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Silk proteins for biomedical applications: Bioengineering perspectives

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ABSTRACT

Biomaterials of either natural or synthetic origin are used to fabricate implantable devices, as carriers for bioactive molecules or as substrates to facilitate tissue regeneration. For the design of medical devices it is fundamental to use materials characterized by non-immunogenicity, biocompatibility, slow and/or controllable biodegradability, non-toxicity, and structural integrity. The success of biomaterial-derived biodevices tends to be based on the biomimetic architecture of the materials. Recently, proteins from natural precursors that are essentially structural and functional polymers, have gained popularity as biomaterials. The silks produced by silkworms or spiders are of particular interest as versatile protein polymers. These form the basis for diverse biomedical applications that exploit their unique biochemical nature, biocompatibility and high mechanical strength. This review discusses and summarizes the latest advances in the engineering of silk-based biomaterials, focusing specifically on the fabrication of diverse bio-mimetic structures such as films, hydrogels, scaffolds, nanofibers and nanoparticles; their functionalization and potential for biomedical applications.

Contents

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1. Introduction

Fundamental and applied applications in biomedical engineering and healthcare integrate multidisciplinary fields to meet the demands for developing successful treatments. Diverse areas including cellular and tissue engineering, material design and synthesis, image processing as well as biochemical and biophysical characterization come together in an effort to maintain, improve, repair or replace biological function. One of the keys to advancing this field lies in the development of functional materials that can interact with and within biological systems. Such materials can be derived directly from nature, or synthesized in the laboratory in the form of synthetic polymers, metals, alloys and ceramics [1]. However, despite the remarkable potential and diversity of man-made synthetics, their applications have been limited by challenges including biocompatibility, biodegradability and bioresorbability. Due to the intrinsic advantages, natural materials have re-emerged as viable alternatives for such applications in biomedical engineering [2,3].

Natural materials for medical purposes have enjoyed a long and rich history. The use of nacre as dental implants by the Mayans and the utilization of animal sinews as sutures by the ancient Egyptians date back several thousands of years [4]. Within the broad spectrum of naturally derived materials, silks produced by silkworms and spiders stand out as a unique class of structural proteins. The inherent design simplicity of silk at the molecular level has often resulted in its classification as a "model biomaterial" [5]. Being members of a fibrous protein family, silks possess impressive mechanical strength, which make them widely suitable as biomaterials. Indeed, the journey of silks (silkworm silk in particular) in biomedical applications began with their use as sutures in wound treatment [6]. With excellent biocompatibility, absent or minimal immunogenicity [7,8], limited bacterial adhesion [9,10] and controllable biodegradability [11], this natural biopolymer has been found to be suitable for a variety of applications. Until recently, silks from silkworms have been widely reported in comparison to silks from spiders for biomaterial applications. Spider silks tend to display a wider diversity in composition owing to the multiplicity of their functions, including prey capture, sensing and serving as draglines. However, owing to the predatory and territorial nature of spiders, spider silks are difficult to obtain in large quantities and thus tend to be produced by recombinant DNA technology, which is still inefficient. Therefore, silkworm silks, with conserved properties and greater abundance, have found wider acceptance and applications.

Silks from silkworms are broadly classified into mulberry and non-mulberry silks depending upon food sources for the worms. The domesticated mulberry silkworm *Bombyx mori* (*B. mori*) is the most famous member of the family *Bombycidae*. The non-mulberry silkworms of Indian origin all belong to the family *Saturniidae*. These include *Antheraea mylitta* (*A. mylitta*), muga silkworm *Antheraea assamensis* (*A. assamensis*), oak silkworm *Antheraea pernyi* (*A. pernyi*) and *Philosamia ricini* (*P. ricini*) or *Samia cynthia ricini* (*S. cynthia ricini*). Irrespective of their sources (arachnids or insects), silk fibers are primarily composed of proteins associated with certain macromolecules such as polysaccharides and lipids [12]. The two primary proteins that comprise silkworm silk, fibroin and sericin, consist of 18 different amino acids: predominantly glycine, alanine and serine. In contrast, spider silk primarily contains glycine and alanine-enriched fibroin protein. Additionally, the amino acid sequences of silk proteins can vary from species to species, resulting in a wide range of mechanical properties. For example, the hexapeptide sequence Gly-Ala-Gly-Ala-Gly-Ser dominates the β-sheet regions of *B. mori* fibroin, while those of *A. pernyi* and *S. cynthia ricini* are primarily composed of repetitive stretches of polyalanine [13,14]. Details of the wide diversity of structure and chemical nature of silk variants have been extensively reported [15,16].

In previous years several reports have been published describing the use of silks in a large variety of applications. They have been used for the development of vehicles for the delivery of bioactive molecules, growth factors and signaling cues and to support adhesion, proliferation, and differentiation of cells leading to tissue or organ regeneration [17,18]. Several excellent reviews on silk as a biomaterial have been published in recent years [6,19–22]. In contrast to broader articles on the *material* itself and its properties, in this review, we outline the ways by which engineering and manufacturing technologies have been employed to develop versatile structures and morphologies for specific biomedical applications. The focus is on parameters for designing and fabricating different silk-based structures with controlled geometries and properties. These include recent strategies to physically and chemically modify the protein to enhance its versatility and scope. Given the extensive research on silkworm silk to date, this is the primary focus of this review. Some recent advances in adapting spider silk-derived biomaterials in modern therapeutics are presented in Tables 1 and 2.

Table 1
Recombinant production of spider silk proteins in different host systems.

Recombinant protein	Origin	Host
Major spidroin dragline types I – MaSpI Major spidroin dragline types II – MaSpII	*Nephila clavipes*	Mammalian [78] Yeast [197,198] Plant [199,200]
Flagelliform		Insect [201]
Dragline ADF-3 Dragline ADF-4	*Araneus diadematus*	Mammalian [78] Bacteria [202]

2. Properties of silk for engineering applications

Discussions on harnessing biopolymers such as silks for engineering applications begin with a look at the properties desired for such uses. Various parameters are typically considered during the selection, design and development of biomaterials for tissue engineering applications. These include, but are not limited to: (1) improved biocompatibility with minimal adverse reactions *in vivo*, (2) optimized physical properties, in particular mechanical properties mimicking those of target tissues (ranging from soft to hard, *e.g.*, skin to bone), (3) ease of development of topographic and morphological cues for various cellular architectures, (4) biodegradability with the production of non-toxic by-products and (5) control of diffusivity or mass transfer properties. Often, choices have to be made between selecting several or all of these parameters depending on the specific applications. Here, we elaborate on a few of these properties in the context of engineering silk-based biomaterials.

