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Review

# Stem cell-based tissue engineering with silk biomaterials

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**Abstract**

Silks are naturally occurring polymers that have been used clinically as sutures for centuries. When naturally extruded from insects or worms, silk is composed of a filament core protein, termed fibroin, and a glue-like coating consisting of sericin proteins. In recent years, silk fibroin has been increasingly studied for new biomedical applications due to the biocompatibility, slow degradability and remarkable mechanical properties of the material. In addition, the ability to now control molecular structure and morphology through versatile processability and surface modification options have expanded the utility for this protein in a range of biomaterial and tissue-engineering applications. Silk fibroin in various formats (films, fibers, nets, meshes, membranes, yarns, and sponges) has been shown to support stem cell adhesion, proliferation, and differentiation in vitro and promote tissue repair in vivo. In particular, stem cell-based tissue engineering using 3D silk fibroin scaffolds has expanded the use of silk-based biomaterials as promising scaffolds for engineering a range of skeletal tissues like bone, ligament, and cartilage, as well as connective tissues like skin. To date fibroin from *Bombyx mori* silkworm has been the dominant source for silk-based biomaterials studied. However, silk fibroins from spiders and those formed via genetic engineering or the modification of native silk fibroin sequence chemistries are beginning to provide new options to further expand the utility of silk fibroin-based materials for medical applications.


*Keywords:* Silk; Stem cell; Scaffold; Tissue engineering; Mesenchymal stem cell

**Contents**



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## 1. Silk—structure and properties

Silks are naturally occurring protein polymers produced by a wide variety of insects and spiders [1–3]. In nature silks exhibit diverse structures and functions that are evolutionally tailored to the environment inhabited by the silk-producing animals [4,5]. The diverse functions of silks range from web construction and prey capture (spider webs), safety line (draglines) to reproduction (cocoons) [5–7]. Silks provide an excellent combination of lightweight (1.3 g/cm$^3$), high strength (up to 4.8 GPa as the strongest fiber known in nature), and remarkable toughness and elasticity (up to 35%) [8]. For example, while the tensile strength of dragline silk is comparable to that of synthetic high-tenacity fibers like Kevlar 49, its elasticity is 4–7 times higher than Kevlar 49 and the energy required to break dragline silk is 3–4 times higher than that for Kevlar 49. In addition to the remarkable mechanical properties, silks are thermally stable up to ~250 °C, allowing processing over a wide range of temperatures [7]. Details on the structure, mechanical properties and biocompatibility of silks can be found in recent reviews [1,2,4–9].

Silk in its natural form is composed of a filament core protein, silk fibroin, and a glue-like coating consisting of a family of sericin proteins. The most widely studied silks are cocoon silk from the silkworm *Bombyx mori* and dragline silk from the spider *Nephila clavipes* [3,10–13]. Structurally, silk fibroins from these species are characterized as natural block copolymers composed of hydrophobic blocks with highly preserved repetitive sequence consisting of short side-chain amino acids such as glycine and alanine, and hydrophilic blocks with more complex sequences that consist of larger side-chain amino acids as well as charged amino acids [6,14]. The hydrophobic blocks tend to form $\beta$-sheets or crystals through hydrogen bonding and hydrophobic interactions, forming the basis for the tensile strength of silk fibroins [15,16]. These ordered hydrophobic blocks combine with the less ordered hydrophilic blocks to give rise to the elasticity and toughness of silk fibroins [3,12,17].

The insight into how silk fibroin solutions are processed into fibers by various organisms remains an area of intensive study. The process involves the spinning of the highly concentrated silk fibroin aqueous solutions in a non-Newtonian liquid crystalline state, where the silk fibroins are lubricated and stabilized by water and form micelle-like structures through phase separation due to silk fibroin's intrinsic hydrophilic–hydrophobic block structure [3,11]. The process is mediated by the content and location of water [11]. During the process, the concentration of silk fibroin solution in the gland gradually increases to form micelles, which further aggregate to form globule like structures and gels [11]. At this stage, the silk fibroin protein is organized in a metastable state that maintains sufficient water content to avoid premature conversion to the $\beta$-sheet structure. The shear alignment during spinning (head movement of the silkworm, leg pulling by spiders) induces the final assembly of the $\beta$-sheets into crystalline blocks [11]. In the final stages of spinning in silkworms, hydrophilic proteins like sericin form composite matrices with the core fibroin fibers [3,11]. Once formed, silk fibers are insoluble in most solvents such as water, ethanol, dilute acids and bases, unless highly concentrated sulfuric acid, formic acid, hexafluoroisopropanol (HFIP), calcium nitrate or LiBr solutions are used [18,19].

The crystalline region of silk fibroins contains repetitive alanine or alanine–glycine rich sequences (Table 1). These repetitive sequences have been used as the basis for genetically engineering silk fibroin-like polymers in host systems like *Escherichia coli*, yeast, mammalian cells, and plants [13,20–27]. Similar to native silk fibroins, most recombinant silk fibroin-like polymers exhibit low solubility in water due to hydrophobicity [2,15,16,19,28]. Strategies to regulate the self-assembly of recombinant silk fibroin-like polymers to increase solubility typically include: (a) the inclusion of molecular triggers [29], such as

Table 1
Repetitive amino acid sequences in the crystalline regions of silk fibroins from selected silkworms and the spiders

| Species | Core Repetitive Sequence |
|---|---|
| Silk worm *Bombyx mori* | GAGAGSGAAG[SGAGAG]$_8$Y |
| Silk worm *Antheraea pernyi* | GSGAGG(*X*)GGGYGWGDGGYGSDS (*X* = S, A, V, R) |
| Silk worm *Galleria mellonella* | GS(SAA)$_2$(SGA)$_2$GE(VI)$_2$DDRS(SAA)$_2$AASSGASGLGGLG |
| Spider *Nephila clavipes* Major ampullate glands 1 (NCMAG 1 or spidroin 1) | GGAGQGGYGGLGSQGAGRGGLGGQGGAG |
| Spider *Nephila clavipes* Major ampullate glands 1 (NCMAG 2 or spidroin 2) | GPGGYGPGQQGPGGYAPGQQPSGPGS |
| Spider *Argiope trifasciata* | (GP(GG*X*)$_{1-4}$Y)*n* (*X* = Y, V, S, A) |

Modified from Ref. [19,171–174].

reduction-oxidation of methionines to control β-sheet formation [30,31] or kinase sites for phosphorylation/dephosphorylation reactions [32]; (b) the construction of chimeric silk fibroin-like polymers to incorporate α-helical structures [33]; and (c) the inclusion of elastin-like domains (GVGVP) to reduce crystallinity [25,34]. The last approach generates silk fibroin-elastin-like copolymers, some of which form hydrogels under physiological conditions, making them attractive candidates for injectable systems for the controlled delivery of therapeutic agents [19,34–36].

## 2. Silk fibroin as a scaffold/matrix for cell-based tissue engineering

For functional tissue repair, tissue engineering combines cells and bioactive factors in a defined microenvironment created by biomaterial scaffolds that are maintained in bioreactors with controlled environmental stimuli [37,38]. A key component for tissue engineering is the biomaterial scaffold, commonly prepared from natural or synthetic polymers, as summarized in Table 2. Ideally, scaffolds should:

(1) support cell attachment, migration, cell–cell interactions, cell proliferation and differentiation;
(2) be biocompatible to the host immune system where the engineered tissue will be implanted;
(3) biodegrade at a controlled rate to match the rate of neotissue growth and facilitate the integration of engineered tissue into the surrounding host tissue;
(4) provide structural support for cells and neotissue formed in the scaffold during the initial stages of post-implantation and
(5) have versatile processing options to alter structure and morphology related to tissue-specific needs.

