REVIEW ARTICLE

Silk Hydrogel for Tissue Engineering: A Review

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Abstract

Aim: This review aims to explore the importance of silk hydrogel and its potential in tissue engineering (TE).

Background: Tissue engineering is a procedure that incorporates cells into the scaffold materials with suitable growth factors to regenerate injured tissue. For tissue formation in TE, the scaffold material plays a key role. Different forms of silk fibroin (SF), such as films, mats, hydrogels, and sponges, can be easily manufactured when SF is disintegrated into an aqueous solution. High precision procedures such as micropatterning and bioprinting of SF-based scaffolds have been used for enhanced fabrication.

Review results: In this narrative review, SF physicochemical and mechanical properties have been presented. We have also discussed SF fabrication techniques like electrospinning, spin coating, freeze-drying, and physiochemical cross-linking. The application of SF-based scaffolds for skeletal, tissue, joint, muscle, epidermal, tissue repair, and tympanic membrane regeneration has also been addressed.

Conclusion: SF has excellent mechanical properties, tunability, biodegradability, biocompatibility, and bioresorbability.

Clinical significance: Silk hydrogels are an ideal scaffold matrix material that will significantly impact tissue engineering applications, given the rapid scientific advancements in this field.

Keywords: Biomaterial, Scaffolds, Silk fibroin, Tissue engineering.

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Introduction

Some of the most critical health issues, such as damaged and degenerated tissue and impaired organs, have been challenging to treat in modern medicine. For example, the muscular-skeletal tissue such as bone, tendon, cartilage, and peripheral nervous system can be damaged easily by injury and degenerative diseases like osteoarthritis. Millions of people worldwide will be affected severely which will affect their quality of life, and there will be much burden on the healthcare system across the world. The most crucial challenge in the modern healthcare system is to provide a solution to the damaged/degenerated tissue.[1,2] Commonly used clinical techniques are autografts and allografts that restore the injured tissue are restricted due to inadequate tissue that may be taken away from the healthy parts of the patient's body and lack of appropriate donors.[3] The success rate of allografts is relatively low as the tissue may have an immune reaction. For instance, it is difficult to get an appropriate material suitable for highly damaged and defected area in suitable time, resulting in low success rates.[3–5] Due to these reasons, TE has drawn more importance as an alternative method to develop tissues that are related to the patient and to reproduce impaired tissues or organs. TE integrates various principles and techniques to reproduce impaired tissues by replacing, supporting, or enhancing tissue functions. Additionally, the use of biocompatible scaffolds that TE depends on is included with cells and protective moieties like growth factors.[6,7] While designing a scaffold, some key factors, such as biocompatibility, biodegradability, mechanical properties, and structure, should be considered, regardless of reproduced tissue types.[7,8] Extracellular matrix (ECM) is a scaffold material created by tissues and organs, and it is a good choice in TE since it preserves "dynamic reciprocity" with native cells,[9] collagen,[10] fibronectin,[11] laminin,[12] elastin,[13] and glycosaminoglycans[14] are ECM components that were utilized as a

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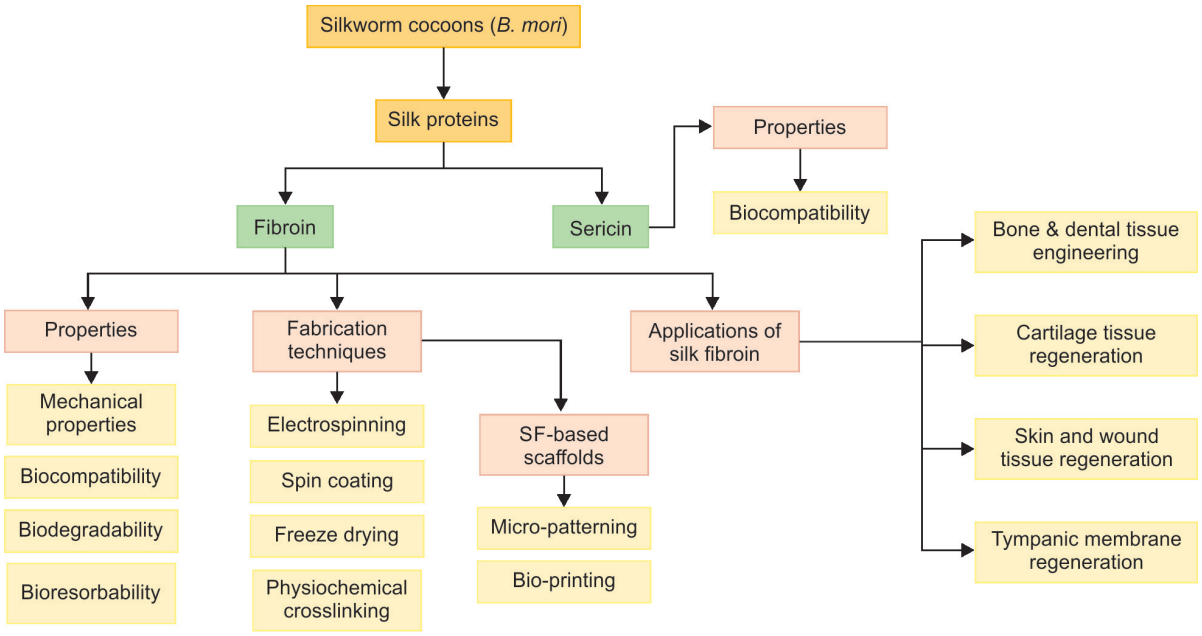
Flowchart 1: Properties, preparation, and potential biomedical applications of silk hydrogel



