Published in final edited form as:

Prog Polym Sci. 2007 ; 32(8-9): 991–1007. doi:10.1016/j.progpolymsci.2007.05.013.

Silk as a Biomaterial

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Abstract

Silks are fibrous proteins with remarkable mechanical properties produced in fiber form by silkworms and spiders. Silk fibers in the form of sutures have been used for centuries. Recently regenerated silk solutions have been used to form a variety of biomaterials, such as gels, sponges and films, for medical applications. Silks can be chemically modified through amino acid side chains to alter surface properties or to immobilize cellular growth factors. Molecular engineering of silk sequences has been used to modify silks with specific features, such as cell recognition or mineralization. The degradability of silk biomaterials can be related to the mode of processing and the corresponding content of beta sheet crystallinity. Several primary cells and cell lines have been successfully grown on different silk biomaterials to demonstrate a range of biological outcomes. Silk biomaterials are biocompatible when studied *in vitro* and *in vivo*. Silk scaffolds have been successfully used in wound healing and in tissue engineering of bone, cartilage, tendon and ligament tissues.

Keywords

Silk; Fibroin; Spidroin; Scaffold; Tissue Engineering; Biomaterial

1. Introduction

Silk, popularly known in the textile industry for its luster and mechanical properties, is produced by cultured silkworms. Silks are produced by members of the class Arachnida (over 30,000 species of spiders) and by several worms of the order Lepidoptera, which includes mites, butterflies and moths. Silk are fibrous proteins synthesized in specialized epithelial cells that line glands in these organisms [1].

Silk fibroin polymers consist of repetitive protein sequences and provide structural roles in cocoon formation, nest building, traps, web formation, safety lines and egg protection [1,2]. Silks are generally composed of β -sheet structures due to the dominance of hydrophobic domains consisting of short side chain amino acids in the primary sequence. These structures permit tight packing of stacked sheets of hydrogen bonded anti-parallel chains of the protein. Large hydrophobic domains interspaced with smaller hydrophilic domains foster the assembly of silk and the strength and resiliency of silk fibers [3].

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Silks from silkworms (e.g., *Bombyx mori*) and orb-weaving spiders (e.g., *Nephila clavipes*) have been explored to understand the processing mechanisms and to exploit the properties of these proteins for use as biomaterials. Silks from silkworms and orb-weaving spiders have impressive mechanical properties (Table 1), in addition to environmental stability, biocompatibility, controlled proteolytic biodegradability, morphologic flexibility, and the ability for amino acid side change modification to immobilize growth factors [2,4–15].

Biomaterial design is an important element of tissue engineering, incorporating physical, chemical, and biological cues to guide cells into functional tissues via cell migration, adhesion, and differentiation. Many biomaterials need to degrade at a rate commensurate with new tissue formation to allow cells to deposit new extracellular matrix (ECM) and regenerate functional tissue. In addition, biomaterials may need to include provisions for mechanical support appropriate to the level of functional tissue development. In general, biomaterials must be biocompatible and elicit little to no host immune response.

Thus, silks have been investigated as biomaterials due to the successful use of silk fibers from *B. mori* as suture material for centuries [16]. Functional differences among silks of different species and within a species are a result of structural differences due to differences in primary amino acid sequence, processing, and the impact of environmental factors [17]. Silks represent a unique family of structural proteins that are biocompatible, degradable, mechanically superior, offer a wide range of properties, are amenable to aqueous or organic solvent processing, and can be chemically modified to suit a wide range of biomedical applications.

2. Silkworm (*Bombyx mori*) Silk

The domesticated silkworm (*B. mori*) silk fibroin fibers are about 10–.25 μm in diameter and consist of two proteins: a light chain (~26 kDa) and heavy chain (~390 kDa) which are present in a 1:1 ratio and linked by a single disulfide bond [18]. These proteins are coated with a family of hydrophilic proteins called sericins (20–310 kDa) [1,18–20]. The disulfide linkage between the Cys-c20 (twentieth residue from the carboxyl terminus) of the heavy chain and Cys-172 of the light chain holds the fibroin together and a 25 kDa glycoprotein, named P25, is non-covalently linked to these proteins [21]. Silk fibroin is purified from sericins by boiling silk cocoons in an alkaline solution (Figure 1A). Twenty-five to thirty percent of the silk cocoon mass is sericin, which is removed during the de-gumming process.

