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Review

Silk Protein as a Fascinating Biomedical Polymer: Structural Fundamentals and Applications

Chang Seok Ki and Young Hwan Park*

Department of Biosystems and Biomaterials Sciences and Engineering, Seoul National University, Seoul 151-921, Korea
Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 151-921, Korea

Hyoung-Joon Jin

Department of Polymer Science and Engineering, Inha University, Incheon 402-751, Korea

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Abstract: Silk is a textile material, as well as one of the oldest biomaterials. However, the recent progress of biomedical science and technology has led to the replacement of silk by various biomaterials based on synthetic polymers. Despite the wide variety of biomaterials available, these materials suffer certain limitations that prevent them from meeting the various demands of the medical field. Therefore, silk continues to attract considerable interest as a promising biomaterial. This paper explains the fundamentals of silk protein, and reviews the many applications of silk biomedical polymers.

Keywords: silk protein, silk fibroin, structure, biomaterial.

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.[1,2] As a substrate, silk protein is good for mammalian cell adhesion and proliferation.[3] Recent studies reported the use of silk in oral administration.[4] The excellent biocompatibility and functionality of silk has led to the development of various biomedical devices.[5-7] For example, in the early era of silk biomaterials, many researchers developed silk films and sponges as would dressing.[8,9] The biological applications for silk now include tissue engineering scaffolds,[10-12] nerve conduits,[13] and artificial ligaments.[14,15] The biocompatibility and functionality of silk is similar to collagen, and the physical and mechanical properties of silk make it suitable in biomedical devices.

On the other hand, the investigations of silk structure and

*Corresponding Author. E-mail: nfchempf@snu.ac.kr

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. In this article, these so far efforts would be introduced and summarized. Based on basic studies and outcomes on the silk structure, various application studies in recent are reviewed.

Fundamentals of Silk Protein on Structural Formation

Two Components of Silk Protein.

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, $(GAGAGS)_n$.[16-18] 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.[19]

In contrast, SS contains a relatively large amount of hydrophilic amino acids and an amorphous structure. Consequently, it has poor mechanical properties and is soluble in aqueous solutions. SS is even soluble in warm water, and its solubility increases with increasing temperature. A SS aqueous solution with a high enough concentration is quite viscous, like an adhesive. This viscosity of SS is most likely how SF strands and raw silk fibers are bound in a cocoon. SS, however, does not contribute to the tensile strength of the silk fiber due to its brittleness. 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.

Spinning Process of Silk Fiber. 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.[17,18,20-22] In the

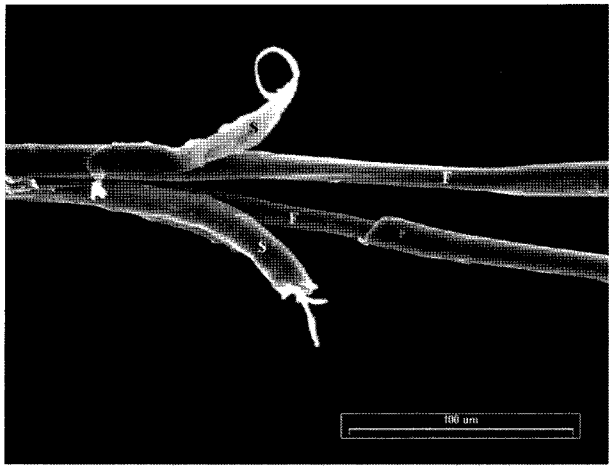
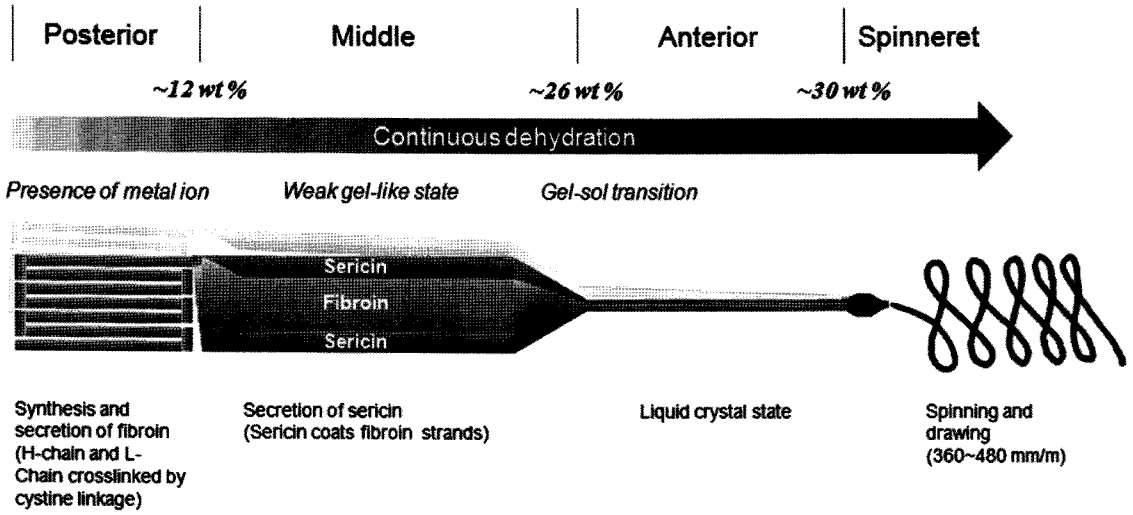


