CHAPTER 1

Silk-based Biomaterials for Tissue Engineering

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Summary

Silks are fibrous proteins, which are spun by a variety of species including silkworms and spiders. Silks have common structural components and have a hierarchical structure. Silkworm silk must be degummed for biomedical applications in order to remove the immunogenic sericin coating. It may subsequently be processed into a variety of forms, often via the formation of a fibroin solution, including films, fibres and sponges, and used in combination with other materials such as gelatine and hydroxyapatite. Spider silks do not have a sericin coating and may be used in natural fibre form or processed via formation of a spidroin solution. Both silkworm and spider silks have been reported to support attachment and proliferation of a variety of cell types. Silks have subsequently been investigated for use in tissue engineering. This chapter provides a general overview of silk biomaterials, discussing their processing, biocompatibility and degradation behaviour and paying particular attention to their applications in tissue engineering.

KEYWORDS: Silk; Fibroin; Spidroin; Tissue engineering.

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1. INTRODUCTION

Silks are fibrous proteins, which are spun into fibres by a variety of insects and spiders [1]. They have a range of functions, including cocoons to protecting eggs or larvae, draglines to support spiders, and capture nets able to withstand high impacts and trap insects [2-4]. Silks are subtly different – individual spiders, for example, may spin up to seven types of silk – but have some common structural elements. They have repetitive protein sequences [5] with a predominance of alanine, glycine, and serine (which is high in silkworm silks but low in spider silks). Silk proteins are comprised of different combinations and four different structural components: (i) elastic β-spirals, (ii) crystalline β-sheets rich in alanine, (iii) tight amino acid repeats forming α-helices and (iv) spacer regions [6-9]. Silks are hierarchically arranged from the amino level [5, 10] up to micro- and macroscopic structures [11-13]. The silks produced by silkworms and spiders are the most comprehensively investigated silks, the former due to its availability, use in textiles and historical medical use as a suture material, and the latter because of its remarkable mechanical properties. Silkworm silks are comprised mainly of fibroin, whereas the major protein of spider silks is spidroin.

2. PROCESSING

Silkworm cocoon fibres are bonded together by a glue-like protein, sericin. Sericin has been linked to *in vivo* inflammation [14] and therefore the silk is 'degummed' before further processing. One of the disadvantages of the degumming procedure is that it results in detrimental changes in the mechanical properties of silkworm silk [15]. Furthermore, the extent to which these properties are affected may depend upon the method used [16]. Silkworm silk is often processed via the production of fibroin solution, where degummed silk is dissolved, thus the inherent mechanical properties of the silk fibres are lost and any damage caused by the removal of sericin becomes insignificant. In its simplest form, fibroin can be regenerated into a film or coated onto other materials [17-21]. The group lead by David Kaplan has investigated porous tissue engineering scaffolds produced using salt leaching [22-30]. Spongy or porous scaffolds can also be produced by freeze drying [31-33], gas foaming [31, 34] and phase separation of silk-solvent solution [35, 36]. Fibroin solution or recombinant silk can also be electrospun to produce fibres [37-41], or wound into yarns [42-47]. It can used as the sole ingredient or blended with

other substances such as gelatine to form hydrogels [48-52]. The manufacture of nano-hydroxyapatite-silk sheets [53, 54] has also been reported.

Relatively few studies reporting the formation of constructs from regenerated spidroin are reported. The absence of the requirement for sericin removal means that spider dragline silk is often left in its original form, although a cleaning protocol may be used for cocoon silk to remove insect debris. Dragline silk can be dissolved to form a spidroin solution and subsequently spin-coated onto a substrate [55] or fabricated into a porous scaffold via salt leaching [56]. Spiders produce relatively small quantities of silk, of which collection of silk is laborious, and there is a lot of variation in the silks, depending on a variety of factors [57-59]. Consequently, methods for artificial synthesis of spider silk are under development (reviewed [60-62]). Recombinant spider silk can be processed to form a film [39] or into hydrogels that are reported to be stable over a few weeks [63].