2.1 *B. mori* silk fibroin structure

The amino acid composition of silk fibroin from *B. mori* consists primarily of glycine (Gly) (43%), alanine (Ala) (30%) and serine (Ser) (12%) [1]. The heavy chain consists of 12 domains that form the crystalline regions in silk fibers, which are interspersed with primary sequence that is non-repetitive and thus forms fewer organized domains in the fibers. The crystalline domains in the fibers consist of Gly-X repeats, with X being Ala, Ser, Threonine (Thr) and Valine (Val) [22]. The crystalline forming domains consist of an average of 381 residues (596 in size in the seventh domain to 36 in the twelfth domain). Each domain consists of sub-domain hexapeptides including: GAGAGS, GAGAGY, GAGAGA or GAGYGA where G is Glycine, A is Alanine, S is Serine and Y is Tyrosine. These sub domains end with tetrapeptides such as GAAS or GAGS [18,22,23]. The less crystalline forming regions of the fibroin heavy chain, also known as linkers, are between 42–44 amino acid residues in length. All the linkers have an identical 25 amino acid residue (non-repetitive sequence) which is composed of charged amino acids not found in the crystalline regions [22]. The primary sequence results in a hydrophobic protein with a natural co-block polymer design. Efficient secretion of fibroin is believed to be due in part to the formation of a disulfide bond between the heavy and light fibroin chains. A naked pupa mutation in *B. mori* has been mapped to the same locus as of the light chain on the 14th chromosome. The resulting fibroin light chain does not have a disulfide

bond with the fibroin heavy chain and the cocoon has less than 0.3% fibroin protein content [24].

A number of silk polymorphs have been reported, including the glandular state prior to crystallization (silk I), the spun silk state which consists of the β -sheet secondary structure (silk II), and an air/water assembled interfacial silk (silk III, with a helical structure) [1,25,26]. The silk I structure is the water-soluble state and upon exposure to heat or physical spinning easily converts to a silk II structure. The silk I structure is observed in vitro in aqueous conditions and converts to a β -sheet structure when exposed to methanol or potassium chloride [27]. The β -sheet structures are asymmetrical with one side occupied with hydrogen side chains from glycine and the other occupied with the methyl side chains from the alanines that populate the hydrophobic domains. The β -sheets are arranged so that the methyl groups and hydrogen groups of opposing sheets interact to form the inter-sheet stacking in the crystals. Strong hydrogen bonds and van der Waals forces generate a structure that is thermodynamically stable [1]. The inter- and intra-chain hydrogen bonds form between amino acids perpendicular to the axis of the chains and the fiber [1]. The silk II structure excludes water and is insoluble in several solvents including mild acid and alkaline conditions, and several chaotropes.

2.2. Control of morphology of silk biomaterials

Due to the well-established sericulture process, 400,000 tons of dry silkworm cocoons are available worldwide per annum for the textile industry [28] and thus for biomaterials applications. Several different material morphologies can be formed from aqueous or solvent formulations of the natural fiber form of silk for utilization in biomaterials for biomedical applications (Figure 1B and Table 2). The fibers must first be dissolved in aqueous systems, followed by reprocessing into desired material formats.

2.2.1 Silk fibers—Silk fibers can be obtained by reeling from cocoons [1]. Sutures braided from silk fibers have been used for centuries in gummed (virgin) and de-gummed (black braided silk) forms as sutures for surgical options [4]. A thorough review of the utilization of virgin and black braided silk (coated with silicone or wax to prevent fraying) and associated immune responses has been previously described [4]. Silk sutures have been used for tendon tissue engineering [29]. Sutures modified with immobilized Arg-Gly-Asp (RGD) peptide to increase cell attachment, were cultured with human tenocytes and supported increased adhesion after 3 days when compared with unmodified silk fiber and tissue cultured plastic [29]. An increase in collagen type I and decorin transcript levels was observed on the RGD-modified sutures compared with unmodified silk and tissue culture plastic at six weeks [29]. Textile engineering techniques with silk fibers were used to generate biomaterial replacements for ligaments. A wire rope design was studied to generate silk protein devices with mechanical strength equivalent to the human anterior cruciate ligament (ACL). Human bone marrow stromal cells (hMSCs) and fibroblasts obtained from ligaments seeded on these silk fibroin wire ropes attached and proliferated [30]. Cells cultured with mechanical stimulation expressed collagen types I and III and tenascin C characteristic of human ligaments [4,31].

2.2.1 Non-woven silk fibroin mats—Non-woven mats are of interest as biomaterials due to the increased surface area and rougher topography for cell attachment. Silk fibroin has been used to generate non-woven silk mats from reprocessed native silk fibers or by electrospinning. Non-woven silk fibroin mats were prepared by partial solubilization of native silk fibers, usually in formic acid and small amounts of calcium chloride. The mats were washed and planted subcutaneously in rats, where they demonstrated good biocompatibility. Histology, mRNA transcript levels, and immunohistochemistry all suggest that the silk fibroin non-woven mat guided the formation of vascularized reticular connective tissue [32].

Silk fibers can also be treated by homogenization to achieve similar outcomes [33]. Silk fibroin mats 10–30 μm in diameter and pores of about 300 μm in diameter were obtained. These non-woven mats were studied with a variety of cells, including keratinocytes, fibroblasts, osteoblasts, and cell lines from epithelial lung, colon, and cervical carcinomas for up to 7 weeks. No degradation of the silk fibers was observed during culture [33], possibly due to low infiltration of the cells within the matrix. Endothelial cells (both primary and transformed), when cultured on the silk fibroin mats, attached and proliferated. This outcome improved with a coating of collagen type I or fibronectin, most likely due to the presence of RGD sequences available for cell binding. Endothelial cells cultured for a week formed microvessel-like structures on the nonwoven mats [34].

