

Chapter 6

Advances in Nanotechnologies for the Fabrication of Silk Fibroin-Based Scaffolds for Tissue Regeneration



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Abstract Silks are protein fibers produced by silkworms whose architecture is based on two proteins: fibroin and sericin. Because sericin has been recognized as the main cause of silk's poor performance due to its antigenicity, fibroin alone has now remained popular as a biomaterial, also due to its strength and mechanical properties. Other advantages of this biological product are the water-based processing, biodegradability, and the presence of easily accessible chemical groups for functional modifications. Due to its versatility, fibroin is now widely considered for use in the manufacture of many biological devices and substitutes in different medical fields, with very different biological, physiological, and mechanical properties. In recent years, nanomaterials have gained considerable attention also in tissue engineering, because they exhibit properties that are significantly different to corresponding bulk materials, such as large surface area, increased strength, and enhanced surface reactivity, thus improving material performance. Reviewed studies, mainly in the regeneration of the musculoskeletal system, have been outlined the advantages of fibroin as a scaffold, and the technologies adopted for the nanostructure development of this protein. Further advancements will open up new perspectives in the use of this product in tissue regeneration. Silk-based materials are of particular interest where controlled biodegradation and good mechanical properties are required, such as in tissue engineering of musculoskeletal tissues. Their versatility in processing, biocompatibility properties, ease of sterilization, thermal stability, possibility for surface chemical modifications, and controllable degradation therefore make silk-derived proteins promising biomaterials for many

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clinical functions. Since research into these applications is quite new, we can expect interesting future developments, in which the nanotechnologies might play a decisive role.

Keywords Silk fibroin · Nanotechnologies · Nanomaterials · Biomaterials
Biocompatibility · Composite materials · Preclinical studies · Hard tissues
In vitro study · In vivo study

Silks are protein fibers produced by silkworms (*Bombyx mori*), but also by spiders and others arthropods. The architecture of silk is based on two proteins: fibroin, which is the core filament, and sericin, which is a protein that glues fibroin fibers together.

Although silk was used extensively in surgery for making suture threads, this application has now been replaced by synthetic materials with greater histocompatibility than natural ones. Benefits and drawbacks of silks for biomaterial applications have been well detailed by Altman, Vepari, and other authors and are summarized in Table 6.1 [1, 2]. More recently, because sericin has been recognized as the main cause of silk's poor performance due to its antigenicity, fibroin alone has remained popular as a biomaterial.

Fibroin is made of highly organized crystals and semi-crystalline regions that account for its elasticity. The primary structure is mainly composed of the amino acids glycine, alanine, serine, valine, and tyrosine with characteristic sequences. These structural elements produce the strength and resiliency of silk fibroin [3]. Indeed, silk has interesting mechanical properties regarding its use as biomaterial. Other advantages of this biological product are its water-based processing, biodegradability, and the presence of easily accessible chemical groups for functional modifications [4].

Nowadays, tissue engineering procedures have become widespread in regenerative medicine for the treatment of diseases when standard medical procedures fail. Regenerative strategies are based on the combination of three main tools: cells (differentiated and not differentiated), scaffolds, and growth factors. Silk fibroin also

Table 6.1 Benefits and drawbacks of silks for biomedical applications

<i>Benefits</i>
Mechanical properties
Prolonged history of use in clinical applications
Manufacturing in water solution and easy insolubilization
Easy chemical modifications
Slow in vivo degradation
Easy functionalization
<i>Drawbacks</i>
Adequate removal of sericin required
Slow degradation of crystalline regions
Possible formation of granuloma
Sensitization and possible allergic response

has received much attention as a scaffold material, due to its above-mentioned biocompatibility, processability, biodegradability, and mechanical and thermal properties [5]. Scaffolds should mimic tissue extracellular matrix (ECM) in biological and chemical composition and physical structure [6]. Mimicking the nanofibrous structures of ECM to achieve better biocompatibility remains a challenge [7].

In recent years, nanomaterials have gained considerable attention also in tissue engineering, because they exhibit properties that are significantly different to corresponding bulk materials, such as large surface area, increased strength, and enhanced surface reactivity, thus improving material performance. The definition adopted by the International Organization for Standardization (ISO/TS 27687:2008) is: “Material with any external dimension in the nanoscale or having internal structure in the nanoscale” defining “Nanoscale” as a size range from approximately 1 to 100 nm. Polymeric nanofiber matrix is similar to fibrous ECM proteins and is thus a candidate as ECM-mimetic biomaterial [6].