native scaffold material to help in tissue repair. Alginate,[15] cellulose,[16] and chitosan[17] are other natural polymers that are observed to be used in TE applications. Even though the natural polymers have been observed to give favorable results, they are some disadvantages, such as high cost, lack of mechanical properties, and different from one another, which make it difficult to use in clinical applications.[18] In addition, synthesized polymers that include polylactic acid (PLA), polyurethane (PU), poly(lactide-co-glycolide) (PLGA), and polycaprolactones (PCL) are extensively utilized since synthesized polymers had better mechanical properties and decomposition rates in TE.[19] A large percentage of these polymers' byproducts are acidic chemicals, which can be dangerous and cause undesired immunological reactions. Due to the limitations in the existing polymeric scaffolds, for the past 10 years, researchers have aimed at synthesizing biomaterials which are natural and synthesized polymeric materials.[20] According to recent research, it is observed that silkworm silk is an outstanding biopolymer for TE scaffolds. Silkworm silk was first commercialized in the manufacturing industry about 4000 years ago, due to its physical qualities of brightness, lightness, elasticity, and hardness.[21] Furthermore, Food and Drug Administration (FDA) has recognized silk which can be used in sutures and in biomedical fields for last 20 years.[22,23] Silk fibroin is a synthetic protein derived from silkworm silk. Because of its physiochemical features, including as strong biocompatibility, biodegradability, bioresorbability, minimal immunogenicity, and tuneable mechanical qualities, it is utilized as a promising biopolymer for TE.[24–27] SF in combination of other polymers can produce SF-based composite scaffolds which may improve cellular actions such as differentiation, growth, and adhesion.[28–30] Films,[31] hydrogels,[32] sponges,[33] 3D structures,[34] and nanoparticles[35] can all be made using SF-based biomaterials. The resources, material characteristics, manufacturing methods, and uses of silk scaffolds are covered in this paper, focusing on the bone, cartilage, ligament, tendon, skin, and wound tissue formation.

There is a need to identify an ideal biomaterial suiting varied TE applications. One such versatile biomaterial is silk fibroin. The unique feature of silk includes its tunability into different morphological forms, economical manufacturing process, superior mechanical properties, biocompatibility, biodegradability, and bioresorbability. Hence there is a great need for silk fibroin to be used in tissue engineering (Flowchart 1). This review delves into each of these aspects.

Origin of Silk Fibroin

Silk proteins are produced in the glands after synthesis in the epithelial cells. It is estimated that more than 20,000 various silk-producing arthropods live in the natural world.[35] Caterpillars, spider, lace wings, glow worms, and bugs are just a few of the scientific silk-producing families that can twirl into the fibers throughout their metamorphosis.[36,37] Furthermore, recently identified that Psychidae family (bagworm moth) can produce a solid form of moth silk according to Yoshioka et al.[38] For biological applications, silk from silkworms and spiders is used more frequently.[39–41] Once the spider silk is spun and when it comes in contact with air that hardens and limits producing spider silk in large amounts. When compared to a spider's ampullate gland, one silkworm cocoon yields around ten times more threads.[36,42] Even though researchers have utilized a biomimetic spinning process to mimic spider silks, developing spider silk-like fibers with mechanical characteristics that are comparable to native spider silk fibers is difficult.[43] Furthermore, by using bacterial shake-flask culture, chimeric recombinant spider silk was made to manufacture more artificial spider silks, according to Andersson et al.[44] The native spider silk mechanical properties are reproducible and have the highest tensile strength and toughness, when compared with natural spider silk threads. Silkworms in the families Bombycidae and Saturniidae feed on mulberry trees or other sources of food, with some being categorized as nonmulberry (Saturniidae) silks. *Bombyx mori* (*B. mori*) is a silkworm that feeds on mulberry and produces higher quality fibers than Saturniidae silkworms.[45,46] Furthermore, over the last 5000 years, unlike different silk moths, *B. mori* was widely raised all

