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***In Vivo* Bioresponses to Silk Proteins**

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Abstract

Silks are appealing materials for numerous biomedical applications involving drug delivery, tissue engineering, or implantable devices, because of their tunable mechanical properties and wide range of physical structures. In addition to the functionalities needed for specific clinical applications, a key factor necessary for clinical success for any implanted material is appropriate interactions with the body *in vivo*. This review summarizes our current understanding of the *in vivo* biological responses to silks, including degradation, the immune and inflammatory response, and tissue remodeling with particular attention to vascularization. While we focus in this review on silkworm silk fibroin protein due to the large quantity of *in vivo* data thanks to its widespread use in medical materials and consumer products, spider silk information is also included if available. Silk proteins are degraded in the body on a time course that is dependent on the method of silk fabrication and can range from hours to years. Silk protein typically induces a mild inflammatory response that decreases within a few weeks of implantation. The response involves recruitment and activation of macrophages and may include activation of a mild foreign body response with the formation of multinuclear giant cells, depending on the material format and location of implantation. The number of immune cells present decreases with time and granulation tissue, if formed, is replaced by endogenous, not fibrous, tissue. Importantly, silk materials have not been demonstrated to induce mineralization, except when used in calcified tissues. Due to its ability to be degraded, silk can be remodeled in the body allowing for vascularization and tissue ingrowth with eventual complete replacement by native tissue. The degree of remodeling, tissue ingrowth, or other specific cell behaviors can be modulated with addition of growth or other signaling factors. Silk can also be combined with numerous other materials including proteins, synthetic polymers, and ceramics to enhance its characteristics for a particular function. Overall, the diverse array of silk materials shows excellent bioresponses *in vivo* with low immunogenicity and the ability to be remodeled and replaced by native tissue making it suitable for numerous clinical applications.

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Conflict of Interest

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Keywords

tissue engineering; silk fibroin; immune response; inflammatory response; foreign body response; vascularization

Introduction

Silk has long been used in its native fiber form as a suture material, and more recently is gaining popularity for use in numerous additional applications from tissue engineering to drug delivery to implanted devices. One reason for this widespread appeal is the ability to fabricate silk into a wide range of material formats with tunable mechanical and degradation properties. The natural silk polymer in fiber form has both great strength and elasticity, a combination of properties not matched by current synthetic polymers (1). Silk proteins are produced by an enormous variety of insect and spider species including ants, fleas, and crickets (2). For biomedical applications silk is sourced primarily from the textile industry silkworm *Bombyx mori*, and occasionally spiders. Silk from spiders has superior strength and elasticity but the significantly greater ease of cultivating *B. mori* silk has made it more popular. Most silk suture material is made from *B. mori* silk, but it is important to note that many silk suture materials are not purely silk. Frequently they are coated with waxy materials or contain contaminants, and thus studies assessing the *in vivo* bioresponse and degradation of silk sutures do not reflect purely silk creating confusion in the literature (see Altman et al. (3) for review).

B. mori silk consists of two main components, fibroin proteins and sericins, a family of glue-like proteins that coat the fibers and hold them together. In its original use as a suture material, silk was typically used in its virgin form. Many patients exhibited a significant inflammatory response and some patients became sensitized leading to severe allergic reactions. While there has been some confusion over time as to the true source of the allergic response, recent careful studies have come to the consensus that the allergic response is elicited by the native combined fibroin-sericin structure, but that either fibroin or sericin alone does not elicit an allergic reaction (4, 5). Given that sericin has only recently been exonerated for its role in allergic responses, it has been used in limited *in vivo* studies. While fibroin does not induce an allergic response, as with any biomaterial introduced into the body, it induces a biological response that must be understood to improve its use in clinical applications. The rest of this review will focus on studies utilizing purified fibroin from *B. mori*, referred to simply as ‘silk’, as this has been by far the most commonly used form of silk for biomedical applications.

The key attribute that allows silk to be processed into such a wide variety of materials is its ability to be solubilized in certain high ionic strength or acidic solutions and then remain in solution when exchanged with less harsh solutions. Once solubilized, any one of numerous fabrication methods can be used to form films, gels, solid porous scaffolds, or other materials. Some common fabrication methods include casting and drying for films, sonication to form hydrogels, and lyophilization or salt leaching to form porous solid scaffolds (Figure 1). Next, crystalline beta-sheet formation is induced, usually by either heat

or exposure to solvents, which causes the silk material to become water insoluble. Finally, the material must be sterilized prior to implantation. In every method, the mechanical and degradation properties can be modulated by changing the silk processing conditions, silk concentration, and method of inducing β -sheet (crystal) formation. It is important to note that conversely, each step of the processing method affects the final material properties and even small changes in the protocol can greatly alter the material. For example, the method of sterilization can change the molecular weight distribution and degree of beta-sheet formation and therefore change the material stiffness and degradation (6). For a more thorough review of common processing methods and how the processing affects the final material properties see Rockwood et al. (7).

In order to utilize silk biomaterials in more clinical applications, it is important to understand the biological responses to silk. The goal of this review is to summarize the current understanding of how silk protein-based biomaterials interact with the body *in vivo*. A summary of papers that have characterized the inflammatory response and vascularization of implanted silk materials is provided in table 1. More extensive reviews on silk sources, silk structure and properties, fabrication of various biomaterial and tissue constructs, and interactions with cells *in vitro* can be found elsewhere (1, 3, 7, 11).

Mechanism of Silk Degradation

In the body, silk degradation depends on many factors including the degree of β -sheet formation, the material structure (e.g., gel vs fiber, porosity), and location of implant in the body. For use as a biomaterial, silk fibroin is generally first solubilized in a strong ionic solution, such as 9 M lithium bromide, and then processed to induce β -sheet formation making it water insoluble. Silk primary structure is comprised of repeating hydrophobic bulk domains interspersed by hydrophilic regions (34). Processing of the silk induces the hydrophobic bulk domains to form crystalline β -sheet regions with the degree of β -sheet formation dependent upon the method of processing, thus giving a mechanism to control material properties. For use as a biomaterial, most processing methods induce sufficient β -sheets such that no appreciable silk degradation occurs in water or biological salt solutions (35, 36). However, silk is sensitive to degradation by proteases. *In vitro* studies have demonstrated that many proteases including protease K, collagenase, and alpha-chymotrypsin are able to cleave silk and cause a decrease in the material weight and strength over time (36–38). In contrast, MMPs, including MMP-1 and MMP-2 exhibit lower degradation activity towards insoluble forms of silk compared to soluble silk (38). In general, increased β -sheet content has a protective effect on silk degradation, which is likely in part due to the fact that most proteases act outside of the β -sheet regions (38). Denser structures such as silk fibers or films have longer degradation times suggesting silk is degraded by surface erosion and not bulk degradation (35, 38). Degradation of silk *in vivo* is also dependent on β -sheet content with higher β -sheet scaffolds showing less degradation (15). Interestingly, a direct correlation between immune cell invasion into the silk scaffold and degradation was also found. Regions accessible to macrophages showed visible evidence of degradation while cell-free regions did not and scaffolds implanted in immune-compromised nude rats were significantly less degraded than those implanted in Lewis rats

(39). While these results suggest immune cells are primarily responsible for silk degradation *in vivo*, other cell types are also capable of degrading silk.

