Recent Innovations in Silk Biomaterials

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Abstract: Silk contains a fibre forming protein, fibroin, which is biocompatible, particularly after removing the potentially immunogenic non-fibroin proteins. Silk can be engineered into a wide range of materials with diverse morphologies. Moreover, it is possible to regenerate fibroin with a desired amount of crystallinity, so that the biodegradation of silk materials can be controlled. These advantages have sparked new interest in the use of silk fibroin for biomedical applications, including tissue engineering scaffolds and carriers for sustained release of biologically active molecules. This article summarizes the current research related to the formation of silk materials with different morphologies, their biocompatibility, and examples of their biomedical applications. Recent work on the preparation of silk particles by mechanical milling and their applications in silk composite scaffolds is also discussed.

Keywords: Silk fibroin, powder, composite, morphology, application.

1. Introduction

Silk consists of fibrous proteins that are stored as a liquid in silk producing arthropods (such as silkworms, spiders, scorpions, mites and bees) and are spun into fibres at secretion [1]. Amongst various silk species, silkworm and spider silk fibres have been widely studied for their structure, processing, and functional properties. About 90% of commercial silk fibres used in the textile industry come from Lepidopteron silkworms from the family, *Bombycidae* [2]. In the textile industry, it is commonly referred to as Mulberry silk. *Saturniidae* is another class of Lepidopteron silkworms that produce commercial silk fibres such as Tasar, Eri and Muga.

Apart from their long history as luxury textiles, silk has also been used in the human body in the form of suture material [3]. A study by Minoura *et al.* [4,5] has suggested much wider application prospects for silk. Their study showed that silk fibroin of *B.mori* and *A.pernyi* was equal to or even better than collagen in supporting attachment and proliferation of murine L-929 fibroblast cells. The results have generated enormous interest in the ensuing years to examine silk fibroin for various biomedical and healthcare applications through *in-vitro* or animal model studies. Native silk fibre suture is a Food and Drug Administration (FDA, USA) approved biomaterial. Recently, regenerated silk has also been approved by the FDA for human clinical tests.

2. Different forms of silk biomaterial

Silk biomaterials can be prepared directly from silk fibres, or reconstituted from silk fibroin solution. An alternate way is to convert silk fibres into ultrafine particles through milling. Figure 1 shows a schematic diagram of processing silk fibres into various forms of diverse morphologies [6].

2.1 Native Silk fibre based structures

Degummed silk filaments in the form of twisted structures, such as wire-rope, cable, braided, and textured yarns have been analysed for potential biomedical applications [7]. Silk filaments can also be used to construct nonwoven structures as a cell supporting template, by partially dissolving silk fibres [8,9]. Random arrangements of filaments in a cocoon can be preserved during degumming to design a porous nonwoven silk mat for cell seeding [9]. An alternate way of using silk filaments directly in tissue engineering is making a knitted structure [10]. Such knitted structures have been used to reinforce 3-D porous tissue engineering scaffolds for improved mechanical properties [11].

2.2 Regenerated Silk Biomaterials

Silk fibres can be dissolved by using a highly concentrated solution of chaotropic salts followed by dialysis to prepare an aqueous silk solution. Alternatively, ionic solvents have been used to dissolve silk fibres [12-14]. Silk aqueous solutions form gels during prolonged storage (months), particularly when silk concentration is high [15]. Hence, silk aqueous solutions are normally lyophilized for the purpose of

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storage and further processing. Lyophilized silk is amorphous in nature and can be reconstituted into dope using different organic [16-20] or acidic solvents [21]. A number of different regenerated products can be formed using silk fibroin aqueous solutions or reconstituted dope. The following forms of regenerated silk have been examined in recent years:

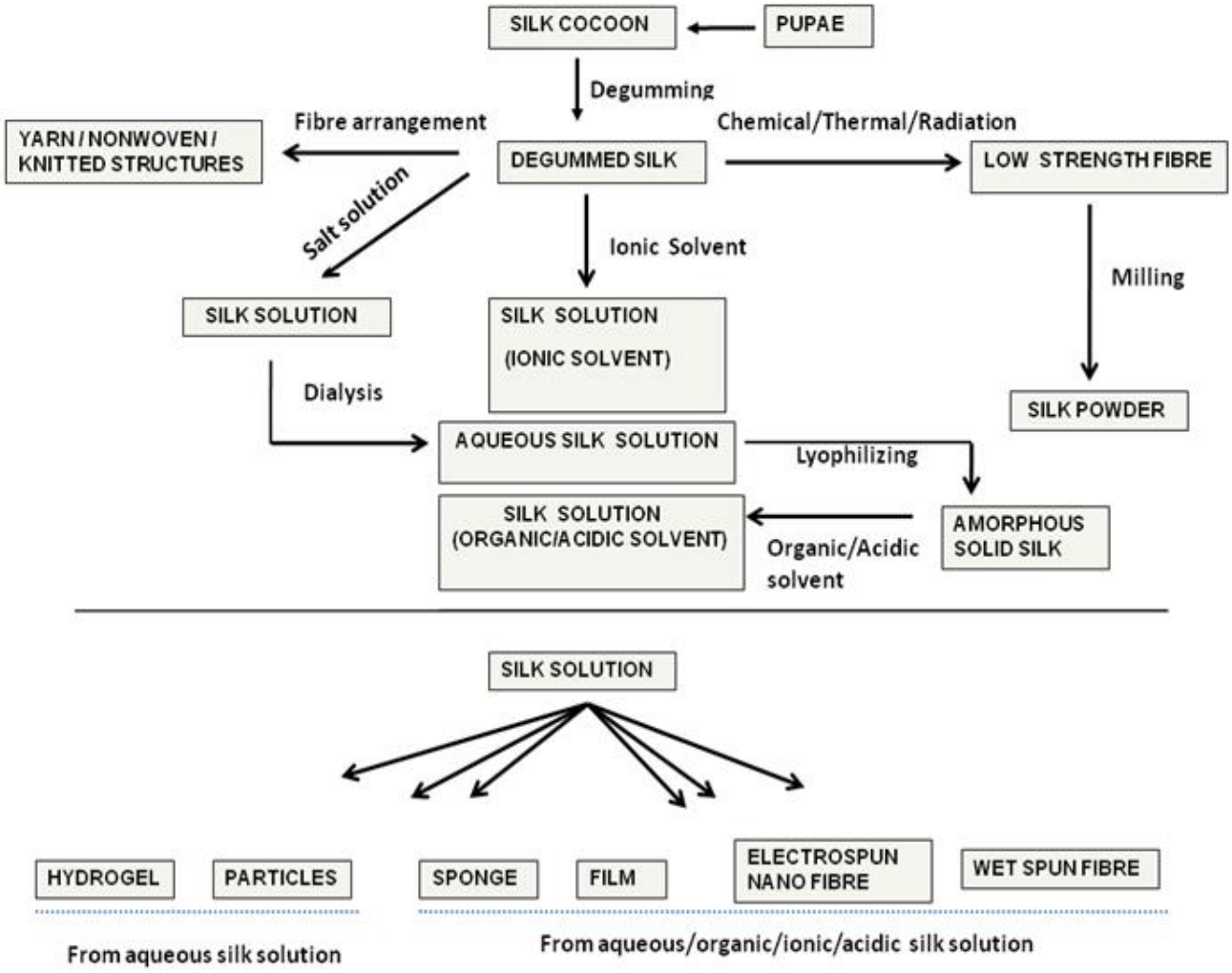

Figure 1 Process pathways for the different forms of silk biomaterial

2.2.1 Silk films

Silk fibroin films can be prepared by casting [13,21-24], spin coating [13], Langmuir-Blodgett (LB) [25] and layer by layer deposition [26-28]. Recently, patterned silk films have been developed as a cell supporting template for improved cell proliferation [13,29]. High oxygen and water vapour permeability of silk films is important for their wound healing applications [30,31].