Silk fibroin solution has no secondary structure – this is imparted by the processing method. In order to manipulate the structure of regenerated fibroin, various treatments have been investigated, particularly those involving organic solvents. Methanol treatment of regenerated fibroin in various forms is widely reported, resulting in β-sheet formation [41, 64-66], although α-helix and random coil regions do not disappear completely [67, 68]. Spidroin experiences a similar change in secondary structure [39, 69]. Transition to β-sheet structure is dependent on the time of exposure to the solvent and solvent concentration [64]. The transition induces aggregation of the fibroin molecule chains [65, 68], alters thermal degradation behaviour [37, 50, 66] and reduces the amount of water that fibroin absorbs from its surroundings [66]. Oxygen and water vapour permeability are high for methanol-treated fibroin membranes [70, 71] and mechanical properties are also improved [3, 37, 66, 72]. Transition to β-sheet structure also increases the resistance to degradation of both fibroin and spidroin [67, 69, 70, 72, 73]. Improvement of mechanical and degradation properties is significant for some biomaterial applications, such as tissue engineering scaffolds for load-bearing sites. One study reported that manipulation of the amount of β-sheet crystallisation (which affects flexibility) and surface roughness can be varied by the following of different methanol treatment protocols [68]. Ethanol treatment has been reported to have a similar effect to methanol in some studies [65], having a three-phase action [74], but others suggest that ethanol has only a minimal effect on secondary structure [68]. Water vapour treatment can be used to elicit the transition to β-sheet

secondary structure, with the time and temperature of treatment being crucial in terms of the amount of change [72].

2.1. Biocompatibility

B. mori silk fibres have been used as commercially-available sutures since the end of the 19th century, and have proved to be effective biomaterials. Evidence of adverse biological reactions, however, raised concerns about biocompatibility. Sericin, a glue-like protein that holds the fibroin fibres together, has been identified as the source of immunogenic reactions [14, 75].

Numerous *in vitro* studies have demonstrated that once sericin is extracted, fibroin supports cell attachment and proliferation for a variety of cell types [19, 33, 68, 76-79]. Silk from the wild silkworm, *Antheraea pernyi*, contains the RGD sequence, and has been reported to support cell attachment and growth to a greater extent than *B. mori* silk [77]. The use of water vapour rather than methanol to induce β-sheet transition may support better cell attachment and proliferation [72]. One study suggested that sulphonated fibroin inhibits the replication of human immunodeficiency virus (HIV) *in vitro* [80].

In vitro studies examining macrophage response to fibroin concluded that silk in film [81] or fibre form [75] did not elicit any significant macrophage activation. In particulate form, however, there was macrophage activation [75], although the authors speculated that the size of the particulate was the cause.

The *in vivo* inflammatory reaction to fibroin films has been reported to be similar to that of collagen [18]. Another study suggested that *B. mori* silk braided into yarns elicited a mild inflammatory response after seven days *in vivo*, whereas sericin-coated silk yarns and polyglycolide (PGA) caused an acute inflammatory response [47]. Gelatin-coated *B. mori* silk has also been reported to initiate a minimal inflammatory response [82]. Spider silk has been found to invoke a similar inflammatory response to materials used clinically such as collagen and medical-grade polyurethane [83].

Sericin has been identified as the cause of the inflammatory response to un-degummed silkworm silk, but it appears that only when associated with fibroin does it bring about a significant activation of macrophages [75]. Sericin films support the attachment and growth of L929 murine fibroblasts [84] and human skin fibroblasts [85]. The latter of these studies identified one particular component of sericin, sericin M, that enhanced cell attachment. Sericin-

S has also been proposed as a potential supplement for serum-free culture medium, following results demonstrating cell proliferation and mitogenic activity [86, 87] in serum-free culture conditions.

