Silk fibroin as biomaterial for bone tissue engineering

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Review article

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Silk fibroin as biomaterial for bone tissue engineering

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Abstract

Silk fibroin (SF) is a fibrous protein which is produced mainly by silkworms and spiders. Its unique mechanical properties, tunable biodegradation rate and the ability to support the differentiation of mesenchymal stem cells along the osteogenic lineage, have made SF a favorable scaffold material for bone tissue engineering. SF can be processed into various scaffold forms, combined synergistically with other biomaterials to form composites and chemically modified, which provides an impressive toolbox and allows SF scaffolds to be tailored to specific applications. This review discusses and summarizes recent advancements in processing SF, focusing on different fabrication and functionalization methods and their application to grow bone tissue \textit{in vitro} and \textit{in vivo}. Potential areas for future research, current challenges, uncertainties and gaps in knowledge are highlighted.

Keywords: Bone tissue engineering, silk fibroin, regenerative medicine, drug delivery, scaffold

1. Introduction

Bone tissue engineering (TE) is a promising strategy to regenerate bone and is regarded as a future alternative to current clinical treatments. Not only can tissue engineered constructs be transplanted as a graft, but also pave the way for three-dimensional (3D) tissue models which help to investigate tissue abnormalities and to analyze them both at the cellular and molecular level [1,2]. In this whole process, the patient’s own cells could be used which would be a key tool for personalized medicine. The goal is to create 3D bone tissues by combining cells, scaffolds and to some extent also growth factors or mechanical stimuli. One of the main challenges is the choice of an appropriate biomaterial which can mimic the natural bone tissue matrix with its mechanical and biological characteristics to support tissue development. Various materials have been tested for bone TE purposes [3,4]. Silk fibroin (SF) has been proven to be a promising biomaterial for scaffold fabrication in general and its remarkable mechanical properties predestine it for bone TE applications [5]. There is a steady increase in number of publications and citations on the use of SF as scaffold material for bone TE applications over the last 10 years which
supports its significance and potential as a biomedical material for bone TE. This review seeks to highlight its characteristics and the various modification options of SF as a biomaterial in bone TE.

2. Bone biology

Bone TE relies on our knowledge of bone structure, composition, mechanics and tissue formation which makes it crucial to have a fundamental understanding of bone biology. Approximately 35% of the bone tissue is made of an organic part while the remaining 65% is inorganic matrix [6]. The organic extracellular matrix (ECM) of bone consists of complex self-assembled macromolecules such as collagens, which make up 90-95% of the organic ECM, osteocalcin, osteopontin, osteonectin, bone sialoprotein, hyaluronan and proteoglycans [7]. The inorganic mineral phase of bone consists of hydroxyapatite (HA) as well as carbonate and inorganic salts [8]. Guiding stem cells along the osteogenic lineage is a critical step in bone regeneration and it is well known that the ECM plays a main role in regulating the stem cell fate [9–11]. Not only does this network serve as a scaffold for cells; it also helps in immobilizing growth factors and cytokines [12]. The water content in cortical bone can vary between 18 to 30 % and shows a direct relationship to bone porosity, which increases through aging and osteoporosis [13]. Nano-composite structures made of collagen and HA contribute to the strength and hierarchical architecture of bone [14]. The overall bone structure is divided into cortical bone, which is more compact, and cancellous bone, which appears more sponge-like and whose pores are filled with bone marrow or fat. Bone is a highly dynamic tissue which is continually renewed and remodeled through formation and resorption processes of bone-forming osteoblasts and bone-resorbing osteoclasts as an adaption to mechanical loads, regulatory factors such as hormones and cytokines, and other environmental parameters [15]. Osteoclasts are multinucleated cells derived from hematopoietic stem cells [16], which dissolve crystalline HA through release of hydrochloric acid and a mixture of proteases which degrades the organic bone matrix rich in collagen fibers [17]. Their role in bone remodeling involves the removal of cracks after fracture or micro damage occurs but they also function as immune cells and can secrete cytokines that can affect surrounding cells [18,19]. Their differentiation and function is controlled by
osteoprotegerin, receptor activator of nuclear factor (NF)-kappaB (RANK) and RANK ligand (RANKL) [20]. Osteoblasts are derived from mesenchymal stem cells (MSCs) located in the bone marrow and differentiate towards the osteogenic lineage [21]. Once they become encapsulated within their own matrix, they acquire a stellate morphology and are referred to as osteocytes [22,23]. Osteocytes comprise more than 90% of all bone cells in adult animals and are considered to be the major cell type responsible for sensing mechanical strain and translating it into biochemical responses which affect bone remodeling [24]. In vivo, osteocytes communicate to adjacent osteocytes via a dense and interconnected canalicular network containing cell processes for rapid signal transduction [25]. When mechanically activated, they produce bone morphogenetic proteins, Wnts, prostaglandin E2 and nitric oxide, which can modulate the recruitment, differentiation, and activity of osteoblasts and osteoclasts [26–30]. This ability makes osteocytes the essential mediator in orchestrating bone remodeling in response to mechanical stimulation [31]. MSCs are a valuable cell source for tissue regeneration because of their ability to self-renew and to differentiate along the osteogenic lineage [32]. Their recruitment, homing and subsequent differentiation also plays an important role in the repair of bone fractures [33]. Readily accessible sources of adult MSCs are bone marrow, adipose tissue or peripheral blood, of which bone marrow mesenchymal stem cells (BMSCs) have been most studied [34]. BMSCs can be expanded in vitro to 50 population doublings [35] which allows to increase their number such that a sufficient amount can be prepared for terminal differentiation and tissue regeneration.

3. Silk fibroin as a biomaterial

The behavior of cells which adhere to a surface is strongly influenced by the biomaterial characteristics. Molecular structure, mechanical properties and surface topography on micrometer and nanometer scale will influence adhesion, migration, proliferation, differentiation, and cell signaling [10,36–42]. In addition to soluble factors, sensing a biomaterial’s physical properties plays a decisive role in cellular function and fate. The following paragraph will address the biomaterial characteristics of SF.
3.1. Structure and mechanical properties

Silk is composed of two major proteins: SF (fibrous protein) and sericin (globular protein). SF is a protein isolated from different animals in the form of an aqueous protein solution. The ability to produce silk has evolved multiple times among insects such as B. mori, spiders, mites and beetles with diverse functions [43]. With such a variety of silk, it is not surprising that its molecular composition and structure can vary among the different species. SF of B. mori, the african wild silk moth Gonometa postica and G. rufobrunnea were compared amongst each other and revealed a significantly higher amount of polar (serine), acidic (aspartic acid) and basic (arginine) amino acids in Gonometa SF which can influence cell attachment and also alter chemical reactivity for side chain modifications [44]. Indian tropical tasar silk of Antheraea mylitta or chinese oak tasar silk of A. pernyi, possess Arginine-glycine-aspartic acid (RGD) motifs in their amino acid sequences which is known to mediate cell attachment (reference: GenBank: AY136274.1, Submitted (on JUL-2002) to the EMBL/GenBank/DDBJ databases). There is a growing interest for silk from genetically engineered organisms. Monster Silk™ of Kraig Biocraft Laboratories, Inc., a composite fiber of spider silk proteins and silk from B. mori, is spun by transgenic silkworms. This chimeric silk has been reported to be stronger and more flexible than commercial grade silk [45]. Such diversities should also be taken into account as they can play a role in scaffold fabrication and the ultimate cell response in TE applications. Most studies so far have focused on SF from B. mori as it has been used for textiles over thousands of years and most of our knowledge on how to process silk is from this species. It is also easy to obtain in large quantities. Therefore only few studies have investigated the biomedical application of genetically engineered SF or SF from other species.