2.2.2 Regenerated silk fibres

Electrospinning has been used to create silk nonwoven mats with a large surface area and a porous structure that are useful for cell seeding [32,33] and as separation membranes [34]. More recently, 3-D constructions of silk nanofibres have been used as blood vessel grafts and nerve guides [35,36]. Likewise, wet spinning of silk has been examined to produce silk fibres with physical and mechanical properties otherwise not available in its natural form. Producing regenerated silk with properties comparable to native silk fibres has been a major challenge. However, in recent years, it has been shown that by appropriate post spinning drafting, regenerated silk fibres having superior mechanical properties could be produced [37]. Likewise, it was also demonstrated that by optimising the rate of coagulation during extrusion and through post spinning stream annealing of extruded fibres, the

fibre properties could be significantly improved. Fibres which are finer and tougher than native silk have also been prepared [38].

2.2.3 Silk hydrogel

Hydrogels are three-dimensional polymer networks useful for encapsulation and delivery of biologically relevant agents. Silk hydrogels can be formed through sol–gel transition of aqueous silk fibroin solutions in the presence of acids, dehydrating agents, and ions [39-42]. Recently, silk hydrogels have been formed using ultrasonic energy [43].

2.2.4 3-D porous scaffold

Porous 3-D sponges are ideal structures for tissue engineering scaffolds as they provide an environment quite similar to the *in-vivo* microenvironment for cells to grow into various tissues. Silk 3-D porous scaffolds have been prepared using freeze drying, porogen leaching, and solid freeform fabrication techniques [44-48]. Due to the success in achieving good control over porosity and pore size, the development of porogen leached 3-D silk scaffolds has continued during the last few years for a number of tissue engineering applications, predominantly for bone and cartilage applications [49-57].

2.2.5 Silk particles

In the past, silk particles were prepared from aqueous silk solutions either by dehydration followed by pulverisation [58] or by spray drying [59,60]. In order to control the particle size and morphology and also to further reduce particle sizes, new techniques have been examined in recent years. These include self assembly [61-63], phase separation [64,65], or rapid expansion of supercritical fluid solution.

3. Biologically relevant properties of silk biomaterials

In addition to the ability to fabricate diverse morphologies, studies in recent years have confirmed that the following properties of silk biomaterials are important for their applications as advanced biomaterials.

3.1 Slow and tuneable biodegradation

Silk is a biodegradable material. The rate of biodegradation of silk depends on its structure and morphology. For example, Arai *et al.* [66] demonstrated that native silk fibres degrade slowly and the degradation depends on the diameter of the silk fibre [67]. Wang *et al.* [68] examined *in-vivo* degradation behaviour of silk porous scaffolds. Their studies revealed that by changing the processing method (aqueous vs. organic solvent) and processing variables (silk fibroin concentration and pore size in scaffolds), short-term (up to 2 months) and long-term (up to 1 year) *in-vivo* degradation could be altered. Other studies on the degradation of silk showed that processing solvents [24,46] and the annealing methods [69] could also influence biodegradation of silk. These studies indicate predictable degradation behaviour of silk which is highly desirable for biomedical applications.

3.2 Biocompatibility

All non-autologous biomaterials result in some foreign body response when interacting with living tissues. Despite their longstanding use as suture materials, including the successful embolization of cerebral arteriovenous malformations [70, 71], some adverse immunological events have been associated with silk proteins. Such problems are now linked to the presence of silk gum (sericin) [72] or one or more non fibroin components, which can be removed while preparing silk based biomaterials [73]. There are still some concerns that fibroin itself may also cause delayed hypersensitivity, but admittedly only in rare cases [74]. Multiple studies with silk biomaterials, such as fibres [75], micro-fibrous meshes [8], nonwoven mats [8,76], films [25,77,78], hydrogels [42], and 3-D sponge [68], have shown only mild foreign body response in some cases when studied *in-vitro* [8,25,42,72,75,77,79]. Similarly, cytotoxicity was found to be very low in animal model studies [8,68,76,78]. Overall, there has been widespread acceptance that properly degummed and sterilised silk products are biocompatible and comparable with other commonly used biomaterials, such as polylactic acid (PLA) and collagen [72,80]. Some studies even suggest that, compared to collagen coatings, silk coatings have superior ability over synthetic biomaterials to reduce the expression of pro-inflammatory cytokines, like IL-1β, TNF-α, or TGF-β1 [81,82], and also reduce thrombogenesis [83].