Figure 1. Scanning electron microscopy (SEM) of raw silk cocoon fiber. Sericin (S) covers two fibroin strands (F) of triangular cross-section.

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.[17,18,20,22] 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.[23,24]

Scheme I describes the silkworm glands and spinning process. Fibroin is secreted from the posterior gland, at a concentration of approximately 12%. In the middle gland, sericin is secreted to cover the fibroin solution stream, and the fibroin solution is concentrated to approximately 25% at the end of the gland. In the anterior gland, the concentration reaches 30% and a gel-sol transition occurs. Fibroin molecules form a liquid crystalline state in the highly concentrated solution.[25-29] The presence of metal ions and the pH affects the structural formation and folding process of fibroin. Metal ions in the dope, such as Ca^{2+} and Cu^{2+}, are added through the posterior and middle glands. Very few metal ions, however, are found in the anterior gland. In the posterior gland, the pH of the dope solution is approximately 6.9. As the protein passes through the three glands, the pH value decreases steadily to 4.8. Previous studies suggested the effect of the changes of metal ions and pH on the fibroin structure.[30-32]

Many parameters affect the formation of silk fiber in complicated manner. One important factor affecting the spinning process is shear. Shear stress is applied to all the glands, as a side-to-side movement of a silkworm's head occurs. The shear appears to cause a molecular orientation affecting the β-sheet formation of fibroin.[27,33-37] Recently, several research groups attempted to elucidate the spinning mechanism using artificially organized silk-like peptides, synthesized both



Scheme I. *Bombyx mori*'s glands and the spinning process.

chemically and biologically. The synthetic peptides provided information on the relationship between the amino acid sequence and structure formation, because they consisted of a more regular structure than natural silk fibroin.[38-41]

Silk fiber is considered an ideal fiber. The properties, structure and spinning process of silk fiber is the model for various synthetic fibers, such as nylon and polyester fibers. The silk worm uses little energy and no harmful chemicals in the spinning process, and manufactures a stronger and more crystalline fiber compared with that produced by artificial spinning. Many scientists and researchers have attempted to elucidate the silk fiber spinning process for application to fiber and polymer processing. However, the nature of silk and the spinning mechanism are not yet fully understood.

Conformation and Crystalline Structure of Silk Fibroin. 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.[42] 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.[39,41,43-48]

Circular dichroism (CD) is the most powerful method for determining the secondary structure of a protein. However, it is generally not suitable for analyzing the conformation of silk in a solid. Although the molecules in a fibroin solution are entangled randomly, there are few reports of CD analysis for solid silk.[27,32,49,50] Instead, FTIR and NMR are used to examine the secondary structure of silk protein. The peak position of amide I at approximately 1650 cm^{-1} is a guide for determining the secondary structure by FTIR. Peaks at 1650 cm^{-1} and 1630 cm^{-1} indicate the random coil and β-sheet conformations, respectively. Amides II (1540 cm^{-1}) and III (1230 cm^{-1}) are also used to determine the conformation of SF. In the case of the β-sheet, the amide II peak shifts to a lower wave number with the concomitant development of a shoulder peak at 1260 cm^{-1} in the amide III peak. According to a recent report, the red shift of the amide II peak is caused by a change in the hydrogen bond of tyrosine in the fibroin molecular chain.[51]

Asakura *et al.* examined the characteristic β-sheet structure of silk using NMR spectroscopy. A peak in the 15-22 ppm range of the ^{13}C NMR spectrum, which was attributed to alanine C_β, indicates the conformational structure of fibroin. The appearance of a peak at 21 ppm indicates the formation of a β-sheet structure. An additional shoulder peak at 23 ppm corresponds to a so-called 'distorted β-sheet', which is caused by tyrosine. These results were reconfirmed by investigating the model peptide prepared by artificial synthesis. In the fibroin molecular chain, 3% tyrosines caused an irregularity of the β-sheet structure, and triggered crystallization from an aqueous solution of random coil peptide.[37-41,44,47]

It is believed that the formation of a silk crystal is similar to that of other polymers, particularly synthetic polymers, such as polyethylene, where the molecular chains are entangled and form a semi-crystalline structure. In 2003, however, Jin *et al.* reported that fibroin molecules can form a micelle structure due to the amphiphilic nature of fibroin in

aqueous solution.[17] Their article suggested a novel model to explain the crystallization of fibroin. A single fibroin molecule or assembled multiple fibroins form a micelle, which then forms a hierarchical structure.