Conventionally, one of the first steps in the fabrication of SF scaffolds is the removal of the sericin component. Allergic reactions to silk have been reported that were attributed to sericin, which first resulted in its exclusion from biomedical applications [46–49]. However, it seems that these adverse reactions were caused by the combination of sericin and fibroin as mere sericin got established as a biocompatible material in the last years [50,51]. The glue-like protein sericin which coats fibroin, is removed by a thermochemical treatment of the cocoons, also known as degumming. In the degumming
process, cocoons of *B. mori* are immersed in boiling water, sometimes with salt or detergent to increase the efficiency, which leaves the fibroin fibers behind. The SF amino acids sequence consists of two different subunits, a light and a heavy chain, which are connected through a disulfide bond. On both chains, the glycoprotein P25 is noncovalently attached to a set of 6 light and heavy chains [52,53]. While the light chain consist of a non-repetetive, more hydrophilic sequence, the amino acid sequence of the heavy chain consists of repetitive hydrophobic blocks of Gly-Ala-Gly-Ala-Gly-Ser and repeats of Gly–Ala/Ser/Tyr dipeptides, which form 12 crystalline domains [54]. This high glycine content allows for a tight packing into extremely stable \( \beta \)-sheet nanocrystals, which can be induced e.g. by potassium phosphate or methanol treatment [55,56]. The crystalline domains have a header and terminal sequence and are linked by 11 spacers with 42-43 residues each which are nearly identical [54]. The main molecular interactions in these crystalline \( \beta \)-sheets are hydrogen bonds. Even though they are one of the weakest known interactions, hydrogen bonds contribute greatly to the rigidity and tensile strength of silk. Through silk’s nano-confinement, the crystals are loaded in uniform shear and rupture is followed by a slip–stick motion as the strand slides and reforms hydrogen bonds which leads to their most efficient use [57]. Reforming those weak hydrogen bonds gives SF the ability to self-assemble and self-heal. SF can self-assemble into larger fibrous structures which are characterized by a high degree of hierarchical molecular order and also their enhanced mechanical properties [58–60]. Silk of *B. mori* obtained in the laboratory by forced silking of silk worms has a young’s modulus of 12.4–17.9 GPa and an ultimate tensile strength of 360–530 MPa at an elongation of 18–21% [61]. Since the degumming process exposes silk to an unnatural environment, the SF structure and its mechanical properties change slightly. Removal of the water-soluble sericin protein coat from the cocoon yields degummed silk fibers with a similar Young’s modulus but an altered tensile strength of 450 to 700 MPa at an elongation of 12-24% [62]. These remarkable mechanical characteristics and its high resistance to deformation make SF a favorable biomaterial for load bearing composites. However, the material properties of SF fibers cannot be directly compared with the processed scaffolds as their mechanical properties will vary depending on different parameters such as processing techniques, composition, matrix stiffness, \( \beta \)-sheet content as well as scaffold morphology and topology.
3.2. Biocompatibility

Due to its chemical composition and structure, silkworm SF has been shown to be highly biocompatible [63]. The role of SF as biomaterial was first assessed in 1995, showing the attachment and growth of fibroblast cells on *B. mori* SF matrices [64]. Years later it has been demonstrated that a negligible inflammatory response was exhibited with a high biocompatibility with blood [65–67]. SF has been recognized by the US Food and Drug Administration (FDA) as a biomaterial in 1993 and is widely used as suture material. Different silk-based medical devices have been approved by the FDA by now such as the long term bioresorbable surgical mesh *Seriscaffold®* and *SeriACL™*, a SF based ligament graft. Since silk can easily be absorbed by the human skin, it has been investigated as a biomaterial for wound dressing and products for clinical application are being developed (Akeso Biomedical Inc.) [68,69].

3.3. Degradation

Degradation plays an important role for biomaterials used in bone TE. Depending on the target tissue, scaffolds should degrade at a controlled degradation rate to support new bone formation. Therefore the controlled manipulation and understanding of degradation rates is one of the main goals in materials science. Being a protein, SF has been shown to be susceptible to proteolytic degradation by various enzymes such as protease XIV, alpha-chymotrypsin and collagenase IA [70,71]. Concerns have been raised about the degradation of the SF β-sheet structure since amyloid β-fibrils are associated to Alzheimer disease. However, SF degradation products showed no significant cytotoxicity to neuronal cells in *in vitro* assays [72]. As SF is being degraded, the resulting amino acids can be absorbed *in vitro* or *in vivo* which is favorable in biomedical applications. SF degradation can be influenced by the content of water-insoluble silk II and water soluble silk I structures, the former being induced e.g. during methanol treatment or stretching. With increasing silk II amount, and therefore increasing amount of β-sheet structures, the degradation time has been shown to increase as well [73]. A faster degradation of SF films was achieved by preparing water insoluble SF film with lower β-sheet content from a concentrated aqueous solution by either subsequent water-based annealing or a very slow, controlled drying process [73,74]. Of these two methods, SF films prepared by a slow drying process, showed the lowest β-sheet
content which resulted in the fastest degradation [74]. The degradation behavior is also influenced by the SF source. When comparing SF from mulberry and non-mulberry silkworms, different degradation rates have been observed. Non-mulberry SF was less prone to degradation due to the compact crystal structure and high α-helix and β-sheet contents [75]. However, so far only a few studies compared the degradation of SF among different species or genetically modified organisms which produce SF. Using transgenic silk fused with a matrix metalloproteinase (MMP-2) cleavage site, a faster degradation by matrix metalloproteinase-2 was achieved [76]. To slow down degradation, it was shown that protease inhibitors integrated into silk based systems, can decrease the degradation rate [77]. This enhanced regulation of degradation also opens up new possibilities for controlled drug release applications.