4. Targeted biomedical applications

4.1 Wound healing and tissue engineering

In 2000, Sugihara *et al.* [84] showed that wound healing was much faster with silk films than conventional hydrocolloid dressing (Duro Active). Their histological findings revealed greater collagen regeneration and less inflammatory response in the case of silk. Other silk materials, such as nonwoven mats [76], hydrogels [85], and electro-spun silk fibroin mats [86] have also shown promises as wound healing materials. In recent years, silk has been widely studied for tissue engineering applications. Host cells are pre-seeded in scaffolds followed by implantation in the damaged site of the body. Silk scaffolds have been extensively used for the regeneration of musculoskeletal tissues, such as bone and cartilage with promising results [87-89]. For example, Meinel *et al.* [53,55] showed that silk 3-D scaffolds with pre-differentiated (osteogenic) hMSCs could support healing critical size femoral segment defects and calvarial critical sized defects in nude rats and mice, respectively. 3-D scaffolds exhibited good osteogenic potential, almost bridging the defects with new bone after 8 weeks and exhibited good load-bearing capabilities and torque when compared to their other experimental and control groups [53]. Incorporation of BMP-2 in scaffolds could further accelerate the healing of bone defects in animals with or without pre-differentiated hMSCs [54]. In addition, 3-D porous scaffolds showed promising results for cartilage tissue engineering [9,56,57,90] and generation of bone and cartilage at the same time [91].

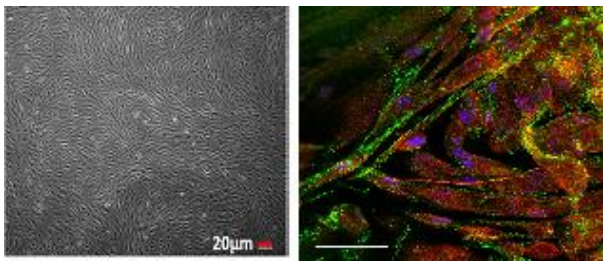

Figure 2 Human Tympanic membrane keratinocytes growth on silk fibroin membrane: (i) Light microscopy image of day 18 [99]; (ii) Confocal microscopy image of cells immunofluorescently stained with occludin (green) and ZO-1 (red). Cell nuclei are counterstained with DAPI (blue) [100].

Other tissue engineering studies with silk for repair of specific defects include regeneration of ligament [7,11,92], peripheral nerve [79], skin [93,94], cornea [29,95], ear drum [96], and trachea [97]. Recently, Lovett *et al*. used silk fibroin for the construction of microvascular grafts [98]. Tissue engineered silk sling for the treatment of stress urinary incontinence has also been reported [10]. Silk membranes have been used for the growth of human tympanic membrane (TM) kerotinocytes [99]. Figure 2 presents images of TM keratinocytes grown on such silk films.

4.2 Immobilisation and delivery of drugs/enzymes

Mild aqueous processing of silk is advantageous for loading sensitive drugs without affecting their functions. For example, Lu *et al*. studied glucose oxidase, lipase, and horseradish peroxidise entrapped in silk films and found that for over 10 months, enzymes retained their significant activity, even when stored at 37^0C, and in the case of glucose oxidase, they did not lose any activity [101]. Release kinetics of drugs and enzymes is reported to depend, to a large extent, on the content of crystalline β-sheets [28,61, 102,103].

Silk has been used for the control release of specific drugs for targeted clinical needs. Uebersax *et al.* [104] and Szybala *et al.*[105] used silk matrices as adenosine-releasing bioincubators that may be useful in the management of epilepsy. Recently, Pritchard *et al*. demonstrated that slow (up to 2 weeks) and linear release of adenosine was possible by controlling the thickness and cystrallinity of silk coatings [105]. Bayraktar *et al.* [106] used aqueous silk solutions to coat theophylline tablets. It was shown that slow release by multilayered silk coatings could enhance the efficacy of delivering emodin, an anti breast cancer and anti tumour drug [107,108]. Numata and Kaplan have recently reviewed silk-based multi-block copolymer systems as vehicles for sustained release of small molecule drugs, proteins and genes [109].