2.1. Biocompatibility

Silk-based biomaterials exhibit processing-dependent biocompatibility (process of extraction or purification) [6]. Issues of biocompatibility are visible in virgin silks used as suture material, with reactions ranging from delayed hypersensitivity to acute and chronic inflammatory processes [23]. However, in the absence of the silk sericin component (degummed silk fiber), fibers demonstrate a minimal inflammatory tissue reaction, which enables successful implantation and cell culture [24,25]. Degummed silk fiber may be incorporated directly into biomaterial architectures. Further processing steps enable the production of "regenerated silk fibroin solution" for an easier fabrication of biomaterials (Fig. 1). These processing steps require different strategies for different silkworm sources. For instance, in the case of *B. mori*, the degumming process results in the extraction of the water soluble protein sericin. The remaining material can efficiently be dissolved in lithium bromide (LiBr) resulting in a silk fibroin solution. In contrast, silk fibroin solution from *A. mylitta* cannot be produced efficiently with this protocol. Thus, silk fibroin of *A. mylitta* is usually obtained in higher yields from the silk gland. *In vitro* studies using silk fibroin films largely demonstrate the absence of significant macrophage spreading, which along with favorable infiltration of fibroblasts, indicate minimal inflammatory potential and a favorable degree of biocompatibility [26]. The absence of significant macrophage activation by degummed silk fibers corroborates previous observations that sericin-free fibers are immunologically inert and possess low inflammatory potential [27,28]. Interestingly, sericin by itself has also been observed to be minimally inflammatory [29]. When separated from silk fibers, solubilized sericin exhibits minimal macrophage response, suggesting that it may be the *co-existence* of the sericin-fibroin strands that evoke such responses. Thus, the use of silk as a biomaterial requires the separation of these two proteins, leaving the sericin and fibroin independently with favorable biocompatibility. As with most materials, incorporating physical cues such as micro-/nanoscale topography that mimic natural tissues favorably enhance biocompatibility of silk biomaterials. For example, surface topography and mechanical properties play a role in adhesion and differentiation of cells [30]. Surface morphology can be precisely tailored to specific properties through the use of chemical treatments [31]. Collectively, silk fibroin and sericin exhibit a variety of characteristics making them highly biocompatible.

2.2. Mechanical properties and degradability

Measures of strength and durability are derived from mechanical properties such as tensile strength and Young's modulus. These properties may be obtained by elongating silk fibers or *via* techniques such as nanoindentation [32,33]. Silk fibers exhibit high tensile strength, flexibility and resistance to compressive forces, which makes

Table 2
Biomedical applications of recombinant spider silk protein biomaterial.

Applications	Structural design
Engineering of cartilage or load bearing tissues	Hydrogels [203]
Adhesive fillers	Chimeric spider silk proteins [204]
Wound dressing	Films [69]
Enzyme immobilization	Films [74]
Drug delivery	Microcapsules [65] Genetically engineered block copolymers [188] Microparticles [205]

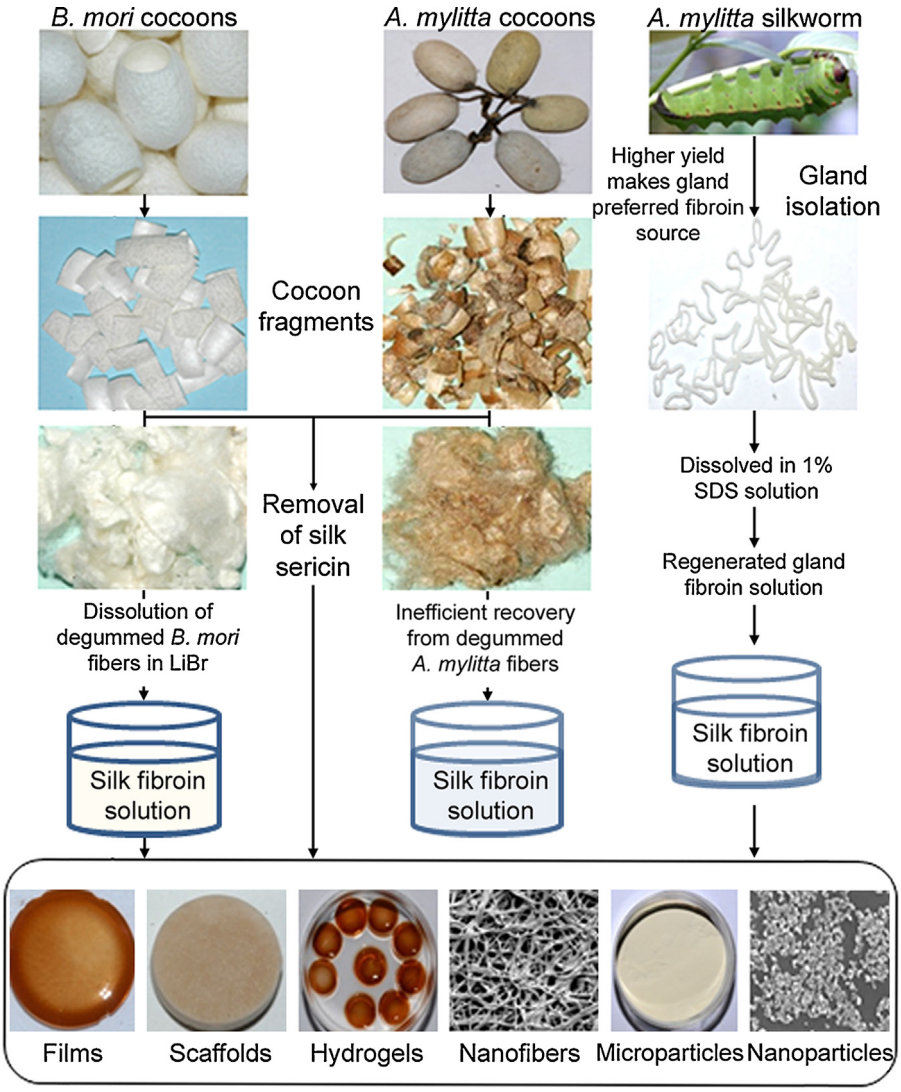

Fig. 1. Extraction of silk proteins leading to the structural design of biomaterials.

