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Review Article

Silk biomaterial for skeletal tissue engineering

Monica P Sikka[a], Siddhi V S Rao & Samridhi Garg

Department of Textile Technology, Dr B R Ambedkar National Institute of Technology, Jalandhar, 144 011, India

Increased fracture rates and problems connected with missing bones as a result of accidents or various illnesses cause serious socio-health issues. Tissue engineering strives to provide a technique for promoting the healing or restoration of injured tissue as closely as feasible to the original tissue. The construction of scaffolds made from recombinant proteins is one of the most important alternatives proposed by this specialisation. Silk in bone tissue engineering has been extensively studied and the current study summarises the literature on such materials and their fabrication.

Keywords: Bone repair, Bone tissue engineering, Silk, Silk-based scaffold, Skeletal scaffold, Tissue engineering

1 Introduction

Skeletal structure of human body is responsible for body's structural stability as well as mobility. Skeletal tissue health is important for day-to-day life. Frequent accidents and growing age are the major reason why skeletal tissue engineering is important. Polymers, bioactive metals, collagens, etc. have been used in bone tissue engineering scaffolds. Among above listed materials, collagen is mostly preferred for bone tissue engineering scaffolds but it loses its mechanical stability and solidarity over the time[1]. Silk shows better results because of its biocompatibility as well as tuneable degradability[2,3]. Additionally, its mechanical behaviour is superior. Among the two popularly known silks, viz silkworm and spider silk, spider silk is more suitable for such application but combative nature of spiders holds the researches back. Silk is an easy-to-process biopolymer, allowing it to be moulded into various shapes and structures, affecting degradability. Silk-based scaffolds can be used for a range of bone restoration and regeneration procedures. Silk surfaces provide active locations for bioactive compounds to mineralize and/or bind, promoting bone repair. Silk has been mixed with polymers and minerals to enhance or introduce new qualities. Also, silk-based scaffolds have been used successfully *in vitro* and *in vivo* to restore bone tissues and other skeletal tissues like cartilage and ligament. The usage of mineralized and nanofibrous scaffolds is increasing as technology improves control over scaffold construction, biodegradability, and sustained release. Skeletal tissue engineering includes three major components, namely bone, cartilage and tendon/ligament[4]. Accidents directly affect all three components whereas age has more effect on cartilage tissues[5]. Natural degradation of cartilage over an age leads cartilage tissue engineering into spotlight for many researches. For skeletal tissue engineering scaffold, silk is not used directly in filament form. So, it is dissolved in a solvent and formed into desired construction according to utility[6-8]. A cross-disciplinary coalition of tissue engineers, material scientists, and manufacturing engineers hopes to accelerate the development of silk-based scaffolds for bone tissue engineering in the near future. Current study summarises the literature on such materials and their fabrication.

2 Skeletal Structures

Skeletal structure helps with mobility, provides rigidity and structure to the body, and protects organs with managing mineral reserves and blood *p*H. Bones are made of collagen, hydroxyapatite, carbonate and some other minor components[9-11]. Normally orthopaedic/skeletal tissue engineering deals with four types of muscles, viz bone, tendon, ligament and cartilage as shown in Fig. 1. Bones are the most rigid tissues that provide structural stability to body. Tendons are stiff cord like structures that connect bone to muscles. Ligaments are elastic structures that connect bone to bone. It holds the bone together as well as allows mobility. Cartilage is a smooth tissue that covers the bone at the joints. It reduces the friction between bones [4].

3 Orthopaedic Surgeries

Orthopaedic surgeries are related to bones and its connecting tissues. In orthopaedic surgeries tissue

[a]Corresponding author.
E-mail: sikkam@nitj.ac.in

engineering stands for putting artificial tendons, ligaments, cartilages or bones in order to develop actual tissues *in vivo* or *in vitro*. Scaffolds are needed to perform such functions.

