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Review Article

Scaffolds Based on Silk Fibroin for Osteochondral Tissue Engineering

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Abstract

Silk fibroin protein from the silkworm Bombyx mori (B. mori) is a natural biopolymer that has extensive structural capabilities for chemical and mechanical modifications for applications in the biomedical field. The SF is versatile in its processing since it can be manufactured into different forms such as gels, films, foams, membranes, scaffolds, and nanofibers; making it an attractive material in a variety of applications that require mechanically superior, biocompatible, and biodegradable biomaterials. In this review, we present an overview of the main chemical and structural features that make silk fibroin a potential biomaterial for its wide application in tissue engineering. We discuss and summarize about different structural designs and methods for the assembly of fibroin-based 3D scaffolds emphasizing the biomedical applications of this biomaterial. Finally, we highlight the most current works in which 3D scaffolds of fibroin are used for cartilage and osteochondral tissue regeneration.

Keywords: Cartilage tissue engineering; Osteochondral tissue; Regenerative medicine; Scaffold; Silk fibroin

Introduction

Silks are polypeptides produced by members of the class Arachnida and several silkworms of the order Lepidoptera [1] that have been used in the textile industry [2], as sutures [3], in cosmetics [4], in enzymatic immobilization [5], to cover lesions [6], as a substrate for cellular growth [7,8], in systems of drug delivery [9-11], as scaffolds for tissue engineering (TE) [12], and in various advanced biomedical applications using transgenic silkworms [13,14]. Finally, silks have been considered for their application as biodegradable adhesives and sealants [15]. The use of silk as a TE biomaterial has been investigated, showing a high biocompatibility, as well as the ability to support cell growth and differentiation, in challenges as demanding as the regeneration of injured articular cartilage. The present review addresses the discoveries in the use of scaffolding systems based on silk fibroin (SF), emphasizing its application for chondral and osteochondral TE.

Physical and chemical features of silk fibroin as a biomaterial

SFs from the cocoons of the silkworm *B. mori* are the most-used and most-studied SFs in TE; South Korea is home to over 300 varieties of this species [16]. Silkworm cocoons primarily consist of 2 bio-macromolecules: fibroin (fibrous protein) and sericin (globular protein). The silk of *B. mori* is synthesized in a group of specialized salivary glands, and fibroin (comprising 60-80% of the silk) is synthesized exclusively in the posterior region of the gland. The fibroin fibres are covered with sericin (15-35%), which is synthesized in the walls of the medial regions of the gland. Between 1 and 5% of the silk consists of non-sericin components, such as pigments, wax, sugars, and other impurities (Figure 1), [1,17,18].

Most lepidopterans produce fibroin that consists of 3 protein components arranged as a single element of silk: heavy-chain (~391kDa) and light-chain fibroins (~25kDa), which are linked by a disulphide bridge, and P25 (~25kDa), also known as fibrohexamerin (Fhx), These components are present at a 6:6:1 ratio, respectively [19-21].