them suitable for applications requiring considerable tensile strength such as sutures or flexibility such as load bearing composites [25]. In addition, they possess unique properties including resistance to cumulative deformation. For example, *B. mori* silk fibers demonstrate a remarkable tensile strength of 0.5 GPa at an elongation of 15% [34]. Removal of the water-soluble sericin protein coat from the cocoon yields degummed silk fibers, which exhibit up to a 50% increase in tensile strength [35]. While the excellent mechanical properties of silk fibroin make it well-suited for load-bearing biomedical applications, the presence of a non-uniform cross-sectional area, along with micro-structural defects, typically decrease fiber reliability and reproducibility. Analysis of elasticity yields a range of moduli, which are best explained by considering differing cross-sectional areas [32]. A comparison of 'force reeled' silk fibers, where silk is collected from the silkworm directly during the spinning process, to cocoon fibers reveals that controlling the process of silk collection yields higher reproducibility in tensile strength [35]. This provides a strategy for avoiding the lower range of mechanical properties that make silk unsuitable for implant applications, which often demand precise deformation properties and compressive resistance against exterior stresses [36]. Another strategy to circumvent non-uniformity in fibers is developed using a step-wise twisting method, which enables single fibers to be bundled *via* sutures into larger 'building blocks' in a hierarchical geometry. This has been employed in anterior cruciate ligaments (ACL) replacements that require a viscoelastic material capable of resisting fatigue over extended time periods [25]. This technique of bundling effectively averages otherwise non-uniform mechanical properties by incorporating many fibers and provides an excellent example on how to effectively utilize fibroin in fibrous biomaterials requiring high tensile strength.

Mechanical properties can be further controlled by engineering the degradation of silk. Most commonly, this

degradation occurs due to proteolytic activity, yielding a loss of silk mass and tensile strength over time. The spectrum of degradation properties may range from tissue engineering applications, where the degradation rate of the scaffold is desired to be essentially proportional to the rate of tissue growth, to sutures requiring relatively static mechanical properties prior to complete dissolution [3]. For instance, controlled silk fibroin biodegradation advances implant technology and the creation of bio-interfaced devices by enabling flexible electrode arrays that can be resorbed over time [37]. For silk fibroin, chemical treatment techniques that delay protein degradation are associated with an increase in β-sheet crystallinity [38]. These changes in turn, favorably alter permeability of fibroin membranes to oxygen, as well as induce architectural changes such as a decrease in membrane density [39]. Varying crystallinity thereby provides diffusive control to small molecular species, which is important in precisely controlling drug elution in areas such as bioactive stent design [40]. The control of degradation therefore delivers control of the transport properties of silk architectures, providing a pathway for drug delivery and controlled release applications.

3. Enhancement of function by chemical and physical methods

While silk proteins offer many of the unique properties discussed in the above section, several applications have necessitated modifications to the native proteins to offer further avenues to engineer desired properties. Such changes can be achieved by tinkering with the intrinsic protein architecture itself, or utilizing the ability of other materials to form composite architectures with synergetic properties. While we discuss these in the context of chemical and physical methods to enhance silk function, often a combination of strategies is adopted to engineer specific properties in biomaterials.

3.1. Chemical modification strategies

Chemical functionalization can be employed to yield an improvement of existing properties (physical or biochemical), or to expand the library of potential functionalities. These can range from chemical *modification* of amino acid residues (Fig. 2) to *functionalization via* insertion of chemical species into silk. The diversity of chemical groups on silk proteins enables site-specific reactions, which allow the addition of unique chemical moieties. For example, the covalent decoration of silk fibroin fibers with the integrin-recognition sequence Arg-Gly-Asp (RGD) and parathyroid hormone (PTH) improve cytocompatibility of silk [41]. Interestingly, the RGD motif is naturally present in non-mulberry silk fibroins, making this modification applicable only to mulberry silks. Such alterations take advantage of the low abundance of aspartic and glutamic residues (2–3%). Upon activation with 3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS), these residues react readily with primary amines in RGD and PTH, allowing for chemical conjugation. The functionalized silk conjugate acts as an effective bone-inducing matrix *in vitro*, demonstrating higher osteoblast adhesion and overall calcification, when compared to other tested matrices. A method of arginine masking in fibroin (i.e. conversion to uncharged imidazolidinone) minimizes

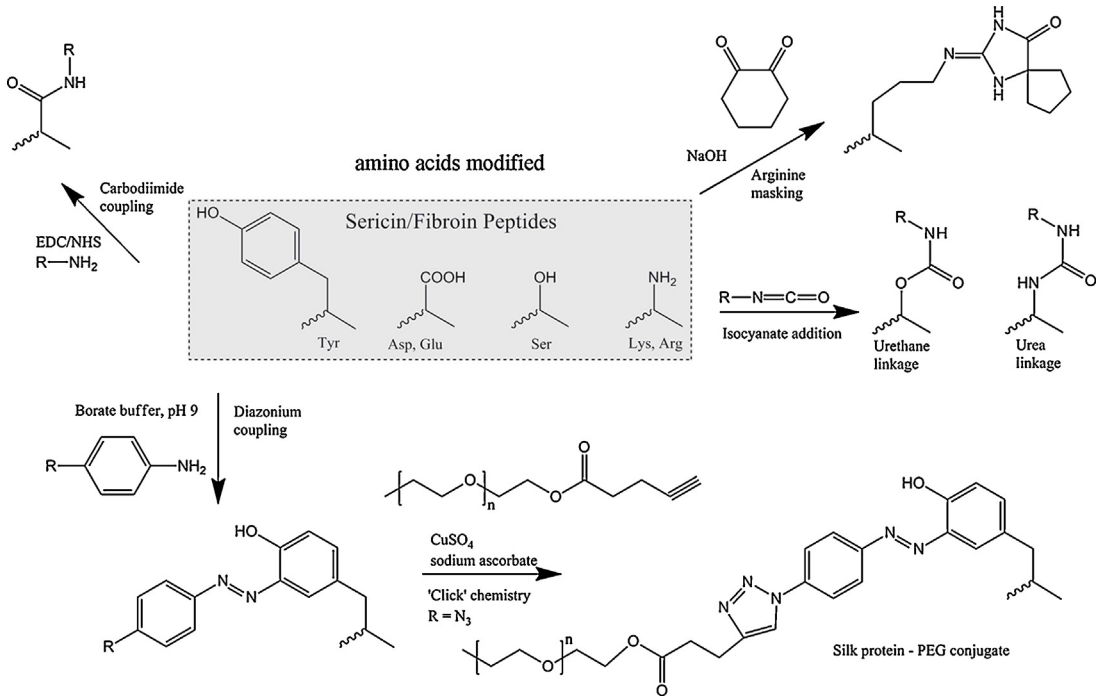

Fig. 2. Possible routes towards chemical modification of amino acid resides of the two silk proteins, sericin and fibroin.

the interactions of positively charged residues, potentially improving compatibility as cell culture substrata [42].