Using scaffolds in skeletal tissue engineering offers various advantages over scaffold free tissue engineering, including better filling of injured sites, less complicity, easier implantation and faster recovery[12]. In most of the cases human stem cells are put into the scaffold to be colonized as illustrated in Fig. 2. Materials that are mostly used for orthopaedic scaffolds are bionic proteins like collagen. Collagen was found to be better than synthetic materials, because it reflects similarity to the bones as the proteins present in the bones have 90% type I collagen[13]. But collagen has drawback of mechanical strength[14]. That's why silk stands as a suitable candidature for this application

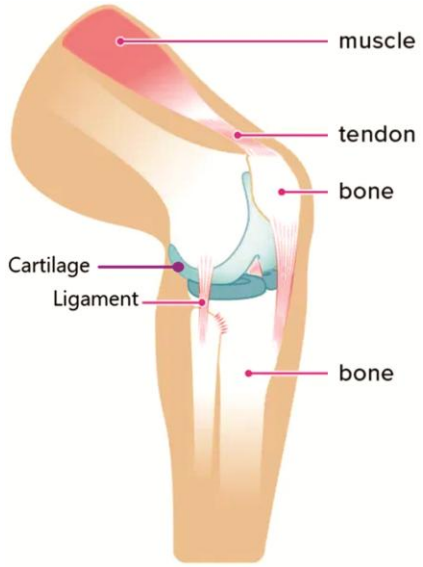

Fig. 1 — Components of skeletal structure in human body

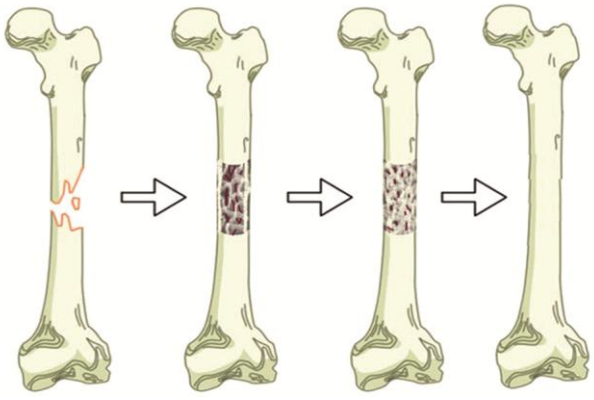

Fig. 2 — Bone recovery by cell proliferation into scaffold

because of its biocompatibility as well as excellent mechanical strength[15]. *Bombyx mori* silk possesses 0.6 GPa strength, 0.18 extensibility and 70 MJ m^{-3} toughness, whereas spider silk possesses 1.1 GPa strength, 0.27 extensibility and 160 MJ m^{-3} toughness[16]. Additionally, degradation rate of silk is slow, that allows better and homogenous culturing of cell matrix. A basic illustration of bone recovery is shown in Fig. 2.

4 Designing and Processing of Silk as Scaffolds

Two types of silks are used in the medical textile. One is silkworm silk and other is spider silk. Silkworm silk is made of two components, viz fibroin and sericin, whereas spider silk is made of spidroin as major substance. Two types of spidroin protein are found in the spiders, namely spidroin protein-I and spidroin protein-II from various glands present in spiders. Among them, spidroin proteins produced by major ampullate glands, namely MaSp-I and MaSp-II are mostly studied. Its mechanical strength, extensibility and toughness are better than native *Bombyx mori* silk, tendon collagen and even bones[16]. To use silk fibroin, sericin must be removed first because it causes negative immune response towards the implant. But degumming also deteriorates mechanical properties of the silk[17]. Silk is preferred to be dissolved in a solvent and respun, it gives opportunities to add some additional characteristics[6-8]. Farming of spiders is a difficult task and harvesting in bulk is next to impossible. That's why regenerated silk is preferred to be used as silk protein. Hosts like bacteria, yeast, eukaryotic cells and insect cells are used to regenerate Spidroin protein directly[1]. To use fibrous silk, electrospinning is the most preferred method to develop such form[18,19]. Nano silk fibres for membrane or yarn in the tissue engineering can be developed easily by electrospinning[20,21]. Bones have a perforated structure that's why for skeletal tissue engineering, perforated material is more preferred.

The process of skeletal tissue engineering starts with selection of osteogenic cells. These cells proliferate and form a neo-matrix along with blood vessels for bone regeneration. The architecture of the scaffold, including pore size and pore density, plays an important role in cell migration. For sufficient oxygen and nutrition supply, pore size >100 µm is necessary. For skeletal tissue regeneration, 200-350 µm pore size is preferable[22]. Additionally, a scaffold should also possess necessary mechanical stability until tissue regeneration is done.