over the world to acquire its silk and was cultivated from an inherited species in China.[47] Silkworm (*B. mori*) comprises SF (75–83.3%) and sericin (16.7–25%).[48] Sericin is a gumming agent that is made up of amorphous protein-polymers. SF is a load-bearing protein with a semicrystalline structure.[49] When compared to sericin-encased fibroin, sericin-free fibroin fibers have been proven to have good mechanical characteristics. Tensile strength was raised by 50%, the modulus was increased to 15–17 GPa, and strain at breakage was about 19%.[50] *In vivo* and *in vitro* the sericin-free fibroin showed good biocompatibility, according to the earlier reports.[51] Additionally, it is observed that sericin can cause inflammation.[52] Hence, to ensure the biocompatibility of SF in TE, sericin proteins are eliminated through the degumming process, and sericin is separated from SF fibers. The degumming technique is done under boiling alkaline conditions.[53] Researchers require reagents and organic solvents to improve the degumming process and achieve pure SF quality. Sodium carbonate (Na_2CO_3) degumming is now utilized, although it is being moved out in favor of a more widely used process, which is the traditional Marseilles soap procedure, which is faster (30 minutes) and less expensive.[54,55] The degumming method decreased the overall thickness of silk strands to 10–25 µm.[56]

Silk Fibroin Characteristics

SF Structure

It is made up of two major chains, one heavy (H-)chain (390 kDa) and the other light (L-)chain (26 kDa). The H-L complex is formed when these chains are joined together by disulfide bonds.[39,40,57,58] Glycoprotein P25 (25 kDa) is composed of oligosaccharide chains as n-linked amino acids joined chemically to form an H-L complex.[59] Three polypeptides such as H-chain, L-chain, and P25 which form *B. mori* cocoon and observed to have a molar ratio that is found to be 6:6:1, respectively.[60] In the sequence of amino acids that constitutes the H-chain, glycine (45.9%), alanine (30.3%), serine (5.3%), valine (1.8%), and other 15 amino acids are present. About 60–70% of the H-chain is the Gly-X (GX) dipeptide pattern. The hydrophobic residues in the dipeptide repeats can form stable antiparallel–β layer crystals. About 70% of the GX dipeptide sequence is taken up by the two hexa-peptides, therefore, Gly-Ala-Gly-Ala-Gly-Ser and Gly-Ala-Gly-Ala-Gly-Tyr are the peptide sequences.[61–64] The primary crystalline structures of SF are silk I and silk II, with silk I being a metastable crystalline structure containing attached water molecules. Because of hydrogen bonding between neighboring peptide blocks, silk II is the most stable state, leading to improved mechanical qualities such as rigidity and tensile strength.[39,65,66] Regeneration silk fibroin (RSF), the secondary structure, is composed of crystalline and amorphous constituents, as explained here. Insoluble solutions give rise to β-turns (silk I) and β-sheets (silk II), whereas the solid form of silk contains α-helices, bends, and irregular curled frameworks in the crystalline structures.[67] It is observed that silk I may be changed to silk II using potassium chloride or methanol, which is extensively applied for biomaterial engineering.[34] RSF structures generate silk III (irregular crystalline form) at the air-water interface.[68]

Mechanical Characteristics

SF fibers were shown to have excellent mechanical characteristics,[56,69,70] including high breaking stress (4–26%), high stiffness (300–740 MPa), and hardness (70–78 mJ m^{-3}).[70] Synthetic fibers such as Kevlar (50 mJ m^3) and carbon fiber (25 mJ m^{-3}) and collagens such as tendon and collagen (7.5 mJ m^{-3}) have higher toughness than SF fibers.[43,71] Wool, resilin, elastin, byssus, cotton, synthetic rubber, and viscose rayon have all been examples of SF fibers with ideal properties of any natural or synthetic fibers.[43] Considering that SF has strong mechanical properties, SF as a scaffold material has been used by the researchers for applications of TE for load-bearing such as musculoskeletal TE. SF Scaffolds are weak and delicate in biomaterial engineering and are manufactured from RSF solutions. This is because RSF fibers have less hierarchical and secondary patterns than untreated raw SF fibers.[72] Various approaches have been tried to ensure that RSF has good mechanical properties. For instance, it was observed that the breaking stress of RSF fibers was 252 MPa, by dry-spinning method, which was 28.6% lower than raw SF fibers (353 MPa). However, in the RSF and graphene oxide composite silk fibers, the breaking stress was shown to have 435 MPa.[24] From other cross-linking[32] porogens[73] and 3D bioprinting,[34] advancement can be utilized to enhance mechanical properties of silk scaffolds produced from RSF. Hence, the produced SF-based scaffolds are strong enough to handle during implantation processes and have mechanical characteristics that are similar to the native tissue having been repaired, allowing for favorable repair conditions in the affected area.