Electrospun fibers can be produced in a wide range of diameters, ranging from a few nanometers to a few microns depending on mode of processing [35]. Electrospinning from aqueous silk fibroin solution mixed with poly(ethylene oxide) (PEO) was established and fiber morphology based on scanning electron microscopy (SEM) analysis showed uniform fibers less than 0.8 μm in diameter [36]. hMSC's cultured on these mats showed attachment and spreading [37]. Atomic force microscopy nano-indentation was used to analyze the mechanical properties of the fibers. Electrospun silk fibers prepared from silk fibroin and poly(ethylene oxide) (PEO) had a lateral modulus of 8 GPa, compared with 13.6 GPa for native silk fibers [38]. Silk fibroin mats prepared from formic acid with fiber diameters averaging 100 nm showed a Young's modulus of 515 MPa, ultimate tensile strength (UTS) of 7.25 MPa and strain of 3% [39].

Electrospun non-woven meshes can be prepared as predominately random coil structure from which β -sheet structures can be formed via methanol treatment [40]. The porosity changed from 76 to 68% due to dehydration after methanol treatment of these non-woven meshes [40]. Silk fibroin non-woven mats prepared using formic acid contained 30–120 nm diameter fibers. Fibroblasts were cultured on the non-woven meshes and attached on silk and silk fibers coated with collagen type I, fibronectin, and laminin [40].

Blends of silk fibroin-PEO have been electrospun into nanoscale diameter fibers for the delivery of cell morphogens like bone morphogenetic protein-2 (BMP-2) [11]. BMP-2 is a morphogen which induces osteogenesis from mesenchymal stem cells. Nanoparticles of hydroxyapatite were added with or without BMP-2 and the mats were seeded with hMSCs and grown under osteogenic conditions. Differentiation was evaluated by calcium deposition and transcript levels of osteogenic markers. The silk fibroin mats supported the differentiation of the hMSCs to bone-like cells, and the mats formed with hydroxyapatite and BMP-2 showed the most calcium deposition and upregulation of osteogenic markers. This difference was attributed to the availability and release of BMP-2. Electrospun mats with hydroxyapatite also induced significant osteogenesis and the upregulation of BMP-2 transcript [11]. Silk fibroin non-woven mats electrospun from a 98% silk formic acid solution were implanted in calvarial defects of rabbits for bone regeneration and resulted in complete healing with new bone at 12 weeks [41].

Aside from biologically generated bone as above, options to control hydroxyapatite mineralization on silk biomaterial matrices have also been reported [42]. In nature, the organic/inorganic interface gives rise to unique material properties. Silk fibroin, when blended with added poly(L-aspartate), was successfully used as a template for the growth of apatite crystals [42].

2.2.2 Silk fibroin films—Silk fibroin films have been cast from aqueous or organic solvent systems, as well as after blending with other polymers. Silk films prepared from aqueous silk fibroin solution had oxygen and water vapor permeability dependent on the content of silk I

and silk II structures [43,44]. Alteration of silk structure was induced by treatment with 50% methanol for varying times. Changes in silk structure resulted in differing mechanical and degradability properties of the films [44]. Nanoscale silk fibroin films can also be formed from aqueous solution using a layer-by-layer technique (Wang et al., 2005). These ultrathin films were stable due to hydrophobic interactions and predictable film thickness could be obtained based on control of solution conditions. The films supported hMSC adhesion and proliferation [45].

Microstructures in films, which are advantageous for increasing surface roughness for cell attachment, were formed via blending of silk with poly(ethylene oxide) (PEO) [46]. The rough surfaces were exposed by extracting the PEO with water, after locking in the beta sheet crystallinity with methanol [46]. The roughness was directly related to the content of PEO used in the process.

Fibroblast attachment to silk films has been shown to be as high as for collagen films [12, 47]. Other mammalian and insect cells also showed good attachment on silk fibroin films when compared with collagen films [48]. Silk films, employed for healing full thickness skin wounds in rats, healed in seven days faster with a lower inflammatory response than traditional porcine based wound dressings [49]. Silk films have also been used for improved cell attachment and bone formation, particularly when chemically modified with RGD cell binding domains [50]. Silk fibroin films coupled with BMP-2 showed increased bone formation compared with the same silk fibroin films without the BMP-2 [9].

Transparent films cast from a blend of silk and cellulose showed increased mechanical strength compared with silk films alone [51]. Films cast from blends of silk fibroin and recombinant human-like collagen were seeded with hepatocytes and showed higher cell viability than silk fibroin films alone [8]. Silk fibroin solution, when coated on polyurethane and poly(carbonate) urethane films and scaffolds, increased the adhesion and proliferation of human fibroblasts [52,53]. Films cast from silk fibroin and S-carboxymethyl keratine (SCMK) showed decreased blood coagulation compared with silk fibroin or SCMK films alone [54].