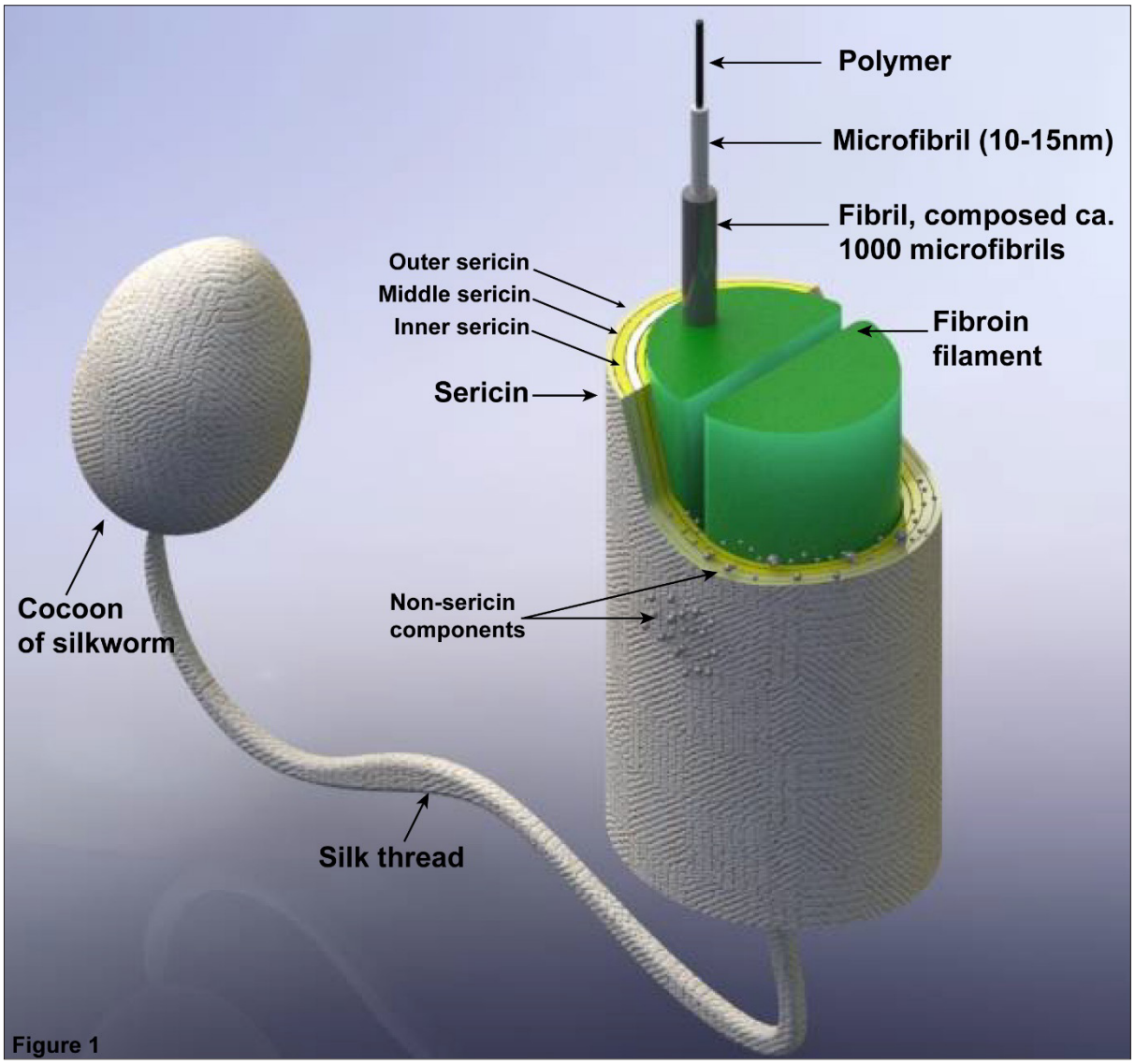

Figure 1: Schematic structure of a silk thread in the silkworm cocoon. Image modified from [11,28,119]..

In the *B. Mori* worm, the structure of heavy-chain fibroin (H-fibroin) is hydrophobic, rich in glycine, and exhibits a hierarchical layout of repeated GAGAGS motifs [21-25] that self-assemble into anti-parallel β-sheets; these structures are highly crystalline and crosslink the protein via hydrogen bonds and van der Waals interactions between β-sheets, conferring robust mechanical properties [26]. Light-chain fibroin (L-fibroin) is predominantly hydrophobic and elastic, whereas P25 consists of an alternating sequence of hydrophobic and hydrophilic regions [20,22,27].

SF is a polymorphic protein, and 3 different types of structural arrangements have been described to date: silk I [28-30], silk II (β-sheets) [23,31], and silk III [32,33]. These structures depend on environmental conditions, such as the temperature, pH, solvents [34], and concentrations of solutions.