Physical Properties of SF

Molecular Weight and Its Impact

The molecular complex of silk fibroin consists of the 350 kDa fibroin heavy chain (H-chain) and two lower molecular mass protein components: the 26 kDa fibroin light chain (L-chain) and the 30 kDa glycoprotein P25.[60] It has been found that the molecular weight of a polymer impacts its mechanical characteristics, biodegradability, and drug delivery area.[74] Natural silks show high molecular weights, and by processing silk, lower molecular weights can be acquired. For example, a frequently used method to remove sericin from fibroin is boiling of silk cocoons for longer time in sodium carbonate solutions that was observed to produce high hydrolytic degradation of SF protein.[75] The use of such replicas like these in load-bearing body areas needs to be investigated further. SF processing can result in variable molecular weights, which can affect bulk viscosity and furthermore drug delivery methods, bulk viscosity, and degradation proportions.[76,77]

Crystallinity and Water Insolubility

Due to their ability to produce beta-sheets, the hydrophobic components of SF constitute the crystalline components of SF. The most common way to enhance SF in beta-sheet structure and hence inducing water insolubility is to treat it with methanol.[78–81] High temperature,[82] a pH near to the isoelectric point, the utilization of salts,[83–86] and shear force are all the factors.[87,88] The stability of SF is based on its crystallinity. Excessive crystallinity decreases flexibility and makes materials increasingly brittle. When compared to methanol treatments, annealing in water or water vapor induces fewer beta-sheets and retains superior flexibility.[78–93]

Solubility

It is observed that most of the solvents used to dissolve polymers are often utilized in drug delivery applications. The salt solutions with high concentrations that are commonly used to dissolve SF are lithium bromide, lithium thiocyanate, calcium thiocyanate, and calcium chloride.[94] These alkaline solutions can destroy the hydrogen bonds that maintain beta-sheets.[95] For instance, early

re-precipitation of aqueous SF solutions into water-insoluble beta-sheets enriched silk can still be processed.

Stability

For the preservation of drug delivery, polymer stability is essential. The enrichment in beta-sheet content during storage causes SF solutions to aggregate and gel. Untreated SF matrices have a low proportion of beta-sheets, are hygroscopic, and so are particularly susceptible to moisture. Untreated SF matrices have a low beta-sheet content, are hygroscopic, and so are extremely sensitive to humidity. SF has excellent thermal stability and is unaffected by temperatures as high as 140°C. The Tg of dry SF films generated from silk obtained from the posterior section of the middle division of the silk gland of the silkworm *B. mori* was around 175°C, above which there is a free molecular movement to transition into the stable β-sheet conformation, exhibiting stability up to around 250°C.[96]

Swelling Properties

The degree of swelling, which is determined by the ionization of the network, degree of cross-linking, and hydrophilic/hydrophobic balance, influences the drug release from matrices such as hydrogels.[97] Swelling levels can be affected by changes in polymer composition.

Mechanical Properties

Numerous polymer-based scaffolds, such as PLGA and collagen, need sufficient mechanical strength for load-bearing applications.[98,99] However, increased mechanical strength becomes essential when a drug delivery system is simultaneously utilized as a load-bearing scaffold, as is typically required in bone healing. Water-stable aqueous-derived SF scaffolds performed porous biodegradable polymeric scaffolds commonly used in bone-related tissue engineering experiments (e.g., collagen, chitosan, and hyaluronan).[100]

Biocompatibility

Biocompatibility is an essential feature in developing successful scaffolds because it allows cells to stick to scaffold surfaces and move into the scaffold, where they can proliferate and differentiate. Furthermore, following implantation, the scaffold may cause no or only a mild immune response.[6] SF is a biocompatible natural polymer that is physiologically inactive.[26] In the year 1989, it has been observed that *in-vivo* experiments SF showed blood compatibility.[101] Food and Drug Administration recognized SF as a biodegradable polymer that is used as a suture material in 1993.[26] In 1995, Minoura et al. have shown that coated SF films effectively grow fibroblasts.[102] Recently, the application of SF films as a collagen alternative in cell culture has helped bone formation in rat calvarias abnormalities, with evidence demonstrating that SF films might modify collagen membranes.[103] *In vitro*, macrophages demonstrated no immune response to the SF films.[104] and fibers.[23] Furthermore, the SF films elicited a collagen-like inflammatory response *in vivo*.[105]