Metal-treated silk fabrics have been demonstrated to have some *in vitro* antimicrobial activity [88-90]. Similarly, sericin coatings on synthetic polymer fibres are reported to have antibacterial and antifungal properties [91]. To date, however, no assessment of the suitability of these materials for biomedical use has been reported.

Silkworm silks have similar structural characteristics to amyloid [74, 92] and dissolved fibroin has been reported to accelerate amyloid accumulation in mice [93]. The presence of amyloids in the body has been linked with neuro-degenerative diseases including Alzheimer's and Parkinson's. The synthesis of spider silks is similar to the formation of amyloid fibrils [94], but also other proteins with a low amino acid sequence complexity such as fibrous collagen. Recombinant spider silk proteins in nanofibrilar form have significant structural differences to amyloid fibrils [95]. There have been no reports to date linking spider silks to amyloid formation *in vivo*. Recent reviews concerning the use of silk-based biomaterials are available [4, 96, 97].

2.2. Degradation behaviour

Tissue engineering scaffolds should ideally degrade at a similar rate as the growth of new tissue, in order that the engineered tissue can be integrated into the surrounding host tissue. Appropriate degradation behaviour can also minimise any risk of stress shielding, which is of particular importance in the engineering of ligaments and tendons.

Cell culture studies have demonstrated that *in vitro* degradation of porous silk fibroin scaffolds is slow, with negligible loss of mass after four weeks in culture [25, 98]. Degradation studies involving the systematic exposure of silkworm silk to enzymes have found that silk will degrade as a result of proteolysis [99], with protease being reported to have the greatest effect [21, 44, 100]. Smooth [21] and porous [100] silk sheets can be significantly degraded by exposure to enzymes even after one day. Silk yarns exposed to protease exhibited a loss of mass of more than 50% and associated loss of mechanical properties after six weeks [44]. The degumming process may cause unwanted degradation of fibroin [16] and the method of silk processing has been reported to affect *in vitro* degradation behaviour. Fibroin films are reported to experience more significant degradation than fibres [21] and aqueous-derived silk fibroin

scaffolds degrade more rapidly than HFIP-derived scaffolds [101], possibly due to increased surface area. Methanol treatment may significantly reduce the rate of degradation [70, 73]. The potential to manipulate the rate of degradation is important for tissue engineering applications, and control over the physical form and post-treatment of a silk biomaterial may allow tailoring of the degradation. In the case of bone, for example, the ability of a scaffold to maintain structural integrity over an extended period of time is crucial as it allows mass transport of nutrients and waste products while bone ingrowth, matrix deposition and remodelling occurs and a vascular network is formed. In other situations, such as wound healing, more rapid degradation may be desirable.

In vivo studies involving silkworm silk have demonstrated slow degradation. Fibroin films were observed to not degrade following implantation for six weeks [18]. Negligible degradation of a non-woven, fibroin-based mesh six months after implantation has also been reported [102]. The stable, slow degrading structure of silk scaffolds compared to collagen-based scaffolds was considered to be the 'single most important factor' in the synthesis of cartilage-like tissue [25] in one tissue engineering study. Spider silk, however, has been found to experience degradation after implantation for fourteen days [83], although it is highly likely that the rate of degradation will be controlled at least in part by the form of the implant. *B. mori* silk is also susceptible to degradation by bacteria, although some resistance to fungal degradation has been reported [103].

TISSUE ENGINEERING

3.1. Skin/wound healing

In vitro studies have reported that dermal fibroblasts spread and proliferate on fibroin coatings and scaffolds without secretion of pro-inflammatory interleukins [104, 105]. Oral keratinocytes also proliferate on woven fibroin meshes [106], a form that is likely to be used for wound healing applications. Fibroin films [107] and fibroin-alginate sponges [33] have been found to enhance skin wound healing *in vivo* compared to clinically used materials. Both studies concluded that fibroin-based materials promoted epithelialisation. A fibroin-chitosan blend has been reported to give superior performance to materials currently in use when tested for repair of ventral hernias [108]. A study investigating wound healing in the presence of spidroin concluded that although the local inflammatory response was comparable to synthetic polymers routinely used in surgical

procedures, such as collagen, there was no evidence to suggest that wound healing was accelerated [83].