4. Silk fibroin processing methods

Not only the choice of scaffold material but also scaffold morphology - and with that the choice of the material processing method - is a decisive factor in bone TE. Scaffolds provide biomechanical support for cells until they are organized into a functional tissue through migration, proliferation, differentiation and deposition of extracellular matrix. Therefore they have to meet certain requirements. Three-dimensional scaffolds need to offer appropriate mechanical properties, pore size, pore orientation, porosity, interconnectivity and surface chemistry to promote the development of osteoblastic cells [78–81]. Also the influence of scaffold design parameters on the transport of nutrients and metabolic waste products has to be taken into account. The application of SF as sutures and wound dressings has inspired materials scientists to develop a wide range of SF scaffolds. SF can be processed into hydrogels, sponges, fibers, particles, microspheres, tubes and electrospun fiber mats with tunable properties to study cell-cell and cell-biomaterial interactions. The most relevant processing methods for bone TE (Table 1) will be discussed in the following sections.

4.1. Gels

Hydrogels are water-swollen three-dimensional polymer networks which provide remarkable options for the delivery of cells and cytokines in TE. Especially for clinical applications they are favorable as they
offer the advantage of being injectable. The hydrogelation of SF is induced in aqueous SF solutions through high temperatures, low pH, high ionic strength, vortexing, sonication, freeze gelation or electrogelation [82–86]. During the gelation process, structural changes of the SF occur from a disordered state to a β-sheet conformation which physically cross-links and stabilizes the gel [87]. Electrogelation is an exception as it leads to random structures and α-helices rather than β-sheets and is temperature reversible [88]. Also sonication-induced gelation can be reversed to some extent. Sonication imparts local vibrational movement of SF macromolecules, which alters the hydrophobic interaction and facilitates self-assembly of SF macromolecules to form β-sheet crystals. After gelation, further sonication can convert the gel back into a solution due to the cleavage of disulfide bonds, modulation in hydrophobic inter- and intra-chain interactions and β-sheet conformations [89]. However, a gel is formed again shortly after the sonication stops. SF hydrogels with interesting mechanical properties can be formed through freeze gelation techniques, which use a frozen regenerated SF solution that is immersed into a suitable solvent while being kept below its freezing temperature. After several hours, gelled scaffolds form [86]. SF cryogels were obtained from frozen SF solutions at subzero temperatures by adding ethylene glycol diglycidyl ether which induced β-sheet crystallization, inducing gelation. These gels exhibited remarkable mechanical properties such as an elasticity that allowed them to resist complete compression without any crack development (Figure 1B), while hydrogels formed at 50°C fractured already under low deformation (Figure 1A). Cryogel scaffolds produced from a 12.6% SF solution showed a very high compressive modulus of 50 MPa which makes them a potential scaffold for bone regeneration [90].

Sonication-induced SF hydrogels have been shown to successfully encapsulate human MSCs while supporting their proliferation, growth and cellular functions [84]. The aqueous SF solution was shortly sonicated, mixed with the hMSCs and then incubated at 37°C to allow for complete gelation. In a similar approach, hMSCs were encapsulated in a blend of SF and SF-poly-L-lysine. The hydrogel induced osteogenic differentiation of hMSCs even when no osteogenic stimulants were added to the cell culture medium [91]. Furthermore, injectable SF hydrogels have been demonstrated to accelerate the remodeling processes in rabbit distal femurs [92]. These osteogenic features were improved by using sonication-induced SF hydrogels with incorporated vascular endothelial growth factor (VEGF) and bone...
In vivo studies in rabbits demonstrated that VEGF and BMP-2 in these hydrogels promote angiogenesis and new bone formation, respectively, and that the combinations of those two factors had an additive effect on bone regeneration [93]. Such gels could be used to deliver various growth factors in a minimally invasive approach to regenerate irregular or poorly accessible cavities in bone. Finally, injectable fibrin network containing activated platelets have also been combined with SF. The resulting gel showed increased mechanical properties, slower degradation, and more controllable growth factor release of VEGF, platelet-derived growth factor and transforming growth factor beta 1 (TGF-β1) as compared to control groups without SF [94]. It is known that platelet-rich plasma with bone grafts significantly improves the quality of bone [95–98]. Therefore, the combination of platelet-rich plasma with SF could be a beneficial bone TE strategy.

### 4.2. Porous sponges

Porous sponge-like scaffolds have been widely used in bone TE. Their 3D porous structure allows for cell attachment, proliferation, and migration, and facilitates nutrient and waste transport either by diffusion in a static environment or perfusion e.g. in bioreactor setups. The pores can be formed in different sizes using porogens, gas foaming or freeze-drying [99,100]. Processing parameters can be varied by using different solvents for regeneration of SF including organic solvents such as hexafluoro-2-propanol (HFIP) or aqueous solution. HFIP-SF scaffolds are relatively persistent towards degradation and may take up to two years to degrade in vivo. Whereas scaffolds derived from aqueous solutions can degrade completely in 2 to 6 months [101]. There are different opinions on which fabrication method is most suitable for bone TE. It was reported that HFIP-derived SF scaffolds pre-seeded with adipose-derived stem cells (ASCs), demonstrated a better bone tissue formation with increased levels of osteopontin, collagen type I, bone sialoprotein, increased calcium deposition and mineralized ECM volume. In this study, the amount of alkaline phosphatase (ALP) activity and calcium deposition after 7 weeks were comparable to cells cultured in decellularized trabecular bone [102]. However, different results were obtained using scaffolds with lyophilization-induced bone lamellar-like structure [103]. In this study, methanol treatment was compared to water annealing and steam sterilization for the induction of β-sheet structures. Cell culture of
hMSCs under osteogenic conditions showed higher ALP levels as well as mineralized matrix after 42 days in scaffolds derived from aqueous solutions. Also, Kim et al. reported enhanced osteogenesis of hMSCs on water-based scaffolds as compared to HFIP-derived scaffolds after 28 days of culture [104]. It was also reported that aqueous-based SF scaffolds exhibited higher osteoconductivity compared to SF dissolved in HFIP when they were implanted into cortical defects of sheep. After 4 weeks of implantation, new trabecular bone formed inside the pores of the aqueous SF scaffold while the HFIP-SF scaffolds mainly contained necrotic cells and no trabecular bone [105]. It is argued that these observations might be associated with an increased surface roughness of the aqueous SF scaffolds at a micro-scale. This is probably due to the partial dissolution of the NaCl particle surface as well as different degradability and structural changes [104,106].

Using porous SF scaffolds, it was shown that the osteogenic differentiation of hMSCs can be enhanced by a combination of low oxygen levels (5%) and increased lysine and proline concentrations in the cell culture medium which altered the metabolic rate of the differentiating cells [107]. Changes in glucose consumption rate, lactate synthesis rate, and the use of lysine, proline, glutamate, and glutamine seemed to be coupled to osteogenesis of hMSCs. The progressing mineralization of ECM on RGD decorated SF scaffolds with small (106–212 µm), medium (212–300 µm), and large pore sizes (300–425 µm) was demonstrated by weekly micro computed tomography (µCT) imaging [108]. The scaffolds were seeded with hMSCs and cultured in bioreactors which allowed to aseptically scan the constructs repeatedly. The hMSCs were differentiated along the osteogenic lineage and produced bone-like tissue which resembled the initial scaffold geometries and featured trabecular-like structures (Figure 2). In a similar setup, it was shown that initial cell pre-cultivation can maximize ECM mineralization by hMSCs on SF scaffolds, but only with smaller pore diameters in the range of 112–224 µm [109].