Although silk has been used clinically as sutures for centuries, only recently has it been exploited as a scaffold biomaterial for cell culture and tissue engineering in vitro and in vivo. Like most biomaterials used in tissue engineering, silk was first evaluated for cellular responses such as attachment and proliferation on 2D film in tissue culture wells. Minoura et al. observed that films formed from native silkworm fibroin collected from glands of *B. mori* domestic silkworms and *Antheraea pernyi* wild silkworms were comparable to collagen films in terms of supporting attachment, spreading and proliferation of murine L-929 fibroblasts [39,40]. Inouye et al. and Gotoh et al. later found that films formed from regenerated silk fibroin prepared by dissolving silkworm cocoon fibers in 9–9.5 M LiBr supported the attachment and growth of human and animal cell lines [41,42]. The authors attributed this cell attachment to the presence of positively charged residues like arginine near the C-terminus of the non-repetitive (hydrophilic) regions of the silk fibroin sequence, considering the surface of mammalian cells are predominantly negatively charged [39,41]. Minoura et al. observed a stronger cell adhesion on films formed by silk fibroins from *A. pernyi,* the wild-type silkworm, than those from *B. mori* domestic silkworms [39]. The difference was attributed to the presence of the tripeptide Arg(R)-Gly(G)-Asp(D), a recognition site for integrin-mediated cell adhesion [43–45], in the silk fibroin sequence from the wild silkworms, but not the domestic silk worms [39]. The effect of the RGD sequence on the attachment of mammalian cells to silk fibroins was confirmed by Sofia et al. and Chen et al. through surface modification experiments with human osteoblasts, fibroblasts and bone marrow derived stem cells [46,47]. The enhancement of cell binding due to coupling RGD on silk fibroin may result from a combination of specific interactions mediated by integrin interactions and increased hydrophilicity on the otherwise highly hydrophobic silk fibroin materials. Interestingly, films formed from sericin, the glue-like coating protein found in naturally spun cocoon silk, also supported the attachment and growth of murine L929 fibroblast cells

Table 2
Some common polymeric materials used in tissue engineering and some of the tissues targeted with these materials

| Polymeric materials | Target tissues | References |
|---|---|---|
| Native/denatured collagen and collagen-containing copolymers/composites | Skin, bone, cartilage, tendon, ligament, lung, nerve | [137–139,145,154,175–196] among many |
| Polysaccharides (alginate, chitosan, hyaluronate) | Skin, cartilage, tendon, ligament | [164,197–212] among many |
| Native silk fibroin | Ligament | [148,150] |
| Regenerated silk fibroin | Skin, bone, cartilage | [52,53,153–159,163,166,167,213] |
| Poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and their copolymers PLGA | Skin, bone, cartilage, tendon, ligament, nerve | [180,196,212,214–222] among many |
| Poly(ε-caprolactone) (PCL) and its composites | Skin, bone, cartilage, tendon | [220,223–244] among many |
| Polyhydoxyalkanoates (PHA) and its composites | Skin, bone, cartilage, tendon | [245–253] |
| Tyrosine-derived polycarbonates | Bone | [254,255] |
| Poly(propylene fumarate) (PPF) | Bone | [256–260] |
| Poly(glycerol sebacate) | Neural reconstruction | [261–263] |
| Poly(phosphoester) | Bone, nerve | [264–266] |
| Poly(phosphazene) and its composites | Bone | [267–269] |

Table 3
Processing of regenerated silk fibroin and related biomedical applications

| Material format | Processing method | Features | Applications | References |
|---|---|---|---|---|
| Film | Casting | Biocompatible | Coating materials | [39–62,66–82,270] |
| | Layer-by-layer deposition | Good oxygen and water permeability | Wound dressing/skin repair | |
| | | Diverse surface modification options | Biosensors | |
| Hydrogel | Sol–gel transition in the presence of acid, ions, and other additives | Biocompatible | Guided bone repair | [83–91,97–101,271][a] |
| | | Diverse formulation for gelation | Drug release/delivery | |
| | | Easy delivery (injectable) | Cartilage tissue engineering | |
| Non-woven mat/net/membrane | Fiber deposition | Biocompatible | Guided bone repair | [102–112] |
| | Electrospinning | High strength | Wound dressing/skin repair | |
| | | Diverse surface modification options | Tissue engineering | |
| 3D porous sponge | Salt leaching | Biocompatible | Bone tissue engineering | [52,53,153–159,163,166,167,213] |
| | Gas foaming | High porosity (up to 99%) and pore interconnectivity | | |
| | Freeze drying | High strength | Cartilage tissue engineering | |
| | Freezing and thawing | Diverse surface modification options | | |

[a]Genetically engineered silk-like polymers have also been used to prepare hydrogels for biomedical applications like controlled drug release/delivery [36,93–96].

[40] and human primary skin fibroblasts [48]. Sericin also promotes cell proliferation if used as a medium supplement [49,50]. However, sericin has been identified as the major cause for adverse immune responses associated with silk materials [5], obviating its utility for tissue engineering. A number of studies have demonstrated that, upon sericin-removal, regenerated silk fibroin has good biocompatibility [5,51–53], hemocompatibility [54], as well as oxygen and water permeability [55,56]. Collectively, these studies established the basis for the utility of silk fibroin from silk worms as a potential scaffold/matrix biomaterial for cell culture and tissue engineering.

Over the past few years, numerous studies have explored the potential of native and regenerated silk fibroin-based biomaterials in various forms, including films/membranes, micro-/nano-fiber mats/nets, hydrogels, and porous sponges, which are reviewed in the following paragraphs in the context of biomedical applications (summarized in Table 3). It is worth mentioning that, although so far the majority of research activity has been focused on silk fibroin from *B. mori* domestic silkworms, very recently recombinant spider dragline silk-bearing RGD binding domains has also been produced and subsequently processed into films and fibers for applications in cell culture and tissue engineering (unpublished data). This opens exciting new possibilities to expand silk-based materials for cellular therapeutic applications.

## 3. Silk fibroin films/membranes

### 3.1. Regenerated silk fibroin films and coatings

Silk fibroin has been used as coating material for polymer scaffolds designed for cell culture and tissue engineering [57–61]. Cai et al. reported that coating poly(D,L-lactic acid) films with regenerated silk fibroin improved interactions between osteoblasts and the polymer films [60,61]. Petrini et al. coated the surface of 2D and 3D polyurethane scaffolds by dipping the scaffolds in 3–4% w/w silk fibroin solutions obtained from *B. mori* [57]. Stable silk fibroin coatings with a thickness of 200–600 nm were formed. Methanol treatment further stabilized the coatings by inducing a transition to the beta sheet crystalline silk structure, also referred to as the silk-II structure. Chiarini et al. examined the effect of silk fibroin coatings on 2D poly(carbonate)-urethane substrates on attachment, proliferation, metabolism and ECM synthesis of four strains of human fibroblasts [58]. The silk fibroin coating improved cell attachment by 2.2 fold, which resulted in a 2.5 fold increase in total cell number by day 30 in culture. Concurrently, the silk fibroin coating significantly affected the metabolism of fibroblasts, inducing higher glucose uptake and lower glutamine consumption per cell in the initial stages of cultivation. The coating also enhanced the extracellular assembly of collagen type I (Col-I), the major ECM contribution from fibroblasts.