Silk is a reasonable selection for this purpose because of its controllable mechanical behaviour and bio compatibility and degradability. It also allows the transfer of nutrition and oxygen necessary for the growth. It can be made into porous sponges, hydrogels, fibrous matrix and films. Various processes of scaffold formation are shown in Fig. 3.

Electrospinning provides the capability of producing fibres of diameter ranging from 50 nm to 1100 nm by varying different parameters. For cell adhesion, larger surface area of the membrane is more suitable. That's why electrospun silk fibres are preferred[23]. It was seen in some researches that, when *Bombyx mori* silk was electrospun with calf skin collagen or chitin, it showed better performance *in vitro* tissue development[24,25]. Electrospinning is more utilized in artificial spider silk spinning. But its mechanical behaviour couldn't compete with that of natural spider silk. Wang *et al*.[26] showed that by using electro spinning, web like nano-nets made of diameter even less than 20 nm can be produced. Fibres of diameter in the range of micrometres can be produced by wet spinning[27]. Electrospinning has a drawback of high scale production, because the volume output from a single jet is limited.

A silk solution is made by dissolving it in LiBr followed by dialyzing and making films. These films are then again dissolved in hexafluoroisopropanol (HFIP). That solution is ready to be spun. This regenerated silk has lesser strength than native silk. That's why it is more and controlled biodegradable and hence becomes more suitable for tissue engineering. Electrospun fibre properties can be managed by controlling various parameters, like viscosity, polarity, conductivity, feed rate, collector distance, type of nozzle, etc[28-30]. But electrospinning has a limitation of producing larger 3D scaffolds to heal bigger sized bone defects.

4.1 3D Porous Sponge

3D porous sponge scaffolds are needed for bone tissue development. There are three basic processes to develop these 3D matrixes, viz freeze drying, salt leaching and gas foaming. Before using all these methods, silk solution is made by dissolving in suitable solvent (mostly LiBr).

NaCl can be used for salt leaching method. Porogens (salt) is added to the mould followed by silk solution. Solution is placed in a fume hood for overnight to let the solvent evaporate. Then this composite can be compacted by pressing. This compact silk/salt composite can be exposed to ethanol followed by demineralized water immersion or direct immersion into demineralized cold water to leach salt out and dried. This dried porous 3D scaffold has structure similar to bones[31]. A salt leached scaffold structure is shown in Fig. 4. This method provides excellent control over pore size and porosity. Porosity can be controlled by adjusting silk-to-salt ratio. Pore size can be managed by using different salt particle size[32].