Due to its above-mentioned properties, fibroins now being widely considered for use in the manufacture of many biological devices and substitutes. Table 6.2 shows several experimental and clinical studies on the possible use of this product in different medical fields.

Such a wide range of applications, with very different biological, physiological, and mechanical properties, requires of course an equally wide range of ways to manage the product to make it suitable for soft and hard tissue substitution. Following the physicochemical characterization, appropriate preclinical investigations, with both *in vitro* and *in vivo* tests, must be planned to validate novel production, based on fibroin alone or as a composite. Some studies mainly in the

Table 6.2 Applications of silk fibroinbiomaterials for tissue regeneration

Organ/apparatus	Applications ^a	Authors
Vascular tissue	Flow diverting devices, stents (C)	[8, 9]
Neural tissue	Peripheral nerve conduits (E)	[10]
Skin	Composite scaffolding membranes	[11]
Bone	Composite scaffolds with/without addition of biological factors (E)	[12, 13, 14]
Cartilage	Scaffolds to support chondrocytes (E)	[15, 16]
Ligament and Tendon	Scaffolds and scaffold-free ligaments, composites (E)	[17, 18]
Cardiac tissue	Cardiac patch composite + mesenchymal cells (E)	[19, 20]
Ocular tissue	Cornea replacement (E)	[21, 22]
Hepatic tissue	Silk fibroin–collagen blended films (E)	[23]
Intervertebral tissue	Porous scaffolds (E)	[24]
Bladder	Wall repair and reconstruction (E)	[25]
Eardrum tissue	Silk-based membranes (E)	[26]

^aC *clinical*; E *experimental*

regeneration of the musculoskeletal system have been reviewed to focus on the use of fibroin as a scaffold and the technologies adopted for the nanostructure development.

Following a preclinical protocol, for example, in an in vitro and in vivo study Fini et al. [27] evaluated the behavior of an injectable silk fibroin hydrogel through osteoblast cultures and after implantation in critical size defects of rabbit distal femurs, using synthetic poly (D, L-lactide–glycolide) copolymer as control material. In vitro biocompatibility was evaluated by measuring cytotoxicity and cytocompatibility on human osteoblast-like cell line (MG 63), whereas in vivo the bone defect healing rate and quality of the newly formed bone inside the defects were determined by measuring histomorphometric parameters, such as trabecular bone volume, trabecular thickness, trabecular number, and trabecular separation. In vitro tests indicated that both materials significantly increased cell proliferation in comparison with the negative control. Both materials promoted bone healing when used to fill critical size defects in rabbit femurs, but the histomorphometry showed better results in new-formed bone of the silk fibroin hydrogel-treated defects in comparison with the control gel. The regrown bone of the Silk fibroin hydrogel-treated defects appeared to be more similar to normal bone than that of the control synthetic polymeric material-treated defects, in comparison with controls treated with a synthetic polymeric material, thus suggesting that silk fibroin hydrogel can accelerate remodeling processes. Like this study, which is aimed at hard tissue repair, many others describe a range of scaffold preparation procedures and the tissues to be replaced.

Electrospinning technology, which uses an electrical charge to draw very fine fibers on the micro- or nanoscale, enables porous nanofibrous scaffolds to be obtained, which are able to mimic the ECM. Considering the physical–chemical properties and the structure of the scaffolds, micro- and nanoparticles can be obtained from silk solutions by various procedures, such as freeze-drying and grinding procedures, spray drying, jet breaking, self-assembly, and freeze-thawing. Milling of silk fibers is also an option to obtain silk particles using any chemicals. According to Kundu et al. [4], these particles can play the dual role of improving mechanical properties of scaffolds and at the same time act as a carrier of growth factors for rapid tissue regeneration. Indeed to improve mechanical and biological performances, inorganic or organic fillers have been incorporated in silk 3D scaffolds during or after fabrication to obtain composites. The main advantage is in this case the compatibility between the components. Consequences of a poor compatibility may result in inhomogeneous mixtures, phase separation, and adverse tissue reactions. To obtain better compatibility, silk–silk composite scaffolds are made by incorporating milled silk particles in porous silk sponge, resulting in a significant improvement in mechanical properties.

