Silk Protein as a Fascinating Biomedical Polymer: Structural Fundamentals and Nanofibrous Scaffolds

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1. INTRODUCTION

Silk has been used as a textile and a fibrous material in many industries for a long time, because of its excellent mechanical properties. Recently, silk and silk-based materials have attracted renewed interest, because of their biological applications. According to early records, silk fibers have been used for wound closure by surgeons for at least 3,000 years. This reflects the high biocompatibility of silk, despite silk being a foreign protein to mammals. The biomedical applications of silk protein have been studied since the 1960s. Various studies have confirmed that silk does not cause severe inflammation or elicit other tissue responses in mammalian tissue. As a substrate, silk protein is good for mammalian cell adhesion and proliferation. Recent studies reported the use of silk in oral administration. The excellent biocompatibility and functionality of silk has led to the development of various biomedical devices. For example, in the early era of silk biomaterials, many researchers developed silk films and sponges as wound dressing. The biological applications for silk now include tissue engineering scaffolds, nerve conduits, and artificial ligaments. The biocompatibility and functionality of silk is similar to collagen, and the physical and mechanical properties of silk make it suitable in biomedical devices.

Electrospun nanofiber has received much interest as a novel scaffold for tissue engineering because nano-fibrous structure is very similar to collagen fibrous structure of natural extracellular matrix (ECM). Many recent studies have demonstrated that the cells seeded on nanofibrous scaffolds were adhered well and grew onto their surfaces due to structural characteristic of large specific surface area. However, nanofibrous scaffold so far developed through electrospinning method might have structural limits for cell proliferation. Furthermore, the pore size is too small for the cells to grow inside and the shape is limited only in a two dimensional non-woven sheet form. Therefore, it is not possible to regenerate a suitable three dimensional tissue with this form of scaffold. In this presentation, we will introduce a novel fabrication method of real three dimensional electrospun silk nanofibrous scaffold with high porosity as well as the control of pore size (100-400 μm), in order that seeded cells can migrate into the inner space of the scaffold.

On the other hand, the investigations of silk structure and spinning process are steady and progressed. The complete comprehension of the nature of silk is a key factor for the successful application of silk protein. Specially, a modulation of silk structure as well as diverse fabrication techniques to fulfill a wide variety of uses is considered importantly.

2. FUNDAMENTALS OF SILK PROTEIN ON STRUCTURAL FORMATION

Silk fiber consists of two proteins, silk fibroin (SF) and silk sericin (SS), both from the silk worm. In a raw silk fiber, two SF strands, which each has a triangular cross-section, are covered by SS. The weight fraction of SF reaches approximately 75% with SS comprising the remainder. There is also a very small amount of lipids and polysaccharides in the silk fibers. SF is a highly crystalline and fibrous protein, imparting high strength and resilience to the silk fiber. Amino acid analysis shows that SF is mainly composed of glycine (G), alanine (A) and serine (S). With the exception of serine, these hydrophobic amino acids comprise almost 75% (mol%) and are sequenced and repeated quite regularly, $(GAGAGS)_n$. Furthermore, such a structure has high crystallinity with the SF chains oriented along the fiber axis. These features of the structure give SF excellent mechanical properties (ultimate tensile strength: 3.6-4.0 gf/d), additionally, SF is insoluble in water. In order to use a silk fiber, SS is generally removed by a degumming process. After degumming, the weight of the silk fiber is reduced by 25%, and its surface texture becomes softer and smoother, and the tensile strength of the silk fiber is maintained.

Silk spinning can be divided into three phases: synthesis and secretion of silk protein, concentration of a silk dope solution, and drawing. Figure 1 shows a pair of *Bombyx mori*'s glands. Amino acid sequences of the silk proteins, SF and SS, have been determined by DNA sequencing of *Bombyx mori*. A fibroin molecule contains one heavy and one light chain, coupled by a disulfide bond (cysteine linkage). Their

molecular weights are approximately 390 and 25 kDa, respectively. In the heavy chain, two segments appear in an alternating manner. One segment is mainly composed of simple hydrophobic amino acids in a regular order, e.g. GAGAGS, and another consists of hydrophilic amino acids. Therefore, SF is amphiphilic. On the other hand, SS is composed of three proteins with different molecular weights and has a large amount of hydrophilic amino acids, compared with SF.