Immune and Inflammatory Responses

The immune response can be broken down into two main components, the innate immune response, which is activated by anything recognized as ‘not-self’, and the adaptive immune response which is activated by specific molecules that were previously recognized as harmful. The adaptive immune response can be severe and it is critical that biomaterials not be targeted by adaptive immune antibodies. Studies have demonstrated that while native fibroin-sericin proteins can activate the adaptive response, purified fibroin does not (4, 40). The innate response, which includes the inflammatory response, can enact a range of symptoms from mild to severe. A mild innate immune response can often be beneficial as it activates many healing processes. However, more severe responses can be detrimental leading to destruction of local tissue or even systemic problems (41). The innate immune response can be further broken down into the acute and the chronic response. The innate response is typically initiated by macrophages, which are located throughout healthy tissues (41). Macrophages express pattern recognition receptors (PRR) that bind to molecules recognized as being produced generically from pathogens, such as particular sugar or fatty acid structures that are only produced by bacteria or fungi. When a PRR binds a target molecule, it initiates a signaling cascade that leads to the activation of many pathways including the release of inflammatory cytokines, initiation of phagocytosis, and recruitment of additional immune cells. This response, known as the complement system, does not involve recognition by specific antibodies. It is activated entirely by receptors that generically recognize ‘non-self’. Thus, this system is often activated by non-native implanted materials (42). Activation of the complement system leads to the release of cytokines, including interferon- β which activates transcription of the inflammatory cytokines tumor necrosis factor- α , interleukin-1 β , and interleukin-6 (41).

In addition to direct interactions between immune cells and silk proteins, another major factor determining the immune response is interactions between immune cells and proteins adsorbed on the implant. Every implanted material will be exposed to extracellular fluid as well as blood proteins due to capillaries or larger vessels damaged during the injection or surgical implantation process. Proteins adsorb rapidly to the exterior of the implant material forming a provisional matrix. The surface chemistry, morphology and structure of the implant will determine the degree and composition of this provisional matrix (43). The proteins that make up the matrix include adhesion proteins and signaling molecules that can allow immune cells to attach and modulate their response. This provisional matrix is often degraded during the wound healing process and can therefore act as slow release mechanism for inflammatory factors. Thus, the surface chemistry, morphology and structure of an implant play major roles directing the immune response by virtue of the proteins adsorbed to the material.

Indeed, most forms of silk fibroin used in biomedical applications activate the complement system. Silk films, fabricated by hexafluoro-2-propanol (HFIP) induced β -sheet formation, implanted intramuscularly attracted activated macrophages to the implant-tissue interface

(12). Silk tubes used for vascular grafts and constructed by winding silk fibers that were then coated in a fibroin solution supported the infiltration of macrophages performing phagocytosis on the fibroin (44). Similarly, porous scaffolds prepared from silk solution by formic acid evaporation and implanted in a bone defect also supported infiltration of activated macrophages (30). Silk-mediated activation of the complementary system is generally short lived and decreases after 14 days. Lyophilized silk sponges implanted subcutaneously had far fewer infiltrating immune cells 4 weeks post implantation as compared to 2 weeks (32). As a different material format in terms of fabrication method and mechanical properties, silk gels implanted subcutaneously also induced mild complement system activation at days 7 and 14 after implantation with the presence of macrophages and neutrophils. But by 4 weeks post implantation the inflammatory response was greatly reduced with far fewer inflammatory cells and no inflammatory cells could be detected 12 weeks after implantation (16).

The inflammatory cytokines, released by the complement response, signal to nearby cells enacting a cascade that leads to local and systemic responses (41). The cytokines act on nearby endothelial cells to slow blood flow and change the expression of adhesion molecules to attract immune cells. Some of the cytokines are also chemokines that create a concentration gradient for neutrophils, macrophages, and leukocytes to follow to the site of the immune response. As increased numbers of immune cells are attracted and activated by interactions with the foreign material, more cytokines are released which can lead to systemic effects including a rise in body temperature and activation of the adaptive immune response. Activation of lower levels of inflammation increase remodeling by inducing vascularization and the release of proteins involved in tissue remodeling and can therefore be beneficial to incorporating and degrading implanted biomaterials as new tissue forms (45). However, biomaterials must be designed to avoid over-activation of inflammation to the point of releasing inflammatory signals into the bloodstream and activation of systemic responses, which will lead to rejection of the material and possible severe side effects.

The acute immune response is generally short lived and dissipates after 7–14 days. However, it can go on to activate the chronic innate immune response which can last months or years. The chronic response is characterized by the presence of monocytes, leukocytes, and most notably, foreign body giant cells (FBGC). Multinuclear giant cells (MNGC) are one cell type of the FBGC family and are commonly referred to in the literature. The chronic response is activated by cytokines released during the acute response, especially interleukin-4 (IL-4) and interleukin-13 (IL-13) (43). In addition to direct interactions with the implanted material, whether a chronic response occurs can also be traced back to the composition of the provisional matrix formed around the biomaterial because it also affects cell adhesion and cytokine release. Macrophages already activated by inflammatory signals will then respond to IL-4 and IL-13 by fusing to form multinucleated FBGCs. Both IL-4 and IL-13 have been shown to be secreted in larger quantities by macrophages cultured on electrospun silk scaffolds *in vitro* (24). Not surprisingly therefore, the implantation of silk materials often induces the formation of FBGCs. This has been observed in a wide range of silk materials implanted in various locations including in scaffolds used to improve healing

of bone defects (22, 30), gels injected in the cervix (25), and macroporous silk constructs implanted in fascial defects (14).