Concurrently, there have been a variety of modifications proven to *add* new characteristics to silk proteins. In tissue engineering applications, cell attachment onto a substrate or scaffold is notably dependent on surface chemistry. Conjugation between fibroin and poly(D,L-lactic acid) is achieved by employing a similar carbodiimide technique to that of RGD conjugation, utilizing EDC and 2-(N-morpholino) ethanesulfonic acid (MES), to react carboxylic acids with peptide amine groups. These poly (D,L-lactic acid)-silk fibroin conjugated scaffolds achieve a higher degree of attachment and proliferation of osteoblasts [43]. Reaction of silk fibroin with 2-methacryloyloxyethyl isocyanate provides a stepping stone for further modifications through the addition of terminal vinyl groups [44]. This technique has been used to introduce bone morphogenic protein-2 (BMP-2) onto fibroin scaffolds to induce osteogenesis, improving the versatility of silk fibroin scaffolds for bone tissue engineering [45].

By taking advantage of the relatively high number of tyrosine residues in silk fibroin (~5%), a tyrosine-specific diazonium coupling chemistry can be used to engineer the protein surface chemistry and hydrophilicity for proper cell adhesion and proliferation in cell culture scaffolds [46]. Tyrosine residues on fibroin can therefore be effectively converted into terminal carboxylic acids, amines, ketones, sulfonic acids, or short-chain alkyl functionalities. Human bone marrow-derived mesenchymal stem cells (hMSCs) attach, proliferate and differentiate on such modified fibroin scaffolds, yielding a cellular growth rate that depends on scaffold hydrophilicity. Expanding on this protocol, a terminal azide can be conjugated to tyrosine, enabling 'click' chemistry to be conducted on fibroin [47]. The high efficiency of click chemistry bioconjugation (88% conjugation of azide-containing tyrosine residues) is capable of yielding bio-hybrid fibroin-poly(ethylene glycol) (PEG) conjugate films. The PEGylation process reduces the nonspecific protein binding and cellular adhesion in addition to increasing the hydrophilicity of fibroin, thereby making the bio-conjugates better suited to biomedical applications.

The physical and chemical properties of sericin have also been altered, taking advantage of the abundance of nucleophilic hydroxyl-containing serine groups [48]. Bioactive proteins can be formed *via* conjugation of the amino acids in sericin with terminal iso-cyanate-containing groups, leading to the formation of urethane linkages with hydroxyls in the native protein structure (Fig. 2). It is important to note that major structural changes to the physical and chemical conformation of silks for the sake of improving one property may lead to deleterious effects in other areas. In such cases, it may be necessary to either optimize the modification with the property or design alternative strategies to circumvent these issues. For instance, methods to increase fiber yield have unintended effects of greatly reducing the ultimate tensile strength and elastic modulus. A workaround for this problem involves utilizing strategies such as pulsed laser irradiation during the fiber spinning process. This allows material properties to be tuned by taking advantage of the differential wavelengths of vibration of secondary structures [49].

3.2. Silk composites

An alternative and more widespread approach is the creation of physical blends of silk proteins with other materials to incorporate different properties into one composite. These composites with different architectures may be created by techniques such as self-assembly, co-polymerization forming interpenetrating networks, co-fabrication and direct inclusion. We confine our discussion to a few examples in this section as several of these blends have widespread applications and are discussed in subsequent sections. Self-assembly of nano-structured silk on poly(ε-caprolactone) (PCL) in conjunction with a ceramic scaffold was demonstrated in a high porosity, durable composite framework for bone regeneration [50]. The formation of the silk blend greatly increases the elastic modulus [25 MPa *vs.* 5 MPa for biphasic calcium phosphate (BCP) scaffold alone], the compressive strength (0.42 MPa *vs.* 0.07 MPa for BCP) as well as the biocompatibility. Cultured human osteoblasts indicate that the BCP/PCL–silk scaffold supports a greater extent of osteoblast attachment and differentiation over ceramic alone. Electrospinning of chitosan biocomposites with fibroin was used to improve the processing and stability over pure-chitosan nanofiber wound dressings. Such blends form highly porous, water-stable scaffolds for tissue re-growth with an ~10-fold increase in tensile strength over chitosan alone (10.3 MPa *vs.* 1.3 MPa), promoting the culture of fibroblasts [51] and hepatoma cells [52]. *In situ* polymerization of conductive monomers like pyrrole, aniline and 3.4-ethylene-dioxythiophene with fibroin enable the coating of silks with electrically-conductive layers, leading to potential use in nerve regeneration applications [53]. Mimicking the finding that natural systems harbor co-coordinating metal ions inside protein matrices, metal ion inclusion in silks has also been used as a method to increase tensile strength and stiffness by swapping hydrogen bonds with metal–organic coordinated bonds [54]. Infiltration of metal ions deeply within the silk using atomic layer deposition enhances amorphous regions. This results in an overall increase in both elastic modulus and maximum stress for treated silks, allowing for example, spider silk to be utilized in a greater diversity of applications.

4. Structural design of silk biomaterials

The versatility of silk protein processing allows the fabrication of a wide range of functional morphologies and architectures that take advantage of the inherent and engineered properties outlined above (Fig. 3) [55–58]. Native and engineered silk proteins obtained from different sources in nature including silk cocoons and/or glands of silkworms as well as recombinant spider silks from several hosts (Table 1) have been solubilized using water, organic solvents or high molarity chaotropic salt solutions to enable facile transformations to form diverse structures in all dimensions [21]. Here, we present a brief overview of