Silk particles have also been examined for potential drug delivery applications. *Lorenz Meinel's* group reported encapsulation and release of horseradish peroxidase (HRP) using self assembled silk microspheres [61]. They also reported encapsulation of salicylic acid, propranolol hydrochloride, and recombinant human insulin growth factor I (IGF-I) in silk microspheres. Encapsulation efficiency of IGF-I was close to 100%. They also used BMP-2 and IGF-I loaded silk microspheres in silk porous scaffolds and obtained enhanced osteochondral tissue engineering outcomes [91].

Silk's ability to hold therapeutic enzymes can be combined with the change in optical response of silk to design biodegradable and chemically responsive implantable optofluidic devices for applications such as monitoring changes in blood glucose. Domachuk et al. incorporated hemoglobin into free-standing silk diffraction gratings and simultaneously immobilized enzymes to examine such prospects [110].

5. Recent advances in silk milling and applications of milled silk particles

Developments of silk particles for biomedical applications have been mostly restricted to bottom up approaches as discussed already. However, top down approach of particle preparation through milling has comparative advantages as it can avoid the use of chemicals and the long dialysis time associated with the bottom up methods. The silk powder thus prepared should enhance the application scope of silk based materials also.

5.1 Silk milling

Silk has a hierarchical structure with fibroin chains forming nano fibrils, bundles of which constitute the fibre [111-117]. This suggests an opportunity to defibrillate silk into nano fibrils and fine particles. However, silk being a strong viscoelastic material is difficult to mill into fine particles with a controlled morphology and a narrow size distribution. Hence, a very long milling time (up to 48 hours) is usually necessary to obtain maximum possible fineness of around 5 μm [118-120]. To overcome such problems, pre-treatments such as alkali degradation [118], exposure to high temperature [121], radiation [122,123], and steam explosion [124,125], have been used to improve the milling efficiency. New developments have been reported to improve fine silk powder production in recent years. Li *et al.* discussed preparation of nano silk particles through a multistep process which includes a special high pressure device and filtering large particles during the process [126,127].

We have recently examined a number of standard milling devices to prepare ultrafine silk particles from Mulberry and non-Mulberry silk fibres. Milling sequences using a combination of milling systems such as a cutter mill, a rotary mill, different forms of ball mills and an air jet mill were examined [128,129]. We observed that non-Mulberry silk fibres which have a more porous structure and lower strength could be milled more efficiently than mulberry silk [129]. It was shown that by appropriate selection of milling sequence and milling parameters, milling time could be reduced to about 6 hours without any pre-treatment. A final step using air jet milling could achieve dry silk powder with a volume based median particle size of less than 1 μm. Figure 3 shows images of such particles.

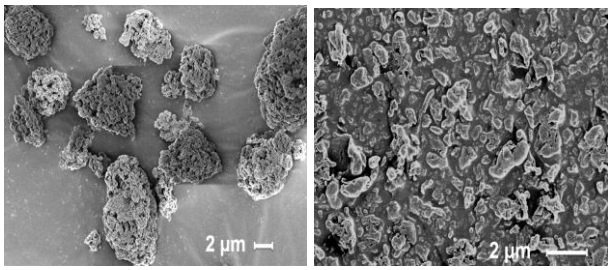

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Figure 3 Wet milled and spray dried silk particles (i) before and (ii) after air jet milling.