The role of FBGCs is to destroy pathogens and other harmful materials that are unable to be cleared by phagocytosis. These cells are generally found adjacent to the biomaterial where they release reactive oxygen intermediates and proteases (41). In a full blown foreign body response the biomaterial becomes surrounded by multiple cell layers of FBGCs and activated macrophages. This can lead to the formation of granulation tissue comprised of macrophages, fibroblasts, and new vasculature and is the precursor towards a fibrotic capsule (43). While silk implants have frequently been found to induce the formation of FBGCs, the response generally subsides before the formation of permanent fibrotic tissue. In a study where a silk scaffold was implanted on a heart to aid in cardiac repair after myocardial infarction, fibrosis was seen two weeks after implantation but had disappeared after 8 weeks (24). Some granulation tissue was seen at both time points, but it remained mild as compared to other scaffold materials being tested including poly-lactic acid. Similar results were found in a study that investigated the biological response to silk scaffolds in a bone defect (22). At four weeks post implantation granulation tissue could be seen within the scaffold pores including the presence of FBGCs and lymphocytes. By 8 weeks post implantation the granulation tissue was still present but the number of inflammatory cells had decreased indicating a diminishing foreign body response. To our knowledge, no studies utilizing silk purified of sericin have reported the formation of fibrotic capsules.

The lack of fibrotic encapsulation is a significant improvement over many synthetic polymers including some of the most commonly used polymers in tissue engineering poly lactic acid (PLA), poly glycolic acid (PGA) and their mixture, poly(lactic-co-glycolic acid) (PLGA). These polymers degrade by hydrolysis of the ester bond into lactic and/or glycolic acid monomers and the acidity of the degradation products activates inflammation (46). The severity of the inflammatory response varies greatly and is thought to be directly related to the accumulation of degradation products (47). The severity is also affected by the location in the body of the implant with soft tissue generally having a larger response than bone and cartilage (48). Degradation of the polymer has an autocatalytic effect wherein the acidic degradation products cause further increased degradation of remaining polymers. Thus, the structure of the material and the ability of degradation products to diffuse away from the bulk material greatly affects the inflammatory response (49). Numerous studies using PLGA implants have reported fibrous encapsulation, including as early as one week after implantation (50–52). Fibrotic encapsulation decreases vascular growth into scaffolds, and in at least two cases, was sufficiently severe to lead to necrosis of cells growing within the scaffold (49, 51). The need to reduce the inflammatory response and avoid fibrotic encapsulation limits the potential structures and, by association, material properties of biomaterials made from these polymers making it unsuitable for many applications.

Looking at natural polymer alternatives to silk, collagen has also been used extensively for tissue engineering. Collagen has very good cell interaction properties with some cell types having higher proliferation on collagen substrates than tissue culture plastic (53). Collagen from various animal sources is also well tolerated *in vivo* with only a moderate immune response (54, 55). Collagen implants activate macrophages and MNGCs are often found

surrounding and invading the material (56, 57). The macrophages are likely involved in remodeling and the number of immune cells decreases over time (58). However, a major drawback for many clinical applications is that collagen has weak mechanical properties and is degraded very quickly. Collagen scaffolds implanted subcutaneously in rats degraded completely within one month of implantation. The tensile strength of processed collagen is less than 10 MPa, significantly less than the roughly 150 MPa required for tendons and bone (3). This makes native collagen on its own unsuitable for any weight bearing applications, or applications requiring that the material remain intact for greater than 15 days. To increase the strength and degradation time, various cross-linking methods have been devised. Depending on the method of crosslinking, the tensile strength can be increased up to 57 MPa (59). However, cross-linking changes the biological response *in vivo* often in negative ways. Most methods of cross-linking are associated with mineralization whereby calcium deposits begin forming in the implanted collagen within as little as 7 days and mineralization increases with time (60). While this can be beneficial for bone tissue engineering, it has been a major problem for many clinical uses of collagen, particularly for heart valve and other vascular applications. Interestingly, cross-linking can decrease the immune response possibly because it makes some of the foreign epitopes inaccessible. Thus, while collagen provides a good substrate for cell adhesion, proliferation, and differentiation, it usually must be combined with additional materials for most applications in order to combat its lack of mechanical strength, rapid degradation, and propensity to be mineralized.

Very few studies have directly compared silk to either natural or synthetic alternatives without the addition of extraneous variables making comparisons of specific aspects of the biological response challenging. However, there are a few direct comparisons worth noting. In a direct comparison of silk, cross-linked collagen, and PLA films implanted intramuscularly in rats, silk induced the smallest inflammatory response (44). After 6 weeks silk films were surrounded by a layer of fibroblasts and macrophages 3–4 cells thick with macrophages located only adjacent to the film and no MNGCs were present. In contrast, collagen films elicited a slightly stronger inflammatory response with a layer of fibroblasts and macrophages 12–20 cells thick, and PLA had the strongest response with an even thicker layer of fibroblasts and macrophages as well as the presence of MNGCs. Furthermore, the collagen film was nearly completely degraded 6 weeks after implant while both the silk and PLA films remained intact. In a study investigating electrospun sheets made of silk, collagen, or the synthetic polymers Poly (3-hydroxybutyrate) (PHB), poly(caprolactone), polyamide, or PLA, implanted on the epicardial surface of rats, silk elicited a mild inflammatory response with a less severe granulomatous response than the synthetic polymers with the exception of PHB (24). Collagen had the mildest inflammatory response but had degraded significantly by 8 weeks. Silk and all of the synthetic polymers except PHB elicited a foreign body response with MNGCs but the reaction to silk was the least severe. PHB was the only synthetic polymer with a milder immune response as compared to silk and also had increased vascular ingrowth.

Depending on the material and function of the biomaterial, the foreign body response can be more or less detrimental. Because of the increased release of reactive oxygen intermediates and proteases, the foreign body response creates a harsher environment for the biomaterial

to withstand (43). This can lead to premature degradation and device failure in some applications. However, in other applications the degradation can be beneficial as it allows formation of replacement tissue or release of factors embedded in the construct. Silk is primarily degraded via the action of phagocytic cells, especially MNGCs (15). Thus, an increased presence of these immune cells increases degradation allowing for more and faster tissue ingrowth. In porous silk scaffolds implanted in bone, immature bone tissue and calcified tissue were found within the scaffold in close proximity to MNGCs (23). The pattern of MNGC-mediated degradation followed by extracellular matrix deposition and finally mineralization is the same as for healthy bone remodeling. In another example, a knitted silk tube filled with a porous silk scaffold designed for anterior cruciate ligament regeneration had partially been replaced by native tissue at 24 weeks post implantation allowing it to retain the necessary mechanical strength and also undergo remodeling that is required for long-term functionality (61). Much of the degradation of the silk material that allowed for formation of new tissue was likely caused by the activity of FBGCs.