The sequence of amino acids influences the structural properties of fibroin [17,24,35]. In silk II, alanine and serine affect the rigidity of β-sheets; alanine provides stability to the sheets, whereas serine is responsible for hydrophobicity [36]. A recent study of the differences in the structural characteristics and properties of cocoons produced by varieties of *B. mori* concluded that the molecular weight of regenerated SF, viscosity in solution, and mechanical properties depended on the variety of the silkworm [37]; however, months later, Jang et al. [38] reported that the differences observed in mechanical properties could be attributed to the techniques used to measure them, such as wet spinnability [38].

Although the structural characteristics and polymer properties depend on the specific variety of silkworm, the silkworm variety is rarely considered in biomedical applications. Despite the diversity in the composition of these silks, these biomaterials tend to have similar functionality.

SF; Suitable features for biomedical applications

The use of biomaterials for scaffolding purposes is a fundamental component of TE efforts to replace injured tissue with functional reconstructed tissue. Ideal scaffolding biomaterials

should support cellular adhesion and migration; promote cell-cell interaction, proliferation, and differentiation; be biocompatible; exhibit controlled biodegradation; match the growth rate of the newly formed tissue; and be processed such that it supports structural and morphological modifications suitable for the needs of the native tissue [39]. Numerous studies have demonstrated that SF is according to these requirements, moreover, SF possesses key features for OC tissue: mechanical elasticity and resistance.

Biocompatibility

In 1993, the Food and Drug Administration (FDA) recognized SF as a biomaterial due to its prolific use in sutures. Two years later, SF was studied for the first time to test its adhesive and cellular growth capabilities by cultivating fibroblasts in matrices formed from the SF of *B. mori* [40]. Scaffolds fabricated from SF generally lack sericin; it tends to be discarded in biomedical uses because it may produce an allergic reaction [41,42]; however, recent studies have demonstrated it to be safe for biomedical uses [18,43], illustrating that it better promotes cellular adhesion than fibroin, among other superior qualities [44]. Additionally, Liu et al [45]. show that immunogenicity does not significantly differ between SFs and sericin.

SF has been used both in raw form and in combination with other biomaterials to optimize the characteristics of each combination and create more efficient scaffoldings for biomedical uses. One such combination consists of SF and chitosan; this combination is biocompatible and permits the growth and migration of mesenchymal stem cells (MSC) on a 3D scaffold [7,46]. A later study by Vishwanath et al. [47] tested different proportions of SF and chitosan, concluding that a respective 80:20 ratio provided the best conditions for cartilage regeneration using MSCs derived from umbilical cord blood. Samal et al. [48] also tested this combination of biomaterials, using ultrasonics to eliminate the use of organic solvents or chemical crosslinking during the production of 3D SF hydrogels. This combination has also been shown to support chondrogenic phenotypes in 3D scaffolds [49]. Tamada et al. [50] used 3D SF scaffolds that were also biocompatible for the growth of MC3T3 cells. One unique characteristic of the SF molecule is the presence of 2 different active sequences, VITTDSDGNE and NINDFDED, both of which are recognized by an integrin. Combined with the ability to promote cellular growth, this characteristic endows SF with the capability of biorecognition [51].

MG-63 [52] cells, mesenchymal stem cells derived from bone marrow (BMSC) [53-56], mesenchymal stem cells derived from adipose tissue (ADSC) [57], chondrocytes [58-60] (including immortalised chondrocytes) [61], nucleus pulposus cells [62], and fibroblasts [63] have also been cultivated in SF-based scaffolds.

Another intriguing combination was studied by Jaipaew et al. [64], who combined SF with hyaluronic acid (HA) and obtained elastic scaffolds; the cells grown on these scaffolds showed the ability to express chondrogenic markers. Li et al. [65] obtained similar results, producing nanofibre scaffolds by electrospinning SF with poly(L-lactic-acid). Other materials that have been used in combination with SF to create scaffolds include agarose [66], type II collagen (col II) [67], type I collagen (col I) [68], and hydroxyapatite (Hap) [69]. The biocompatibility of SF has also been demonstrated in applications associated with the repair of bone [70-77], meniscus [78], skin [79], and tendon defects [3].

