will continue to evolve for producing silk fibroin sponge scaffolds for a variety of applied tissue engineering applications.

3.3.6 Hydrogels

Hydrogels offer a tissue culture system where interconnected filaments aggregate and stabilize water within a confined volume to produce a gel. Gelation of the silk fibroin solution can be controlled by temperature, calcium ion concentration, pH, and polymer blending with materials like PEO to produce a hydrogel (Kim et al., 2004). Results indicated that gelation time decreased with an increase in protein concentration, decrease in pH, increase in temperature, addition of calcium, and addition of PEO. Gelation was linked to beta-sheet secondary structure formation throughout the hydrogel structure (Kim et al., 2004; Matsumoto et al., 2006). It was shown that above 15% beta-sheet content gelation time increased linearly with fibroin protein concentration in solution (Matsumoto et al., 2006). Such results indicate that silk fibroin hydrogels offer a number of control points for defining material properties.

Building upon this work, the silk fibroin gelation rate was highly controlled through the use of sonication (Wang et al., 2008a). Gelation can be induced from minutes to hours depending on sonication parameters, such as power output and time, and silk fibroin concentration. Interestingly, the sonicated silk solution had human mesenchymal stem cells (hMSCs) added while in the solution state, which then encapsulated the cells within the hydrogel matrix. The cells were found to grow and proliferate within the gel over a 21-day time period, where cell viability was highest for lower silk fibroin concentration gels. A subsequent in vivo study implanted silk fibroin hydrogels subcutaneously within nude mice (Etienne et al., 2009). Results indicated that human fibroblasts embedded within the material had a 68% survival rate after 12 weeks post-implantation. Revascularization had occurred and no inflammatory response was observed after the 3-month implantation period. These hydrogel materials are anticipated to have uses in periodontal, maxillofacial, and dermal filling applications.

3.3.7 Microspheres

Microspheres describe a general class of particulates that have diameters in the high nanometer to micron range and assume a spherical shape, which can
be used for a variety of biomedical purposes such as controlled drug release applications. Silk microspheres can be readily produced by mixing regenerated fibroin solution with lipid vesicles that act as templates to efficiently load biological molecules in an active form for sustained release (Wang et al., 2007b). The lipid could then be subsequently removed by methanol or sodium chloride treatments that produced silk fibroin microspheres with beta-sheet structure, and measured 2 μm in diameter on average. Studies were then undertaken using horseradish peroxidase (HRP) as a model therapeutic molecule that was encapsulated within the silk microspheres using freeze–thaw cycles that showed enzymatic activity post encapsulation and continually released over a two-week time frame (Wang et al., 2007a, 2007b). Additionally, silk microspheres could be produced by using a fibroin and polyvinyl alcohol blend methodology to avoid the complications from the freeze–thaw cycle and use of organic solvents (Wang et al., 2010). Fibroin/PVA blended films were first produced by drying the solution into film form, the dried film was then rehydrated in water, and residual PVA was removed by centrifugation. Controlled microsphere diameters ranging from 300 nm up to 20 μm were achieved.

An additional processing method was developed in which drug-loaded silk fibroin microspheres were produced using a laminar jet break-up of aqueous solution (Breslauer et al., 2010; Wen et al., 2011; Wenk et al., 2008). Sphere diameters ranging between 100 and 440 μm were produced depending on the diameter of the nozzle and treatment to induce water insolubility using either methanol or water annealing methods (Jin et al., 2005; Wenk et al., 2008). Insulin-like growth factor (IGF) was used as a model drug for delivery characterization, and showed a seven-week delivery period. In vitro results indicate that silk microspheres are highly biocompatible, and released IGF increased MG-63 cell line growth over time (Wenk et al., 2008). Additionally, smaller sphere sizes ranging in the hundreds of nanometers to the lower micron size range can be similarly controlled through the use of salting-out techniques of potassium phosphate solutions (Lammel et al., 2010). Silk fibroin microspheres may offer much promise for drug delivery applications for a variety of applications, and future in vivo studies will further aid in promoting their translation to clinical use.

### 3.4 Conclusion and future trends

The functionality of silk fibroin in the production of biomedical structures is only limited to the variety of ways to controllably process the varying properties of the material. As with most silk proteins, the highly modifiable secondary structure of fibroin in solution can be manipulated to form any variety of three-dimensional structures (Rockwood et al., 2011). In addition, use of fibroin as a biomaterial post-sericin extraction is well documented and
therefore will continue to find itself as a material of choice for biomedical applications (Altman et al., 2003; Vepari and Kaplan, 2007). Future developments of silk fibroin based scaffolds will benefit from further studies focused on improving material properties and assessing these materials in vivo. In addition, promising work utilizing composite methodologies is being undertaken that has shown much progress in producing fibroin devices for use in peripheral nerve and intervertebral disc regeneration (Ghaznavi et al., 2011; Nectow et al., 2012; Park et al., 2012). Progress will continue in utilizing silk for in situ sensors and electronics for monitoring a variety of medical conditions (Hwang et al., 2012; Kim et al., 2010). Although 40 000 other varieties of silk proteins exist, no other is as highly produced or ubiquitously utilized as silk fibroin derived from the domesticated B. mori silkworm (Shear et al., 1989). As the world continues to look for alternatives to synthetic based polymers, fibroin may be a hopeful candidate for the future development of biologically based building materials for medical applications and beyond.

3.5 References


Processing of *Bombyx mori* silk for biomedical applications