The exact bioresponse to implanted silk materials depends on the interrelated factors of the location of the implant within the body, the material format, and the degradation time. As with most implanted materials, silk implants with longer degradation times or implanted in soft tissues tend to induce a larger response than those with shorter degradation times or located within hard tissues. Unfortunately, despite the extensive number of *in vivo* studies, insufficient data exists to establish more detailed relationships between location of implant, silk format, and degradation time with specific aspects of the inflammatory response. Very few studies have performed more than a minimal analysis of the inflammatory response, and those with detailed characterization utilize different sets of metrics making aggregation of the findings challenging. More studies are needed that analyze quantifiable metrics over time to give a complete picture of the severity of the response and also the specific cell types and regulatory pathways that are most affected. Only this level of meticulous characterization will allow for future rational design of materials that decrease the inflammatory response or modulate it in ways that are beneficial to the implant function. Liu *et al.* (2014) provides an excellent example of the type of studies that are needed (28). In their work, activation of specific cytokines and the number of invading inflammatory cells was monitored over 3 months post implantation. With this information, one could make modifications to the implant that specifically target the cytokine pathways or specific cell types induced by the original material.

Vascular Ingrowth Into Silk Materials

One of the largest challenges facing the field of tissue engineering is the need to develop strategies that increase vascularization to improve oxygen and nutrient diffusion. Passive diffusion is limited to a few hundred microns and any cells further from a blood vessel than the diffusion limit undergo necrosis (62). This places a severe size limitation on tissue engineered constructs as necrosis increases the inflammatory response and inhibits tissue integration.

The ability of blood vessels to grow within silk scaffolds depends significantly on the silk processing method and scaffold properties such as pore size and pore interconnectivity (31,

63). Blood vessels are physically able to grow into silk scaffolds implanted *in vivo* but generally require biological signals to invade beyond the surface and into the bulk of the scaffold. Most silk scaffolds used for tissue engineering are designed to have a high porosity allowing vessels to form without the need to degrade the material. A study of acellular scaffolds implanted subcutaneously in mice found small vessels surrounding and on the periphery of the scaffold within 14 days (30). The growth may be induced by the mild inflammatory response to silk. After longer *in vivo* culture periods, there was very little ingrowth of the vessels beyond a few hundred microns of the exterior of the scaffold. However, vascularization was much improved in scaffolds pre-seeded with osteoblasts prior to implantations. This is likely due to vascular endothelial growth factor (VEGF) and other angiogenic factors secreted by the cells.

Unger et al. (29) further improved vascularization by pre-seeding scaffolds with endothelial cells as well as osteogenic cells that secrete angiogenic factors which lead to the endothelial cells self-organizing into capillary-like structures. Upon implantation, the vessel structures remained intact and became integrated with the host vasculature such that red blood cells were found within vessels composed of the pre-seeded cells. The ability to integrate pre-developed vasculature with host vessels creates important opportunities because, if designed properly, it could allow near immediate blood flow throughout a large construct. Many tissues rely on blood flow not only to avoid necrosis but also for important signaling factors such as those regulating differentiation.

One drawback of pre-vascularized scaffolds is that they require donor cells and long culture times making them unsuitable for many clinical needs. Alternative approaches have been investigated to improve vascular ingrowth from the host by modifying the scaffold structure, or by adding biological signaling molecules. Even highly porous scaffolds limit cell infiltration as demonstrated by the difficulty of evenly seeding scaffolds *in vitro*. To combat this issue, scaffolds have been designed with large, 250–500 micron diameter, arrayed channels to allow cells to quickly infiltrate deep within the scaffold (64). *In vivo*, these scaffolds had increased vascularization by 14 days post implant as compared to scaffolds without channels (32). Cells also occupied the space within the channels indicating the ability of the host form new tissue within the relatively large open space. Thus, the channels are able to provide increased access to the bulk of the scaffold without detracting from the overall tissue integrity.

Other groups have focused on decorating the scaffold with pro-angiogenic factors, most frequently VEGF. VEGF is released from the scaffold and has a chemoattractant effect on nearby endothelial cells leading to increased vascular ingrowth. VEGF doped silk scaffolds had a significantly increased number of vessels as well as vessel surface area compared to untreated silk in a critically sized calvarial defect in rabbits (33). The VEGF scaffolds also had increased mineralization, likely a direct result of the increased vasculature access.

Clinical Application: Small Diameter Vascular Grafts with Improved Patency

Vascular grafts for small vessels (less than 6 mm diameter) must meet a stringent set of requirements in order to maintain long-term functionality. The graft must have sufficient biocompatibility with the surrounding tissue, flowing blood cells, and adjacent vessels such

that it does not induce thrombosis while also having appropriate mechanical strength and elasticity to withstand systolic blood pressure. Autologous vessel transplants have thus far been the most clinically successful treatment. However, many patients requiring a vascular graft suffer from conditions that also cause deterioration of vessel integrity and therefore do not have a suitable donor vessel. For this reason, numerous tissue engineering strategies have been developed. Initially, non-natural polymers including polytetrafluoroethylene (PTFE, Teflon) and poly(ethyleneterephthalate) (PET, Dacron) were chosen. These materials performed well for large vessels but had poor patency for small vessels, often less than 50%. More recently, *in vivo* studies with silk tubular scaffolds have shown improved results as compared to PTFE and PET (see Table 2 for a list of studies characterizing silk vascular grafts *in vivo*).

PTFE and PET grafts in small vessels have a tendency to develop occlusions often due to thrombosis shortly after implant. One cause may be the large difference in mechanical properties between the graft and the adjacent vessel. PTFE and PET have significantly higher tensile strength and elastic modulus as compared to small vessels (65). Silk, with tunable mechanical properties, can be fabricated to have more similar strength and elasticity to the adjacent vessel. With a smoother gradation between the native vessel and the grafts, there is decreased disruption of the fluid flow through the vessel leading to a lower thrombotic response (65). This is supported by *in vivo* studies where PTFE scaffolds often formed occlusions within 24 hours and about half of the grafts failed within 4 weeks. Two groups using different fabrication methods showed that 1–1.5 mm diameter silk grafts implanted in the abdominal aorta of rats had much improved outcomes with 100% patency over 1 month in one study and 85.1% patency after 12 months in the other (44, 65).

In contrast to non-natural polymers, silk can be remodeled allowing cell attachment and infiltration which leads to degradation of the scaffold and replacement by native tissue over time. Endothelial cells begin covering the luminal surface of a silk scaffold within two weeks and cover over 90% of the luminal surface in 12 weeks (44). Smooth muscle cells also migrate into the scaffold in a similar time frame and form a thickened layer after 12 weeks. Thus, the silk scaffold provided a material in which the cells can self-organize into vessel-like structures. This was in contrast to PTFE scaffolds where neither cell type infiltrated through the graft. Additionally, CD68 (lysosomal/endosomal-associated membrane glycoprotein 4) positive macrophages were found within the silk scaffold and contained cell structures indicative of phagocytosis, suggesting the cells were degrading the scaffold. Indeed, the silk content of the graft decreased 20% over 12 weeks and 60% at 48 weeks. At the same time, collagen was deposited on the scaffold to replace the lost silk material. The enhanced ability of silk to be remodeled is significant as remodeling of the scaffold towards native tissue decreases the chance of infection, host rejection, and thrombosis thus increasing the long-term patency of the graft.