2.2.4 Silk fibroin hydrogels—Hydrogels are three-dimensional polymer networks which are physically durable to swelling in aqueous solutions but do not dissolve in these solutions. Hydrogel biomaterials provide important options for the delivery of cells and cytokines. Silk fibroin hydrogels have been prepared from aqueous silk fibroin solution and are formed from β -sheet structures [55,56]. The pH of the silk fibroin solution impacted the rate of solution gelation. Gelation of a 3% solution was obtained in two days at pH 3–4, compared with eight days as required from a solution with pH 5–12 [56]. Other factors important in gelation included silk polymer concentration and Ca^{++} [55]. An increase in silk fibroin concentration, increase in temperature, decrease in pH, and an increase in Ca^{++} concentration decreased the time of silk fibroin gelation. Hydrogel pore size was controllable based on silk fibroin concentration and temperature [55].

Supplementation of silk solutions with poloxamer 407 (a nonionic surfactant) induced gelation; however, additional poloxamer reversed the sol-gel transition [57]. Semi-interpenetrating polymer networks (SIPNs) formed by mixing poloxamer 407 and silk fibroin solution increased the mechanical properties of the hydrogel [58]. A hydrogel blend of silk fibroin and gelatin showed a temperature dependent helix coil transition of the gelatin that impacted the rheological and mechanical properties of the gel. Composition and temperature dependent properties of gelatin-silk fibroin hydrogels were examined for drug delivery purposes [59, 60]. The release of benfotiamide for oral delivery was dependent upon the concentration of fibroin in silk fibroin-glycerol hydrogels [61]. The hydrolysis of trichlormethiazide in silk fibroin hydrogels prepared in various monosaccharides (ribose, fructose, glucose, and

mannose) was dependent upon the number of hydroxyl groups on the various monosaccharide molecules [62].

Osteoblasts-like cells that attached when cultured on 2% (w/v) silk fibroin hydrogels showed adherence and biocompatibility [13]. Addition of 30% glycerol to the hydrogel increased the proliferation of the cells [13]. Silk fibroin hydrogels injected in critical-sized femur defects in rabbits resulted in greater trabecular bone volume and thickness, significantly higher mineral and rate of bone formation when compared to poly(D,L lactide-glycolide) [63].

Hydrogels combining the properties of silk and elastin were formed to generate biomaterials called silk-elastin-like protein polymers (SELPs). The water content in SELP hydrogels could be managed by time of gelation and concentration of polymer, while the properties were not affected by pH, ionic strength, or temperature [64,65]. SELP hydrogels have been employed for the release of small molecules like theophylline, vitamin B₁₂, and cytochrome c [65]. SELP hydrogels were also used for the controlled release of DNA. Size, conformation, and concentration of DNA determined release rates of DNA from SELP hydrogels. The transfection efficiency was 1–3 orders higher than DNA delivered without hydrogel [66].

2.2.5 Silk fibroin porous sponges—Porous sponge scaffolds are important for tissue engineering applications for cell attachment, proliferation, and migration, as well as for nutrient and waste transport. Regenerated silk fibroin solutions, both aqueous and solvent, have been utilized in the preparation of porous sponges. Sponges have been formed using porogens, gas foaming, and lyophilization [67]. Solvent-based sponges were prepared using salt (e.g., sodium chloride) or sugar as porogen. Solvents such as 1,1 3,3 hexafluoropropanol (HFIP) do not solubilize salt or sugar; therefore, pores sizes in the sponges reflect the size of the porogen used in the process [67]. A gradient of pore sizes can be generated by stacking porogens of different sizes (Figure 2). Sponges with varying porosity can be controlled by stacking variations of salt/HFIP-silk solutions. Solvent-based porous sponges can also be prepared by addition of a small amount of solvent (ethanol, methanol, DMSO) into aqueous silk fibroin solution before pouring into a mold and freezing [68].

Aqueous based porous silk sponges can be prepared using variable size salt crystals as porogen, with control of pore sizes from 490 to 940 μm , by manipulating the percent silk solution and size of salt crystals. Pore sizes are 80–90% smaller than the size of salt crystals due to the limited solubilization of the surface of the crystals during supersaturation of the silk solution prior to solidification [10]. Aqueous-based sponges have rougher surface morphology, based on SEM, than solvent-based sponges due to this partial solubilization. Aqueous silk fibroin sponges demonstrated improved cell attachment than the solvent-based porous sponges, likely due to these rougher surfaces. Sponges with high porosity and better mechanical strength were obtained with aqueous-based processing. Stiffness, compressive strength, and modulus were elevated with an increase in percent silk fibroin solution utilized in the process (Table 3) [10]. Enzymatic degradation of aqueous based sponges was more rapid than the solvent-based sponges [10].

A phase diagram for the processing of aqueous- and solvent-based porous sponges has been developed. The concentration of silk fibroin solution and size of sodium chloride crystals (porogen) can be related to stable sponge formation with predictable morphological and structural features [69].