Biodegradability and Bioresorbability

The unique characteristic features of scaffold material are biodegradability and bioresorbability. These scaffolds should be eventually restored by with cells and ECM of patients' in the process of recovery.[106] As a result when biodegradation products are processed in the body, they are not hazardous and do not hinder tissues, organs, or functions. Because SF is a chemically degradable polymer, it should not trigger an immune response.[107] The proteins are adsorbed onto the SF scaffold surface through surface-bonding areas when the degradation process starts. Then the proteins absorb the SF scaffold through hydrolysis of ester bonds.[19,107–109] Noncrystalline SF structures were destroyed in an enzyme solution to produce hydrophobic crystal structures, which were then dissolved in enzyme solutions. The α-chymotrypsin, protease XIV, and collagenase IA are the enzymes that can break down the SF.[19,107,110] It was revealed that protease XIV in *Streptomyces griseus* had a greater degradation effect than α-chymotrypsin and collagenase IA. Hence, when protease XIV degraded SF resulted in smallest molecular weight of SF remnants[111] due to which it is a frequently used protein for silk degradation. The degradation process during the preparation procedures of SF may result in various morphologies of SF, which is disintegrated in the proteins.[109] *In vivo*, amino acids and peptides are the degradation products of SF that are simply absorbed.[107] *In-vivo* experiments involving SF porous scaffolds when placed into Lewis rats revealed that the scaffolds degrades after 8 weeks. Due to macrophage degradation, the administered scaffolds are completely eliminated after 1 year.[112] This indicates the biodegradability and bioresorbability of the SF scaffold. The degradation of native silk fibers was found to be less than that of RSF silk scaffolds. Because the RSF structures have more β-sheet complex over natural silk fibers,[113] the rate of SF degradation is dependent on the number of β-sheet complex structures formed. RSF films, for instance, are made by converting water-soluble silk I structures to water-insoluble silk II structures, which results in a greater content of β-sheet structures after treatment with methanol. The RSF films generated using the delayed air-drying method, on the other hand, had a lower number of β-sheet structures,[114] resulting in faster degradation rates. It was shown that γ-radiation enhances the degradation of SF fibers this is because of changing of silk II to silk I.[115]

Hydrogels: Polyester
Gel formation
Biopolymers

Silk Fibroin Application in Tissue Engineering

Development of Bone Tissue

Bone is a layer of connective tissue comprising 35% organic material and 60% inorganic grid. The remaining 10% of the ECM of organic origin in bone is made up of hyaluronan, proteoglycans, bone sialoprotein, osteopontin, osteonectin, and osteocalcin.[116,117] At the stage of bone production, hydroxyapatite (HA) makes up the majority of the inorganic minerals, with inorganic sodium and carbonate contributing to the remaining.[118] Hence, the major components are collagen and HA of the bone tissue that improves the strength and hierarchical structure of bone.[119] Scaffold materials for bone tissue development are designed to provide matrix strength while also allowing for ECM accumulation. The enhanced hardness, mechanical characteristics, and biocompatibility of SF have been observed and investigated in bone tissue engineering.[120] Using RSF scaffolds, human mesenchymal stem cells have been observed to grow into osteoblasts *in vitro* (HMSC). Furthermore, *in vivo*, conducted in rat experiments, these modifications have been reported to heal femoral deficiencies.[121] Meinel et al.[122] demonstrated that following a 5-week incubation period in bioreactors, *in vivo*, SF-based scaffolds may be inserted into rat cranial defects and accelerated bone formation in 5 weeks.