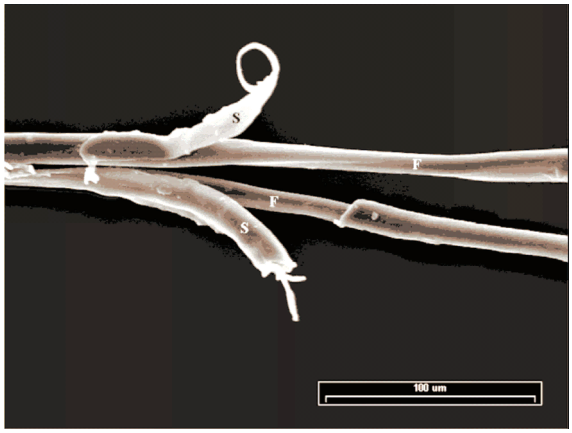

Fig. 1. Scanning electron microscopy (SEM) of raw silk cocoon fiber. Sericin (S) covers two strands (F) of triangular cross-section.

The silk structures are classified as silk I and silk II, which correspond to the characteristic structures of a SF solution in the gland and silk fiber, respectively. At silk I state, the molecular chains of SF are coiled randomly and form an amorphous state. In the glands, with the exception of the anterior gland, the conformation of SF is mostly a random coil. Some reports insist that in the glands, silk I has a crystalline structure and an intrinsic α-form conformation. However, this is inappropriate because silk I is defined as a structure in a silk dope solution in the gland. The secondary structure of a silk fiber, or the re-crystallized regenerated fibroin, is known as a pleated anti-parallel β-sheet. This structure originates from a small repeating peptide unit, glycine-alanine (GA), within the essential peptide sequence generating the structure, GAGAGS, as mentioned above. The hexa-peptide is the most common sequence in the hydrophobic segments of fibroin, and forms β-sheet structure via intra- and inter-hydrogen bonds.

3. BIOMEDICAL APPLICATIONS FOR TISSUE ENGINEERING SCAFFOLD

Since the 1990s, tissue engineering has attracted considerable interest as a promising technique to aid in the healing of many diseases. Materials researchers have focused on scaffold materials, and have attempted to develop many different types of polymers for a cell culture substrate. Silk is considered to be an excellent substrate for mammalian cell cultures. Minoura et al. confirmed that mouse fibroblasts seeded on SF films attached well and proliferated. Since then, many other scientists have reported that silk has good cytocompatibility and cell adhesion ability, making it a good scaffold. As a scaffold material, silk is similar to collagen, which is a main component of the natural extracellular matrix. It was confirmed that silk scaffolds exhibit similar or superior performance to other polymeric scaffolds. Silk is more biocompatible and does not cause inflammation by the biodegradation of byproducts, unlike conventional aliphatic polyesters, e.g. PLA, PCL, PLGA and other similar polymers. According to a recent study, silk is believed to be a promising material for regenerating bone and cartilage because it is quite compatible to skeletal cells, osteoblasts, and chondrocytes.

A SF scaffold can be fabricated into a porous sponge by lyophilization or salt-leaching methods. For lyophilization, degummed silk cocoons are dissolved in highly concentrated metal salt solutions, such as $CaCl_2$ or LiBr, and subsequently dialyzed, to a final SF aqueous solution of 2-6 wt%. The pore structure is varied by controlling the solution concentration and freezing temperature. Generally, the porosity decreases with increasing concentration. In addition, the pore size decreases with decreasing temperature. The pore structure is easily controlled in salt-leaching methods. NaCl particles are often used as a porogen, and the pore size and porosity are dependent on the size and quantity of NaCl particles, respectively. (Fig. 2)

The SF scaffold prepared by salt-leaching has been mainly investigated for bone regeneration. The SF, with similar biocompatibility, has superior mechanical property to collagen-made scaffold which is commonly used for bone healing. According to related reports, the regenerative SF material provides cells participating in osteogenesis with suitable environment. Furthermore, it seems that SF scaffold affects the differentiation of stem cell and osteoblast under appropriate circumstances. Meantime, researchers are trying to fabricate SF/hydroxyapatite (HA) composite scaffold by biomineralization.