Porous three-dimensional silk sponges have been utilized in a number of studies with cells to generate various connective tissues. RGD coupled silk sponges seeded with hMSCs that have been cultured in osteogenic media resulted in differentiation of the cells, deposition of hydroxyapatite, and upregulation of bone markers *in vitro* [70]. Tissue engineered silk

sponges were useful for healing critical size femur defects in rats [71]. Aqueous porous sponge sponges with large pore sizes (900 μm) were used for bone tissue engineering. Structures similar to trabecular bone were observed after 28 days of hMSC differentiation in osteogenic media [72]. Solvent-based silk sponges were cultured with hMSCs in chondrogenic media and collagen type II and glycosaminoglycan transcripts were upregulated to a higher degree than sponges composed of collagen or cross-linked collagen [73]. The structural integrity of silk sponges compared with rapidly degrading collagen-based sponges was in part responsible for these differences [73]. Aqueous-based silk fibroin sponges seeded with chondrocytes also supported cartilage tissue engineering [74]. Chondrocytes originating from New Zealand white rabbits were cultured in silk fibroin sponges and proliferated faster in the silk fibroin sponges and generated a higher content of glycosaminoglycan compared with collagen sponges [75]. Porous silk fibroin scaffold sponges seeded with rabbit chondrocytes [76], cultured in chondrogenic media, yielded a frictional coefficient similar to that of native cartilage after 28 days of culture [77]. Sponges formed from a blend of poly(vinyl alcohol) (PVA), chitosan, and silk fibroin showed the best healing of the epidermis and dermis of rats when compared to the paired and single polymers [78].

2.3 Surface modification

Surface modification can be used to alter cell attachment and impact cell proliferation. Surface modification with the integrin recognition sequence RGDS can increase cell attachment [79]. Modification of silk fibroin with poly(ethylene glycol) (PEG) showed decreased attachment of fibroblasts [80]. Silk fibroin surfaces coupled with PEG also showed contact angle, protein adsorption, and hMSC attachment related to the amount of PEG on the surface of the fibroin [81]. Surface modification includes physical adsorption or chemical immobilization of a protein or ligand. Silk surfaces are hydrophobic, and attract and repel proteins depending upon the pI and hydrophobicity of the protein and pH of the solution. Differential adsorption of horseradish peroxidase (HRP) to silk fibroin porous sponges depended on the pH of the solution; HRP precipitated on the surface when the pI and solution pH were similar [14]. Chemical immobilization of HRP increased the amount of HRP available on the silk fibroin surface [14].

Silk fibroin can be functionalized using the amino acid side chain chemistry. The limitation to using silk fibroin for chemical modification is the limited total content of modifiable amino acid side chain groups: 3.3% of the amino acids contain carboxyl side groups [1] compared with 9.5% found in bovine collagen [82]. Carbodiimide chemistry, which uses amine or carboxyl groups on silk for modification, has been used to modify the surface of silk fibroin biomaterials or to react in solution followed by biomaterials formation. Glucose-oxidase was immobilized on silk fibroin films for use as a glucose sensor [83]. Arginine residues of silk fibroin were modified by 1,2-cyclohexanedione, altering cell attachment and proliferation [84]. Silk fibers modified with RGD (covalently coupled) resulted in improved cell attachment and proliferation and subsequently increased collagen type I production by hMSCs and ligament fibroblast cells [30]. Covalent attachment of RGD to silk fibroin films increased the number of mineral modules formed by Saos-2 cells in osteogenic media [50]. Interestingly, some wild type silkworms like *Antheraea pernyi* have RGD, an integrin recognition sequence, in their silk fibroin sequence, which improves cell adhesion [47]. BMP-2 immobilized vs. adsorbed on silk fibroin films showed increased inducement of bone markers from hMSCs, including alkaline phosphatase, calcium deposition, and transcripts for collagen type I, bone sialoprotein, osteopontin, and BMP-2 [9].

Tissue development occurs in response to gradients of morphogens during embryogenesis and is recapitulated during adult tissue regeneration [85]. These signaling cues required by cells to develop into tissues would be useful to incorporate into biomaterial designs. Silk fibroin

sponges were used to form immobilized gradients of morphogens [14]. Covalently coupled and adsorbed BMP-2 gradients within three-dimensional silk fibroin sponges were studied with hMSCs and a gradient response of calcium deposition was observed (Vepari et al., unpublished results). Immobilized gradients provide new options for biomaterial scaffolds for the generation of more complex cell and tissue outcomes *in vitro* and *in vivo*.

Organic-inorganic composites can also be generated through covalent linkage. Nanoscale particles of hydroxyapatite, less than 200 nm in diameter, were chemically bonded to silk fibroin [86]. Vinyl groups were introduced on silk fibroin by treatment with 2-methacryloxyethyl isocyanate. Subsequently, the modified silk fibroin surface was grafted with poly γ -methacryloxypropyl trimethoxysilane (polyMPTS). Hydroxyapatite particles were reacted with alkoxysilyl group of MPTS to form a siloxane bond. The crystallinity of silk fibroin remained constant during the coupling procedure and resulted in a silk-hydroxyapatite composite with properties of both components [86].