Structural designsof SF-based biomaterials

SF is easy to chemically modify and can later be processed into various forms, such as fibres, gels, films, microspheres, tubes, and sponges (aqueous or with organic solvents) [19,80]. Moreover, 3D biomaterials should provide appropriate mechanical properties, such as a porosity, size, orientation, and interconnectivity between pores specific to tissue type; the ability to culture cells on these biomaterials is equally important.

Despite the existence of different techniques for processing SF, the methods most utilized for developing 3D scaffolds to repair cartilage and bone are HFIP-sponges (1,1,1,3,3,3-hexafluoro-2-propanol; HFIP), aqueous sponges, and electrospinning-derived fibres. Electrospinning can produce small-diameter, large-surface area fibres, which yield a nonwoven isotropic mat of silk fibres. SF sponges possess porous 3D scaffolds; aqueous sponges exhibit excellent interconnectivity between pores and can incorporate bioactive molecules, whereas HFIP-sponges have smooth pore surfaces and greater mechanical resistance, the latter of which can be increased by adding reinforcing agents [80].

One valuable advantage of SF in creating 3D scaffolds is pore size modulation. Kim et al. [81] developed 3D scaffolds derived from aqueous fibroin using NaCl particles of different size for pore formation and observed that salt particles between 470 and 940μm yielded highly homogenous and interconnected pores. Similar results were obtained by Wang et al. [82] for 3D fibroin scaffolds using 500-1000μm salt particles; subsequent to the biological characterization of the scaffolding, they observed high expression levels of chondrogenic genes as well as abundant cartilaginous extracellular matrix (ECM) production. Conversely, Han et al. [83] suggest that the use of 90-180μm salt particles affords a better environment for chondrocyte adhesion and proliferation, yielding an ECM rich in glycosaminoglycans (GAGs) and type II collagen.

In addition to NaCl, other types of particles have been used to promote porogenesis. For example, Moon et al. [84] used a mix of NaCl and sucrose as a porogen to generate 3D scaffolds with different characteristics and suggested that these scaffolds were applicable for TE. Makaya et al. [85] compared SF-based scaffolds derived from combinations of NaCl/water and sucrose/HFIP, using particles that varied between 300 and 500μm in size. The NaCl/water combination yielded an average pore size of 108.2μm and produced much more resistance in compression tests than sucrose/HFIP, which resulted in an average pore size was 329.8μm. Importantly, this study could not definitively attribute the results to either the porogen or the solvent used. In another study, Nazarov et al. [86] used ammonium bicarbonate particles as porogens and gas foaming to produce 3D scaffolds, noting high resistance to compressive forces and interconnected pores greater than 100μm in diameter.

One of the challenges in creating OC scaffolds is in the union of 2 different biomaterials. To solve this problem, Foss et al. [87] recommend the use of genipin as a natural crosslinker for forming HA and SF sponges because these materials remained joined, unlike other spongers that were not created using this crosslinker. Moreover, Zhou et al. [88] attribute to genipin higher mechanical properties when is used as a crosslinker in silk fibroin-chondroitinsulfate scaffolds, due to it provides positively charged amino groups that could adsorb chondroitin sulfate through electrostatic interaction.

Degradation rate

According to the definitions of the United States Pharmacopeia, SF-based sutures are not considered degradable because the suture maintains 50% of its tension *in vivo* after 60 days. However, the literature demonstrates that SF is generally degradable over prolonged periods *in vivo* despite the existence of multiple forms and processing techniques [41,89,90]. Essentially, SF has been shown to induce a mild inflammatory response *in vivo*. Specifically, activating macrophages detect SF as a foreign body, which promotes the formation of multinucleated giant cells [16]. The immune response depends on the structure, implantation site, and fabrication method of the SF materials.