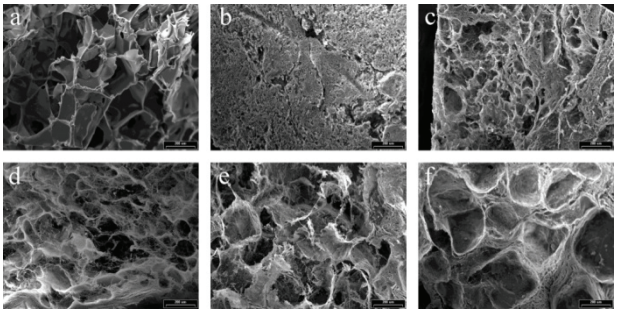

Fig. 2. Scanning electron microscopy images of 3 dimensional silk fibroin nanofibrous scaffold prepared with various pore sizes.

In order to deposit HA crystals on the surface of biomaterial, simulated body fluid (SBF), mainly composed of calcium and phosphate ions, is used. However, it is difficult to deposit HA on pure SF surface because it does not have sufficient electrical charge to induce calcium ions in SBF. Therefore, a surface modification, blending or addition technique is used. For example, Kim et al. prepared SF scaffold containing polyaspartic acid (PA). With negative charge of PA, the surface of the scaffold was successfully coated with HA particles. On the other hand, Mouney et al. evaluated SF scaffold for the generation of adipose tissue. They seeded adipose-derived and bone marrow mesenchymal stem cells on porous SF scaffolds and validated that the cells differentiated to adipocytes and formed adipose tissue by animal experiments. Such a tissue engineered filler, composed of cells and SF scaffold, is a promising candidate in cosmetic surgery.

Artificial ligament fabrications were attempted using twisted or knitted silk fibers in order to regenerate a cruciate ligament. Fini et al. reported on the healing ability of SF hydrogel for a cancellous defect. There have been attempts to develop a SF blood vessel scaffold. In case of large diameter blood vessel, polyurethane (PU) and poly (tetrafluoroethylene) (PTFE) vessels are already commercialized and widely used. But, small diameter (~< 6 mm) blood vessels of those materials cause low patency by thrombosis on inner surface and have poor mechanical property. Therefore, it is expected that the tissue engineered blood vessel can solve these problems. Lovett et al. reported the fabrication of SF conduits and Zhang et al. investigated the feasibility of SF scaffold for vascular cells. Besides, the film type scaffold for cornea tissue engineering was tried. In this study, the transparency as well as biocompatibility of silk film is very advantageous. Yang et al. prepared the SF conduit for the regeneration of peripheral nerve. They expected the SF nerve conduit is a candidate to compete with a commercialized collagen nerve conduit.

An electrospun SF nanofiber assembly has recently attracted interest as a scaffold. The electrospun SF nanofibers can provide cells with an ideal structure for their growth, in the same manner as natural extracellular matrix (ECM) collagen fibers. It was reported that various cell types were successfully proliferated on the electrospun SF nanofiber mat. Jin et al. prepared electrospun SF mat from SF/PEO aqueous solution. They compensated the poor electrospinnability of SF aqueous solution by blend PEO and extracted PEO after spinning. Finally, they made the electrospun SF fiber using only water without any toxic solvent. Meinel et al. observed cell adhesion and morphology on aligned electrospun SF fibers. They reported the cells seeded on the electrospun fibers were largely affected by the orientation of the fibers. Nevertheless, the electrospun silk scaffold is hardly used because of the sheet-like shape of the electrospun mat. Ki et al. suggested a novel method to fabricate 3D electrospun SF scaffold. After dispersing electrospun silk nanofiber in methanol coagulation bath, the silk nanofiber assembles were shaped into a foam by molding. Using this method, various shapes of 3D silk nanofibrous scaffold can be fabricated with controllable pore structure and it was possible to culture osteoblasts inside the scaffold. (Fig. 3)