2.4 Degradation

The degradation of biomaterials is important in terms of restoring full tissue structure and function *in vivo*. Control over the rate of degradation is an important feature of functional tissue design, such that the rate of scaffold degradation matches the rate of tissue growth [87]. Silk fibroin fibers retain more than 50% of their mechanical properties after two months of implantation *in vivo*; thus, they are defined as a non-degradable biomaterial by the United States Pharmacopeia [7]. Other polymers like poly (lactic acid) (PLA), poly (glycolic acid) (PGA), and PLGA have degradation rates related to hydrolysis based on the polyester composition, purity, and processing conditions. The degradation of these polymers is usually controlled by varying ratios of polymers with different degradation rates or by altering molecular weight of the polymer [88]. These polymers only exhibit limited degradation by enzymes, such as metalloproteinases (MMPs). Furthermore, the degradation products of synthetic polymers like PLA decrease local pH and result in inflammation [89].

Natural polymers like collagen and silks degrade via the action of proteases. Typically, the rate of collagen degradation is altered by cross-linking in order to reduce enzymatic degradation. Cross-linking of collagen may also reduce immunogenicity [90]. The degradation byproducts of collagen are peptides and amino acids. The rate of silk fibroin degradation depends upon the structure, morphology, and mechanical and biological conditions at the location of implantation. Degradation of silk fibroin films and fibers has been explored using several types of proteases, including α -chymotrypsin and collagenases [5,7]. Interestingly, a 6 kDa trypsin inhibitor isolated from the water extract from silkworm cocoons, termed cocoon shell associated trypsin inhibitor (CSTI), protected the light chain of silk fibroin against tryptic degradation [91]. This inhibitor may be a useful tool in biomaterials design for spatial restriction of the degradation process.

A correlation between *in vitro* and *in vivo* rates of degradation of silk fibroin fibers has also been established (Horan et al. unpublished). Arai *et al.* [5] compared degradation of silk fibers with silk films when exposed to different amounts and types of enzymes. Exposure to similar enzymes resulted in faster film degradation than the fibers based on weight loss. The weight loss was accompanied by a change in average molecular weight of silk from 120 kDa for control silk films to 53 kDa for silk films degraded with α -chymotrypsin for 17 days. An increase in the crystallinity of the silk was observed with degradation. Tensile properties of silk fibers decreased without a large change in molecular weight of the silk fibers [5].

Silk fibroin porous sponges from regenerated *B. mori* fibers degraded differently with different processing conditions [10]. Aqueous processed 3D sponges, with similar pore sizes, degraded more slowly upon exposure to proteases with an increased percent silk present during the

processing. Solvent-processed sponges degraded more slowly than the aqueous-processed systems with similar pore sizes: 65% mass remained after 21 days as compared to aqueous-based sponges, which degraded completely in 4 days. The difference in degradation was due to increased surface roughness or differences in content or distribution of crystallinity [10].

Silk fibroin degradation could be regulated by changing crystallinity [44], pore size, porosity, and molecular weight distribution (MWD) of the silk fibroin. A change in MWD can be achieved by treating silk fibroin under alkaline conditions and heat. A decrease in MWD may disrupt ordered structures and reduce cross-links, potentially resulting in faster degradation. It will be useful to understand the mechanism and correlation of silk fibroin degradation with mechanical properties. Poly(D,L-lactic acid) surfaces coated with silk fibroin of different MWD resulted in differential attachment of osteoblasts. The adhesion of osteoblasts could have been a result of differences in surface hydrophilicity [92].

2.5 Immunological responses

Immunological reactions to biomaterials are an important consideration. Sutures made from virgin silk compared with sutures from de-gummed silk showed differences in hypersensitivity [4]. The inflammatory response of de-gummed silk fibroin *in vitro* compared with polystyrene and poly(2-hydroxyethyl methacrylate) showed less adhesion of immuno-competent cells [93]. Silk films implanted *in vivo* induced a lower inflammatory response than collagen films and PLA films [94]. Silk fibroin non-woven mats implanted subcutaneously in rats induced a weak foreign body response and no occurrence of fibrosis. There was little upregulation of inflammatory pathways at the implantation site and no invasion by lymphocytes after six months *in vivo* [32].

Sericins, which have been known to cause hypersensitivity [4], have been used to increase cell proliferation and attachment. Sericin M, a 400 kDa protein, supported cell attachment of skin fibroblasts to collagen [95]. Sericin-S (5–100 kDa) have been used to increase proliferation of mammalian cells like T-lymphocytes and hybridomas [96]. Fibroblast cells cultured on sericin matrices showed non-elongated morphology, unlike the normal spindle shaped morphology observed on silk fibroin and collagen matrices [12]. Biomaterials like tricalcium phosphate have also been coated with sericin to improve biocompatibility [97].

2.6 Sterilizability

An important feature of silk as a biomaterial, compared with other fibrous proteins such as collagen, is the versatility of options for sterilization [49]. Sterilization of silk fibroin scaffolds by autoclaving does not change morphology [73] or β -sheet structure when heated to 120°C [86]. Comparatively, collagen denatures at these temperatures [98]. Silk fibroin scaffolds can also be sterilized using ethylene oxide [4], γ -radiation, or 70% ethanol [9,11].