RSF scaffolds with added biomaterials like collagen or calcium phosphate-based inorganic substances are used to increase osteogenic characteristics.[28,123] The HA-RSF porous scaffold, for instance, were manufactured by immersing it in $CaCl_2$ and Na_2HPO_4 on a regular basis, or by combining NaCl particles and HA and further linking it to RSF solutions.[124,125] It was observed that these composites had higher osteoconductivity and tissue-engineered bone development than unmodified RSF scaffolds. The FDA has recognized growth factors including bone morphogenetic protein BMP-2 and BMP-7, which can aid in bone development and regeneration.[126] The RSF with these growth factors and HMSCs showed increased adhesion of osteoblast and improved formation of bone *in vivo*.[127,128] Li et al.[129] further showed how BMP-2 and HA nanoparticles might be added to electrospun RSF mats to aid in HSMC differentiation and growth. This showed that there was an increased deposition of calcium than RSF mats. Collagen and BMP, both of which are osteoinductive and osteoconductive, were also present in demineralized bone matrix (DBM) granules or particles. RSF as a carrier, according to Ding et al.,[130] aids in the building of DBM. Furthermore, the RSF carrier aids in the development of strong porous materials and has been demonstrated to stimulate osteogenesis in BMSC-treated rats. Additionally, for more bone regeneration, fast and rigorous vascularization is required. For example, *in vitro* RSF matrices before incubation with osteoblasts and *in vivo* when inserted into mice revealed increased vascularisation.[131] Furthermore, the RSF revealed the distribution of vascular and prevascular structures *in vitro* using endothelial cells and osteoblasts.[132,133] In mice with compromised immune systems, microcapillary structures were embedded but effectively incorporated with host vasculature and triggered host capillaries for vascularization.[134] Furthermore osteoblast differentiation could not be promoted with vascular endothelial growth factor (VEFG) but produced neovascularization.[135] According to Farokhi et al.,[136] RSF calcium phosphate–poly(lactic-co-glycolic acid) scaffolds preserved 83% of their bioactivity after releasing VEGF *in vitro* for 28 days. The development of neo-bone in rabbit deformities following implantation for 10 weeks was studied *in vivo*. After implanting a sonicated silk hydrogel carrier containing BMP-2 and VEGF into rabbit's maxillary sinus wall, Zhang et al.[137] observed improved osteogenesis and angiogenesis.

Cartilage

Regeneration of Cartilage Tissue

Cartilage which is lacking blood vessels, nervous tissue, and containing connective tissue is encompassed by a thick ECM and does not have the inborn capacity to heal by itself after wound degeneration. The main parts of the cartilage ECM are collagen and proteoglycans, which exert mechanical properties for tissues *in vivo*.[138,139] Hence, the vital aspect of tissue engineering is maintaining and preserving this tissue. SF scaffolds may be utilized to increase cartilaginous ECM production,[140] and because of its outstanding properties, the scaffold can be made into various morphologies.[141] Following 3 weeks of incubation, Wang et al.[141] demonstrated that porous RSF scaffolds containing HMSCs can produce zonal patterns comparable to those obtained in native cartilage tissue.[142] It was shown that insulin-like growth factor I (IGF-I) can promote the development of various progenitor cells, which can then be placed into porous RSF scaffolds to promote HMSC chondrogenic differentiation.[143] RSF can be combined with other natural biopolymers to generate biocompatible cartilage structures. Due to glycosaminoglycan residues, chitosan, for instance, may provide adequate support to the chondrocytes.[144] Chitosan can increase chondrocyte cell attachment, growth, and chondrocyte agglomeration, according to Bhardwaj et al.[145] and Silva et al.[146] Another biopolymer such as RSF combined gelatin has been studied. Collagen and gelatin were found to enhance chondrogenic differentiation.[147] When compared to collagen thick mats, RSF-collagen thick mats were produced using electrospinning and implantation, which demonstrated greater chondrogenic growth of MSCs and showed improved results when the cartilaginous matrix was used.[148] This could be attributed to the scaffold's increased strength. Additionally, Wang et al.[30] developed the porous RSF–collagen scaffolds using poly-lactic-co-glycolic acid (PLGA) microspheres, which increased cell adherence and articular cartilage in rabbits. According to Shi et al.,[149] with a 1:2 weight ratio of SF solution (6.9% w/v) to gelatin solution (6.9% w/v), RSF–gelatin scaffolds with satisfactory degradation and mechanical properties for cartilage regeneration. After 21-day *in vitro* incubation period, SFG scaffolds were shown to be capable of differentiating bone marrow stem cells (BMSC) toward chondrogenic cells. After 24 weeks, the SFG scaffolds were found to be effective in repairing affected sections of rabbit cartilage. To promote chondrogenesis, RSF can be combined with various biodegradable polymers including cellulose, hyaluronic acid, agarose, and poly (D,L-lactic acid).[150–153] Argon plasma treatment has been found to improve the mechanical and structural characteristics of RSF-based scaffolds.[154–156] Baek et al.,[154] for example, looked at porous RSF scaffolds and investigated that the porous RSF scaffolds showed remarkable high adherence of chondrocytes and proliferation when treated with microwave-induced argon plasma. Cells implanted on RSF scaffolds and cultured in bioreactors that are physically active under physiological circumstances have been found to improve cartilaginous structures.[157,158] HMSCs are seeded into porous SF scaffolds and incubated for 4 weeks in perfusion bioreactors. For example, glycosaminoglycan, overall collagen, collagen II, and DNA production in cells, and also cartilage-related gene function were increased. It was shown that there was higher mechanical stiffness of activated scaffold when compared to a static culture in the same study.[157] These findings showed that hydrodynamic parameters, cell types,[159] and scaffold designs, such as pore size and distribution, are essential components optimizing cartilage TE structures.[36]