In bone TE, micro-CT imaging gives the opportunity of monitoring 3D mineralization without destroying the bone tissue engineered construct. However, when using SF as scaffold material, the underlying scaffold structure remains invisible as the material is not radio-opaque. SF scaffolds cannot be segmented from the cell culture medium after taking up water. Therefore it is not possible to monitor degradation processes or to investigate the influence of single scaffold structures on ECM mineralization over time.
Several studies have shown that porous SF scaffolds can mineralize spontaneously in the presence of culture media, which strongly affects the comparability of studies analyzing how cells form bone-like tissue [110,111]. The choice of FBS has been shown to be a decisive factor in this process for both acellular and cell-seeded SF scaffolds [112]. Many theories concerning the mineralizing mechanism of SF have been put forth. Vetsch et al. have attributed the inherent mineralization of silk to the abundant β-sheet crystalline regions present in the structure which act as nucleating sites to facilitate HA deposition, similar to the mineralizing mechanism of collagen in native bone tissues [112]. On the other hand, there is evidence that the amorphous linkages within the β-sheets of SF, which resemble the anionic, non-collagenous proteins, facilitate the deposition of HA on these nucleation sites [113]. The hypothesis was supported by in vitro evidence showing extensive apatite formation only in the low molecular weight (2-10 kDa), electronegative fragments of the fibroin chain. Another supporting study by Jung et al. has demonstrated enhanced mineralization on such hydrophilic, electronegative fragments of SF by seeded hMSCs [114]. Furthermore, this pro-osteogenic property of the low molecular weight fibroin fragments has been attributed to the suppression of the Notch signaling pathway [114].

Improved mineralization in vitro and in vivo was observed with genetically modified SF which had a polyglutamic acid site and was produced in transgenic silkworms through systematic transformation [115]. In bone, mineralization occurs by self-assembly at the charged acidic domains of non-collagenous proteins [116,117]. Upon implantation of these modified, porous scaffolds into epicondyle defects in rabbit femurs, mineralization and bone formation occurred earlier as compared to native SF scaffolds.

### 4.3. Electrospinning

Electrospinning allows for the production of polymer fibers with diameters on a micrometer and nanometer scale which are able to mimic nanoscale properties of fibrous ECM components. It is a simple and inexpensive process as its setup consists only of a syringe pump, a high voltage source, and a collector. With this technique, fine mats from B. mori SF with a fiber diameter of less than 800 nm were formed by electrospinning with polyethylenoxide [118]. Electrospinning with formic acid as solvent resulted in fibers with an average diameter of 80 nm [119]. Cell culture experiments showed that after 14
days of incubation, the electrospun SF mats supported extensive BMSC proliferation and matrix coverage [108]. To mimic the ECM for TE applications, a 3D environment is desirable. The importance of this was shown by the use of electrospun 3D SF scaffold which increased the adhesion and proliferation of preosteoblasts and the ALP activity of osteoblasts [121]. *In vivo* studies in rats demonstrated an increased bone regeneration in 3D porous electrospun SF scaffolds compared to non-porous controls and commercially available porous three-dimensional polylactic acid (PLA) scaffolds [122]. It is assumed that the 3D fibrous structure provides a more natural surface for cell adhesion and allows easier circulation of nutrients and waste compared to sponge-like structures which only offer a 2-D surface in a microscopic view and usually have lower porosities.

4.4. 3D bioprinting

Current designs and fabrication methods fail to control the material’s resorption properties and lack pre-defined internal geometry. Most scaffolds produced these days are randomly porous with no defined internal architecture which hinders vascular ingrowth, subsequently resulting in poor osseo-integration within the construct. Also *in vivo* experiments to investigate the influence of this internal geometry on cellular behaviour are therefore limited. This is mainly because the scaffold architecture produced by conventional strategies such as porogen leaching, gas foaming, freeze drying and melt molding is often process driven, rather than design driven. Advanced rapid prototyping has developed a whole array of approaches such as 3D bioprinting which creates arbitrary shaped, customised scaffolds with pre-defined internal architecture using the solid freeform approach that might help to overcome the above-mentioned drawbacks [123].

Bioprinting is regarded as the future technique for combining biomaterials, cells and to some extent also supporting components into 3D biological constructs to reconstruct deficient tissues or to model tissues and organs in a healthy and diseased state. The goal is to plan the precise positions of cells with computer aided design and then print them individually or layer-by-layer. Bioprinting is a relatively new method and mostly uses biocompatible hydrogels as they allow cell encapsulation in a gelated, hydrated and mechanically supportive 3D environment [124]. Only a few studies have been conducted so far using SF
as a material for bioprinting processes. Recently, an inkjet printing process was developed and used to fabricate cell hosting SF nests [125], where patterned SF nests measuring 70-100 µm in diameter and modified with anionic and cationic side chains acted as anchored nests for the incubation and proliferation of *E.coli* cells. Using 3D printing, material stiffness can be modified effectively by altering the β-sheet content which consequently affects material degradation. It was shown recently that direct write silk-gelatin scaffolds (Figure 3A) processed by sonication, possessed higher β-sheet (25.4%) content over cross-linked structures using tyrosinase enzyme (14.2 %) (Figure 3B) [126]. Subsequently, biological activity of the material was assessed by culturing human nasal inferior turbinate tissue-derived mesenchymal progenitor cells which demonstrated enhanced osteogenic differentiation only on sonicated scaffolds possessing higher β-sheet content (Figure 3C-E), whereas their tyrosinase cross-linked counterpart demonstrated chondrogenic differentiation as a result of the lower β-sheet content and consequently lower material stiffness. This study suggested a strong significance of controlling the structural parameters of SF scaffolds such as their secondary conformation and consequently matrix stiffness to direct the cell population towards a specific lineage.

With the recombinant spider silk protein eADF4(C16), which mimics the repetitive core sequence of dragline SF, 3D printing without crosslinking agents or thickeners was shown by pre-gelling the solution overnight at 37°C and 95% relative humidity. The adhesion of different cell types which were seeded after the printing process, was tested and revealed that osteoblasts showed a much better adhesion than fibroblasts, myoblasts, HeLa cells or keratinocytes [127]. Thus, in the future this recombinant spider silk protein could prove useful for 3D bioprinting of cells for bone TE.