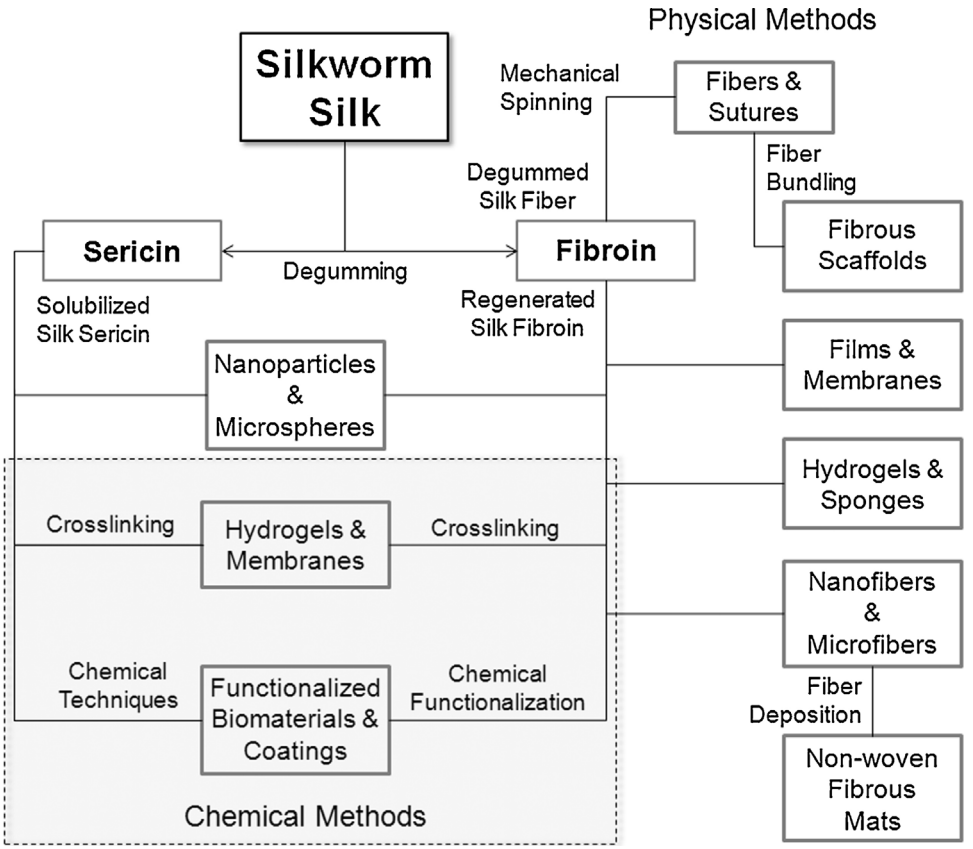

Fig. 3. A variety of physical assemblies and chemical approaches towards the formation of a diverse set of biomaterial architectures from the two silk proteins, sericin and fibroin.

specific silk architectures leading to strategic applications in the subsequent section.

4.1. Particulate architectures

Micro- and nanoparticles with highly controlled diameters are formed from homogenous mixtures of polymers and active agents and exist in a variety of geometries, including spheres, cubes and prisms [59]. These submicroscopic particles have unique properties including sub-cellular size, stability, high surface to volume ratio, high carrier capacity for the entrapment of bioactive molecules *via* enhanced drug solubility, as well as the ability to deliver them to target sites [59,60]. Microparticles may be fabricated by spray-drying [61] or using lipid vesicles as templates [62]. Microspheres from engineered spider silks have also been reported; low molecular weight protein MaSp1 of *N. clavipes* forms microspheres in water using a water-isopropanol solvent, in a process strictly controlled by the solvent environment [63]. Spheres of the recombinant protein, ADF-4 are generated by a salting-out process based on liquid-liquid phase separation by rapid emulsification of an aqueous solution of the protein in toluene [64]. This process is reversible and ADF-4 can be recovered by centrifugation, resulting in stable microcapsules 1–30 μm in diameter [65]. Techniques for forming nanoscale particles include emulsification, desolvation, coacervation and electrospray drying [59]. The cellular uptake of nanoparticles tends to be more efficient than that of microparticles. For instance, the uptake efficiency of 100 nm particles is 2.5× higher than that of 1 μm particles and 6× than 10 μm particles [66]. *B. mori* and *A. mylitta* silk fibroin nanoparticles (150–170 nm) are stable, non-toxic and can therefore act as sustained release delivery systems for bioactive molecules in 3D culture systems both *in vitro* and *in vivo* [67].

4.2. Two-dimensional architectures

Structures in two-dimensions (2D) primarily cover films, membranes and mats. Films are optically transparent 2D matrices typically prepared by casting and air-drying the aqueous or organic solvent-based silk protein solutions [68]. Once the solvent has evaporated, the films can be peeled off easily for further applications. Precise film thicknesses can be obtained by controlling the solution conditions, while modifications are achieved chemically (*e.g.*, cross-linking) or *via* solvent treatment [69]. Solvent treatments (*e.g.*, immersion in alcohols or kosmotropic salts, or water annealing) enable tuning of the ratio of silk I (α-helix) to silk II (β-sheet) secondary structural content [12,68,70,71]. Specifically, induction of the β-sheet crystalline state in fibroin significantly decreases water solubility, allowing for higher aqueous stability

of fibroin architectures. Such treatments are also able to influence degradation properties, mechanical properties as well as oxygen and water vapor permeability [68]. Methanol treatments are most frequently used for inducing crystalline architectures; however this transition yields brittle, optically opaque films. The resulting films also display hydration-dependent mechanics when hydrated, the brittle fibroin films are able to tolerate greater strain in comparison to dehydrated counterparts. Water annealing provides an alternative to this technique, to enable the production of flexible, optically transparent films [72]. The physical architecture of fibroin matrices can be scaled down to nanoscale-thickness (45 ± 5 nm) and can be prepared using a layer-by-layer technique, wherein the stability of the ultrathin films is due to the presence of significant hydrophobic interactions [73]. Dragline spider silk proteins ADF-3 and ADF-4 have also been used to generate transparent films (thickness from 0.5 to 1.5 mm) for biomedical applications by casting using hexafluoroisopropanol (HFIP) as solvent [69,74,75]. The water insolubility of these films is induced by kosmotropic salts (*e.g.*, potassium phosphate) or methanol treatment.

Silks exist in nature in the form of fibrous architectures. In order to utilize them for applications, microfibers (diameters on the order of several micrometers) and nanofibers (diameters less than 100 nm) can be formed using different strategies. Micro- and nanofibers can mimic native tissue, while providing topographical and biochemical cues for healing. Such fibers have been incorporated into 2D mats with a high surface area to mass ratio that provide strong mechanical properties [76]. From an engineering perspective, electrospinning is one of the most widely adopted techniques for fiber and mat formation owing to its versatility and flexibility. This highly scalable method produces nano- and microfibers of pure silk as well as silk composites [*e.g.*, silk fibroin blended with poly(ethylene oxide)] [76]. Concurrently, electrospinning has been employed to obtain fibers of spider silk from MaSpI protein analogs [77] and recombinant ADF-3 protein [78]. Smaller scale nanofibers may be prepared by techniques such as phase separation [79], self-assembly [80] or electrospinning [81,82]. Phase separation is based on the thermodynamic de-mixing of a homogeneous solution into two phases – a polymer-rich phase and polymer-poor phase. This is achieved by exposure of the silk solution to another immiscible solvent or by cooling of the solution [83]. Self-assembly on the other hand, occurs by the spontaneous hierarchical organization to higher order structures. This has the ability to form very fine fibers, which can often act as precursors to macroscopic silk fibers [84,85].