Fibroblasts seeded on silk fibroin-coated substrates did not secrete appreciable levels of cytokines like IL-1$\beta$, TNF-$\alpha$, or TGF-$\beta$1, all of which are implicated in inflammation reactions and tissue repair during wound healing. However, the secretion of IL-6, another important cytokine involved in inflammation reactions and wound healing, was detected and enhanced by silk fibroin coating after 2 weeks. Using similar methodology, Dal Pra et al. investigated the cellular response of human fibroblasts seeded on silk-fibroin coated 3D polyurethane scaffolds [59]. The coating affected the cell attachment, proliferation, and cellular metabolism in a similar fashion as on the 2D substrate. Cytokines like IL-1$\beta$, TNF-$\alpha$, and TGF-$\beta$1 were also undetected in this system. In comparison to the 2D substrates, silk fibroin coated on 3D scaffolds did not significantly affect the expression of IL-6 or the extracellular assembly of Col-I. These differences indicate the complexity of transferring information obtained in 2D formats to 3D biomaterial structures. Regardless, these studies provided an experimental basis for the potential of silk fibroin as a coating material for tissue-engineering scaffolds on a variety of underlying material substrates.

Wang et al. recently employed an all-aqueous stepwise (layer-by-layer) deposition technique to assemble nanoscaled thin film silk fibroin coatings on a number of substrates and evaluated the response of human bone marrow mesenchymal stem cell (MSC) to the coatings [62]. Mechanistically, hydrophobic interactions and partial electrostatic interactions were the main driving forces for the deposition and stabilization of the silk fibroin on the solid substrate surfaces. Therefore, both hydrophilic and hydrophobic materials could be coated. The thickness of the multilayered film coatings was linearly correlated with the number of layers, each of which had a controlled thickness in the range of a few to tens of nanometers depending on the concentration of silk fibroin and salt in the solution used in the process. During the process, silk fibroin undergoes a structural transition from a mixture of random coil and $\alpha$-helices (silk I) to organized $\beta$-sheets (silk II structure) based on FTIR analysis. The silk fibroin films were stable and supported the attachment, proliferation, and differentiation of the human bone marrow MSCs. This simple, yet versatile, technique has the potential to be used to generate silk fibroin films with controlled morphological and structural features for clinical applications such as drug delivery and tissue engineering. The process also allows for conformal coatings of various articles.

### 3.2. Silk fibroin films with surface modifications

The biomedical applications of silk fibroin films could be broadened by surface modifications with RGD or specific growth factors. As mentioned earlier, Sofia et al. and Chen et al. showed the benefit of RGD coupling (via carbodiimide chemistry) to silk fibroin films and fibers on the attachment, spreading, proliferation and differentiation of human Saos-2 osteoblasts, fibroblasts and bone marrow stromal cells [46,47]. Similarly, Kardestuncer et al. showed that RGD modification of silk fibroin enhanced the adhesion and proliferation of human tenocytes and supported their differentiation as evidenced by elevated transcript levels for decorin and Col-I [63]. The enhanced differentiation of cells on RGD coupled silk matrices is likely due to an increased cell density, which enhances cell–cell interactions [47].

Sofia et al. also showed that surface modification with parathyroid hormone (PTH), which affects the differentiation of osteoblasts in vitro [64] and in vivo [65] if used in soluble form, may enhance cell attachment but not differentiation of human Saos-2 osteoblasts on silk fibroin films [46]. More recently, Karageorgiou et al. showed that silk fibroin films decorated with bone morphogenetic protein-2 (BMP-2) via covalent coupling enhanced osteogenic differentiation of human bone marrow stromal cells [66]. Compared to adsorbed BMP-2, covalently coupled BMP-2 was retained on the surface at a significantly higher level for a longer period in culture media. Within a week, 70% of the adsorbed BMP-2 was released from the film surface. By the end of week 4 only 10% of the adsorbed BMP-2 remained while 50% of the coupled BMP-2 still present. More importantly, both covalently coupled and surface-adsorbed BMP-2 remained active and enhanced the osteogenic differentiation of the bone marrow stromal cells. And the covalently immobilized BMP-2 was more effective than soluble BMP-2, likely due to a slower degradation and a higher protein concentration in the local microenvironment.

Overall, these studies demonstrated that the diversity of amino acid side chain residues contained in silk fibroin provides useful and accessible options for surface decorations with adhesion ligand and specific growth/morphogen factors. In most cases, biological activity was retained and in some cases improved. These strategies open up further options for selective chemical enhancements of the silk fibroin biomaterial to encode functions related to directing cell and tissue outcomes in a tissue-engineering context.

### 3.3. Biomaterial films by blending silk fibroin with other natural or synthetic polymers

The structure and properties of silk films can be further modified by blending with other natural and synthetic polymers such as cellulose [67,68], chitosan [69,70], poly(ethylene oxide) [71], polyacrylamide [72], poly(ethylene glycol) [73–75], poly(vinyl alcohol) [76], poly($\varepsilon$-caprolactone-*co*-D,L-lactide) [77], collagen [78], polyallylamide [79], *S*-carboxymethyl keratin [80,81], and other systems. Although most of these materials have not been fully tested in vivo for biocompatibility and degradability, a few reports have shown that silk fibroin films and some blend/composite materials promote in vivo healing when used as a wound dressing [52,82].

## 4. Regenerated silk fibroin hydrogels

Hydrogels can be formed from regenerated silk fibroin solution by a sol–gel transition in the presence of acid, ions, or other additives [70,83–87]. Besides these additives, other factors such as temperature, silk fibroin concentration, and pH significantly affect the gelation process. Generally, gelation time decreases with an increase in silk fibroin concentration, temperature, concentration of additives like $Ca^{2+}$, glycerol and poly(ethylene oxide), or a decrease in pH [84,85]. During the gelation process, silk fibroin experiences a structural transition from random coil to $\beta$-sheet due to enhanced hydrophobic interactions and hydrogen bond formation [84–86,88,89]. Regenerated silk fibroin can also be blended with other biopolymers like chitosan and gelatin to form hydrogels [70,90,91] and scaffolds [92]. In addition, genetically engineered silk fibroin-like polymers have been used to prepare hydrogels [36,93–96]. Silk fibroin hydrogels have been studied for controlled release/delivery of bioactive agents such as plasmid DNA, viruses, and growth factors [19,36,97].

Recently, silk fibroin hydrogels were explored for their potential in guided tissue repair. Fini et al. reported the repair of confined, critical-sized cancellous bone defects in a rabbit model using silk fibroin hydrogels [98]. The hydrogels were prepared by adding 1 M citric acid to a 2% w/v regenerated silk fibroin aqueous solution until passing the isoelectric point (3.8), followed by an overnight treatment at 50 °C. Since the acidity of resultant silk fibroin hydrogels (pH = 3.3) was not suitable for cell culture, the hydrogel was extracted using a 0.9% NaCl solution at 37 °C for 3 days. The resulting extract (pH = 5.8) was subsequently used for in vitro cytotoxicity and cytocompatibility evaluations using a human osteoblast-like cell line (MG63). The silk fibroin hydrogels showed cytocompatibility comparable to poly(D,L lactide-glycolide), based on cellular responses such as cell proliferation, differentiation, and the release of inflammation-related cytokine IL-6. Despite the apparent low pH, the silk fibroin hydrogels supported the healing of critical sized cancellous bone defects in vivo in 12 weeks with no obvious inflammatory reactions.