3. Spider (*Nephila clavipes*) silk

Spider silk from *N. clavipes* has been studied extensively and is characterized by its remarkable mechanical strength and thermal stability in fiber form [2]. The different types of silks formed by spiders serve various functions. The mechanical properties of the different silks are due to structural differences derived from different amino acid compositions and sequences. Dragline silk for safety and web construction is one the strongest natural materials and is composed of two proteins: major ampullate spidroins protein 1 and 2 (MaSp1 and (MaSp2). A putative molecular weight of 275 kDa based on gel electrophoresis of MaSp1 [1] and 740 kDa based on the major ampullate gland silk by size exclusion chromatography [99] this sentence sounds a little strange, but I'm not sure why, so I left it alone. The molecular weight of minor ampullate gland silk was determined to be 290 kDa, as measured by size exclusion chromatography

[99]. Unlike silkworms, spider silks characterized to date do not indicate the presence of sericin proteins. The dragline silks from different species of spiders have different mechanical properties [100] (Table 4).

The superior mechanical properties of dragline spider silks can be used as a template for developing specific structures for various biomaterial needs. Spider silks have not been commercialized in a similar fashion as has been done with silkworm silk due to the lack of domestication and lower productivity of spiders. However, genetic engineering provides the opportunity to produce spider silks, both native and hybrids, for biomaterial applications.

3.1 Structure of Dragline silk

The amino acid composition of dragline silk, MaSp1 from *N. clavipes*, consists mainly of the amino acids glycine and alanine, like silkworm silk, while glutamic acid, proline, and arginine are also significant in content [1]. This silk consists of repetitive blocks of peptides which give rise to the unique structural properties. The crystalline domains, which contribute to the tensile strength, contain repeats of alanine or glycine-alanine in MaSp1 and MaSp2. Another motif consisting of GPGXX (where X is most likely Glutamine and P is Proline) found only in MaSp2 is responsible for β -turn spiral and results in the elasticity of silk. Flagelliform silk from *N. clavipes* is rich in this motif and is highly elastic to serve its function in prey capture. Another motif, GGX, a glycine helix found in MaSp1, is responsible for the less crystalline regions of the silk structure. These domains also give rise to elasticity of dragline silk. At the carboxy- and amino- termini of the protein, non-repetitive sequences are found which have been proposed to have a role in assembly of the protein [101].

3.2 Large scale production of spider silk

MaSp1 from *N. clavipes* was expressed in *E. coli*; however, gene stability and complications in processing long repetitive sequences were encountered [102]. Therefore, alternative host systems have been explored for the cloning and expression of MaSp1- and MaSp2-like proteins, such as transgenic tobacco [103]. Spider dragline-like protein with yields of 1 g/L were expressed in the yeast, *Pichia pastoris* [104]. MaSp1 and MaSp2/ADF-3 were cloned and expressed in baby hamster kidney cells and bovine mammary epithelial alveolar cells [105]. Proteins of 60–140 kDa were produced and spun into fibers with reasonable mechanical properties [105]. Genes encoding spider silk have also been expressed in mouse milk [106]. Despite the above efforts, no substantial effort at commercialization of spider silk via genetic engineering has been successful to date, a required step to realize biomaterials applications from spider silks.

3.3 Morphology of recombinant spider silk

Environmental factors such as pH, water content, concentration of protein, salt, and charge impact the processing and assembly of spider silk *in vivo* and *in vitro*. For example, interchain interactions increased with enhanced calcium concentration and low water content [107]. Films cast from dragline silk proteins of *Araneus diadematus* in HFIP exhibited α -helical structure which dissolved in aqueous solution. Insoluble films were prepared by treatment with methanol and resulted in β -sheet structures. These films could be chemically coupled with fluorescein and β -galactosidase, which showed higher activity in chemically coupled than adsorbed forms [27]. A higher percentage of aspartic acid and glutamic acid found in MaSp1 (11.7%) than fibroin (3.3) [1] provides enhanced options for the chemical modification or coupling of factors to MaSp silk when compared to silk fibroin from silkworm. Hydrogels have also been prepared from recombinant spider silk and were found to be stable for several weeks and demonstrated high elastic modulus [108].

4. Conclusion

Silks are a unique group of fibrous proteins with unusually high mechanical strength in fiber form. The clinical success of silk sutures and availability of silkworm silk have encouraged a recent expansion of new biomaterials generated from the original suture-based protein harvested and processed from silkworms. Native silk fibers can be processed into wire ropes and non-woven silk mats. The same fibers can be solubilized and regenerated in aqueous solution, then further processed into sponges, films, hydrogels, and nanoscale electrospun non-woven mats. Surface modification of silk fibroin biomaterials can be used to alter cell responses. Cell culture on silk-based biomaterials has resulted in the formation of a variety of tissues including bone, cartilage, and ligament, both *in vitro* and *in vivo*. The degradability of silk fibroin can be altered by processing conditions. Molecular biology has been used to generate spider silks, although reasonable quantities are still not available. These genetically engineered spider silk proteins which have been formed into films can be chemically modified [27]. The unique structure of silk, versatility in processing, biocompatibility, availability of different biomaterial morphologies, options for genetic engineering of variations of silks, the ease of sterilization, thermal stability, surface chemistry for facile chemical modifications, and controllable degradation features make silks promising biomaterials for many clinical functions. Since the exploration of biomaterial applications for silks, aside from sutures, is only a relatively recent advance, the future for this family of structural proteins to impact clinical needs appears promising.