While silk has made a significant improvement over PTFE and PET, further modifications could be made to improve the mechanical properties, interactions with cells, and overall patency. The best surface for minimizing negative biological responses is the body's natural surface consisting of a layer of endothelial cells. Thus, one way to improve current silk scaffolds is to make modifications that decrease the time until the luminal surface is

completely covered by endothelial cells. Recently, groups have investigated the effect of adding biological factors and cell binding sites to stimulate endothelial cell infiltration and proliferation (66). Other groups have added anti-thrombic factors such as heparin to decrease the initial thrombic response following implantation (67). Finally, the mechanical properties of the scaffold can be more closely matched to the surrounding vessel by combining silk with other materials (68). In order to move silk-based constructs into the clinical setting, studies using large animal models are needed to demonstrate efficacy under conditions that more accurately replicate the human body, particularly as it relates to the mechanical stresses induced by blood pressure.

Clinical Applications: Ligament Reconstruction

Numerous studies have investigated silk materials as a potential replacement to current clinical tendon and ligament reconstruction strategies. Tendon and ligament injuries including anterior cruciate ligament (ACL), rotator cuff, and Achilles tendon tears are quite common affecting over 500,000 people per year in the United States alone (73). Current clinical methods to repair tendon injuries typically utilize autografts, allografts, xenografts, or suture repair. However, each of these strategies has major drawbacks, including donor site morbidity, risk of disease transmission, and high risk of re-injury, and often does not lead to a complete return to pre-injury strength and range of motion. In the case of ACL repair, current clinical strategies also lead to the development of osteoarthritis. Thus, there is a need for tissue engineering strategies that would allow a complete recovery from injury without increasing the risk of additional health problems. The ideal material would have equivalent mechanical properties to the native tissue, allow remodeling, promote regeneration so that new ECM and tendon tissue could replace the material over time without scar tissue, and have a slow degradation rate in order to maintain the necessary mechanical properties until the replacement tissue could be developed.

In vitro studies (8) first demonstrated the feasibility of using silk scaffolds for tendon replacement. A method of weaving silk fibers was developed such that the final mechanical properties of the cord were similar to human ACLs and the scaffolds were shown to support progenitor cell growth and differentiation towards tendon cell types. Initial studies testing silk scaffolds in *in vivo* models of tendon injuries demonstrated clinical potential for silk scaffolds. In both rabbit and pig models of ACL reconstruction, the silk scaffolds did not rupture and were able to maintain joint stabilization (61, 74). By 6 months post-surgery scaffolds contained fibrous tissue ingrowth and the original silk fibers of the scaffold could still be detected demonstrating a slow degradation profile. However, scaffolds made simply of silk alone were unable to induce complete tendon healing. While the mechanical tensile strength was sufficient to prevent rupture, it was less than 50% compared to the native ACL and decreased over time suggesting the scaffold was degrading more quickly than it was replaced by new tissue (74). Tissue ingrowth into the scaffold was incomplete and after 6 months spaces between the silk fibers of the scaffold existed. There was also incomplete formation of the bone-tendon junction, an important indicator of long-term positive clinical outcomes, with the presence of aligned collagen fibers but little to no mineralized fibrocartilage and poor mineralization and graft incorporation into the bone tunnel (74).

Following on to these initial studies, many groups have made improvements to silk tendon replacements by incorporating additional materials or pre-seeding the scaffold with tendon cell types. Pre-seeding scaffolds with mesenchymal stem cells (MSCs) dramatically increased tissue regeneration, collagen deposition and blood vessel growth within a scaffold used for ACL repair (74). A similar increase in tissue growth and collagen deposition was seen in silk scaffolds pre-seeded with tendon stem/progenitor cells (TSPCs) used for rotator cuff repair (75). The TSPCs also caused a decrease in the inflammatory response with more fibroblasts and less lymphocytes growing within the scaffold as compared to unseeded controls. Pre-seeded scaffolds were better able to maintain mechanical strength, likely because of the increased amount of deposited ECM and organized collagen fibers and decreased inflammatory response (61).

While pre-seeding of silk scaffolds has led to improved tendon regeneration, it is not ideal due to long culture times and increased risk of disease transfer, and did little to improve the bone-tendon junction. Thus, other groups have focused on improving the scaffold material. As an alternative means to encourage increased cellularity within the scaffold, silk scaffolds have been coated with a collagen film or filled with collagen sponges. Reconstituted collagen lacks the mechanical properties required for tendons and degrades quickly, but has improved cell attachment, growth, and differentiation compared to silk (53, 76). Thus, combining the mechanical benefits of silk with the cell compatibility benefits of collagen can make an ideal blend for many tissue engineering applications. Indeed, silk-collagen scaffolds greatly improved tendon reconstruction (27, 75, 76). Silk-collagen scaffolds had significantly increased regenerated tissue and organized collagen fibers compared to silk only scaffolds (75). The silk-collagen scaffolds also had increased vasculature at 2 months post surgery, which is an integral aspect of tendon healing and likely contributed greatly to the improved phenotypes seen at later time points. The ligament-bone junction had more fibrocartilage and calcified fibrocartilage compared to silk only scaffolds demonstrating that it more closely resembled the native transition. Interestingly, the silk of silk-collagen scaffolds showed less degradation at 18 months than the silk of silk-only scaffolds. The initial collagen scaffold was degraded quickly suggesting the protective effect on silk degradation may be due to the additional vasculature, cell growth, and provisional matrix provided by incorporation of collagen. The regenerated tendons still lacked the tensile strength of native tendons, but were stronger than silk-only scaffolds (27). As an alternative means to improve the bone-tendon junction of silk scaffolds, tricalcium phosphate (TCP) has been incorporated at the tips of the tendon scaffold (77). Addition of TCP significantly increased fibrous tissue ingrowth surrounding the bone insertion site and Sharpey's fibers could be detected (78).

The more recent improved silk scaffolds with additional materials or pre-seeding show clinical promise for improving some of the negative aspects of current clinical strategies (see table 3 for list of studies). Unfortunately, at this time it is difficult to determine how close silk-based approaches are to current clinical standards because none of the *in vivo* studies directly compare tendon reconstruction with silk scaffolds to current clinical methods, and very few of the studies compare the reconstructed tendon to the native tendon. Thus, it is difficult to determine whether any of the newly designed silk scaffolds are likely to improve

clinical outcomes. Further studies are needed in large animal models that directly compare silk-based scaffolds with routinely used autograft and allograft methods. Ideally, these studies should investigate both early tissue ingrowth and vasculature as well as long-term development of mature structures, maintenance of mechanical properties, and preservation of surrounding cartilage.