With further processing, such as freeze-drying, microporous silk fibroin sponges can be formed from hydrogels and used for cell culture and tissue engineering [85,99–101]. Morita and Aoki et al. combined microporous silk fibroin sponges with freshly isolated rabbit chondrocytes for cartilage tissue engineering [99–101]. Throughout the cultivation, the chondrocytes proliferated and maintained the differentiated phenotype in the silk fibroin sponge better than in collagen gels used as a control. The mechanical properties of the regenerated cartilage tissue demonstrated culture time-dependent changes that correspond to the temporal and spatial deposition of cartilage-like extracellular matrix [100,101]. These results suggest the potential of hydrogel-derived silk fibroin sponges as 3D porous scaffolds for chondrocyte-based cartilage regeneration. There remain a series of questions regarding: (a) whether these sponges will be able to support the differentiation of culture-expanded chondrocytes, as freshly isolated chondrocytes are often in limited numbers and quickly de-differentiate during in vitro expansion; (b) whether sufficient cell condensation and cell–cell interactions needed for chondrogenic differentiation can be achieved in these sponges; and (c) whether cartilage-like tissues with more uniform extracellular matrix deposition can be regenerated by overcoming the mass transfer constraints in the rather small pores in the spongy scaffolds. Putatively, the mechanical performance of the generated cartilage tissue would be improved if these issues were fully addressed. In general, standard protocols to assess mechanics of tissues generated from silk-based biomaterial matrices are employed, including mechanical compression via Instron sytems, for cartilage-like tissues.

## 5. Non-woven silk fibroin micro-/nano-fibrous nets/mats/membranes

Non-woven fibrous silk fibroin nets/mats/membranes can be prepared using degummed silk fibroin fibers with diameters in the range of several to tens of micrometers in their native or partially dissolved forms [102–104]. Finer meshes can be obtained by electrospun silk fibroin fibers with diameters in the range of tens to hundreds of nanometers [105–111]. Unger et al. reported that non-woven micro-fibrous nets support the adhesion, proliferation, and cell–cell interactions of a wide variety of human cell types including epithelial cells, endothelial cells, glial cells, keratinocytes, osteoblasts, and fibroblasts [104]. A follow up study from the same group showed that, if precoated with fibronectin, these micro-fibrous nets supported in vitro endothelialization, an essential step for vascularization [103]. After seeded in fibronectin-coated silk fibroin nets, primary human endothelial cells of macro-/micro-vascular origin exhibited normal structure, proliferative activity, migration, cell–cell interactions and other phenotypical features. Cell cultivation did not alter the structural integrity of the non-woven nets. In addition, the good cytocompatibility of these non-woven nets to keratinocytes and osteoblasts suggested potentials for skin or bone repair, which would have to be evaluated through further studies. Recently, Dal Pra et al. evaluated the biocompatibility of non-woven micro-fibrous meshes composed of partially dissolved native silk fibroin fibers [102]. After implanting subcutaneously, the non-woven micro-fibrous meshes induced a mild foreign body response without fibrosis. Among 23 proinflammatory genes evaluated by microarray, only migration inhibitory factor showed a transient intense expression at the mRNA level in implantation sites with the silk fibroin mesh. No appreciable infiltration of lymphocytes was observed six months after implantation. These results suggest good biocompatibility. These silk fibroin mesh implants supported the regeneration of vascularized reticular connective tissue

based on the temporal evaluation of cytokeratins, vimentin, and Col-I; and based on morphological, histological, and immunohistochemical evaluations of the regenerated tissue at different time points after implantation. Within 6 months of implantation the silk fibroin mesh implants were integrated with the surrounding tissue while no apparent degradation was observed. This study and the in vivo study by Sugihara et al. [52] identified silk fibroin-based membranes/meshes as promising materials for skin regeneration.

Non-woven nano-fibrous nets/mats prepared by electrospinning regenerated silk fibroin solution are of interested for biomedical applications because of the high surface area of these materials. Upon electrospinning and treatment with methanol, nanofiber solubility in water can be negated thus the mechanical properties can be improved [105,107,108]. Jin et al. and Min et al. reported that the non-woven silk fibroin nano-fibrous mats/nets support the attachment, spreading and proliferation of human bone marrow stromal cells, keratinocytes and fibroblasts in vitro [108,109,112]. Kim et al. examined the in vivo biocompatibility of silk fibroin non-woven nanofiber membranes/nets and their effect on guided repair of critical-sized calvarial bone defects in a rabbit model [106]. The nanofiber membranes/nets were formed by electrospinning regenerated silk fibroin solution in 98% formic acid on a grounded target drum and subsequently treated with 50% methanol for 60 min at room temperature before drying for 24 h under vacuum. The resulting non-woven nanofibrous membranes contained randomly deposited fibers with diameters ranging from 150 to 300 nm. The membranes supported the in vitro attachment, spreading, proliferation and differentiation of MC3T3-E1 osteoblast-like cells. When evaluated in vivo in a rabbit calvarial bone defect model, the silk fibroin non-woven nanofibrous membranes showed good biocompatibility and structural stability. The membranes were able to enhance bone formation over 12 weeks with no evidence of inflammatory reactions. This study suggests that non-woven silk fibroin nano-fibrous nets/mats/membranes have the potential to be used for guided regeneration of bones at non-weight bearing sites. The repair of weight bearing bones, such as femur and tibia, requires scaffolds with good mechanical strength [113–115].

## 6. Silk fibroin-based 3D scaffolds for stem cell-based tissue engineering

Cell-based tissue engineering requires a reliable cell source to respond properly in terms of morphology, proliferation and tissue-specific differentiation to biomaterial scaffolds and other biochemical/physical signals. Embryonic stem cells are capable of giving rise to cell types of all tissue lineages; however their applications in cell-based tissue engineering are constrained by a lack of fundamental understanding and control of their differentiation toward desired specific tissue lineages in vitro and in vivo. There are also legal restrictions and ethical concerns surrounding their use for medical applications. In contrast, adult stem cells can only differentiate towards a limited number of tissue lineages. The isolation, expansion, genetic manipulation, and clinical application of adult stem cells must follow appropriate local and federal regulations. However, it is generally acceptable from the public and federal government funding perspectives to use adult stem cells for clinical applications. For these reasons, adult stem cells have emerged as an attractive alternative to embryonic stem cells as a cell source for tissue engineering. One such example is MSCs, which can be isolated from a wide variety of tissues including bone marrow [116,117], periosteum [118,119], synovium [120], muscle [121–123], adipose tissue [124], lung [125–127], bone [128], deciduous teeth [129], dermis [130], and articular cartilage [131]. MSCs can be expanded and differentiated into cells of different connective tissue lineages including bone, cartilage, fat, and muscle upon proper stimulation [132]. These cells also have the potential for a wide range of therapeutic applications through autologous, allogeneic or xenogeneic stem cell transplantation [132,133]. Bone marrow is the major source of MSCs and bone marrow-derived MSCs have been used to treat a variety of defects and diseases, including critical size segmental bone defects [134–136], full thickness cartilage defects [137–139], tendon defects [140], myocardial infarction [141] and even nerve defects [142,143]. In the following context, this review will focus on the potential of combining bone marrow derived MSCs and silk fibroin-based 3D scaffolds for tissue-engineering applications.