### 4.5. Silk fibroin composite scaffolds

For bone TE applications, SF is often used in combination with other biomaterials which have been shown to be beneficial for bone regeneration such as calcium-phosphate based inorganic components or collagen which are both found in bone *in vivo* [128–130]. The signaling cascades in response to HA are not yet completely understood. So far it is known that, upon interaction with HA, MSCs trigger signaling cascades such as ERK/Sox9, BMP/Smad, Wnt, TGF-β, MAPK, and Notch signaling pathways [131].
Recently it was suggested that nano-HA induced osteogenic differentiation and inhibited adipogenic differentiation of seeded BMSCs via upregulation of IL-1α; an autocrine/paracrine soluble factor that promotes bone formation by regulating collagen and osteopontin synthesis [132]. HA can be incorporated into porous SF scaffolds by direct deposition using an alternate soaking process of CaCl$_2$ and Na$_2$HPO$_4$, by mixing it with a porogen like NaCl or by mixing HA with the regenerated SF solution [133,134]. The osteoconductivity of the resulting composite materials leads to higher formation of tissue engineered bone as compared to unmodified SF scaffolds. This can be attributed to the osteoconductivity of HA as well as the presence of nucleation sites for new mineral. Nanohydroxyapatite (nanoHA) has also been incorporated into SF hydrogels using ethanol as gelling agent. The resulting SF/nanoHA hydrogels had a higher compression modulus as well as increased osteogenic potential and could serve as filling material for small bone defects [135]. The incorporation of HA, due to its osteoconductivity and scaffold reinforcing properties, has also found application in electrospinning [136–139]. NanoHA was surface-modified by γ-glycidoxypropyltrimethoxysilane to prevent aggregation and mixed with aqueous SF solution, resulting in beadless nanofibers with peak strengths at a nanoHA content of 20 wt.% [139]. Modification of electrospun mats with BMP-2 and/or nanoparticles of HA supported growth and osteogenic differentiation of hMSCs when cultured in osteogenic medium whereby the combination of BMP-2 and HA resulted in the highest calcium deposition [140]. Next to HA, different composites containing SF have been investigated and proven to be osteoconductive, including chitosan and aloe vera [137,141]. An interesting biomaterial combination for bone regeneration and tissue engineering is SF and demineralized bone matrix (DBM) which mainly consist of collagen and BMP-2 and has been widely used for bone regeneration in clinical applications. DBM powder or particles are difficult to handle which impedes its clinical application and usually requires the use of a biocompatible, viscous carrier such as putty. Better handling can be achieved through the incorporation of DBM into 3D SF scaffolds which also promoted osteogenesis in rat BMSCs and showed increased toughness and strength, probably through the particle incorporation [142].

To meet the compressive properties of bone e.g. to function as direct load-bearing support, without adding a different biomaterial to SF, self-reinforced SF composites have been explored. SF microfibers as well as
SF microparticles have been used to increase the mechanical strength and robustness of SF scaffolds [143–146]. SF scaffolds containing microparticles (~ 3 µm) had a compressive modulus of 2.82±0.40 MPa and showed slower enzymatic degradation as well as increased surface roughness [144]. SF scaffolds with incorporated large (400-600 µm) and medium (150-200 µm) fibers even achieved compressive modulus values in the range of approximately 10 MPa [145], which was comparable to the compressive modulus of cancellous bone (~10 MPa) and about 10 times lower than cortical bone (~100 MPa), thus forming the toughest silk-based regenerated material developed so far. It is argued that the scaffold’s strength is a result of the fibers bonding to the matrix and transferring load during compression which decreases stress buildup. These incorporated fibers not only increased the rigidity but also the surface roughness of the scaffolds and enhanced the differentiation of hMSCs towards the osteogenic lineage.

5. Interaction of osteoclasts with silk fibroin

The use of SF based scaffolds for bone TE is strongly connected to showing that MSCs can differentiate along the osteogenic lineage on them and mineralize their ECM. However, for a functional tissue-engineered system which is intended to serve as a human in vitro model for drug discovery or testing, not only bone forming cells have to be taken into account but also bone resorbing cells. Only a few publications have dealt with the interaction of osteoclasts and SF so far. With SF films it was demonstrated that vapor stabilized SF and methanol stabilized SF support the growth of murine osteoblasts and osteoclasts in both single and co-cultures (Figure 4) [147]. The study also showed that when monocytes were cultured as a single cell type, cells that were positive for the osteoclast marker tartrate resistant acid phosphatase (TRAP), formed aggregates. However, osteoclasts and osteoblasts co-cultures formed a homogeneous layer with interspersed TRAP positive cells. This indicates that SF may be a favorable biomaterial to study the cell-cell communication of osteoclasts and osteoblasts. On the contrary, it was reported that SF hydrolysate inhibits RANKL-induced formation of TRAP in RAW 264.7 cells, which is a murine monocyte macrophage cell line, in a time and dose dependent manner. It is not clear to which extent SF lysate would be present during a cell culture of osteoclasts on SF scaffolds.
However, SF lysate may be used as a natural compound to prevent bone loss by reducing osteoclastogenesis.

6. *In vivo* bone regeneration with silk fibroin

The ability of a biomaterial to perform specific functions in patients cannot be evaluated only with *in vitro* tests as it is difficult to extrapolate from the *in vitro* to the *in vivo* situation. Therefore, in bone TE, both biocompatibility and bone promoting activity of the orthopaedic material should be demonstrated in *in vivo* studies as well. To assess its bone regeneration capacity, SF has been tested as porous scaffold, electrospun material and hydrogel in different animal models comprising mainly mice, rats, rabbits and sheep with calvarial, mandibular or femoral defects.

Studies were conducted to explore differences between collagen and silk proteins in bone regeneration. The presence of GER peptide sequence (GFP*GERGVEGPP*GPA) present in collagen I which acts as essential recognition site for the binding of α2β1 integrin, important in mediating osteoblast differentiation, could not be identified on *B. mori* SF [148]. However, when directly compared *in vivo*, in terms of their efficacy in promoting guided bone regeneration, it was revealed that the absolute volume of new bone formation did not vary significantly in SF and commercial Bio-Gide® collagen membranes (8.75 ± 0.80 and 8.47 ± 0.75 mm³ respectively) after 8 weeks of implantation in critical size rat calvarial defects [149]. Moreover, the SF-derived membranes reduce the risk of transmitting infections, providing a viable alternative for facilitating bone regeneration.

Porous SF scaffolds exhibit architectures with hierarchical organization which is comparable to cortical and trabecular bones and can facilitate osteogenesis within the defect site. For instance, 3D electrospun SF scaffolds resembling ECM-like structure with high porosity and controlled pore sizes (200–400 nm) showed significantly higher bone coverage over commercial PLA scaffolds possessing comparable morphology [122]. After 7 weeks of implantation in a critical sized rat calvaria defect, mean normal bone area of 78.30% was attained in SF scaffolds as opposed to only 49.31% in 3D PLA scaffolds, marking the efficiency of electrospun SF scaffolds over commercial PLAs for the treatment of large bone defects.
Another group prepared nanofibrous membranes of such electrospun SF and demonstrated excellent biocompatibility, with complete defect coverage after 8 weeks of in vivo implantation in rabbit calvarial defects [150]. Also recently prepared resorbable screws of SF targeted for maxillofacial regeneration promoted satisfactory bone remodeling in rat femoral defects 8 weeks post-implantation comparable to commercially available poly-lactic-co-glycolic acid fixation systems [151].