4.3. Three-dimensional architectures

Three-dimensional structures include hydrogel matrices and sponges that may be built using a wide variety of techniques. Hydrogels are chemically or physically cross-linked three-dimensional networks of hydrophilic polymers, capable of absorbing a large amount of water without losing their structural integrity. Hydrogels are advantageous over porous sponges or scaffolds because they provide benign environments for the entrapment of cells resulting in a homogenous cell distribution throughout the matrix [86]. The commonly employed approaches for silks include cell-laden hydrogels and self-assembled hydrogels. The beauty of the utilization of silkworm silk proteins for self-assembled gelation is that the silk solutions produced *in vivo* are stored within the silk glands in form of a gel. This gelation process can be replicated *in vitro*, by shearing, sonication, osmotic stress and heat or solvent treatment [87]. Silk fibroin protein is rich in glycine, serine and alanine [88], which results in the easy gelation of fibroin without the need of any gelling agent. On the other hand, the high water solubility of silk sericin results in poor stability in water, therefore requiring cross-linking during the design of hydrogels to enhance the stability of the matrix [89].

The stability of silk fibroin hydrogels primarily depends upon their β-sheet content as the gelation process is a result of β-sheet transformation [90]. At the onset, weak interactions between the protein chains occur without any change in secondary structure [91]. The rate of gelation is governed by parameters including the protein concentration, temperature and pH. An increase in protein concentration or temperature results in faster gelation while a decrease in pH toward the isoelectric point of fibroin ($pI = 3.8–3.9$) has the opposite effect [92]. Conversely, the effect of temperature and pH on sericin-based hydrogels is different, and varies with the nature of the composite partner in the hydrogel. Harsher extraction methods for sericin (like treatment with heat or urea) can cause degradation of the isolated products, resulting in reduced sensitivity to self-gelation. Chemical cross-linking using ammonium peroxodisulfate and tris(2,2′-bipyridyl) dichlororuthenium(II) increases the mechanical stiffness and stability of these hydrogels. Silk hydrogel matrices with controlled degradation properties can be developed such that breakdown *in vivo* occurs once a desired function is achieved. The rate of degradation of these stable hydrogels is controlled *via* labile bonds incorporated within the matrix. The bonds are susceptible to degradation under physiological conditions (chemical or enzymatic) and broken down, mostly by hydrolysis [93]. By controlling the rate of degradation, it is possible to affect timed-release of bioactive materials or to form scaffolds that disappear once the supported cellular architectures are fully formed. Silk fibroin-based hydrogels have proven useful in enabling osteogenic differentiation of human bone marrow stem cells [57] and effectively healing critical sized bone defects [7].

Porous sponges of silk fibroin can be obtained by utilizing porogens, gas foaming, and freeze-drying [19,94]. For lyophilization, degummed silk cocoons are dissolved in highly concentrated salt solutions, such as calcium chloride ($CaCl_2$) or lithium bromide (LiBr) and dialyzed to a 2–8 wt% silk fibroin aqueous solution. The pore architectures and sizes of porous sponges can be manipulated by controlling the protein concentration [92], freezing temperature [95] and salt leaching [94,96]. As an example of this salt-leaching technique, pore sizes ranging from 200–900 μm can be achieved using NaCl as porogens, where the pore sizes and porosity are solely controlled by the size and quantity of particles [94,96]. Gas foaming

is another method for the formation of sponges involving the addition of ammonium bicarbonate (NH_4HCO_3) into silk fibroin aqueous solutions followed by sublimation of NH_4HCO_3 in hot water [94]. The fibroin sponges thus formed are very stable and sterilization by autoclaving does not change the morphology or the β-sheet content even when heated to 120 °C. An added advantage is that unlike their collagen counterparts, 3D silk fibroin matrices can be sterilized using methods such as ethylene oxide [6], γ-radiation or 70% ethanol [6,45,97].

5. Applications of silk biomaterials

Following the consideration of material properties and diverse architectures that can be fabricated, we consider specific biomedical and bioengineering applications of silks. Paralleling the discussion of structures across dimensions above, applications ranging from two-dimensional cellular surface coatings to three-dimensional scaffolds and delivery vehicles are outlined. By presenting various examples, we establish the connection between application and the biofabrication (biochemical and physical) processes involved in forming specific morphologies. In contrast to the widespread availability of silkworm silk that allows extensive exploitation of these proteins both *in vitro* and *in vivo*, the use of natural spider silk-based materials is largely in its infancy (some key examples have been summarized in Table 2). The typically used fabrication strategies, the resultant architectures and the relevant length scales at which these tend to be applied are summarized in Table 3.

5.1. Two-dimensional coatings and films

The functionality and life span of biomedical devices and/or implants can be enhanced *via* various surface modification strategies including the development of biocompatible coatings. Silks, when used as coating materials, can influence the anticoagulant properties and cell compatibility of bio-devices [98]. Biocompatible coatings of silk fibroin have been shown to promote adhesion of human fibroblasts on polyurethane and polycarbonate urethane surfaces [76,77] and osteoblasts on multilayered hydroxyapatite-fibroin films [99]. Blends of silk fibroin with polyallylamine, a polycationic polymer widely used in biomedical and pharmaceutical industries, increase the processability while enhancing water stability [100]. Similarly, fibroin-carboxymethyl keratin blends provide improved anti-thrombogenic effects and cause lower inflammatory response in wound dressings, compared to traditional dressings [101,102]. Silk sericin also possesses anticoagulant activity [103] and has been reported for wound dressing [104].

Coatings and films of silk fibroin may themselves be coated with nanoparticles (*e.g.*, hydroxyapatite, metallic or polymeric) to enhance biocompatibility, for example to mouse fibroblast cell lines (L929) [105], mouse pre-osteoblast cell lines (MC3T3-E1) and human mesenchymal stem cells [106]. While fibroin films are typically used for their *in vitro* anti-bacterial effects [9], combinations with silver [107] or titanium dioxide nanoparticles [53] strongly inhibit *in vitro* bacterial attachment, leading to applications as bone implants, surgical coatings as well as wound dressings and cosmetics. Nanohydroxyapatite/fibroin composites Ti and alloy substrates can improve biocompatibility and guided bone formation [108].

Films of silk fibroin are capable of supporting cell growth in cancer cell lines with therapeutic implications. For instance, the chemotherapeutic impact of doxorubicin (DOX)-loaded silk films on primary tumor growth and metastasis were examined in mice using a humanized adenocarcinoma model. DOX-loaded silk films not only had a significantly greater primary tumor response than the equivalent dose administered intravenously, but were also not associated with any local or systemic toxicity [109]. Studies with the L929 fibroblast cell line demonstrate that silk fibroin, sericin and collagen films are equivalent in their ability to support cell attachment, physiological morphology and growth [68,110]. Silk films can also effectively induce the elongation of corneal fibroblasts with strong expression of corneal ECM including collagen V, decorin and biglycan [111]. Films made by vapor- and methanol-stabilized *B. mori* silk fibroin enhance the growth of osteoblasts and osteoclasts in both single as well as co-culture conditions as compared to PLLA films [112]. Porous fibroin films are able to be used in a biomimetic approach to replicate corneal stromal tissue architecture, enhance trans-lamellar diffusion of nutrients and cell–cell interaction [113] while supporting the ocular reconstruction using human limbal epithelial cells [114,115] and corneal endothelial cells [116,117]. Blending of fibroin with recombinant human like collagen [118] or functionalization with the RGD sequence [119] provides better response as compared to the individual components alone. The incorporation of the RGD motif in mulberry silk for instance, increases the production of mineral modules formed by Saos-2 cells [120].