### *6.1. Native silk fibroin fibers for stem cell-based ligament tissue engineering*

Over 200,000 Americans require knee ligament reconstruction annually [144–146]. The ACL and the posterior cruciate ligament (PCL) are the major intra-articular ligaments connecting the femur to the tibia to stabilize the knee. Damages to these ligaments render the knee unstable and susceptible to further injury, which can eventually cause the knee to lose its normal function. The ACL is the most commonly injured ligament with a higher frequency occurring in females than males [147]. The normal ACL is a dense, cable-like tissue with a complex but highly organized ECM containing collagen, elastin and proteoglycans. If severely damaged, the ACL tissue has poor self-healing capacity due to limited access to the blood supply [144]. The traditional treatment for severe ACL injuries using biological substitutes (autografts, allografts and xenografts) has been associated with disadvantages such as limited donor tissue supply, potential disease transmission, infection, and immune rejection [144,146]. As an alternative to biological substitutes, synthetic material-based ligament replacements have had only limited success due to material fatigue, debris generation, inflammatory reactions, poor

tissue ingrowth, and damage to the anchor sites in the femur and tibia [144,146]. These limitations have prompted interest in ligament tissue engineering strategies based on biomaterials and autologous cells, especially adult stem cells.

Altman et al. first explored the potential of native silk fibroin fibers (yarns) as 3D scaffolds for tissue engineering of ACL in cultures with dynamic mechanical loading [5,148–150]. After sericin extraction, the silk fibroin fibers were cabled into 6-cord wire-rope matrices with improved elasticity without sacrificing tensile strength when compared to an equivalent matrix formed from parallel fibers. This matrix had a hierarchal structure similar to that of collagen fibers in the native ACL and the mechanical properties were comparable to that of the native human ACL with respect to strength, stiffness, yield point, and percentage elongation at break. In addition, the wire-rope geometry increased surface area for cell attachment and ECM deposition and minimized mass transfer limitations, all of which contribute to an enhanced neotissue formation. The silk fibroin scaffolds supported the attachment, spreading, proliferation and differentiation of adult human MSCs [148]. During 3 weeks in static culture, the silk fibroin scaffolds retained mechanical strength. At week 2, the expression levels of ligament-related transcripts (tenascin-C, collagen type III (Col-III) and Col-I) were significantly higher in cells seeded on the silk fibroin scaffolds. In comparison, the expression of bone or cartilage related genes was not significantly affected, suggesting the silk fibroin scaffolds enhanced the ligament-specific differentiation of adult human MSCs [148]. This tissue-specific differentiation was further enhanced in a computer-controlled bioreactor that imparted complex mechanical forces to the silk matrices, in conjunction with improved fluidic control ([149,150] and Chen et al., unpublished data).

Recently Horan et al. systematically investigated the effect of yarn design on the mechanical properties of these silk fibroin scaffolds [151]. Extracted silk fibroin yarns were fabricated using 4 textile methods (twisted, cabled, braided, and textured) to form several geometries (Fig. 1). The mechanical properties of the yarns were significantly affected by the fabrication methods when tested in hydrated condition used to mimic physiological conditions (summarized in Table 4). Based on the mechanical features, braided and textured yarns were not suitable for tissue engineering applications where regular loading/un-loading and tissue ingrowth are needed (Table 4). Among the four textile methods, the cabled yarns possessed a highly organized hierarchal structure and allowed the most flexibility in controlling mechanical outcomes. Surface modifications such as RGD coupling and plasma treatment had significant influence on the mechanical strength of the yarns. Plasma treatment with $NH_3$ and $N_2$ decreased the yarn strength by 7.2% and 3.5%, respectively, but did not affect the stiffness. The RGD surface modification resulted in a 13.1% increase in mechanical strength and an 11.4% decrease in stiffness of the yarns [151], in addition to the positive effect of this treatment on cell attachment, proliferation and differentiation [47]. In summary, when intended for tissue-engineering applications, yarn designs should take the following features into considerations: (a) size and physiological environment of implants; (b) mechanical properties (strength, stiffness, yield, and fatigue) under regular loading/un-loading conditions; (c) surface properties (surface area and surface modifications with functional ligand like RGD); (d) void volume/length if tissue ingrowth is desired; and (e) biocompatibility and in vivo degradation rate [151].

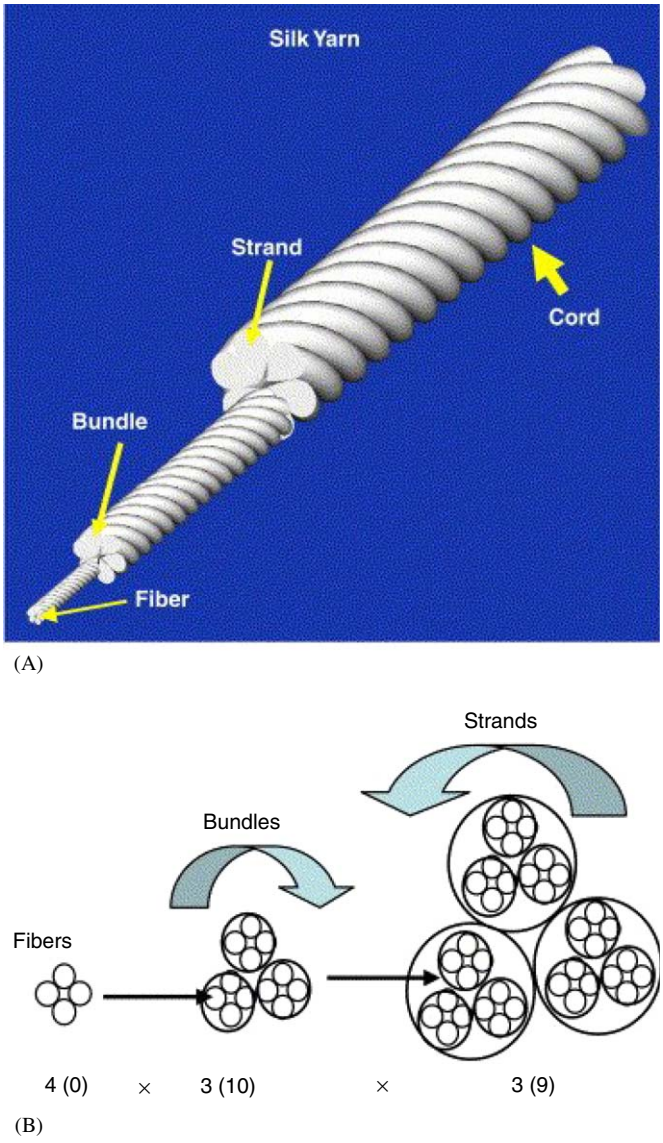



Fig. 1. Hierarchal organization of a twisted or cabled yarn. Fibers are combined to form bundles, bundles to form strands, and strands to form cords. The yarns are labeled in the following format: A(a) × B(b) × C(c), where A, B, C… represent the structural levels such as number of fibers, bundles, and strands and a, b, and c… represent the number of turns per inch on each of these levels. For example, Fig. B shows a configuration of 4(0) × 3(10) × 3(9). From Ref. [151] with permission.