It was demonstrated that native B. mori SF scaffolds without any pre-seeded osteogenic cells, showed insufficient bone regenerative potential required for complete in vivo healing of large femoral defects in mice [152]. A similar outcome was observed when these scaffolds were pre-seeded with un-differentiated hMSCs and implanted. However, with pre-differentiated mesenchymal stem cells, a major portion of the defect was healed with newly deposited bone tissue. Therefore, a series of subsequent studies investigated the osteogenic potential of premineralized or apatite-coated SF scaffolds in the mandibular defects of rats [153] and canines [154]. Increased bone formation was observed in premineralized constructs over uncoated ones in all the studies. Another observation was that the addition of BMP-2; a growth factor which plays a significant role in promoting osteogenic mechanisms in skeletal development [155], either exogenously incorporated on the scaffold [156] or transduced in the pre-seeded osteogenic cells [153], further accelerated the amount of new bone formation and increased the bone mineral density of the implanted constructs. Karageorgiou and co-workers demonstrated the upregulation of the osteogenic marker genes cbfa-1, osteopontin and osteocalcin together with a considerably higher bone formation (0.28 ± 0.15 mm² vs. 0.12 ± 0.05 mm²) on SF scaffolds with incorporated BMP-2 and pre-seeded hMSCs as compared to only pre-seeded hMSCs without BMP-2, when implanted in mice calvarial defects [156]. Besides BMP-2, epigenetic modification of osteogenic precursors with additional sequences such as PHF8, a major H4K20/H3K9 demethylase involved in craniofacial and bone development [157], and SATB2 (special AT-rich sequence-binding protein 2) modified induced pluripotent stem cells [158] on SF constructs have been shown to promote healing of critical size defects of mice calvariae.

Though pre-culturing of osteogenic cells on SF has demonstrated excellent osteoinductivity in vivo, the procedure might not be as suitable for clinical use. The isolation protocols for autologous cells are invasive with lengthy culture times, making it rather inconvenient for patients. Therefore to further
accelerate bone-related outcomes with cell-free naive SF matrices, surface properties of SF matrices have been altered. For instance, rather than loading with pre-differentiated cells, rapid mineralization of scaffolds was induced by incorporation of mesoporous bioactive glass [159], known to accelerate apatite deposition in contact with fluids in situ [160]. When implanted in mouse calvarial defects in vivo, bioactive glass incorporated in SF scaffolds facilitated the mineralized tissue formation (7 mm$^3$), compared to unmodified SF scaffold where only peripheral bone deposition occurred (2.5 mm$^3$). A similar in vivo response of unmodified SF was observed by Meinel and co-workers when B. mori SF scaffolds were implanted in mice calvarium [161]. The enhanced osteo-regenerative ability of the bioactive glass/SF scaffolds is mainly attributed to the consistent release of silica from bioactive glasses which subsequently up-regulates the bone-related markers in osteoblasts [162]. Others included polyaspartic acid in SF scaffolds followed by controlled calcium phosphate deposition to develop pre-mineralized SF scaffolds, which resulted in increased expression of BMP-2 in seeded human bone stem cells in vitro [163]. However, when implanted in the mandibular defects of canine, polyaspartic acid/SF scaffolds formed nominal traces of bone deposition along the defect periphery, while substantial bone formation could only be achieved with the addition of pre-seeded autologous BMSCs [153].

Recent advancements in the bone regeneration field have highlighted the absence of the RGD peptide motif in mulberry B. mori silk, a cell adhesion ligand typically present in nonmulberry A. mylitta [GenBank: AY136274.1], as one of the probable reasons for the low bone promoting ability of B. mori SF. Cell-free A. mylitta derived 3D SF sponges implanted in premaxillary defects of rat model demonstrated considerably higher bone volume over empty control defects (64.89 ± 7.46 mm$^3$ vs. 27.28% ± 9.2% mm$^3$) after 6 weeks of implantation [164]. In agreement with the above argument, a recently conducted study compared the repair of critical size rat calvarial defect with A. mylitta and B. mori lyophilized scaffolds (Figure 5) [165]. The results provided evidence of progressive mineralization with bony union in A. mylitta implanted defects within 6 months, while the B. mori scaffolds only provided nominal traces of mineral deposition within the same time span. However, a shortcoming in the study was that B. mori scaffolds were fabricated by dissolving the cocoons while A. mylitta SF proteins were directly isolated from the gland due to the difficult dissolution of A. mylitta cocoons in common solvents [166]. The
translation of \textit{A. mylitta} scaffolds to clinical settings for bone regenerative procedures still remains a challenge due to limited pre-clinical trials and the debate about the existence of RGD motifs [166].

For facilitating a congruent bone ingrowth, vascularization of the construct seems to be a pre-requisite. The development of angiogenesis concomitantly with osteogenesis will not only aid in the provision of oxygen and nutrients for osteogenic and endothelial cells, but also contribute towards the survival and differentiation of their counterpart [167]. Sun and co-workers efficiently demonstrated the \textit{in vitro} coexistence and extensive matrix formation by hMSCs and human microvascular endothelial cells on SF/HA direct write composites with controlled pore morphologies [168]. When SF scaffolds with pre-formed microcapillary-like networks developed \textit{in vitro} by co-culturing human dermal microvascular endothelial cells (HDMECs) and human osteoblasts were implanted in the subcutaneous tissue of mice, an extensive infiltration of host capillaries, identified with histological evaluation and immunohistochemical staining with human-specific antibodies, were distinctively visible in the construct [169]. Significantly reduced capillary invasion from the host occurred in SF scaffolds when HDMECs alone were cultured (8.81 ±2.11 vessels/mm$^2$) which clearly suggested the synergistic relationship of osteoblasts with endothelial cells.

Determining the underlying key signaling mechanisms that trigger such cross-talks between the two cell types can prove to be very crucial for the development of novel scaffold materials.

SF has largely been favored in \textit{in vivo} bone regenerative studies and its immuno-inflammatory response after implantation has therefore been investigated as well. For instance, when RGD-decorated SF was implanted in mouse calvarial defects, new bone deposition was evident consisting of both mature lamellar and immature woven bone [108]. A moderate level of typical immuno-inflammatory cells including macrophages, giant cells and foreign cells (lymphocytes and neutrophil granulocytes) were interspersed with the fibrous tissue and new bone tissue was observed via histopathology. However, this moderate range of immune reaction juxtaposed with the SF material falls within the expected range for such a defect. A comparable level of immuno-inflammatory response was observed when \textit{A. mylitta} SF scaffolds with natural RGD sequences were implanted within a similar rat calvarial model. Moderate levels of immune cells, typically macrophages and giant cells penetrated during the initial period, did not impede infiltration of osteogenic precursors and subsequently a mature lamellar bone was deposited within 6
months [165]. This moderate immunogenicity attained in SF scaffolds has been attributed in vitro to the silk II conformation possessing a high β-sheet content in 3D scaffolds when compared to silk I conformation in 2D SF films, as well as differences in hydrophilicity-hydrophobicity, topographical features, matrix stiffness and exposed surface area [170].