With respect to the material porosity, different methods of processing are presented in the literature. The importance of the processing method is highlighted by Kuboyama et al. These authors evaluated the porosity of scaffolds prepared using regenerated *Bombyx mori* silk fibroin dissolved either in water (AF) or in hexafluoroisopropanol (HFIP). The two preparations were comparatively analyzed

in an animal model in which the formation and growth of new bone in the implantation site (rabbit femoral epicondyle) was examined by means of micro-CT and histology. The AF scaffold exhibited significantly greater osteoconductivity than that obtained by the protein dissolved in HFIP. Micro-CT analysis showed that the morphology of the newly formed bone differed significantly in the two types of silk fibroin scaffold. After 4 weeks of implantation, new trabecular bone was seen inside the pores of the AF scaffold implant, whereas the HFIP scaffold only contained necrotic cells. No trabecular bone was seen within the pores of the latter scaffolds, although the pores of these did contain giant cells and granulation tissue [28].

Lin et al. [29] evaluated silk fibroin with different nanostructures, self-assembled in aqueous solution to improve porous structure formation. In comparison with scaffolds derived from fresh solution, the nanofibrous silk scaffolds showed better cell compatibility *in vitro*. These observations suggest that the control of silk nanoscale can regulate matrix features including porous structure and nanostructure, which are important in regulating cell and tissue outcomes.

In the specific field of bone tissue engineering, silk fibroin gained much interest as a scaffold material and various strategies were developed to create a three-dimensional (3D) structure with high porosity and osteoconductivity. Salt-leaching or freeze-drying methods were used to create porous scaffolds. Moreover, in attempts to mimic bone properties, the incorporation of ceramic components into silk fibroin scaffolds has also been shown. Fibrous silk fibroin scaffolds were obtained using the electrospinning technique and the bone regeneration in the scaffolds was confirmed; they are considered to be effective in replacing collagen for many tissue engineering applications [30].

The use of non-mulberry tropical silk fibroin as a bioactive polymer in blended nanofibrous matrices resulted in osteoconductive scaffolds. The blending of 2 wt% silk fibroin exhibited higher cell attachment, growth, and ECM formation, when compared to unmodified polyvinyl alcohol as control and the other blend ratio. The mechanical robustness of constructs resembled the original bone tissue, thus indicating a promising future for this blend in bone regeneration and reconstruction. This *in vitro* study, therefore, lays the foundation for designing clinically relevant orthopedic grafts *in vivo* [31]. Considering in particular, the behavior of scaffolds interacting with cells, Teuschl et al. [32] found that surface modifications of fibroin with carbohydrate-binding protein lectin enhanced the adhesion of cells, in particular adipose-derived stromal cells; the proliferation and differentiation in osteogenic lineages were however not influenced. Considering that silk fibroin is used to obtain scaffolds for bone tissue engineering, this possibility may be of practical interest. Uchida et al. [33] also showed that plasma-irradiated silk fibroin was a regulator of bone matrix properties in an animal model based on the placement of scaffolds in critical size defects in rabbit femurs, which increased bone matrix, mineral concentration, cortical thickness, and trabecular bone volume.

Fibroin fibers can also be used as composites with other polymers. Yang et al. [34] studied different types of scaffolds based on silk fibroin and poly (*L*-lactide-*co*-caprolactone) blends intended for tendon tissue engineering. Biocompatibility

analysis revealed that bone marrow-derived mesenchymal stem cells exhibited a higher proliferation rate when cultured on nanofibrous scaffolds compared with the other scaffolds. The mechanical testing results indicated that the tensile properties of the nanofibrous scaffolds were reinforced in the direction parallel to the nano-yarns and fulfilled the mechanical requirements for tendon repair.

Teimouri et al. [35] proposed a nanocomposite with silk fibroin–chitosan/Nano ZrO₂ for tissue engineering. The scaffold was found to possess a porous nature with pore dimensions suitable for cell infiltration and colonization.

Kishimoto et al. [36] observed that the incorporation of montmorillonite, a very soft crystalline silicate mineral in silk fibers would improve their physical properties. This nanocomposite might be considered for new biocompatible applications, such as scaffolds for tissue engineering like bone regeneration, because of the osteoinductive montmorillonite properties and biodegradable and biocompatible silk presence.

Hydrogel products constitute a group of polymeric materials, the hydrophilic structure of which renders them capable of holding large amounts of water in their 3D networks. Hydrogels can be formulated in different physical forms, such as micro- and nanoparticles, coatings, and films. Kim et al. [37] studied silk fibroin/hydroxyapatite composite hydrogels obtained with different hydroxyapatite contents in fibroin matrix. Previous studies have shown that fibroin can be easily manufactured into hydrogel without additional crosslinking reagents or toxic chemicals. Therefore, the fibroin hydrogel manufacturing process is not only physiologically safe but also biocompatible. For bone regeneration, this offers interesting properties for silk-based scaffolds to be used in critical size bone defects and cartilage.