Dental Tissue Engineering

Scaffolds and biomaterials are crucial elements in dental tissue regeneration. These components function as an implantation surface for regenerative cells from nearby tissues, a structure for tissue regeneration, an origin of implanted odontogenic cells are capable of differentiating important cellular forms, and an origin of biologically active compounds, mainly growth factors, that aid in regeneration.[160,161]

Silk-gel material is an excellent biopolymer for gingival and maxillofacial treatments since it may be utilized as a cell scaffold or a biomaterial.[162] The use of a scaffold to distribute cells and/or growth factors to the injured area has been proven necessary for stem cell-based therapy.[163] Depending on the success of application of the scaffolds in bone tissue engineering, the researchers have investigated the use of mineralized dental tissue engineering.[164] Bioactive materials may promote osteogenesis by forming strong chemical interactions by increasing the biological response. They are osteoconductive (hydroxyapatite and beta-tricalcium

phosphate), which promote bone formation along the surface, or osteoproductive (bioactive glasses), which promote bone growth distant from the implant or bone interface.[165] Human dental stem cells were cultivated on fibroin films and showed distinct growth and the maintenance of mesenchymal stem marker expression levels.[163] Tissue engineering aims to emulate the extracellular microenvironment of the cells/tissues, promoting cell attachment, adhesion, proliferation, differentiation, and ECM deposition. The native extracellular microenvironment consists of a myriad of biomolecular factors, including molecular structures that make up ECM, chemokines, cytokines, growth factors, and cell surface glycoproteins. These physical and chemical cues within the extracellular microenvironment can be combined to have a synergistic and hierarchical effect on specific cellular processes that affect cell behavior and function.

Skin and Wound Tissue Regeneration

In the first line of defence against infectious germs, the skin performs a crucial role. The epidermis and dermis, which are made up of keratinocytes and ECM, are the two primary layers of skin. In case of complete loss of skin integrity, for instance, severe burns, this can lead to impairment and even death.[166,167] RSF biomaterials have been demonstrated to alter keratinocyte and fibroblast[168] adhesion and are commonly used in TE skin regeneration. According to Zhang et al.,[169] with porcine models, RSF films may be used in rabbit models with abnormalities which considerably decreased healing time and exhibited better skin regeneration than existing commercial wound dressings. RSF films have also been found in clinical trials to dramatically improve healing time and the risk of adverse effects to commercial wound dressings. Furthermore, antibacterial silver particles (AgNPs) that covered RSF mats could be used to limit the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in antimicrobial wound treatments.[136] The hydrophilicity of RSF nanofibers increased after treatment with O_2 plasma, which has been demonstrated to stimulate human keratinocytes and fibroblasts.[170] Skin tissue is complex by appendages such as hormone glands and hair.[36] For its biocompatibility, biodegradability, and antibacterial properties, chitosan has been widely utilized in skin tissue engineering. It also encourages fibroblasts to produce collagen, improving the tensile properties of the tissue that has been healed in the wounded location. Cai et al.[29] used electrospinning to make RSF-chitosan scaffolds, which showed enhanced mechanical strength and antibacterial activity as RSF concentration increased. In *Escherichia coli*, RSF-chitosan scaffolds have been found to increase cellular proliferation and also antibacterial properties.[29,171] When used as a cross-linker, alginate dialdehyde (ADA) demonstrated higher cell growth, bonding, and decreased toxicity.[172] As a result, crosslinking RSF–chitosan scaffolds with ADA improves water accumulation, water conductivity, and cell activity in injured skin.[173] Using a hydrogen-bonding method, chitosan coating on porous RSF scaffolds was used to create 3D RSF–chitosan scaffolds, which were further transplanted into the wound of a rat and then was examined by Guang et al.[174] It was observed that the wound was entirely healed following 21 days but had no teratogenic or pathogenic effects, according to the findings. In contrast to *Antheraea assama* (*A. assama*) nonmulberry RSF, nonmulberry RSF from *A. assama* has been a potential material for skin TE (*A. assama*). This is due to the presence of the RGD peptide sequence, which facilitates cell adhesion.[175] RSF hydrogels were made *in vitro* by combining SF solutions using *A. assama* and *B. mori*, which encouraged the growth of predominantly human dermal fibroblast and keratinocyte cells, according to Chouhan et al.[176] Mixed SF solutions, on the other hand, were administered into third-degree injuries *in vivo*, resulted in gels that tightly adhered to the wounds. Not only does the RSF hydrogel mixture help with tissue repair, but it also reduces inflammation and encourages cell growth (transition stages).