Table 4
Yarn design for tissue engineering

| Yarn type | Mechanical features | Implications | Potential in tissue engineering |
|---|---|---|---|
| Braided | (1) Instantly locked upon mechanical loading, causing a sharp increase in stiffness<br>(2) Permanent locking occurs once a significant load is applied | (1) Stress shielding<br>(2) Permanent deformation<br>(3) Neotissue damage due to scissoring effect | Applications with no regular loading/un-loading and tissue ingrowth |
| Textured | (1) Fibers permanently deformed, resulting in strain hardening of the yarn<br>(2) Increased stiffness<br>(3) Decreased tensile stress/fiber | (1) Increased volume/length for better tissue ingrowth<br>(2) Permanent deformation | Non-loading bearing applications with tissue ingrowth |
| Twisted or cabled | (1) Highly organized geometry<br>(2) Decreased stiffness<br>(3) Significant decrease in tensile strength for yarns with large diameter | (1) Flexibility in mechanical outcome in a wide range<br>(2) Hierarchal organization similar to native tissue | Regular loading bearing applications with highly organized tissue ingrowth |

Summarized based on Ref. [151].

### 6.2. *Regenerated silk fibroin for stem cell-based bone tissue engineering*

The timely repair of critical sized bone defects/damages remains a major challenge for regenerative medicine. As a complex, highly organized tissue with a mineralized extracellular matrix, bone possesses marked rigidity, strength, and some elasticity, all of which are essential to support and protect the body. In addition, as the major source of inorganic ions, bone is essential to calcium homeostasis. Cortical (compact) bone provide mechanical and protective functions while cancellous (spongy) bone mainly provides metabolic functions [152]. The complexity of bone tissues and their morphological, structural and functional diversity impart a great deal of difficulties to the repair of critical sized bone defects/damages. Despite the merit of immune compatibility, bone repair using autologous tissue is often not the best treatment option as it is associated with disadvantages like limited donor tissue supply, repeated surgery, second site morbidity with additional pain, and long rehabilitation time [37,153].

A number of recent studies have explored a tissue engineering approach using silk fibroin scaffolds in various forms for the repair of bones with diverse morphologies [98,106,153–156]. As previously reviewed, silk fibroin hydrogels [98] and membranes/nets [106] without pre-seeded cells have been used for guided bone regeneration. In recent years, techniques have been developed to use 3D porous silk fibroin scaffolds and MSCs for the repair of critical-sized bone defects/damages [153–157]. The 3D porous scaffolds were derived from regenerated *B. mori* silk fibroin solution using either an all aqueous process or an organic solvent (HFIP) process with salt leaching, gas foaming and freeze drying as modes to generate the interconnected pore structures in the 3D matrices [157–159]. The highly porous scaffolds (porosity up to 99%) prepared by salt leaching possess a useful combination of high compressive strength and uniform, interconnected pores with controllable pore size and size distribution (Fig. 2). The morphological and structural features of the scaffolds produced by salt leaching depend on a number of variables including silk fibroin concentration, solid salt particle loading, salt particle size, and the use of aqueous- or HFIP-derived process. A phase diagram for the formation of the aqueous and HFIP-derived 3D porous scaffolds has been generated based on these approaches [157] (Fig. 3). During the formation of these scaffolds, silk fibroin generally undergoes a structural transition from random coil to $\beta$-sheet structures, regardless of the solvent used in the process [158,159]. The HFIP-derived scaffolds can be formed by silk fibroin solutions in a larger range of concentrations (6–20% w/v) than those (4–10% w/v) for the aqueous-derived scaffolds [157]. However, the aqueous-derived scaffolds have better pore-interconnectivity, rougher and more hydrophilic surfaces, and higher mechanical strength than the HFIP-derived scaffolds [158]. In addition, aqueous-derived scaffolds degrades faster than the HFIP-derived scaffolds both in vitro [158] and in vivo (Wang et al., unpublished data). All of these characteristics allow the preparation of scaffolds with controllable morphological and structural features to match diverse needs for the engineering of various tissues with specific functional requirements in vivo such as repair rates and tissue remodeling rates.

Meinel et al. and Kim et al. have systematically investigated HFIP- and aqueous-derived 3D porous silk fibroin scaffolds for MSC-based bone tissue engineering in vitro and in vivo [153–156]. Prior to cell seeding, the MSCs were characterized for the expression of surface markers and the capacity to differentiate into cells of multiple

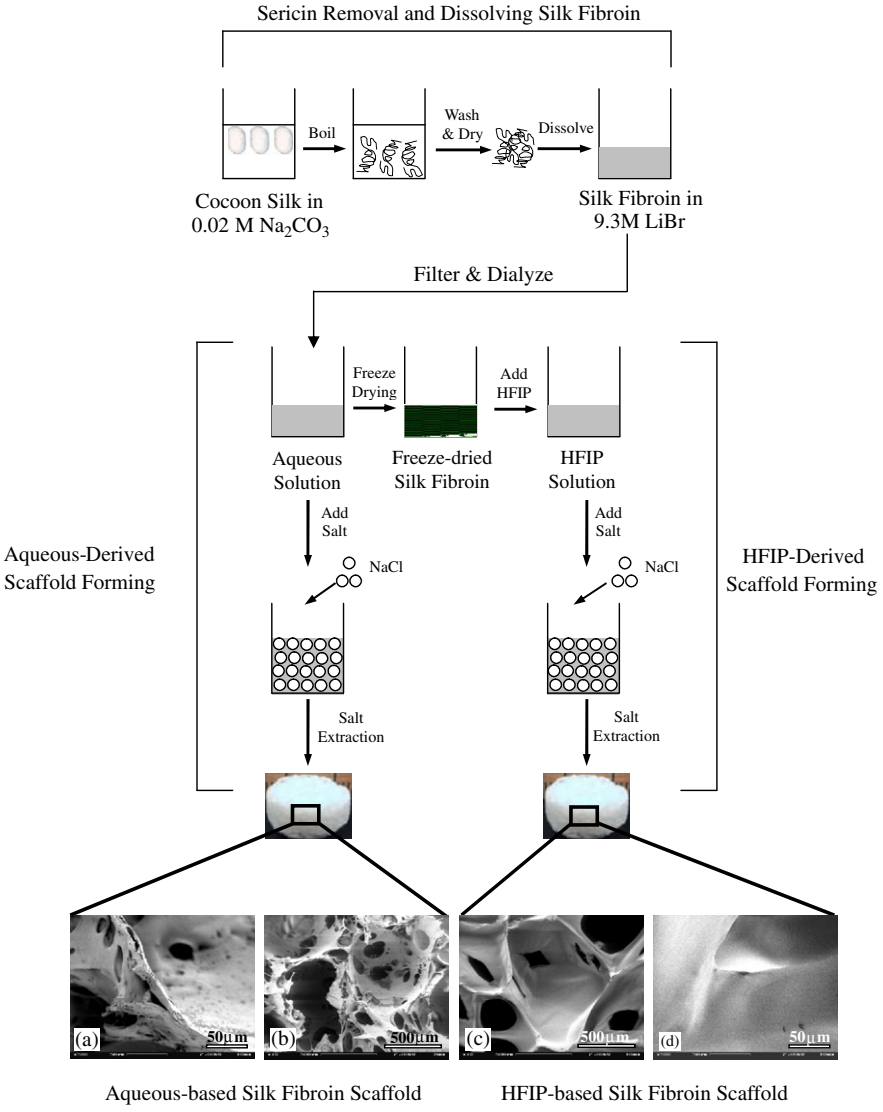
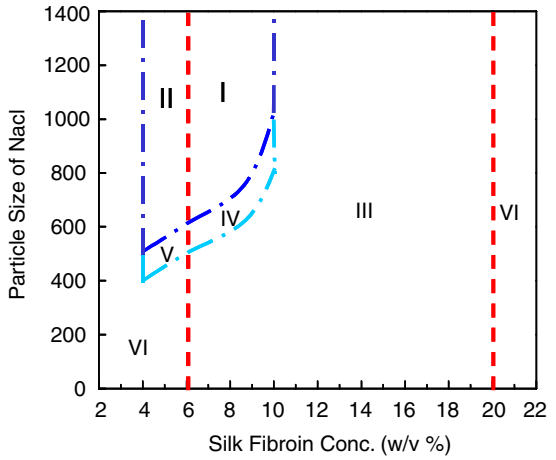