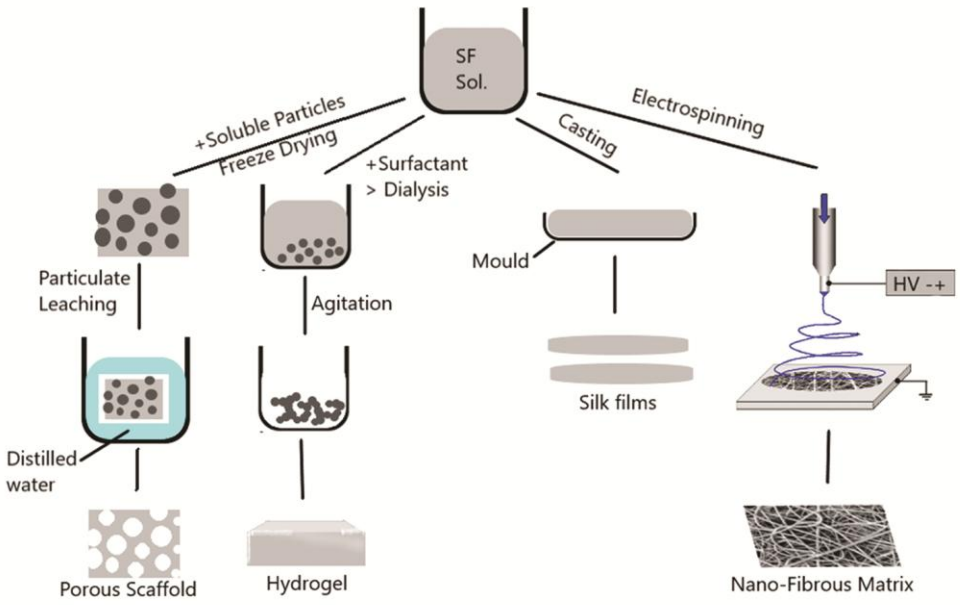

Fig. 3 — Various types of scaffold design for skeletal tissue engineering

Gas foaming can be done by mixing supercritical gases into the SF (silk fibroin) solution in order to make foam like structure. Rapid reduction in the pressure exerted by gas causes the SF material to form foam like structure due to thermo-kinetic instability. Managing the amount of gas gives possibility to manage pore size and porosity. It has better pore inter connectivity and compressive strength. Supercritical CO_2 is the most commonly used gas for gas foaming[33,34]. But super critical CO_2 needs complex machinery to apply high pressure, and pressure control is also not an easy task. Dissolution of $scCO_2$ lowers the pH of water that will lead to gelation of the solution [35]. Maniglio *et al.*[36] used N_2O gas for foaming as shown in Fig. 5(a). It does not alter the *p*H of water. Figure 5(b) shows structure of the scaffold made by gas foaming.

For freeze drying method, a gel like solution is made with silk, alcohol and water. Moulding is done with dry ice. After that it is placed in freezer at a very low temperature (Up to -80°C). Lyophilizing is done to insert pores. β structures are formed using methanol[37]. Pore sizes and porosity can be controlled by controlling cooling temperature and SF concentration, but the strength is not found good enough to perform as cartilage scaffolds. Recently lamellar structure was fabricated using freeze drying method and human MSCs were successfully cultured[38,39].

4.2 Hydrogel

Hydrogels are also a kind of 3D scaffolds having large amount of water in it. Its ability to deliver cytokines and great interaction with cells attracted many researches towards it. For making hydrogel, silk solution is made by dissolving degummed silk fibroin into LiBr solution for 3-4 h and dialyzed in distilled water for 2-3 days. Hydrogelation can be processed by dehydration of the silk solution via vortexing, sonication, *p*H drop, freezing, electro-magnetic fields etc. All these are formed through physical entanglement, and β-sheets are developed. It has good strength and stability, but brittle as well.

Another method of hydrogelation is enzymatic cross-linked silk hydrogelation. Silk solution is reacted with horseradish peroxidase (HRP) in the presence of aqueous hydrogen peroxide (H_2O_2). Free radicals formed in the reaction makes crosslinks with tyrosines in silk fibroin[40,41]. Molecular structure is improved through sonication process. It breaks disulphide bonds and converts gel into aqueous solution. Formation of hydrogel is occurred after sonication[42-44].