SF degradation can be affected by several factors, including the fabrication method, and can last from hours to years. For example, *in vivo* aqueous SF scaffolds degrade quickly (2-6 months) because their hydrophilicity make them more susceptible to enzymatic degradation, whereas scaffolds derived from HFIP degrade much more slowly (1 year) due to their hydrophobic nature [91].

As mentioned previously, biomaterials should degrade at rates like the formation of new tissue to restore physiological function. This requirement necessitates a fundamental understanding of the interactions between physiological factors, such as the routine mechanical loads that each tissue is subject to under physiological conditions, and the effect that these factors have on biomaterial degradation. Kluge et al. [92] developed an experimental mathematical model to characterize the degradation of silk fibres produced after enzymatic treatment (α-chymotrypsin or protease XVI) combined with cyclic loading and unloading to elucidate degradation and develop effective, future clinical applications [92]. Summarizing the above observations, SF was initially demonstrated to be a biomaterial with adequate characteristics for use in biomedical applications, and the selection of the system of dilution used was shown to be critical for the degradation of SF.

Scaffolds based on fibroin for the repair of OCDs

Articular cartilage is a type of connective tissue [93] responsible for distributing loads over the entire joint surface [94]. However, its natural lack of vasculature and the low mobility of its component cells, i.e., chondrocytes, confer limited self-repair capabilities [95], and severe trauma to joints not only produces lesions in the cartilage but may also extend damage to the subchondral bone, resulting in osteochondral defects (OCDs) [96]. These characteristics make cartilage an ideal candidate for cartilage tissue engineering (TE).

Although initial attempts at cartilage reconstruction focused on regenerating superficial layers [97,98], without taking into account subchondral tissue, recent studies have explored the biomechanical structure and properties of osteochondral (OC) tissue to develop biomimetic scaffolds that can imitate the patterns of natural structures in tissue and improve integration and regeneration [99,57,100].

An OCD compromises articular cartilage, subchondral bone, and interphase tissue to affect joint mechanics and create degenerative changes [101]. The creation of scaffolds that can restore normal function to an OCD is complex due to differences in the composition and structure of each segment as well as the mechanical and biochemical needs that must be considered at each phase. Taking into account these requirements, OC scaffolds should generate biomimetic structures that can incorporate bioactive materials that significantly influence osteogenesis and/or chondrogenesis and obtain mechanical and biochemical characteristics similar to those of tissue.

Strategies for constructing OC scaffolds have improved over the years, and Li et al. [102] classify these strategies into the following categories: monophasic scaffolds, osseous phase scaffolds with cells in the chondral phase, an ensemble of scaffolds with both osseous and chondral phases pre-cultured separately, homogenous scaffolds with different cell populations at each phase, homogeneous scaffolds with a continuous gradient of bioactive molecules for one or both phases, and individual scaffolds with chondral and osseous phases integrated during their fabrication.

Three-dimensional porous SF-based scaffolds have been proposed to emulate the collagen fibril network of normal cartilage ECM, where, the porosity in the phases of the scaffolds is critical to achieve efficient OC regeneration. In general, small pores in the chondral phase are desired to induce hypoxia and favour chondrogenesis [103,104], whereas larger pores are more desirable in the osseous phase [105,106] to promote angiogenesis; this different structures simulates physiological conditions, however there are recent designs that propose opposite [107,108]. Furthermore, chondrogenic or osteogenic differentiation is influenced not only by the size of the pore but also by the assembly strategy, the starting cell type, and the culture conditions (Table 1).

Table 1 summarizes the 3D, SF-based scaffolds developed for OC TE applications. Notably, new studies are on-target to develop co-culturing systems that better emulate the native characteristics of each phase of OC tissue. These differences, i.e., modelling the regenerative niche in stratified tissue, such as OC tissue, represent the greatest challenge in the development of biomaterials. The development of co-culture systems seeks to take advantage of heterotopic cellular communication, that is, the capacity of cells from different lines to mutually influence their functions and, ultimately, simulate the native niche [109]. Despite the complexity represented in the development of *in vitro* co-cultures, they are expected to be the most promising clinical option for treating OC lesions.