3.2. Skeletal tissues

3.2.1. Cartilage

The group led by D. L. Kaplan has conducted the majority of research into the use of fibroin-based scaffolds for cartilage tissue engineering. Studies use porous scaffolds, often fabricated by salt-leaching [22-25, 109]. These scaffolds have been demonstrated to result in chondrogenesis (measured by assessing type II collagen and GAG levels) when used to culture human articular chondrocytes [22] and mesenchymal stem cells (MSCs) [24, 25, 98, 109] *in vitro*. In one study, where GAG deposition in the presence and absence of serum on silk and collagen scaffolds was investigated, the surface properties of silk were found to promote chondrogenic events [109]. The synthesis of cartilage on silk scaffolds in serum-free conditions is highly significant in terms of its potential to be taken forward for clinical use. However, currently the mechanical properties of engineered cartilage are inferior to those of native cartilage [24, 109] and further optimisation of culture conditions is required. The team led by Tomita produced sponges by phase separation [35, 36]. These scaffolds can support chondrocyte proliferation for up to 28 days [36], matrix synthesis, positive type II collagen staining and GAG production indicative of hyaline cartilage-like tissue [35].

Recent studies have investigated the use of spider silk (*Nephila edulis* egg cases) for cartilage tissue engineering [110]. Successful engineering of hyaline-like cartilage tissue was achieved, with histological analysis demonstrating comparable results to PGA controls. Porous scaffolds fabricated from regenerated spidroin have also been shown to support chondrocyte attachment and proliferation and ECM production [56].

3.2.2. Bone

The majority of the research carried out into the use of silk-based biomaterials for bone tissue engineering has been performed by the group led by D.L. Kaplan. Fibroin solution used to form films [17, 111], electrospun to form scaffolds [38] or processed into a 3-D porous scaffold by salt-leaching [24, 26-30, 98, 112, 113]. Additionally, RGD functionality [18, 25] or growth factors [29, 30, 38] may be incorporated. Osteoblast-like cells grown on fibroin films coupled

with RGD peptides were found to produced mineralised matrix [17]. Osteogenesis has also been achieved with stem cells grown *in vitro* on thin fibroin films [111], scaffolds electronspun from fibroin-poly(ethylene oxide) solution [38], porous fibroin scaffolds [24, 26, 27, 98, 112, 114]. *In vivo* bone formation in non-load bearing [113, 115] and load-bearing [28] sites has also been reported. Bone morphogenic protein-2 (BMP-2) immobilised on fibroin films and scaffolds has been demonstrated to induce osteogenesis *in vitro* [114, 116] and augment bone formation by pre-differentiated and undifferentiated MSCs seeded onto silk scaffolds and cell-free scaffolds in non-load bearing [30] and load-bearing [29] bone defects.

More recently, spider silks have been investigated for bone tissue engineering. Regenerated spider silk has been found to have similar visco-elastic properties to human bone [55]. *In vitro* studies using RGD-functionalised electrospun recombinant spider silk matrices found that MSCs could be differentiated down osteogenic lineages [39].