7. Drug delivery for osteogenesis

Due to its slower degradation rate, SF has been shown to possess outstanding properties as a carrier for bioactive drug delivery in several therapeutic applications, especially bone regeneration [171–175]. In an attempt to mimic the physiological bone hierarchy, various morphologies of SF have been investigated for the delivery of bone specific growth factors (e.g. BMP-2, platelet-derived growth factor), genes and enzymes which include porous 3D scaffolds, planar films, SF particles and electrospun fibers, with electrospun fibers being considered the most superior as they allow the fabrication of SF into fibers in the range of several nanometers to micrometers [176–180]. SF has been widely used to evaluate its potential for the delivery of bioactive molecules, especially BMP-2, known to play a crucial role in promoting osteogenesis. The delivery of BMP-2 using SF has been extensively evaluated for the application in bone regeneration [155]. BMP-2-loaded SF particles have successfully induced the formation of ectopic bone formation in rats, with increase in bone density concomitant with the delivery rate of BMP-2. Moreover, BMP-2-loaded SF groups showed superior bone formation over BMP-2 alone. The reasons for the observed phenomenon could be many. Direct administration of growth factors without immobilization on a carrier would result in a shorter half-life of the growth factor, slower penetration into the tissue and rapid diffusion of the delivery molecule from the site [181]. Therefore, repeated administration would be needed to consistently maintain the required concentration of the growth factor over long periods of time, which would be clinically impractical and very expensive [181,182]. Moreover, higher concentrations of the drug than required could have deleterious effects on the target tissue as well as ectopic sites [172]. This underlines the indispensable role of SF as an efficient carrier in increasing the efficacy of BMP-2 as a drug delivery molecule to promote bone regeneration. A BMP-2 delivery system composed of electrospun
polycaprolactone nanofiber mesh tubes with the central hollow chamber injected with a SF hydrogel, was developed and tested for the treatment of an 8 mm critical size rat femoral segmental defect [183]. The results demonstrated effective bone formation in BMP-2 containing groups with biomechanical properties (0.030 ± 0.001 Nm/deg torsional stiffness and 0.31 ± 0.02 Nm maximum torque) comparable to native femurs and significant degradation of the SF hydrogels after 12 weeks of implantation. It was further demonstrated that the release of BMP-2 from SF scaffolds pre-seeded with hMSCs led to higher bone formation than with SF alone [156]. In this study, BMP-2 was adsorbed on the surface of SF scaffold by continuous immersion for 6 h in 0.05 mg/ml of BMP-2 solution. These BMP-2-adsorbed scaffolds were subsequently incubated in vitro in media and the release kinetics of the drug was quantified. Results revealed that 75% of BMP-2 was released into the media from the scaffold surface within the 1st week of incubation followed by no release thereafter. Thus it may not be logical to define it as a sustained release method. Clearly, sustained release of BMP-2 from the carrier surface is an important aspect in bone TE and would benefit from a technique that can efficiently immobilize growth factors on the scaffold's surface in a way that leads to a sustained release of the molecule in situ. Another packaging strategy was to prepare SF particles with different sizes and components by a self-assembly procedure, resulting in complex calcium carbonate encrustation particles and functionalized with BMP-2 [178]. This process was used to regulate a sustained release of BMP-2 to the MSCs to trigger osteogenesis. The results showed higher ALP activity and expression of the osteogenesis-related genes Runx2 and osteocalcin, which led to enhanced differentiation of MSCs along the osteogenic lineage. The combination of BMP-2 with calcium components in the SF/calcium carbonate particles specifically induced higher osteogenesis by synchronized activity. This was evident from the comparatively reduced osteogenic ability of control groups carrying BMP-2 carriers under similar conditions.

In another study, BMP-2 was immobilized on SF planar films by utilizing carbodiimide chemistry [177]. When hMSCs were cultured on these BMP-2 decorated films in the presence of osteogenic stimulants, enhanced differentiation towards the osteogenic lineage was evident by the significantly upregulated ALP activity, increased calcium deposition, and higher expression of collagen I, bone sialoprotein, osteopontin, osteocalcin, BMP-2, and cbfa1 genes. On the contrary, nominal traces of osteogenic differentiation could
be identified on unmodified SF films cultured in osteogenic stimulants. The phenomenal tendency of osteogenesis exhibited by hMSCs on BMP-2 loaded SF was primarily a function of the immobilized protein and not the soluble BMP-2 in media as demonstrated by cell culture inserts.

To promote successful bone regeneration, efficient vascularization is a pre-requisite. Therefore, besides BMP-2, the angiogenic growth factor VEGF and its controlled delivery play a vital part in bone regeneration. It has been reported that sustained delivery of VEGF could aid in bone regeneration by promoting neovascularization and osteoblast differentiation [184]. Farokhi and co-workers fabricated electrospun poly(lactic-co-glycolic acid) nanofibers on the surface of freeze-dried SF/calcium phosphate substrates loaded with VEGF [176]. The controlled release profile of VEGF measured in vitro over a 28-day period, classified the setup as a sustained delivery system with 83% sustained bioactivity post-release. The delivery system demonstrated good cytocompatibility in vitro, characterized by improved cell adhesion, proliferation and ALP activity of human osteoblast cells. In vivo, the induced neo-bone formation resulted in coverage of 8-mm critical size calvarial defects of rabbits. As VEGF and BMP-2 are important factors involved in angiogenesis and osteogenesis, Zhang et al. evaluated the potential of sonication-induced SF hydrogels as a carrier to encapsulate dual factors and test their efficacy in the repair of rabbit maxillary sinus floor augmentation [93]. In vivo, while BMP-2 alone mediated great bone formation and VEGF triggered neo-vascularization, cumulatively they promoted both osteogenesis and angiogenesis 12 weeks post-surgery.

8. Conclusion and outlook

SF is a natural biomaterial with unique biomedical and mechanical properties which make it favorable for a wide range of bone TE applications. It can be combined with several other biomaterials such as HA to create composite scaffolds which mimic the natural bone environment and increase the scaffold’s osteogenic potential. Being a protein with tunable degradation and diverse modification options, SF possesses exceptional properties as a carrier for drugs and their sustained release in therapeutic applications. SF can be processed into different scaffolds such as injectable and printable gels, porous
sponges and electrospun 2D and 3D constructs. With novel processing techniques it can be expected that new SF based scaffolds will be developed in the future and open up even more possibilities for bone TE applications. Clinical practice shows that most fractures will heal naturally and do not require TE strategies. Complex or non-union fractures might be more relevant for bone TE with SF. Creating solid organs and achieve reasonable tissue dimensions is a goal we are still far away from. However, these constructs will have to have a structure and SF with its versatile scaffold types offers a broad toolbox to choose from. In vivo studies using SF scaffolds for bone TE applications have proven the material’s osteogenic potential. Nevertheless, these studies were done mostly in small animals that do not sufficiently predict their performance in humans. More research will have to be done before SF can be used for clinical trials and commercialized for bone TE applications. It is likely that SF will contribute to creating sophisticated bone tissue models to study ECM mineralization, mineral resorption and vascularization as well as to investigate bone diseases and possible therapeutic drugs. Most of the in vitro studies have focused on MSCs alone, their osteogenic differentiation and ability to mineralize their ECM. A better understanding is needed regarding 3D co-culture systems to create dynamic tissues which are able to remodel similar to bone. However, the future of SF in bone TE, where mechanically stable and long-term degradable biomaterials are needed, is promising and has great potential to bring viable strategies and innovations.