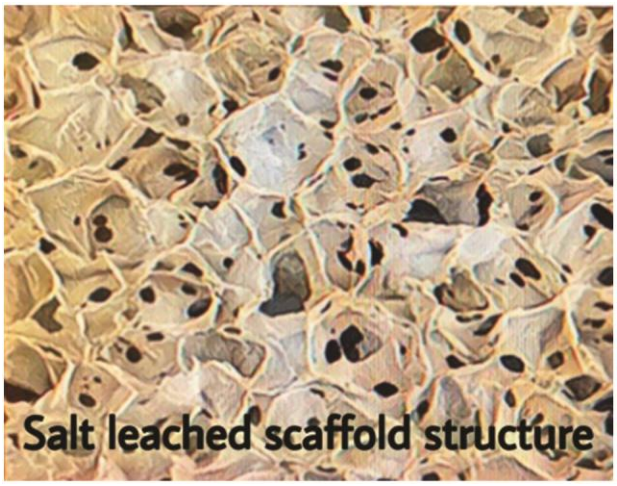

Fig. 4 — Structure of salt leached silk scaffold

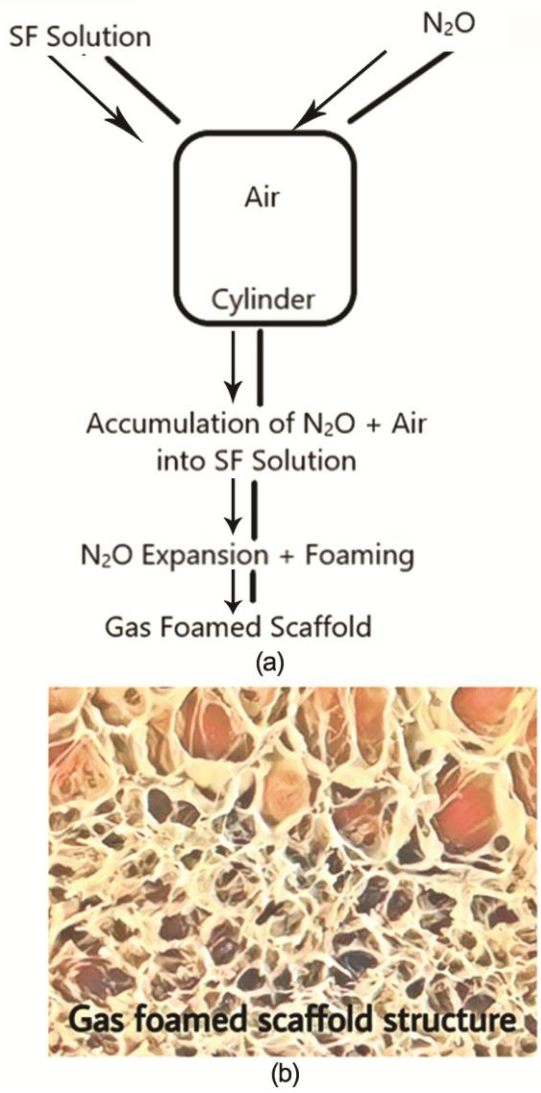

Fig. 5 — (a) SF foam preparation using N_2O gas foaming technique and (b) Structure of gas foamed porous silk scaffold

Another method of forming hydrogel is freezing method. Frozen silk fibroin solution in LiBr is added into organic solvents like methanol, ethanol, propanol, etc. with gentle mixing. This gel-paste mixture of silk/alcohol/water is put into moulds and placed at a very low temperature to develop β structures into gel[37]. Electro-gelation is also a way to produce SF hydrogel. In this, SF solution is given a direct current of 0.5 mA for about 90 min. It converts SF solution into gel form[44].

4.3 Fibrous Matrix

Electrospinning is known to be best method of fibre formation for scaffolds because of its ability to spin fibres from nano to micro sizes. It also allows mimicking the extracellular matrix. Electrospun scaffold structures oriented randomly and in biaxial form are shown in Fig. 6. By controlling various parameters as mentioned above, diameter, strength, porosity and degradability can be varied. It is also relatively faster method of scaffold formation[30,32,45]. Bone marrow derived mesenchyme stem cells showed better proliferation and extra cellular matrix secretion on electrospun SF fibrous matrix[46,47]. To convert silk fibroin α-helix into β-sheets, it's treated with methanol. If freeze drying is done after electrospinning, growth factor is increased, that enhances osteogenic differentiation by mimicking the microenvironment for bone regeneration [41].

5 Applications of these Scaffolds in Skeletal Tissue Engineering

Silk is very well known for its use in biomedical applications. Humans have been using silk as suture for hundreds of years. Modern science brings it to a broader level. Earlier metal and hard plastics were used for many implants but now-a-days soft implants are more preferred. Silk can be used efficiently in skeletal tissue engineering.

5.1 Cartilage Repair

Cartilage has less ability to repair by itself[37].This inability causes osteoarthritis, which is a major issue faced by millions of people over an age[5]. They start feeling pain and inabilities to walk. That's why repair to these tissues is necessary. Various cartilage injuries that can be treated by tissue engineering are shown in Fig. 7. Cartilage is made of avascular, aneural, and alymphatic tissues covered by extracellular matrix[38]. That's why tissue engineering has a wide scope in cartilage repair. Similar to other skeletal repairs, it also requires mesenchymal stem cells for tissue engineering. Silk is preferred for cartilage tissue engineering because of its biocompatibility and controlled degradation as well as excellent mechanical behaviour. Biodegradation rate of the scaffold has to be in proportion of cartilage repair[48]. Silk satisfies this condition and causes homogenous formation of cartilage.