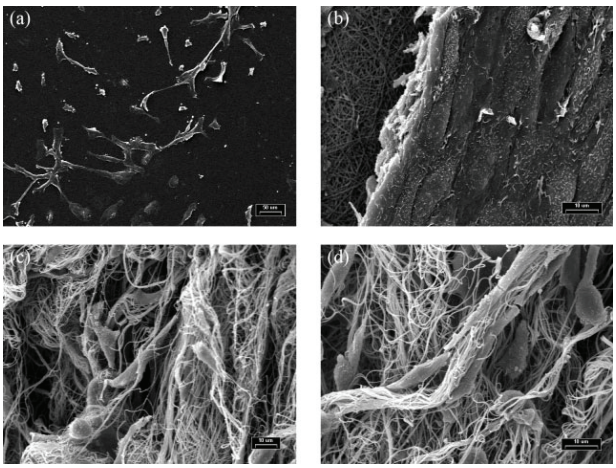

Fig. 3. SEM micrographs of cultured mouse fibroblasts after 7days incubated at 37°C in 5% CO_2 atmosphere on (a) SF film, (b) 2-D SF nanofiber mat, and (c) and (d) 3-D SF nanofbier foam.

Moreover, various modifications of a SF scaffold were attempted in making a scaffold for a target tissue. Chen et al. immobilized RGD peptides, which promote focal adhesion of a seeded cell, on degummed silk fibers for ligament regeneration. Human bone marrow stem cells were also seeded. The number of cells seeded on RGD-immobilized silk fibers increased more rapidly than on non-immobilized silk fiber. Moreau et al. developed and evaluated a growth factor-releasing SF scaffold that enhances the cell proliferation and differentiation. These studies suggest that a growth factor contained in the SF scaffold is quite effective for culturing stem cells.

4. SUMMARY

Silk has always been of great interest to materials researchers because silk has excellent mechanical properties and thermal stability. Many studies and trials are needed to determine the nature of the silk structure and mechanism of formation. And studies on the applications of silk are resulting in the

development of a wide variety of silk based biomaterials (Fig. 4). With the progress of tissue engineering technology, tissue engineering scaffolds and implants are particularly attractive. Silk is considered to be a candidate biomaterial with other synthetic biocompatible polymers. In the case of biomedical application for silk nanofibrous scaffold, we demonstrate that silk-based biopolymers can offer exceptional benefits over conventional synthetic (PLA, PGA, PCL ant etc.) and natural biomaterials (collagen, gelatin, chitosan ant etc.) in generating functional tissue replacement for various cells, in addition to high potential applications of this scaffold for tissue engineering. Accordingly, there will be a need for more study on the structure and spinning process of silk as well as on the production of silk-based biomaterials for use in the biomedical field.

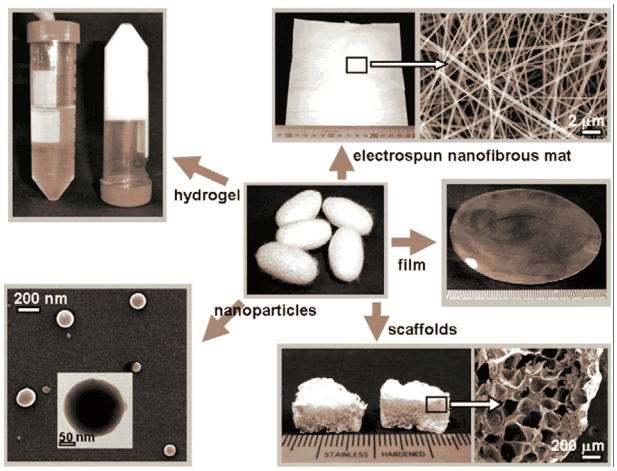

Fig. 4. Various biomaterials based on silk fibroins for biomedical applications. All the samples would be fabricated from the aqueous regenerated silk fibroin solutions.

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