3.2.3. Ligament/tendon

B. mori silk (after sericin extraction) wound into strands wound into yarns, has been investigated for its potential for ligament tissue engineering [42]. These matrices have suitable mechanical properties for ACL reconstruction, and modification to the yarn design and surface modification allows tailoring of the mechanical properties [43]. One design was predicated to fail after one 1 year under cyclic loading, [42], whereas an alternative cord configuration was predicted to lose 100% of its tensile strength five months after implantation [44]. Anterior cruciate ligament fibroblasts [45, 47] and MSCs [42] adhered to and spread on silk yarns and expressed ligament-specific markers, although cells did not infiltrate the centre of individual cords [42]. Coating the cords with RGD increased ECM production by both cell types and allowed MSCs to remain attached and aligned in the direction of a mechanical stimulus. In the absence of RGD, cells were rounded and started to detach. The timing of mechanical stimulation was found to be important, with stimulation after nine days resulting in increased metabolic activity and collagen production [117]. The application of growth factors to MSCs on RGD-coupled cords has been found to further improve cell in-growth and collagen-I production, although the mechanical properties of this tissue were reduced [46]. Gelatin-coated *B. mori* fibres have also been proposed for ligament tissue engineering, as the gelatin coating has been demonstrated to restore the mechanical properties that are reduced by sericin removal [82]. Preliminary investigation has shown that

these fibres are not cytotoxic and do not cause an *in vivo* inflammatory response, but no ligament-specific cell work has been performed. Similarly, the use of pressed silk sheets [118] formed into a vitrigel scaffold retained mechanical strength and prevented cell damage following mechanical stimulation. The authors speculate that this approach may be suitable for tissue engineering hard connective tissues such as ligaments and tendons.

Silk-based materials have also been investigated for tendon tissue engineering. Human tenocyte attachment and proliferation was increased on silk-RGD films compared to unmodified silk films and TCPS [119] and mRNA levels for decorin (the most abundant proteoglycan in tendon tissue) and type-I collagen were highest on silk-RGD and higher on silk films than on the control surface.

3.3. Vascular tissues

Recent evidence suggests that sulphonated and heparinised silk fibroin films have suitable mechanical properties for use as artificial blood vessels [120, 121]. These studies demonstrated that these films have good anticoagulant activity and platelet response and support endothelial cell spreading and proliferation. Preliminary work into the design of collagen gel-silk filament composites for vascular tissue engineering has also been reported [122]. The blood compatibility of silk fibroin may also be further improved by the grafting of water-soluble polymers such as 2-methacryloyloxyethyl phosphorylcholine (MPC) onto the surface [123] or by blending with S-carboxymethyl kerateine [124].

Silk fibroin nets have been reported to support endothelial cell attachment, maintenance of phenotype and the formation of microvessel-like structures when the fibroin is coated with fibronectin or collagen [125, 126]. Subsequently, co-culture of endothelial and osteoblasts cells on silk fibroin nets has been demonstrated to result in the formation of microvessel-like structures [127], even in the absence of pro-angiogenic factors and the fibronectin coating. Another group has reported that vascularised reticular connective tissue was formed within a non-woven fibroin mesh in a six month implantation study [102]. It is essential for the success of many types of engineered tissues that vascularisation occurs and the ability of silk fibroin to support this process is encouraging for its potential use for engineering of bone and other tissues.

3.4. Other tissues

There is recent evidence emerging that collagen-fibroin blends [128, 129] and lactose-fibroin conjugates [130] support the proliferation of hepatocytes. One study reported that cells were able to eliminate ammonia and synthesis urea [129], suggesting potential applications for liver tissue engineering. *B. mori* silk has also been demonstrated to support the growth and proliferation of Schwann cells and dorsal root ganglia [131] and has, therefore, been proposed as a potential scaffold for the tissue engineering of nerve grafts.

4. CONCLUSION

The silks produced by silkworms and spiders have fibrous proteins that may have several conformations. Silks can be processed into many forms suitable for a variety of biomedical and tissue engineering applications. They can be modified by chemical treatment or used in combination with other materials in order to vary mechanical properties and surface chemistry. By these means, biomaterials appropriate to specific applications can be produced. Silk-based biomaterials are at least as biocompatible in terms of inflammatory response and ability to support cell proliferation as many materials currently in use. The degradation behaviour can be tailored, depending on the application, from a few days to many months. These materials are promising for use in wound healing and as tissue engineering scaffolds, particularly for the development of skeletal tissue. Other applications, such as the use of silk for nerve regeneration, are yet to be fully investigated.

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