The crystalline structure of SF is in a monoclinic space group. The unit cell lattice parameters are a=0.938 nm, b= 0.949 nm and c (chain axis)=0.698 nm.[21,46,52] The characteristic arcs on the 2D wide angle X-ray diffraction (WAXD) pattern appear at $2\theta = 9.34$, 18.93, 20.80 and 25.78°, which are assigned to the reciprocal lattices, [100], [200], [210] and [002], respectively when using Cu K_α irradiation. The crystalline structure and molecular orientation are well developed for a raw silk fiber, though regenerated silk fibroin shows very low crystallinity. Regenerated silk can be recrystallized by alcohol immersion, generally in solutions of methanol or ethanol. These alcohols cause a re-arrangement of the hydrogen bonds between fibroin molecules, leading to a β-transition for even solidified fibroin. The degree and rate of the β-sheet transition depend on the alcohol type and concentration. As the size of the alkyl group of the alcohol increases, the β-sheet transition efficiency and rate decrease due to a lack of accessibility. For example, methanol and ethanol are similarly effective whereas pentanol and hexanol barely induce a conformational change. In addition, solidified SF can crystallize partially in saturated water vapor which may happen a change in hydrogen bonding by water molecules around the SF molecular chains.[53]

However, such artificial crystallization is quite different from the natural spinning process as there is no alcohol in the silk worm spinning process. In natural silk fiber, silk crystallization is driven by a concentration process of the dope as well as shear stress. It has been reported that as a result of the shear stress in a re-dissolved, highly concentrated SF aqueous solution, SF transforms to crystallized precipitates by a β-sheet conformation.[35,37] The mechanism of the shear-induced crystallization of fibroin is unclear despite the large number of studies. Vollrath *et al.* studied the rheological behavior of silk dope from silk glands and re-dissolved aqueous silk solution. In most cases, the shear viscosity of a silk solution increases slightly in the range of 1/1,000-1/100 s^{-1}. In addition, over a certain shear rate, the shear viscosity decreases rapidly with increasing shear rate, and reaches a constant at >500 s^{-1}.[54-56] The initial increase in shear viscosity might be due to the drag force caused by fibroin chain entanglement. The rapid decrease in viscosity, which is referred to 'shear thinning', is caused by the loosening of entangled fibroin chains. In this loosening process, fibroin chains are aligned along the direction of the shear force. Moreover, the process is irreversible and may cause precipitation and crystallization, particularly for SF.

Rössle *et al.* observed by X-ray scattering the transition of the fibroin structure in an aqueous solution under shear.[35] As a shear stress was applied to the SF solution, the shape of the fibroin particles changed to a spindle shape from a sphere. This was attributed to the stretching effect by shear. The sheared SF solution finally formed a crystal, after drying without methanol treatment. Asakura *et al.* attempted to measure the conformational transition of an aqueous SF solution under shear as a function of time using real time Rheo-NMR. They found that shear-induced crystallization should occur after a certain time and shear rate. Once precipitation begins, the conformational transition and crystallization proceed rapidly. However, they could not quantitatively determine the β-transition with time.[37]

Many studies have investigated the mechanisms of silk spinning and structural formation, focusing mainly on the properties and structure of SF because it is the main component of natural silk protein. On the other hand, it has been considered that sericin is not important in the silk fiber spinning process even though it comprises approximately 25% of the total silk protein weight. However, it is believed that SS plays a role in the spinning process, because SF and SS coexist from the middle gland to the final spinning step. In addition, some recent reports have suggested the effect of SS on the formation of the SF structure.

Jin *et al.* reported that the surround SS molecules aid in the micelle formation of fibroin. SS is more hydrophilic than SF, so SS can extract water molecules from SF and assist in fibroin folding, which is induced by hydrophobic interactions between the repeating GAGAGS segments in an aqueous solution.[17] Lee also reported the possibility that SS affects SF structural formation. He insisted that the alcohol-induced re-crystallization process of SF was retarded in a regenerated SF/SS blend.[24] Our previous studies examined the effect of SS in detail. In the process of fabricating a regenerated SF filament, the SS that remains from a degumming process or is added by blending contributes to the crystallinity of a regenerated silk filament and the β-sheet structure.[57,58] According to reports, SS can enhance the SF crystalline structure by dehydration and hydrogen bonding under shear conditions. SS is probably a critical factor that can affect the SF structure during spinning. In addition, a study on SS could provide insight on the mechanism of silk fiber spinning.