Clinical Applications: Breast Implants

The ability of silk scaffolds to maintain their shape over long time periods makes them an ideal material for soft tissue reconstruction. Recently, a silk scaffold commercially produced by Allergan was utilized for breast implants in a preliminary clinical trial(82). The number of breast implant surgeries performed annually is increasing for a number of reasons including an increasing number of mastectomies and improved surgical procedures(83). Silicone breast implants have been in use for over two decades but suffer from capsular contraction and rupture leading to 30–54% re-operation depending on the nature of original surgery(84). Silicone is not able to be remodeled and it was hypothesized that using a material capable of remodeling such as silk could improve long-term outcomes. The preliminary clinical trial investigated 21 cases of silk implant breast reconstruction, all in oncological patients not treated with radiation (82). Six months after surgery only one implant required re-operation and patient satisfaction was similar to silicone implants. This initial study provides encouraging results for use of silk scaffold breast implants, but further investigation of longterm outcomes, use in more complicated cases, and more in depth analysis of patient response is needed before conclusions can be drawn comparing silk to currently used silicone implants. If silk scaffolds continue to perform well for breast implants, they may also prove useful for other soft tissue applications such as dermal layer skin reconstruction to reduce scarring and abdominal wall reconstruction.

Clinical Applications: Skin Graft

Dermal wound healing continues to be a major clinical concern. Whether from major burns, injuries, or diabetic ulcers, improved methods that speed the healing process and cause less scar formation are needed to decrease the risk of infection, dehydration, and painful scars associated with dermal wounds. The process of dermal wound healing involves first platelet aggregation and inflammation, then the formation of granulation tissue, and finally re-epithelialization and remodeling of newly generated tissue. Clinically, many dressings and grafts have been developed to speed and improve the wound healing process. However, negative outcomes including painful scarring, permanent ulcers, and infections causing serious health problems and possibly death remain problems. Various forms of silk including silk wound dressings and silk graft scaffolds have been tested *in vivo* for their efficacy improving healing and the condition of regenerated skin. Many of these studies have demonstrated improved results over clinically used treatments. In an early study, silk film wound dressings outperformed Duro Active, a dressing commonly used clinically, reducing the healing time of a full thickness skin wound in mice by 7 days(85). The rate of healing was similar to another clinically used material AlloaskD, which is made from lyophilized porcine dermis and thus suffers from possible disease transmission risks. Similarly, a silk-gelatin electrospun dressing functionalized with astroglase IV significantly improved healing in a rat full thickness burn model with increased

vascularization, collagen deposition, and tissue organization compared to burns treated with silk-gelatin only dressing and untreated controls(86). A major concern for dermal wound treatments is preventing infection as most morbidity associated with skin wounds is caused by infection. Lan et al. (87) combined antibiotic loaded gelatin microspheres in silk scaffolds creating a slow drug release wound dressing. The dressings were tested on full thickness burn wounds in rats that had been exposed to *P. aeruginosa* bacteria. The antibiotic-loaded dressings had significantly decreased inflammation and improved healing including faster re-epithelialization as compared to dressings without antibiotic and gauze treated controls.

In addition to wound dressings, other groups have focused on silk-based grafts that remain in the wound and become integrated into the healing tissue. Porous silk scaffolds implanted in a rat full thickness wounds outperformed PVA porous scaffolds with a decreased inflammatory response and improved vascular ingrowth (18). Other groups have improved upon this result by functionalizing the silk scaffold with additional factors. Silk combined with alginate, a material purified from algae with excellent hydration properties, improved the healing time in a rat full thickness wound from 12 to 6 days compared to clinically used NuGuoz(88). The silk-alginate treated group also had increased collagen deposition. Electropun silk scaffold functionalized by culturing adipose derived MSCs for 7 days and then decellurizing the scaffold prior to implantation, significantly increased wound healing of a full thickness wound in diabetic mice(13). The size of the wound was decreased by 50% at day 10 as compared to silk only or untreated controls. The decellurized scaffold had more organized tissue regeneration with a clear epithelial-dermal junction and hair follicles within the wounded area.

These studies demonstrate the ability of xeno-free silk materials to improve skin wound healing, often in direct comparison to current clinical treatments, suggesting these materials are ready to enter clinical testing (see table 4 for list of studies). It is interesting to note that the processing and fabrication methods varied widely for the same application. Many of the materials were designed to improve a specific aspect of healing and could possibly be combined to create an even further improvement.

Conclusions

Silk is a versatile natural protein polymer that can be fabricated into many different structures with a wide range of physical properties. Key to its use in clinical application is the favorable short- and long-term biological responses. Silk induces a mild inflammatory response *in vivo*, with recruitment and activation of macrophages, and often a mild foreign body response that includes the formation of MCGCs. The response is affected by the material structure, method of fabrication, and site of implantation within the body. In general, implants with longer degradation times and implanted into soft tissues have a higher response than materials with shorter degradation times or implanted in hard tissues. Importantly, activation of the immune response does not include activation of the adaptive immune response and thus formation of antibodies towards silk and downstream severe reactions do not occur. The inflammatory and foreign body response tends to peak 1–3 weeks post implantation and then decreases over time with fewer immune cells and less

granulation tissue surrounding the implant. A depiction of a typical biological response to silk over time is shown in Figure 2. Activation of the inflammatory response is often beneficial as it leads to increased remodeling and degradation of silk. The enhanced ability to be remodeled at a slow rate is a significant advantage of silk when compared to most synthetic materials and to the rapid degradation of collagens. This feature allows for improved integration with the host tissue and vascular ingrowth, both of which aid in attenuating the foreign body response, preventing fibrous encapsulation, and improving the long-term biocompatibility of the construct. Silk materials also have not been demonstrated to induce mineralization, except when used in calcified tissues. While silk allows for cell attachment, proliferation, and differentiation, some applications require better cell interactions than silk alone can provide. In many of these instances, silk has been combined with additional materials such as collagen or growth factors, to successfully improve or specifically modulate cell response.

The combination of versatility and biocompatibility of silk make it a promising material for many clinical applications beyond a suture material. Indeed, it has recently been used in clinical trials including for breast reconstruction and tympanic membrane repair (84, 90). However, in order for silk to be used more extensively, more detailed analysis of its immune response including more extensive characterization of M1 versus M2 macrophage activation, locally released cytokines, and the time course of activation of the foreign body response are needed. This knowledge will allow for better prediction of the specific bioresponse to a silk construct at a given implantation site, which in addition to improving functionality, would also greatly enhance the translational ability for specific purposes. Rational design of constructs, such as incorporation of pharmacological agents, could then be used to augment particular beneficial aspects of the inflammatory response, such as vascular ingrowth, while simultaneously inhibiting other aspects such as MNGC activation. The use of silk as a biomaterial beyond its role as a suture material has been studied for well over a decade and, with extensive *in vitro* characterization and more recently *in vivo* studies, has the potential of becoming a standard clinical treatment for numerous applications.