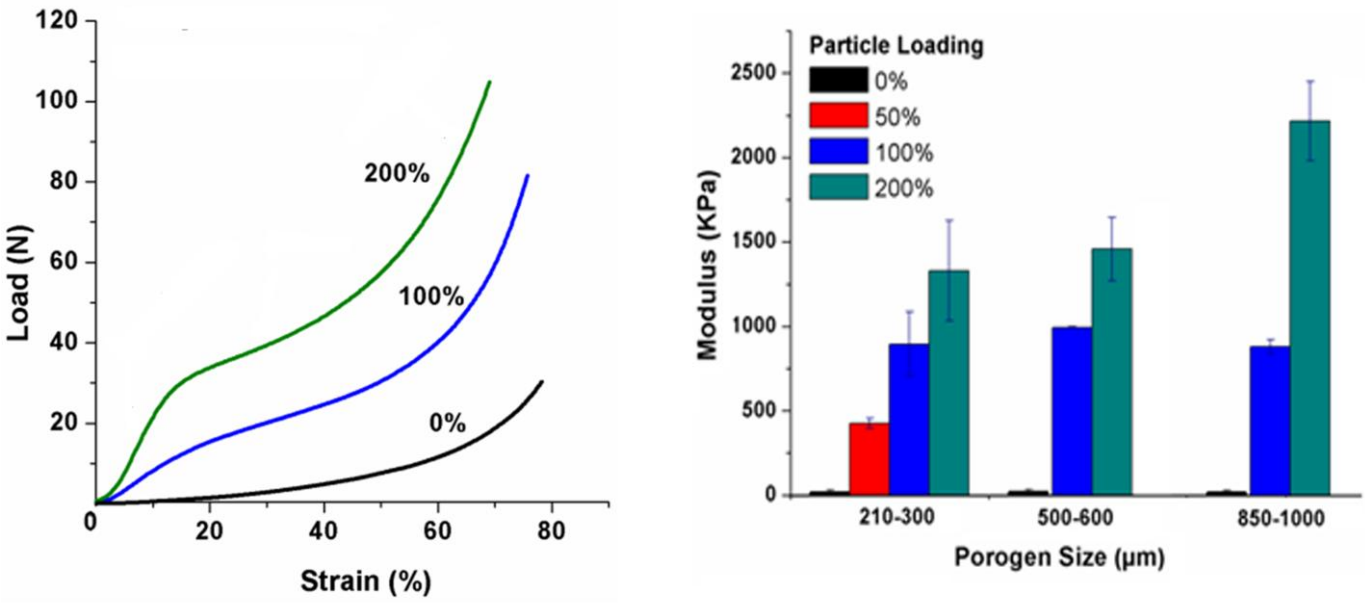
5.2 Silk composites with silk particles

Despite excellent biological outcomes, 3-D silk scaffolds are not strong enough for load-bearing tissue engineering applications. Kim et al. [49] reported that, usually, bone-like tissue ingrowth could be seen after 12 weeks of cell seeding in 3-D silk scaffolds. Such bonny tissue deposition could enhance the strength of scaffolds, but they were still much weaker (wet compressive modulus remained less than 200 kPa and yield strength less than 40 kPa) than the natural bone.

To increase the mechanical properties of silk scaffolds, milled silk particles were used as a reinforcing agent to fabricate a new silk-silk composite system [130]. SEM images of the composites are presented in Figure 4. The reinforcing particles maintained their crystalline structure in the composites [131], but unlike parent fibres, the particles are partially soluble in organic solvent, such as hexaflouroisopropanol (HFIP) [130]. This provides a high interfacial bonding between the particles and the bulk phase prepared using HFIP. This mechanism has resulted in a substantial increase in mechanical properties of silk particle reinforced silk composites (Figure 5). This novel silk processing method therefore provides a promising option for preparing new silk based biomaterials for hard tissue engineering applications. Further work is ongoing in this area in our laboratory in collaboration with Prof Kaplan's group at Tufts University.

(i) HFIP based, reinforced with 100% silk particles; (ii) water based, reinforced with 25% silk particles.

Figure 4 SEM images of 3D silk composite scaffolds



(i) Compressive load vs. strain curves (porogen size: 500-600 μm); (ii) Compressive modulus.

Figure 5 Compressive properties of silk composite scaffolds prepared from 17% HFIP-based silk solution reinforced with different % of silk particles[130].

6. Conclusion

Research into silk materials and their *in-vitro* and *in-vivo* performance for targeted biomedical applications has gained much ground over the last few years. This has resulted in unique innovations in silk materials developed for specific applications. In addition to silk fibres and solutions, milled silk particles are now expanding the application scope for silk based biomaterials.

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