Applications of Regenerated Silk Protein in Biomedical Fields

Wound Dressing. There have been many attempts to develop high performance wound dressings using SF because silk is highly biocompatible and exhibits wound healing effects. SF has a very low inflammatory reaction when in contact with a wound site, whereas conventional cotton gauze occasionally causes severe inflammation and inhibits wound healing. A silk dressing material can be fabricated using a variety of processing methods and the shape of film,

sponge or fabric can be easily controlled.

As previously reported, SF films exhibit excellent oxygen gas permeability compared to typical wound dressings made of synthetic polymer films.[59] To make the SF sponge type dressing, lyophilization is frequently used to create a porous structure that provides gas permeability and exudation absorbability. This provides the advantage that it can be fabricated very easily. In addition, it has been reported that an electrospun SF non-woven mat can be applied as a wound dressing material.[53,60,61] Such studies are based on the excellent biocompatibility and healing ability of silk fibroin. Roh *et al.* reported on the advantages of using a silk fibroin sponge as a wound dressing on a mouse wound the full thickness of the dorsal skin although the healing mechanism of silk is unclear.[9]

There are some problems and limitations, however, in using silk protein in wound dressings. The cost of the entire process to fabricate silk wound dressings is relatively high compared to other materials. Adhesion to the wound surface often occurs due to the good cytocompatibility of silk fibroin; the cells around the wound migrate to the silk dressing. For these reasons, the silk wound dressing is hardly commercialized.

Controlled Drug Release Matrix. Silk proteins can be used as a drug releasing matrix, however, the studies are limited because it is difficult to control the rate of drug release from a silk matrix. Drug release techniques using silk proteins are used for additional functionality, such as gene expression and the release of growth factors.[5] Min *et al.* reported drug releasing profiles from a porous silk matrix with acidic, basic and hydrophobic model drugs.[62] A technique blending silk with other polymers was suggested to control the release properties of silk.[63] In addition, controlled drug release from SF gel was reported by modifying the gelation property of SF. Matsumoto *et al.* reported that the different gel-sol transition behaviors in varying pH conditions can be used to control gelation, which can be applied to a controlled drug release system.[32] According to a recent report, there was an interesting attempt to use SF as a coating material on a blood vessel stent. Wang *et al.* exhibited that the stent surface could be functionalized by drug loaded SF coating. They used a multi-layer coating technique to control a drug release property and this method has overcome the limited SF's characteristics as a drug carrier.[64]

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.[3] 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.[2,5,65] 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 by-products, 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.[10,66-70]

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.[12,62,71]

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.[72,73] Meantime, researchers are trying to fabricate SF/hydroxyapatite (HA) composite scaffold by biomineralization. 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.[74] 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.[75] 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 liga-

ment.[14,66,76,77] Fini *et al.* reported on the healing ability of SF hydrogel for a cancellous defect.[8] 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[78,79] and Zhang *et al.* investigated the feasibility of SF scaffold for vascular cells.[80] 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.[81] 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.[13]

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.[60,61] 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.[68] Meinel *et al.* observed cell adhesion and morphology on aligned electrospun SF fibers.[82] 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.[83-85]

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.[66] Moreau *et al.* developed and evaluated a growth factor-releasing SF scaffold that enhances the cell proliferation and differentiation.[77,86] These studies suggest that a growth factor contained in the SF scaffold is quite effective for culturing stem cells.

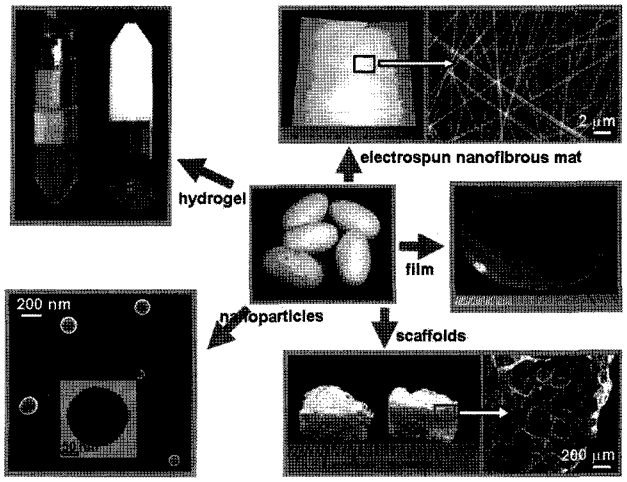


Figure 2. Various biomaterials based on silk fibroins for biomedical applications. All the samples would be fabricated from the aqueous regenerated silk fibroin solutions.

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 (Figure 2). 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. 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.

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