Regeneration of Tympanic Membrane

The tympanic membrane (TM) is a changeable membrane between the outer and inner ear and aids in sound vibration transmission while also preserving the middle ear. Keratinocytes, fibroblasts, and collagen contribute to the epidermal (external surface), fibrous (innermost layer), and mucosal (interior layer) layers of the TM (type II and type III). Middle ear ruptures caused by trauma or mechanical damage and pressure are the most common causes of TM perforations. If the rupture is not corrected within 3 months, it will develop a chronic perforation, resulting in hearing loss and recurring infections.[177–179] RSF is a great material for tympanic membrane TE because of its excellent characteristics, which allow keratinocytes produced from human TM cells to develop and disseminate. Shen et al. used RSF films to regenerate acute in rat and guinea pig models.[180–184] The treatment for TM perforations is onlay myringoplasty. After 7 days, perforation closure was seen in the rat and guinea pig models, but there was no change in the control groups. RSF films have also been proven to repair TM perforation and speed up TM regeneration, resulting in much faster hearing improvement. According to Shen et al.,[185] RSF 19 of 28 films exhibited no observable macrophage activity in living tissues, reduced swelling, and was degradable *in vivo*. RSF membranes have excellent insulation energy transfer capacities and high strength to cartilage, as per Allardyce et al.,[186] indicating that they have a high potential to cure chronic TM holes *in vivo*.

Silk Fibroin Preparation

B. mori cocoons are heated in a mixture of (0.02 M) sodium carbonate for 30 or 60 minutes, then washed and dried overnight at room temperature. The dried fibroin is dissolved in an aqueous lithium bromide (9.3 M) solution at 60°C for 2–4 hours, yielding a 20% (W/V) solution. LiBr is recovered from silk using dialysis cassettes after dialysis with clean water for 2–5 days (MWCO 3500). The silk fibroin concentration is calculated by removing moisture from a known volume solution sample and massing with an analytical balance. The silk solution is stored at a temperature of 4–7°C before being used.[187–189] Cocoons are sliced into 5 × 5 mm^2 pieces and heated in 1% sodium carbonate solutions before being degummed with wasted cocoons. 1:20 Sodium carbonate quality ratio, heated for 30 minutes, then washed and chopped. After four repetitions, the degummed silk was obtained. Two grams degummed silk were mixed with 10 gm calcium chloride solution of various concentrations, agitated for 1 hour at 98 + −2°C, cooled, and suction filtered. The weight of the insoluble compounds is then calculated. The rate of silk dissolution is calculated. The filtrate was collected and immersed in deionized water in a dialysis bag, and the water is drained three times a day. Following dialysis, the regenerated silk fibroin solution is kept at 4°C.[190]

B. mori cocoons are degummed by incubating for 30 minutes at 98°C in a mixture of 0.25% and sodium carbonate (0.25% w/v).

The samples are cleaned three times with deionized water before drying in the night at 65°C, after cooling at room temperature. Silk fibroins are degummed, and sericin, another silk protein, is extracted. Subsequently, the extracted silk fibroins are soaked in strong calcium chloride solution with ethanol/methanol and water (1:2:8 mol) then soaked in strong calcium nitrate tetrahydrate solution combined with ethanol or methanol (1:2 mol) in a water bath at 65°C for 1 hour. Silk fibers and solution are mixed in a 1:20 (M/V) ratio. Dialyzed silk fibroin solutions are lyophilized to create an aqueous solution. As needed, the dried silk powder or pieces are preserved at 4°C.[191]

Conclusion

Tissue engineering will be used to repair and regenerate injured tissue and organs in the future. This explains why implanted scaffolds must be fully assimilated without provoking an immune reaction or causing any undesirable side effects. Silk fibroin is made from silkworm cocoons and has been recognized by the FDA. SF is widely utilized in TE because of its unique biological qualities, mechanical behavior, and tunability. SF has been used in TE applications in the form of films, mats, artificial fibers, sponges, and hydrogels are some morphological forms. Latest advances have focused on bionanotechnology and technologies, including micro-patterning and 3D printing to manufacture SF hierarchical linear structures to the nanoscale. It has been found to be beneficial for cell proliferation, differentiation, migration, and adhesion in several investigations.

Future Directions

SF combining with 2D nanomaterials can improve the mechanical properties of bone, cartilage, and tendon in tissue engineering. The use of SF matrices in a particular form in treating skin and wounds has demonstrated that patients are less likely to develop scar tissue. Furthermore, because clinical use is currently limited, more research is required to advance clinical trials and obtain FDA approval for this unique biomaterial. SF should be combined with specific other components, including ECM or synthetic peptides, to enhance its activity and applicability in diverse TE fields. RSF is a unique biomaterial that may be used in bio-ink formulations and has the potential for 4D bio-printing in the future.

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