Fig. 2. Processing silk fibroin into 3D porous scaffolds. Scanning electron microscopy (SEM) images (a)–(d): aqueous- and HFIP-derived scaffolds prepared from 8% w/v silk fibroin solutions. Modified from Ref. [158,159].




| | Aqueous system | HFIP system |
|---|---|---|
| I | O | O |
| II | O | Δ |
| III | X | O |
| IV | Δ | O |
| V | Δ | Δ |
| VI | X | X |

Structure of Silk Scaffold Formed:
O=homogeneous, Δ=mixed, X=no scaffold

Fig. 3. Phase diagram of silk fibroin processing into 3D porous scaffolds by aqueous- and HFIP-based processes, NaCl particle size in microns. Modified from Ref. [157] with permission.

lineages [154,155]. The MSCs stained positive for CD105, CD44, and CD71 and negative for CD34 and CD31. In pellet cultures, the MSCs were shown to have the capacity to differentiate along chondrogenic and osteogenic lineages [154,155]. When cultured in BMP-2-containing osteogenic medium under static conditions for 4 weeks, MSCs seeded in HFIP-derived porous 3D silk fibroin scaffolds (pore size ~200 μm) showed an enhanced osteogenic differentiation over the control (collagen scaffolds) as evaluated by realtime RT-PCR for bone-related gene markers and by immunohistochemistry and microcomputerized tomography for calcium deposition. The RGD modification of the scaffolds further enhanced the differentiation of MSCs and resulted in more organized extracellular matrix structures under the same culture condition [155]. When cultured under dynamic conditions, the stability of the HFIP-derived silk fibroin scaffolds were beneficial in terms of maintaining high cell density and promoting the differentiation of MSCs [153,154]. Upon 5 week's cultivation in spinner flasks stirred at 60 rpm, the MSCs successfully generated trabecular-like bone networks with an extracellular matrix similar to that of the physiological bone [153]. Subsequently, the engineered bone-like tissue was implanted into critical sized calvarial bone defects in nude mice and compared with MSC freshly seeded scaffolds, scaffolds alone and unfilled defects. Five weeks after implantation, the tissue engineered bone implants and freshly seeded scaffolds integrated well with the surrounding tissue and stained positive in the center regions for bone sialoprotein, osteopontin and osteocalcin, which was not observed in the controls (scaffolds alone and unfilled defects). Compared to MSC freshly seeded implants, the tissue-engineered bone implants showed more substantial bone formation. Within 5 weeks, these tissue-engineered implants started to transform from the trabecular-like bone network to coalescing structures, similar to the physiological healing process of intramembraneous bone [153]. Collectively, these observations suggested that a tissue-engineering approach combining 3D porous silk fibroin scaffolds and MSCs holds promise for the repair of critical sized bone defects, where the contribution of host cells is not sufficient for a proper healing. In addition, Kim et al. recently reported that aqueous-derived silk fibroin scaffolds showed improved bone-tissue engineering outcomes when compared to HFIP-derived silk fibroin scaffolds in vitro [156]. This study suggests important implications for silk protein processing modes related to biomaterial matrix interactions with stem cells for tissue engineering.

### 6.3. Regenerated silk fibroin for stem cell-based cartilage tissue engineering

Healthy articular cartilage is an avascular tissue with a zonal matrix rich in collagen type II (Col-II) and glycosaminoglycans (GAGs) [160]. Adult articular cartilage has limited self-repair capacity due to the low cell density, slow cell proliferation, slow matrix turnover rate, and lack of the vascular supply. Severe damages in articular cartilage tissue caused by developmental abnormalities, trauma, or aging-related degeneration such as osteoarthritis often result in extensive chronic pain, gradual loss of mobility and eventually disability. Current treatment methods are often not sufficient to achieve a timely recovery of normal cartilage functions or to maintain a long-term therapeutic effect [133]. Most synthetic polymers used in cartilage tissue engineering, especially the widely used polyesters like poly(lactide) (PLA), poly(glycolide) (PGA), or the copolymer poly(lactide-*co*-glycolide) (PLGA), induce some inflammation in vivo [161,162]. The use of collagen as a natural polymeric scaffolding material is impeded by fast degradation [163] and a high swelling ratio [158]. Alginate as another popular natural biomaterial also has limitations including fast degradation, insufficient mechanical properties, inhibitory effects on spontaneous repair, and unfavorable immunological responses [164,165]. The useful combination of high strength, porosity, processability, good biocompatibility and ability to support cell adhesion, proliferation and differentiation as reviewed above suggests 3D porous silk fibroin scaffolds as candidates for stem cell- and chondrocyte-based cartilage tissue engineering [53,163,166,167].

Meinel et al. first combined 3D HFIP-derived silk fibroin scaffolds (pore size ~200 μm) and MSCs for in vitro cartilage tissue engineering and compared outcomes with unmodified and crosslinked collagen scaffolds [163]. Similar to the observations in bone-tissue-engineering studies [154,155], the structurally stable, slow degrading scaffolds (crosslinked collagen scaffolds, silk and RGD-modified silk scaffolds) were essential to maintain sufficient cell density and promote the formation of cartilage-like extracellular matrix, as evaluated by total DNA content and glycosaminoglycan deposition. After 4 weeks, MSCs in the porous silk fibroin scaffolds deposited higher amounts of cartilage-specific extracellular matrix proteins (GAGs and Col-II) and expressed higher levels of Col-II mRNA than MSCs in the collagen-based scaffolds.