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Figure 1. Silk Processing

Schematic of common silk material fabrication methods (8–10). Silk materials are made starting from either silk cocoons (**a**) or silk fibers (**b**), both must be boiled to remove sericin. Cocoons are solubilized while fibers are left intact before final processing to form different silk materials. (2 column width figure, color on web black and white in print)

Figure 2. Immune response to implanted silk

Overview of immune response to silk materials *in vivo* using a porous silk scaffold as an example. (2 column width image, color on web black and white in print)

Table 1Inflammatory response and vascularization *in vivo*.

Silk material and processing	Response	In vivo model and tissue site	Time-points, analysis	Ref
INFLAMMATORY RESPONSE				
silk film, HFIP annealed, pre-seeded with rMSCs	mild inflammatory response with layer of macrophages and fibroblasts 3–4 cells thick surrounding implant, no MNGCs	intramuscular implant rats	6 wks H, IC	(12, 13)
woven silk mesh	macrophages and MNGCs found at interface of silk implant at 7 days, and within bulk of implant at later time points, similar amount of macrophages but more MNGCs as compared to polypropylene	abdominal wall facial repair in rats	1, 2, 4, 12 wk H, IC	(14)
water or HFIP prepared porous silk scaffold	granulation tissue, MNGCs and macrophages present for both scaffold types at 2 months in lewis rats, fewer macrophages at 6 months for aqueous scaffold, HFIP more resistant to macrophage degradation	intramuscular and subcutaneous in Lewis and nude rats	2 wk, 2, 6, and 12 month H, IC and QPCR	(15)
sonicated silk gel	mild inflammatory response with neutrophils and macrophages, response decreasing by 4 wks and not detectable at 12 wks	subcutaneous implantation nude mice	1,2,4,12 wk H	(16)
knitted silk fiber scaffold	mild inflammatory response	subcutaneous implantation in rats	1, 8 wk H	(17)
porous silk scaffold	mild inflammatory response which decreased after day 3, less severe in comparison to PVA scaffold	full thickness skin wound repair in rats	3, 7, 10, 18 day H	(18)
98% silk/2% poly(ethylene oxide) porous tube, sonicated and MeOH treated	number of macrophages decreased from 1 to 8 weeks, more macrophages in silk constructs than control collagen or autograft constructs, similar nerve repair in silk and collagen constructs but less than autograft	sciatic nerve repair in rats	1,4, 8 wk H, IC, HM	(19)
porous silk scaffold or cross-linked silk film	inflammatory cells surrounding silk materials, decreased by day 20	buccal mucosa full thickness wound repair in rats	10, 20 day H	(20)
porous silk tube gelled with acetic acid, EtOH treated	macrophage recruitment and functional nerve repair similar to autologous nerve graft	sciatic nerve repair in rats	1, 4 month H, IC, muscle strength testing	(21)
water or HFIP prepared porous silk scaffold	both scaffolds contained granulation tissue and MNGCs at 4 wks, at 8 weeks foreign body response of water prepared scaffold was diminished while it remained high for HFIP prepared scaffolds	femur defect in rabbits	4, 8 wk H, HM	(22)
water or HFIP prepared porous silk scaffold, MeOH treated	lymphocytes, macrophages, and MNGCs found around and within scaffold, no neutrophils, new bone formation in close proximity to MNGCs	tibia and humerus defect in sheep	2 month H	(23)
electrospun silk sheet treated with MeOH	moderate inflammatory response with MNGCs and M1 macrophages	placed on epicardial surface after myocardial infarction	2, 4, 8 wks H, IC	(24)
sonicated silk gel	mild foreign body response, no neutrophils	injection into cervix of pregnant rats	4 day H	(25)
lyophilized silk scaffold	foreign body response with similar number of MNGCs at 4 and 8 wks	calvarial defect in rabbit	4, 8 wk H, HM	(26)

Silk material and processing	Response	In vivo model and tissue site	Time-points, analysis	Ref
braided silk tube	mild inflammatory response to fibroin tube, severe response to fibroin and sericin tube	subcutaneous implantation in mice	10 day H	(27)
HFIP prepared electrospun silk-tropoelastin or silk only scaffold, glutaraldehyde cross-linked, EtOH treated	number of inflammatory cells and inflammatory markers decreased in silk-tropoelastin compared to silk, also less remodeling	subcutaneous implantation in mice	1,2,3, 10, 21 day H, IC	(28)
VASCULARIZATION				
porous silk micronet treated with formic acid, pre-seeded with HDMEC, HDMEC and osteoblasts, or no cells	vascular ingrowth into co-culture but not others, in vitro pre-formed vasculature of co-culture integrated with host vasculature	subcutaneous implantation in SCID mice	2 wk H, IC	(29)
porous silk micronet treated with formic acid, pre-seeded with osteoblasts	pre-seeded vascularized throughout scaffold, unseeded only vascularized on surface	subcutaneous implantation in SCID mice	2 wk H, IC, HM	(30)
silk-RGD porous scaffold MeOH treated, pre-seeded with hMSCs	vascularization improved with larger pore sizes,	calvarial defect in nude mice	8 wk H, HM	(31)
lyophilized porous silk scaffold with linear channels, autoclaved	channels increased the number of blood vessels and depth of blood vessel growth into the scaffold	subcutaneous implantation in mice	2, 4 wk H, IC	(32)
porous silk scaffold, loaded with VEGF, BMP-2, or both	vascularization highest in scaffolds with VEGF and lowest in unloaded scaffold	cranial defect in rabbits	4 wk quant. vasc. analysis	(33)

H = histology, IC = immunohistochemistry HM = histomorphology)

Table 2

In vivo studies of silk materials used for vascular grafts.