Table 1:

	Chondral					Osseous					
Reference	# Phases	Material	Pore size/ Pore formation technique	% Porosity	Cellular source	Material	Pore size/ Pore formation technique	% Porosity	Cellular source	Culture conditions	Assembly
[113]‡	2	SF	300-425μm /Particulate leaching (NaCl)	95.50%	BMSC	SF	300-425μm / Particulate leaching (NaCl)	95.50%	BMSC	Pre-culture separately in each phase of the scaffold with control medium (static culture) and later change to CM or OM (dynamic culture). Co-c in bioreactors after the assembly of both phases with OM, CM or control medium.	Suture
Chen [119]‡	2	SF	150μm / Freeze-lyophilization	NA	BMSC	SF	150μm / Freeze-lyophilization	NA	Osteoblast	Pre-culture separately in each phase of the scaffold with respective CM or OM. Co-c after assembly.	RADA peptide
Saha [96]‡Ý	3	SF Mulberry or no Mulberry + TGF-β3	Mulberry 72μm; No-Mulberry 74μm / Freeze-lyophilization	74-82%	In vivo: BMSC; In vitro: NC	SF mulberry o no mulberry + BMP2	Mulberry 72μm; No-Mulberry 74μm / Freeze-lyophilization	74-82%	In vivo: BMSC In vitro: NC	In vitro: dynamic cultures on scaffolds without separated induction media, later with CM or OM In vivo: NC	Fibrin glue only prior to implantation in vivo
Chen [120]‡	2	SF	150μm /lyophilization	NA	BMSC	SF	150μm / Lyophilization to form pores	NA	BMSC	Pre-culture separately on the scaffold with respective CM or OM. Co-c after assembly	RADA Peptide, self-assembly
Yan [114]‡Ý	2	SF	300-700μm / Particulate leaching (NaCl)	82.02%	BMSC only for characterization in vitro	Silk-nano-CaP	300-700μm / Particulate leaching (NaCl)	62.27%	BMSC only for characterization in vitro	In vivo: NC for implants in OCD lesions	Lyophilization

Ding 57‡	3	SF	112.4µm / Particulate leaching (NaCl)	85.30%	ADSC	SF/Hap	362.2µm / Particulate leaching (NaCl)	90.25%	ADSC	Pre-culture separately in monolayer with CM or OM and subsequent culture separately in each phase of the scaffold with CM or OM.	TIPS Technique
Ghezzi [117]‡	3	HybridCol I-SF-Col I	NA	NA	BMSC	Hybrid Col I-SF-Col I	NA	NA	BMSC	Cells were seeded in the hybrid and each phase separately with non-differentiation medium, CM and OM for each	PC Technique
Li [102]‡	2	SF	100-120µm / Freezingat -20º C	NA	BMSC	SHG-slk	400-500µm / PSM	NA	BMSC	Cells were grown in both phases of the scaffold and cultured as biphasic or separate phases with MC and OM	lyophilization
Chen [112]‡	2	SF	NA / Freeze-lyophilization	NA	BMSC	SF	NA / Freeze-lyophilization	NA	BMSC	Cultivation in chambers for co-cultivation with CM or OM medium for each compartment	RADA Peptide, self-assembly
Çakmak [115]‡	3	Hidrogel-PA-RGDS	NA	NA	Chondrocytes	Silk-Hap	240-585µm / Particulate leaching (NaCl)	90%	BMSC	BMSC were grown on the silk scaffold with OM and chondrocytes were cultured in hydrogels-PA-RGDS with CM, separately. Subsequent assembly and Co-c in osteochondral medium	Through an acellular SF interface, the MEC secreted by the cells in each phase helped to self-adhesion

It is notable that new research is aimed at developing co-culture systems that allow better emulation of the native characteristics in each phase of the osteochondral tissue. Modeling the "regenerative niche" in tissue stratified as the OC tissue represents the greatest challenge in the development of biomaterials. The application of co-culture systems is based on heterotypic cellular communication, this ability of cells of different strains, to mutually influence their functions and simulate the native niche [109]. Thus, Ribeiro et al. [110] in their recent study investigated cell behavior in the complete osteochondral grafts through a chondrocytes and osteoblasts co-culture system, even though the established co-culture system showed the possibility of maintaining for long-term the co-culture of each cell line, the results indicated that the proposed co-culture model may have the potential to induce chondrocytes pre-hypertrophy, so the use of chambers is suggested.