A scaffold for cartilage repair can be made by various methods, e. g. freeze drying, salt leaching, gas foaming, electrospinning, 3D printing, etc. [38, 49-51]. Cartilage tissues are similar to bone tissues but with a difference in structure. Bone tissues are vascularized, whereas cartilage tissues are avascular. That's why most researchers try to develop vessels in bone tissue engineering, whereas in case of cartilage it isn't so[52]. For cartilage TE, 3D sponge structure is more used than any other structures. It provides cell culture in rounded morphology with high cell density so maintain chondrocyte phenotype and support redifferentiation[53]. Many composite materials are also used to enhance the functionality of scaffold for cartilage repair. Zhou *et al*.[54] used silk/chondroitin sulphate composite material to produce scaffold in order to achieve both excellent mechanical behaviour of silk and anti-inflammatory response of the CS (chondroitin sulphate). This enhanced self-healing properties of cartilage. Liu *et al.*[55] developed cartilage

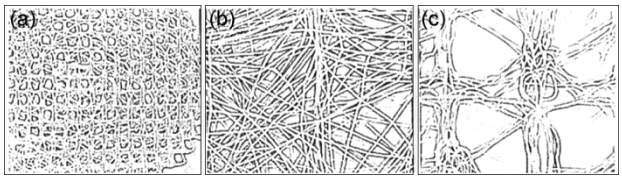

Fig. 6 — Electrospun fibrous matrix scaffold structures (a & b) biaxial orientation and (c) random orientation

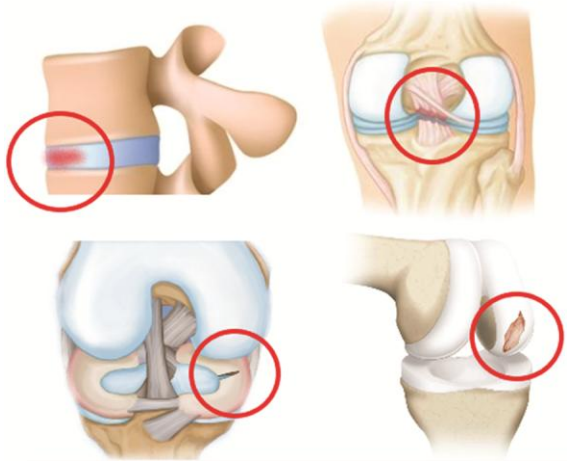

Fig. 7 — Common cartilage injuries where silk scaffolds can be used

hydrogel scaffold with silk/thiolated chitosan composite material and found that it showed better performance in terms of strength elasticity and support to the growth of chondrocytes than both the single component materials. Other studies introduce spider egg case silk as suitable material for cartilage tissue engineering. Hyaline like cartilage was successfully developed by Kearns *et al.*[56]. Regenerated Spidroin has also been successfully used in cartilage tissue engineering[57].