Chen et al. [5] studied the potential of a Silk Fibroin 3D scaffold produced by additive manufacturing technology, which can be used to engineer tissues that are structurally complex, because it is capable of producing precise architectural features using a layer-by-layer approach based on computer-aided design, to obtain a scaffold for cartilage engineering. These authors verified the presence of micropores and interconnected channels within the scaffold by scanning electron microscopic observations. In vitro cell culture within the fibroin scaffold using porcine articular chondrocytes showed a steady increase in cell numbers, thus giving positive indications for the use of the scaffold for cartilage repair.

Again in the field of osteoarticular apparatus, considering its application as a tendon and ligament substitute, Farè et al. [38] performed studies on a novel structure made of silk fibroin able to match the mechanical performance requirements of anterior cruciate ligament. In particular, these authors evaluated in vitro cell ingrowth in sericin-free, silk fibroin knitted sheath with braided core structure. Micro-CT analysis confirmed that the core was highly porous and had a higher degree of interconnectivity than that observed for the sheath. The in vivo cell colonization of the scaffolds is thus expected to penetrate even the internal parts of the structure. Tensile mechanical tests confirmed the scaffold's suitability for anterior cruciate ligament reconstruction. In vitro tests with fibroblasts revealed the absence of cytotoxic substances in the extracts. ESM obtained with human

periodontal ligament Fibroblasts cultured in direct contact with fibroin, compared to control samples, displayed an increased secretion of aggrecan and fibronectin (FBN) at 3 and 7 days of culture, and no change in IL-6 and TNF- α secretion, thus suggesting the usefulness of this novel scaffold for tendon tissue regeneration.

Nanotechnology and tissue engineering are widely involved in the achievement of constructs for skin tissue regeneration. Gandhimathi et al. [39] performed a study to evaluate the applications of composite copolymers of polylactic acid and poly-(ϵ -caprolactone) with silk fibroin, vitamin E, and curcumin, as nanofibrous scaffolds, to assess their potential as substrates for the culture of human dermal fibroblasts for skin tissue engineering. Scaffolds were made by electrospinning and characterized by fiber morphology, membrane porosity, wettability, mechanical strength, and chemical properties. Human dermal fibroblasts were cultured on these scaffolds, and the cell–scaffold interactions were evaluated. The results showed that human dermal fibroblasts cultured on nanofibrous scaffolds proved to be a potential support for skin regeneration.

Suganya et al. [40] also evaluated a hybrid biomaterial for dermal substitutes, based on nanofibrous silk fibroin scaffold. The scaffold was made by the electrospinning technique and the physical–chemical characterization showed improved hydrophilic properties and favorable tensile strain, as well as a favorable cell proliferation.

Finally, magnetic fibroin scaffolds were also evaluated, by integrating magnetic materials and fibroin scaffolds, for potential use in magnetic-field-assisted tissue engineering. Magnetic nanoparticles were introduced into scaffolds at different strengths of magnetization. The scaffolds were evaluated *in vitro* and were found not to be toxic to cells and improved cell adhesion and proliferation. These findings suggest that magnetized silk-based biomaterials can be engineered with interesting features for biomaterials and tissue engineering applications [41].

Besides these applications as a support for tissue regeneration, fibroin shows interesting properties for implantable systems for the controlled release of drugs.

Indeed, nanostructured materials are now frequently used in drug delivery systems.

Achieving efficient drug delivery systems is a way to improve new routes of administration of therapeutic agents. Silk fibroin is a suitable material for incorporation into a variety of drug delivery vehicles capable of delivering a range of therapeutic agents. Indeed, silk fibroin matrices have been shown to successfully deliver anticancer drugs, small molecules, and biomolecules [42]. In a study by Subia [43], silk fibroin–albumin blended nanoparticles were made using the desolvation method and evaluated as carriers for the delivery of methotrexate. They found promising properties as a nanocarrier for the delivery of drugs and other bioactive molecules.

Conclusions

The manifold investigations that span through very different fields of applications concur that fibroin is a promising biomaterial. Further advancements will open up new perspectives in the use of this product in tissue regeneration. Silk-based materials are of particular interest where slow biodegradation and good mechanical properties are required, such as in tissue engineering of bone, ligaments, and musculoskeletal tissues.

Their versatility in processing, biocompatibility properties, ease of sterilization, thermal stability, possibility for surface chemical modifications, and controllable degradation therefore make silk-derived proteins promising biomaterials for many clinical functions. Since research into these applications is quite new, we can expect interesting future developments, in which the nanotechnologies might play a decisive role.

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