Table 3
Typical fabrication strategies for silk proteins and architectures produced.

Technique	Typical architectures	Length scales
Self-assembly	Nanofibers, films, micro and nanospheres [80]	nm–μm
Electrospinning	Fibers, mats [51]	μm–mm
Casting, molding	Membranes, mats, micro needles [68,75]	nm–mm
Spray drying	Micro and nanospheres [61]	nm–μm
In situ polymerization	Fibers, hydrogels [53,87]	μm–mm
Freeze drying	Porous sponges [19,94]	μm–mm
Soft lithography, nano-imprinting, inkjet printing	Nanostructures, wires, photonic elements [122,194]	nm–μm

5.2. Micro-patterning and microfluidics

While typically architectures and morphologies tend toward macroscale applications for silk proteins, recent work at the micro- and nanoscales have produced exciting avenues. The incorporation of microscale physical and chemical features in micro-patterned surfaces provides a means to modulate surface topography to guide cell shape and function [121]. Cast silk fibroin creates optical-grade, high-quality films for use in cell culture applications and biomedical devices requiring optical transparency. This early approach used a simple modified soft lithography technique to crystallize fibroin solution under ambient conditions over a master pattern [122]. Once extracted, films with controlled properties and microscale geometries can be modulated to an appropriate degree for specific biomedical application. For instance, by incorporating methanol treatment or vapor annealing, these films can be rendered insoluble and mechanically strong.

Micro-patterned silk proteins have been used to create robust, micro- or nanoscale biocompatible silk biosensors and silk/metal biohybrids. Free-standing silk-metal patterns may be fabricated from purified fibroin using a transfer-based patterning technique [123]. Metal-transfer and fibroin casting is accomplished *via* standard lithography to deposit a desired pattern of metal on the substrate, enabling *in vivo* use as biosensor. Ti-based implants with micro-patterned fibroin may provide a means for enhancing bone formation. Precise control of surface topography is achieved through use of a patterned hydroxyapatite/silk fibroin composite [108]. The composite material is formed using a co-precipitation technique, followed by template-assisted electrospray deposition of the fibroin composite onto predefined templates to create a variety of biochemical micro-patterns. Silk fibroin-chitosan scaffolds with predefined microfluidic channels exhibit excellent structural property and promote uniform cell growth, making them potentially useful to engineer thick pre-vascularized organs [124]. Fibroin can be patterned into minimally invasive, painless micro-needles (500 μm length) for drug delivery applications [75]. In this process, solubilized silk fibroin is cast and dried over polydimethylsiloxane (PDMS) molds with deep channels to form silk microneedles. This technique enables loading of drug components, allowing the creation of antibiotic-impregnated fibroin films, which inhibits substrate colonization by bacteria. Degradation and drug diffusion kinetics can be controlled by modifying the secondary structure *via* water annealing (high β-sheet content renders the fibroin insoluble), leading to a 5.6-fold decrease in the release rate of post-processed films.

Coupled with micro-patterning approaches, there have been recent inroads into using silk proteins for the isolation of specific mechanical and chemical properties for small fluid volumes that can enable *in vitro* biological analyses in precise spatiotemporal environments. Silk-based microfluidic and micro-electromechanical systems (MEMS) devices are able to provide the required micro-environmental control of various physical and chemical attributes, while offering some of the distinct properties of silk fibroin. These include flexibility, biocompatibility, ease of processing, chemical conjugation and a low rate of biodegradation. Silk microfluidic devices are fabricated by casting silk fibroin solutions on microfabricated PDMS molds and delaminating cured films [125]. Along with micro-patterning, surface treatment with methanol increases hydrophobicity and stabilizes the films against aqueous environments. Such microfluidic devices are highly scalable owing to the bottom-up technique of laminating stacked micromolded fibroin layers in the presence of silk fibroin solution, with treatments enabling bonding between layers (under exterior mechanical pressure at 70 °C). Fibroin-based microfluidic devices show an enhancement in tensile strength and elastic modulus during mechanical tests, in comparison to often-used biopolymers like poly(glycerol-co-sebacate) (PGS) [125]. Cell culture studies with hepatocyte carcinoma cells indicate that silk fibroin-based materials perform similar to typical PGS films in cell growth rate, function and maintain silk's high degree of biocompatibility. Overall, these translations to the micro- and nano-scales are still relatively recent. Combining the chemical functionality and biocompatibility of fibroin with the scalability of bottom-up approaches in generating microfluidic devices has enormous potential in the creation of highly complex, implantable bioactive devices for potential biomedical uses.

5.3. Three-dimensional cellular substrata

One of the goals in tissue engineering is to mimic the natural 3D cellular environment, the extracellular matrix (ECM). The ECM acts not only a physical framework for cells but also as a complex, dynamic and critical environment that regulates cell adhesion, survival, proliferation, migration, and differentiation. The 3D tissue scaffolds tend to more closely mimic natural tissue complexity than 2D constructs as discussed above [126]. Such functional approaches to tissue engineering therefore primarily involve redesigning silk matrices through proper evaluation of structure and structure–functional relationships along with intrinsic evolutionary constraints [127]. A few examples of such adaptations are: (a) immobilization of RGD peptides to promote focal adhesion of seeded cells [128], (b) use of enzyme gradients to control cell and tissue outcomes [19], and (c) reinforced silk sponge matrices with silk microparticles to generate protein–protein composite scaffolds with desirable mechanical properties for *in vitro* osteogenic tissue formation [129]. As a scaffold material, silk is similar to collagen, the main component of the ECM. Silk based 3D matrices are attractive for load bearing tissue engineering applications because of their high mechanical and tensile strength [6,18]. The elasticity of the matrix can direct the differentiation fate of progenitor cells, specifically stem cells that are able to respond to the stiffness of microenvironments and mechano-transductional signals [130].