5.2 Bone Repair

Orthopaedic implants are used for bone repair, and at some places fixation plates are used for fracture stabilization. As previously mentioned, bone tissues are porous vascularized structures. It is broadly made of two parts, namely compact bone and spongy bone also known as cortical bone and cancellous bone[53]. The ratio of these varies site to site. That's why architecture of the scaffold plays a major role in bone regeneration. Pore size and porosity have to be suitable for tissues to culture. Pores larger than 100 mm in diameter and completely linked are essential for tissue regeneration. Both salt leaching and gas foaming produce porosity ranging from 85% to 98%. Among all, gas foaming shows the best mechanical behaviour. Mechanical behaviour of an implant should also be similar to the native organ. Tuneable architecture of silk protein and its superior strength make it suitable to be used in scaffold for bone development. Bone repair includes proliferation of osteoblasts and mesenchymal stem cells (MSCs) migration. Many researches are being done in this area but still an ideal bone regeneration is not done till date. An ideal scaffold should support host integration and manage cell osteogenesis as well as degrade with similar rate to the growth of host tissues[58]. High porosity and bigger pore size are more suitable for this application because it supports nutrition and waste transport. Hence pore size and porosity directly affect the engineered tissues. That's why selection of proper pore size is an important aspect. Higher pore size and porosity also degrade its mechanical behaviour. It was also seen that SF scaffolds has low osteogenic capacity. To overcome these problems, composite structures are introduced. Hydroxyapatite (HAp) bioceramic is known to have good biocompatibility and suitable for bone substitute but has low mechanical strength. Therefore, it can be combined with silk fibroin in order to make a composite scaffold for bone regeneration[59]. Magnesium oxide (MgO) is also used in combination of HAp and SF because Mg^{2+} ions promote bone tissue regeneration. Wu *et al.*[60] made scaffold with combination of SF/HAp/MgO and found positive results in bone repair. In order to mimic bone tissue structure, researchers tried many composite scaffolds and gain good results. Bichao *et al.*[61] developed freeze dried ZnSr.TCP-SF hydrogel composite scaffold that mimicked the porous network of the native subchondral bone matrix. Special photothermal activity in SF scaffolds opens up possibility to cure bone tumours. This ability generates cytotoxicity towards MG63 cancer cells after NIR laser irradiation. That's how silk scaffolds can also be used in photothermal therapy for bone cancer[62]. Salehi *et al.*[1] enlightened research that have been done on fusion proteins based on regenerated spider silk for bone tissue engineering scaffolds. Good results were found related to MSC growth rate and scaffold's mechanical behaviour.

5.3 Ligament/Tendon Repair

Ligaments are cord like tissues that connect one bone with another. It manages the movement of a joint and maintains stability. Tendons also have similar structure but connect bones with muscles. These are responsible to transmit force exerted by muscles to the bone and body movement occurs. Intensity of tendons and ligaments to heal is very low. That's why it's a challenging task to repair ligament and tendon tissues[63]. An ideal ligament scaffold must possess high tensile strength because ligaments face high stresses during movement of muscles and bones. These are positioned parallel to the direction of forces, so the tensile load is applied on the scaffolds[64]. Knitted scaffolds and cords perform best for this purpose as they fulfil the mechanical requirement as well as well as adopts most similar shape in the body[52]. It was seen that corded ligaments show better mechanical properties than other structures and meet the properties required in the body[65]. Another comparative study between porous and nonporous tendon/ligament scaffold was done by Yao *et al.*[66] and it was found that nonporous scaffolds show better regeneration in tendon/ligament TE. Chen *et al.*[67] used human periodontal ligament stem cells (hPDLSC) as seed cells and found that hPDLSC seed cells seeded in aligned SF scaffold enhanced the results and can be used broadly in tendon tissue engineering.

6 Conclusion

Silk sutures have been used in surgery and ophthalmology for many years. Silk is also used in anti-hay fever masks and dermatological gauge pads and bandages. With rapid advancements, it is envisaged

that additional silk-based biomedical products, such as wound dressings or orthopaedic implants, would soon be in use. On the other hand, creating nanofibrous scaffolds to better replicate the basic native bone structure is a huge advancement in musculoskeletal tissue engineering. Silk performs well as scaffold, because of its biocompatibility, tuneable degradability and excellent mechanical strength. In the field of skeletal tissue engineering, it cannot be used as continuous filament form like sutures and artificial skin scaffolds. For bone repair and cartilage, a 3D porous scaffold is needed. Freeze drying, salt leaching and gas foaming are the most commonly known methods for development of porous scaffolds, whereas tendon/ ligament tissue engineering requires nonporous scaffold to mimic the tendon tissue architecture. Braided cord like structure and aligned tubular moulded SF scaffolds are widely used for tendon/ligament tissue engineering. Among the natural silk sources, silkworm silk is the most abundant. Due to their enormous future potential, several spider silk domains may be seriously considered. Despite promising *in vitro* and *in vivo* results, no human studies of silk-based biomaterials have been conducted. Thus, the benefits of silk-based tissue engineering for patients and victims remain distant. This is true for all tissue engineering domains, not only bone tissue engineering. Translational research is urgently needed to address this gap in the near future.

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