Silk material and processing	Response	In vivo model and tissue site	Time-points, analysis	Ref
silk formaldehyde cross-linked or collagen glutaraldehyde cross-linked coated commercial knitted polyester graft	100% patency, silk did not induce MNGCs whereas collagen did, silk tubes 85% and 97% cell coverage of luminal surface at 3 and 6 months respectively	abdominal aorta of dogs	3 days, 2 wk, 1, 3, 6 month H	(69)
wound silk fibers, coated in fibroin solution, EtOH treated	85% patency after 12 months, nearly complete endothelial cell covering of inner tube surface by 12 wks	abdominal aorta of rats	2, 4, 12, 24, 72 wks H, IC,	(44)
gel spun silk tube, MeOH treated	patent after 4 wks, interior surface of tube completely covered by endothelial cell layer at 4 wks	abdominal aorta of rats	2, 4 wk H, IC	(65)
braided silk fiber tube coated with fibroin and cross-linked with poly(ethylene glycol diglycidyl ether)	greater than 80% patency at 8 wks, in depth quantitative analysis of tube remodeling and new tissue growth	abdominal aorta of rats	2, 8 wk, H, IC	(68)
braided silk fiber tube coated with fibroin and EtOH treated	85% patency at 12 months, inner surface of tube mostly covered by endothelial cells at 9 wks	abdominal aorta of rats	9 wk, 12 month H, IC	(70)
silk electrospun tube, MeOH treated, 1.5 mm diameter	endothelial, smooth muscle, and macrophage cells found on lumen and within scaffold, 100% patency at 7 days	abdominal aorta of rats	7 day H, IC	(71)
silk solution coated silk fiber braided tube, EtOH treated	100 % patency in all grafts, silk tubes near complete coating of lumen by endothelial cells at 3 months while PET tubes had only 50% lumen coverage, silk tubes had significantly improved tissue integration compared to PET	abdominal aorta of rats	2 wk, 3 month H, IC	(72)

H = histology, IC = immunohistochemistry

Table 3

In vivo studies of silk materials used for tendon and ligament reconstruction.

Silk material and processing	Response	In vivo model and tissue site	Time-points, analysis	Ref
knitted silk mesh tube filled with porous silk scaffold unseeded or seeded with autologous MSCs	cell ingrowth and ECM deposition minimal in unseeded scaffold, more pronounced in seeded scaffold, tensile strength met requirements but significantly less than reported for native ACL and decreased over time	ACL reconstruction in rabbit	2,4, 6 month H, HM, MT	(74)
knitted silk mesh tube filled with porous silk scaffold unseeded or seeded with pig MSCs	significant ECM deposition and cell ingrowth into seeded scaffold with mostly normal insertion site including Sharpey's fibers, little remodeling of unseeded scaffold and less developed insertion site. Seeded scaffold retained tensile strength but only 53% of native ACL.	ACL reconstruction in pig	6 month H, IC, HM, MT	(61)
braided silk fiber tube filled with collagen-hyaluronan porous scaffold	silk collagen-HA scaffold had increased remodeling including vascular ingrowth and lower inflammatory response compared to silk only constructs	ACL reconstruction in dogs	6 wk H	(76)
braided silk fiber tube filled with porous collagen scaffold and seeded with Tendon stem/progenitor cells	seeding greatly enhanced remodeling including more organized collagen deposition and decreased the immune response	rotator cuff reconstruction in rabbit	1,2, 3 month H, IC, HM	(79)
braided silk fiber tube coated in silk/hydroxyapatite solution, MeOH treated	significant ingrowth of fibrous tissue, significant mineralization including within bone tunnel	ACL reconstruction in rabbit	2, 4 month H, IC, HM, MT	(80)
braided silk fiber tube filled with porous collagen-chondroitin scaffold	tensile strength 76% of native tissue, significant regenerated tissue with replacement of original collagen scaffold with native collagen bundles but also some fibrosis	achilles reconstruction in rabbit	6 month H, MT	(27)
braided silk fiber tube with tricalcium phosphate polyether ether ketone anchor	substantial tissue ingrowth at junction, bone tunnel contained mineralized as well as unmineralized tissue, Sharpey's fibers present	ACL reconstruction in pig	3 month H	(77)
braided silk fiber tube with tricalcium phosphate polyether ether ketone anchor	tensile strength increased with time and similar to autograft studies but less than native ACL, anchor increased silk to bone attachment including regeneration of mineralized tissue and Sharpey's fibers	ACL reconstruction in pig	3, 6 month H, MT	(78)
braided silk fiber tube filled with porous collagen scaffold, or braided silk fiber tube alone	silk-collagen constructs had significantly increased remodeling including more and better organized collagen fibrils, silk-collagen had better bone-tendon transition with mineralized fibrocartilage and better preservation of cartilage within the knee joint	ACL reconstruction in rabbit	2,6, 18 month H, IC, HM, QPCR	(75)
interwoven silk and PLGA fiber mesh filled with porous collagen scaffold, seeded with rabbit MSCs	tensile strength increased from 2 to 4 months, seeded samples significantly stronger than unseeded and 58% of native tendon at 4 months more remodeling and collagen deposition in seeded scaffolds	achilles reconstruction in rabbit	2, 4 month H, MT	(81)

H = histology, IC = immunohistochemistry, HM = histomorphology, MT = mechanical testing

Table 4

In vivo studies of silk materials used for skin grafts and dermal wound dressings.

Silk material and processing	Response	In vivo model and tissue site	Time-points, analysis	Ref
silk film dressing	silk film promoted better healing than clinically used DuoActive and similar healing as AlloaskD	full thickness wound in nude mice	7, 14, 21 day H	(85)
lyophilized silk-alginate homogenous mixture	silk-alginate blended sponge increased speed of re-epithelialization from 12 to 6 days compared to NuGuaz control, more collagen deposition and higher number of dividing epithelial cells	full thickness wound in rat	3, 7, 10, 14 day H, IC	(88)
electrically polarized hydroxyapatite in silk gel fixed with glutaraldehyde and osmium oxide, lyophilized, EtOH treated	scaffolds with polarized hydroxyapatite performed similarly to clinically used IntraSite gel, better than scaffolds with non-polarized hydroxyapatite	full thickness wound in pig	6, 11, 18 day H	(89)
lyophilized porous silk scaffold	smaller inflammatory reaction and improved dermis healing compared to PVA scaffold	full thickness wound in rat	3, 7, 10, 18 day H	(18)
lyophilized porous silk scaffold with gentamycin sulfate-impregnated gelatin microspheres, MeOH treated	decreased inflammation and increased vascularization and speed of re-epithelialization compared to non-antibiotic loaded and gauze control	full thickness burn in rat, bacteria applied to wound 24 hrs after burn	3,7,10, 14, 21 day H, IC	(87)
electrospun silk scaffold, EtOH treated, pre-seeded with adipose MSC followed by decellurization	cell seeded and seeded-decellurized scaffold increased speed of healing as compared to unseeded silk and untreated controls	full thickness wound in diabetic mice	3, 7, 10, 14 day wound healing	(13)
silk-gelatin electrospun dressing with astragaloside IV	astragaloside infused dressing had improved healing, more organized tissue regeneration, and more vascularization compared to dressing alone and untreated control	full thickness burn in rat	5,10,17,24, 31 day H, IC	(86)

H = histology, IC = immunohistochemistry