Wang et al. utilized 3D porous aqueous-derived silk scaffolds (pore size ~550 μm) for in vitro cartilage tissue engineering using MSCs and chondrocytes [166,167]. MSCs successfully adhered, proliferated and differentiated along the chondrogenic lineage in the aqueous-derived silk fibroin scaffolds, based on evaluations using confocal microscopy, real-time RT-PCR, histology and immunohistochemistry. In the 3D cultivation environment created by the highly porous aqueous-derived silk fibroin scaffolds, within 3 weeks the majority of MSCs were embedded in lacunae-like spaces and acquired a spherical morphology, which has been found to be essential for the synthesis of ECM components related to cartilage tissue [168]. In the presence of inducers like dexamethasone and TGF-$\beta$3, the proliferation of MSCs peaked at 7–9 days and switched to a more actively differentiating stage. Within 3 weeks, the MSCs expressed high levels of cartilage-related ECM transcripts (Col-II, aggrecan (AGC), Col-X, and Col-II/

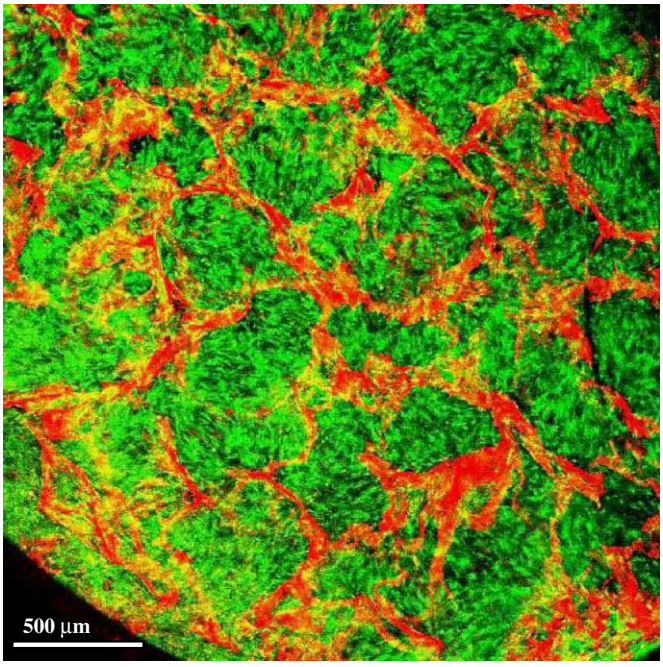



Fig. 4. Three-dimensional distribution of human bone marrow derived mesenchymal stem cells in porous aqueous derived silk fibroin scaffolds at week 3. Cells were stained using 2 μM calcein AM (green for live cells) and 4 μM EthD-1 (red for mostly silk fibroin and also dead cells) (Molecular Probes, Eugene, OR) for 30-45 min at room temperature and evaluated using a Bio-Rad MRC 1024 confocal microscope with Lasersharp 2000 software.

Col-I ratio) and deposited an ECM rich in Col-II protein and sulfated proteoglycans as evaluated by histology and immunohistochemistry. Although Col-I mRNA expression was appreciable, Col-I protein was non-detectable throughout the MSC-silk scaffold constructs at the end of 3 week's cultivation. No calcium deposition occurred in all 3D cultures as evaluated by von Kossa staining, confirming the absence of osteogenesis. These results confirmed the presence of a specific chondrogenesis under the cultivation conditions. A rather homogeneous cell and ECM distribution was achieved thanks to the unique features of these aqueous-derived scaffolds including a rough, hydrophilic surface and an excellent pore interconnectivity [166,167] (Fig. 4). By week 3, the MSCs-silk fibroin scaffold constructs acquired a unique zonal structure with a thin, dense outer layer containing cells of fibroblastic morphology enclosing an intermediate zone and a deep inner zone composed of smaller cells with a more spherical morphology embedded in lacunae-like space in the abundant cartilaginous ECM. The distribution of Col-II protein in the 3D constructs also showed a zonal pattern with more protein deposited in the outer regions, an architecture similar to native articular cartilage tissue.

In a more recent study, Wang et al. combined adult human chondrocytes (hCHs) with aqueous-derived porous silk fibroin scaffolds (pore size ~550 μm) for in vitro cartilage tissue engineering and the results were compared with the previous study using MSCs and the same scaffolds [166]. The hCHs were isolated from adult normal articular tissues and expanded in monolayer culture in the presence of 1 ng/mL TGF-$\beta$1, 10 ng/mL of platelet-derived growth factor BB (PDGF-BB) and 5 ng/mL basic fibroblast growth factor (bFGF) [169]. After cell seeding, hCHs attached to, proliferated and redifferentiated in the scaffolds based on cell morphology, expression of cartilage-related gene transcripts, and the presence of a cartilage-like extracellular matrix rich in GAGs and Col-II. Compared to MSCs, hCHs attached more slowly on aqueous silk fibroin 2D films and 3D scaffolds. Cell density was found critical for the differentiation of culture-expanded hCHs in the 3D aqueous-derived silk fibroin scaffolds. Significant levels of cartilage-related transcripts (AGC, Col-II, Sox 9 and Col-II/Col-I ratio) were upregulated, and uniform deposition of cartilage-specific extracellular matrix components (Col-II and GAGs) were observed, in hCH-silk fibroin scaffold constructs seeded at higher cell densities than observed for the MSC-based constructs. In addition, the hCH-based constructs were significantly different than MSC-based constructs with respect to cell morphology and zonal structure. Almost all hCHs in the porous silk fibroin scaffolds acquired a spherical morphology after 3 weeks of cultivation. This work diversifies cell sources for silk fibroin-based tissue engineering applications. The results suggest fundamental differences between stem cell-based (MSC) and primary cell-based (hCH) tissue engineering outcomes, as well as the importance of suitable scaffold features in the optimization of cartilage-related features. Collectively, these studies demonstrate the potential of porous 3D silk fibroin scaffolds in autologous cell-based cartilage tissue engineering.

## 7. Conclusions

The wide range of molecular structures, remarkable mechanical properties, morphology control, versatile processability and surface modification options make silk fibroin an attractive polymeric biomaterial for design, engineering and processing into scaffolds for applications in controlled drug delivery, guided tissue repair and functional tissue engineering. 3D porous or fiber silk fibroin scaffolds with surface morphology, useful mechanical features, biocompatibility, and ability to support cell adhesion, proliferation, and differentiation have expanded silk-based biomaterials as promising scaffolds for engineering a range of skeletal tissues like bone, ligament, and cartilage as well as connective tissues like skin. The generally slow rates of degradation of silk fibroin in vivo, coupled with the versatile control of structure, morphology and surface chemistry, offer a range of utility for this family of protein polymers in many needs in biomaterials and tissue engineering. In addition, since these structures can be sterilized by autoclaving or ethylene oxide treatment, suitable options are available to prepare the

materials for in vivo studies. To date most of the impact with silk-based biomaterials has been with only one source of silk, the fibroin from *B. mori* silkworm. As new sources of silk proteins become available, such as from spiders and via genetic engineering and modification of native silk sequence chemistries, the range of material properties can be generated and utilized for biomaterials can be expected to further expand options and lead to additional medical impact. For example, genetically engineered nanocomposites of spider silk with mineralizing domains have recently been described and offer new mechanical properties as well as interfacial properties, along with osteoconductivity or osteoinductivity depending on design [170]. Future directions to improve the incorporation and delivery of cell signaling factors via the aqueous processing modes available during the formation of silk biomaterial matrices, or to induce vascular networks in silks in vivo, will further enhance impact for this family of protein biomaterials. Finally, hybrid or composite systems with other biopolymers offer novel options to match complex mechanical and biological functions with tissue-specific needs.

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