As mentioned, most SF-based osteochondral scaffold designs are initially cultured in differentiation inducing media separately for each phase, in contrast, some designs use techniques involving compression [111], lyophilization [102] or temperature gradient [57] to interleave the phases before performing the initial culture. This improves the integration, however, despite having an integrated scaffold, the ability to support differentiation (chondrogenic and osteogenic) is still evaluated in the separated phases instead the interlaced. Recently Chen et al. [112] developed chambers for co-culture, thus allowing the coordinated differentiation of the chondrogenic and osteogenic phenotype. Equally important is the recent research by Liu et al. [113] which achieve isolate the cartilage phase from bone phase developing a layer that mimics the osteochondral tissue calcified layer, being permeable to some molecules with limited molecular weight and able to prevent the

seeded cells from migrating cross the unit when being grown in chambers.

It has been observed that the use of bioactive molecules improves osteochondral differentiation. Saha et al. [96] added TGF-β3 in the chondral phase and BMP2 in the bone phase of FS-based osteochondarl scaffolds, these bioactive molecules induce chondrogenic and osteogenic differentiation, respectively, even when implanted cell-free in femoral Wistar rats. Their results further confirm that depending on the variety of silkworm used to influence the efficiency to route the cells to the chondrogenic or osteogenic phenotype both *in vivo* and *in vitro*. In addition, osteoconductors have been used, such as calcium phosphate [114], Hap [57,115] and strontium-hardystonite-gahnite [102], incorporated into the bone phase of osteochondral scaffolds resulting in better osteogenesis and superior mechanical properties. For example, Ruan et al. [108] achieved high levels of collagen I gene expression incorporating Hap in the phase that mimics bone although the scaffold was cultured in non-osteogenic medium.

Ideally for rapid clinical application would be that the scaffold, alone, without cellular additives or bioactive molecules would be able to influence the chondrogenic and osteogenic process of the OC tissue to restore the native physiological functions [116]. A small number of FS-based OC scaffolds are evaluated by *in vivo* models [96,114], although the results appear to be promising, future research should focus on models where load and joint design resemble the human (horse, pig, sheep) [117-120].

Conclusion and Future Directions

SF as biomaterial is promising for the construction of osteochondral scaffolds due to its good biocompatibility, versatile processing, and varied sterilization options and to support chondrogenic and osteogenic differentiation. Satisfying the physical, biological and mechanical requirements of OC tissue is complicated due to its stratified nature but approaches in the field of tissue engineering and co-culture have achieved promising biomimetic approaches for a possible short-term clinical application.

We consider that the next big step that SF-based chondral and OC scaffolds must give is their application by *in vivo* models; especially in models that represent a challenge for its design and for the potential for chondral and osteochondral regeneration, a characteristic that has been attributed to it throughout its *in vitro* evaluations and that, unlike other biomaterials, *in vivo* evaluations are scarce. Additionally, most of the models used rarely simulate the native design and mechanical needs of the human joint. In order to accelerate the transfer of the multi-phases grafts to the medical area, clinical practice requirements must be taken into account; the direction to achieve this requires greater efforts in a simplified manufacturing, reproducibility of the technique, storage conditions and sterility methods.

The development of scaffolds and physical, biological and mechanical characterization is a multidisciplinary work, so it is essential that ongoing advances in every branch converge to achieve far-reaching results clinically to restore normal functions of osteochondral tissue.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

All authors were involved in drafting the paper, and all authors approved the final version to be published.

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