Acknowledgments

We thank our many colleagues inside and outside of Tufts University for their contributions to the studies on silk over the years. We also thank various funding agencies for support of different aspects of our studies on silks, including the NIH, the NSF, and the DoD.

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Fig. 1.

Fig. 1A: Silk fibroin is purified from sericins via boiling in an alkaline solution. The degummed or purified silk fibers can be processed into silk cords by twisting [4]; non-woven silk mats by partial solubilization [32]; or dissolved in lithium bromide, dialyzed and formed into aqueous silk fibroin solution [67] for preparation of other material morphologies (See figure 1B).

Fig. 1B: Processing of silk morphologies from aqueous silk fibroin solution into non-woven silk fibers [11]; aqueous and solvent based porous sponges [67,69]; hydrogels [108]; and films [46].

Fig. 2. Porous gradient silk sponges prepared by stacking a water soluble porogen from smallest to largest (A). Solvent based silk solution is added and allowed to diffuse through the salt crystals (B & C). The porogen is dissolved in water leaving the sponge with pore gradient (D) [109].

Fig. 3.

Covalent coupling versus adsorption of proteins on silk surfaces. A: Modifiable amino acid side chains; presence of amine, carboxyl and hydroxyl groups. B: Carboxyl side groups activated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) for 10–15 minutes. A protein like BMP-2 can be introduced via amine groups reacting with the activated silk to form amide bonds. Cyanuric activated polyethylene glycol (PEG) reacts with amine and hydroxyl groups on silk fibroin surface. C: Coupling of PEG on silk fibroin films generates a more hydrophilic surface and reduced attachment of human mesenchymal stem cells (hMSCs) [81]. D: Coupling BMP-2 to silk fibroin films via carbodiimide coupling results in increased calcium deposition (increased calcein labeling) by differentiated hMSCs [9].

Table 1

Mechanical properties of biodegradable polymeric materials (modified from [4]).

Source of Biomaterial	Modulus (GPa)	UTS (MPa)	Strain (%) at break	Reference
<i>B. mori</i> silk (with sericin)	5–12	500	19	[110]
<i>B. mori</i> silk (without sericin)	15–17	610–690	4–16	[110]
<i>B. mori</i> silk	10	740	20	[111]
<i>N. clavipes</i> silk	11–13	875–972	17–18	[111]
Collagen	0.0018–0.046	0.9–7.4	24–68	[112]
Crosslinked collagen	0.4–0.8	47–72	12–16	[112]
Polylactic acid	1.2–3.0	28–50	2–6	[113]

Table 2

Cell and tissue applications of silk fibroin scaffolds.

Application	Morphologic form	References
Wound dressings	Film	[49]
	Sponge	[78]
Bone tissue engineering	Sponge	[70–72,114–117]
	Film	[9,50,118]
	Hydrogel	[13,63]
	Non-woven	[11,41]
Cartilage Tissue engineering	Porous sponge	[73,74,76,77,117]
	Hydrogel	[75]
Ligament tissue engineering	Fiber	[30,31,119]
Tendon tissue engineering	Fibers	[29]
Hepatic tissue engineering	Films	[8]
Connective tissue	Non-woven mats	[32]
Endothelial and blood vessel	Non-woven mats	[6,34]
Antithrombogenesis	Films	[54]

Table 3

Comparison of compressive strength and modulus of degradable polymeric porous sponge scaffolds (modified from [10,67]).

Porous sponge material	Compression strength (KPa)	Compression modulus (KPa)	Reference
Silk fibroin (aqueous)	11–320	70–3330	[10]
Silk fibroin (solvent)	30–250	100–1000	[67]
Collagen	15	150	[120]
Fibroin/collagen composite	20–354	430–30000	[121]
Chitosan	45	750	[120]
Crosslinked Collagen/Chitosan	30	500	[120]
crosslinked Hyaluronan		0.6	[122]
Poly(D,L-lactide)		227–301	[123]
Poly(lactic acid)		66–242	[124]
Poly(lactic acid)/poly(glycolic acid)		1.0– 20	[125]
Polyurethane		40–400	[126]
Poly(ϵ -caprolactone)		44–90	[123]
Poly(ϵ -caprolactone/D, L-lactide)		190–900	[127]
Poly(1,8-octanediol citrate)		3.7	[128]
Poly(propylene fumarate)		290,000	[129]

Table 4

Mechanical properties of dragline silk from different species (abridged from [100]).

Species	Young's modulus (GPa)	Toughness (MJ/m ³)
<i>Nephila clavipes</i>	7.38–22	80–111.2
<i>Araneus diadematus</i>	4.0–10	131–160
<i>Argiope trifasciata</i>	6.9–11	90–116.3
<i>Latrodectus hesperus</i>	6–10.2	180.9
<i>Leucauge verusta</i>	10.6	151
<i>Plectreurys tristis</i>	16.1	112.1
<i>Kukulcania hibernalis</i>	22.2	132.2