Acknowledgements

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References


Figure Captions

Figure 1: Silk fibroin hydrogels (A) and cryogels (B) formed at 50 and −18 °C, respectively, undergoing a compression test; CSF = 4.2 %, EGDE = 20 mmol/g, TEMED = 0.10 %. Adapted with permission [90]. Copyright 2013 American Chemical Society.

Figure 2: SEM images (A–C) of silk fibroin scaffolds decorated with RGD sequences with small (106–212 µm), medium (212–300 µm) or large (300–425 µm) pore diameters. Micro-CT images (D–L) taken from the same scaffolds seeded with hMSC after 5 weeks of cultivation under osteogenic conditions in spinner flasks, showing the constructs as seen from the top (D–F), from the side (G–I) and at higher magnification (J–L). Bar length: 500 µm (A–C), 2 mm (D–I), 1 mm (J–L). Reprinted with permission [108]. Copyright 2013 Elsevier.

Figure 3: (A) Schematic showing 3D printed SF-gelatin constructs produced by direct writing. (B) Characterization of SF-gelatin ink demonstrating significantly increased β-sheet content in sonicated constructs over tyrosinase cross-linked constructs. (C) SEM image of custom-made 3D filamentous architecture and osteogenic differentiation of hMSC laden constructs confirmed by alizarin Red S (D) and ALP staining (E). Abbreviations - SF: silk fibroin, hMSC: human mesenchymal stem cells, SEM: scanning electron microscopy, ALP: alkaline phosphatase. Adapted with permission [126]. Copyright 2014 Elsevier

Figure 4: SEM images of single and co-cultures of osteoblasts (OB) and osteoclasts (OC) on silk fibroin (SF), chitosan and polylactic acid (PLLA) after 10 days of culture. (A) Vapor treated SF film with OB:OC, (B) vapor treated SF film with OC, (C) vapor treated SF film with OB, (D) methanol treated SF film with OB:OC, (E) methanol treated SF film with OC, (F) methanol treated SF film with OB, (G) chitosan film with OB:OC, (H) chitosan film with OC, (I) chitosan film with OB, (J) PLLA film with OB:OC and (K) PLLA film with OC, (L) PLLA film with OB, Scale bar = 75 µm. Reprinted with permission [147]. Copyright 2009 Elsevier.
Figure 5: Bone regeneration in Antherea mylitta (Am) and Bombyx mori (Bm) scaffolds. (A) X-ray radiographs 6 months post-surgery showing defect coverage with neo bone formation in Am constructs (left panel) and no bone formation in Bm constructs (right panel). Centre image shows surgical site drilled with 5 mm bilateral calvarial defects. (B,C) Histological analysis of constructs 3 months post-surgery stained with H&E (B) and Alizarin Red S (C) showing moderate immuno-inflammatory responses with enhanced bone deposition in Am (arrows) (left panel). In Bm constructs, profuse infiltration of immuno-inflammatory cells is conspicuous with minimal traces of bone matrix deposition (right panel). M: Month. Adapted with permission [165]. Copyright Wiley 2015.
Table Captions

Table 1: Silk fibroin (SF) scaffold processing techniques for bone tissue engineering applications.
Figure 3

(A) Schematic representation of the process involving B. mori cocoons and gelatin for 3D bioprinting.

(B) Graph showing increased β-sheet content in sonicated hydrogels. The legend indicates:
- Red bar: SF-Gelatin
- Blue bar: SF-Gelatin Tyrosinase
- Green bar: SF-Gelatin Sonication

(C) Microscopic image showing a 1mm scale bar.

(D) Magnified image showing details with a 100 μm scale bar.

(E) Fluorescence microscopy image with a 100 μm scale bar.
<table>
<thead>
<tr>
<th>Format</th>
<th>Fabrication technique</th>
<th>Description</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>References</th>
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<tr>
<td>Hydrogels</td>
<td>High temperatures, low pH, high ionic strength, vortexing, sonication, freeze gelation</td>
<td>Structural changes of the SF in an aqueous solution occur from a disordered state to a β-sheet conformation which physically cross-links and stabilizes the gel</td>
<td>Injectable for in vivo applications, cells can be encapsulated</td>
<td>Small pore sizes</td>
<td>[82–87]</td>
</tr>
<tr>
<td>Electrogelation</td>
<td></td>
<td>An electric fields induces assembly of SF nanoparticles into larger nano- or microspheres which leads to random structures and α-helices</td>
<td>Temperature reversible, gelation of low concentrated solutions (1%), injectable</td>
<td>Electrical impulse might be unsuitable for cell encapsulation, small pore size</td>
<td>[88]</td>
</tr>
<tr>
<td>Sponges</td>
<td>Freeze drying</td>
<td>Ice crystals formed within the aqueous SF solution through freezing are removed through ice sublimation which induces porosity</td>
<td>Good porosity and interconnectivity, also lamellar structures are possible</td>
<td>Pore size is limited</td>
<td>[99,100,103]</td>
</tr>
<tr>
<td>Porogen leaching</td>
<td></td>
<td>A SF solution is poured over porogens and solidified; then the porogen is leached out</td>
<td>Simple, adaptable, easily controllable pore size and geometry</td>
<td>Difficult to design precise pore orientation and pore interconnectivity</td>
<td>[101,102,104–106]</td>
</tr>
<tr>
<td>Fibers</td>
<td>Electrospinning</td>
<td>SF nanofibers are formed by the creation and elongation of an electrified fluid jet and spun into 2D and 3D scaffolds</td>
<td>Tunable fiber thickness and porosity, good interconnectivity</td>
<td>Process depends on many variables which hampers reproducibility</td>
<td>[121,122,129]</td>
</tr>
<tr>
<td>3D printed constructs</td>
<td>3D printing, rapid prototyping</td>
<td>CAD (computer-aided design) software can be used to create 3D models and print them with a 3D printer, the printed constructs can be crosslinked by enzymes or high temperatures</td>
<td>Controllable micro- and macro-geometry, cells can be encapsulated</td>
<td>Low resolution and porosity</td>
<td>[123,125–127]</td>
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