Since the compressive stiffness of silk is significantly lower than that of native bone, native silk is typically considered a suboptimal osteogenic material. To address this limitation, the fabrication of composites such as fibroin/hydroxyapatite (HA) [131], silk/silica [132] and silk protein/clay [133] have been proposed to form materials with suitable compressive stiffness. The cultivation of

hMSCs within fibroin/HA composite scaffolds under perfusion conditions results in the formation of bone-like structures with enhanced mechanical properties. Electrospun fibers of silk fibroin-poly(ethylene oxide) (PEO) blends provide an excellent substratum for human bone marrow stem cells (hBMSCs) [76]. Similarly, growth of hBMSCs on electrospun fibroin/PEO/BMP-2 nanofibrous scaffolds produce bone-like tissue, wherein incorporation of HA nanoparticles within this composite improves osteogenic outcomes [97]. Further, these silk fibroin matrices may be employed for human knee articular chondrocyte repair after treatment with microwave-induced argon plasma [134]. Electrospun poly(lactic-*co*-glycolic acid) (PLGA) nanofibers onto knitted silk microfibers designed for the release of basic fibroblast growth factor, initially stimulate *in vitro* stem cell proliferation and subsequently promote tendon repair with an increase in both type I and type III collagen expression [135]. Other modified cell substrata include electrospun nanofibrous silk fibroin/chitin blends [136], fibroin fibers coated with ECM proteins (*e.g.*, collagen type I, fibronectin, and laminin) [137] and silk fibroin-collagen scaffolds that act as a base for human embryonic stem cell-derived mesenchymal stem cells (hESC-MSCs) for tendon and ligament tissue engineering [138,139]. Composite nanofibrous membrane of carboxyethyl chitosan/poly(vinyl alcohol) with embedded silk fibroin nanoparticles are useful in skin tissue engineering applications, wherein intermolecular hydrogen bonding provides the stiffness to the nanofibers for wound dressings and skin regeneration.

The generation of surfaces with variable adhesive characteristics has also received a lot of attention. Native silk fibers (*i.e.*, silk fibers containing both fibroin and sericin without any post-processing) typically have low compatibility with blood due to strong adhesive properties toward platelets. The compatibility of the degummed silk fibers can be increased using treatment with poly-2-methacryloyloxyethyl phosphorylcholine (MPC), which markedly decreases platelet, macrophage and monocyte adhesion [44]. In contrast, for other applications it may be important to enhance cell adhesion. For example, when the native fibers are partially solubilized by formic acid or salts like calcium chloride, non-woven mats are produced, which greatly promote the proliferation of fibroblasts, keratinocytes, osteoblasts, and carcinomas including epithelial cell lines of lung, colon and cervical cancers [19].

It is also possible to take advantage of species specific properties of silk across different natural sources. As discussed above, woven mats modified with the RGD sequence can further enhance cell adhesion on such structures [58]. Since non-mulberry silks naturally possess RGD motifs [140], silk glands as well as cocoon fibroins from *A. mylitta* have recently been extensively characterized for direct use as cell substrata for mammalian fibroblasts and cardiomyocytes. *A. mylitta* fibroin is superior compared to *B. mori* fibroin with regards to cellular responses like metabolic activity, attachment, cytotoxicity and proliferation [55,141,142]. Other demonstrated approaches employing culturing cells on non-mulberry silk proteins include using silk fibroins of *A. pernyi* (hBMSCs) [143], *A. assamensis* (human lung carcinoma cells) [144] and *P. cynthia ricini* (hBMSCs). Films fabricated from hope-sericin, a new mutant strain of silkworms, also behave like fibrous materials when hydrated and exhibit minimal cytotoxicity [145]. Silk of *B. mori* supports the regeneration of cartilage [128,146–148], adipose tissue [149] and the bladder [150]. The 3D fibroin scaffolds from non-mulberry silkworms like *A. mylitta* are shown to be promising materials to promote regeneration of tissues such as cartilage [151] and adipose [152], while *A. pernyi* silk fibroin scaffolds promote nerve regeneration [153]. Scaffolds of both mulberry and non-mulberry fibroin have also been used for multicellular culture models of human tumor tissues [154,155]. The nanofibrous composite scaffold of PVA and *A. pernyi* silk fibroin is well established for tendon tissue engineering [156,157].

Concurrently, sericin of *A. mylitta* cocoon and gelatin-blended films can be used as substrata for feline fibroblast cells (AH 927) [29]. Films of silk sericin extracted from the silk glands of *A. mylitta* without using any chemical cross-linking show low inflammatory response toward macrophages, along with improved cell attachment and spreading [120]. *B. mori* sericin can be used as a media additive promoting the growth of several human cell lines and mouse hybridoma [158], as substrata for a human cornea epithelial cells (HCE-T) [159] and for rat embryo epithelial cells [160].

5.4. Implant applications

Following the discussion of *ex vivo* growth of cells on silk architectures, we focus specifically on applications where the structures are *directly* in contact with the body in the form of implants. Historically, as well documented, one of the oldest biomedical applications of a silk-based biomaterial was as a suture for wounds [18]. These days however, the use of silk sutures is limited to eye, lip and intraoral surgery. To enhance mechanical properties and reduce the fraying tendency of silk fibers, commercial silk sutures are typically coated with wax or silicone and termed as black braided silk (*e.g.*, Perma-Hand™) [6]. Wet-spinning of silk in formic acid or a solution of formic acid blended with 50 wt% poly(vinyl alcohol) improves the mechanical properties of the suture [161]. Silver nanoparticles may be coated on the suture to enhance the antimicrobial properties of the composite [162].

Silk fibroin films are also used for *in vivo* wound healing, wherein the healing rate is observed to be faster in skin wounds of rats with a lower inflammatory response compared to traditional porcine-based wound dressings [101]. Blending of silk fibroin with chitin (chitin:silk 75%:25%) as a wound dressing, improves the adhesion of both keratinocytes and fibroblasts [136]. Electrospun matrices of collagen and silk may be used for instance, to support human keratinocytes [163]. Other implantable applications of silk fibroin include cruciate ligament repair [25,146], tendon replacements [156], knee meniscus grafts [164] and for the repair and regeneration of buccal mucosa [165]. Silk nerve conduits are viable candidates to compete with commercialized collagen nerve conduits [166]. Fibroin blood vessel constructs are preferred over commercially available

polyurethane (PU) and poly(tetrafluoroethylene) (PTFE) vessels due to larger diameters and higher mechanical strength [167]. It is important to vascularize implants while simultaneously investigating the interactions of the material with endothelial cells. The response of endothelial cells with respect to adhesion, proliferation and retention on both micro- and nanofibrous silk fibroin scaffolds is promising [168]. This fundamental knowledge in vascular development is further exploited by the fabrication of porous tubular structures of silk fibroin nanofibers [169]. The co-culture of human aortic endothelial and coronary smooth muscle cells on such constructs has therefore resulted in successful development of small diameter vascular grafts [10].

5.5. Delivery vehicles

Architectures of silk are ideal as vehicles to transport and deliver bioactive molecules across boundaries. As evident from the discussion above, 2D and 3D architectures of silk used in some applications also tend to be useful for others, with overlap of properties and processing. Control of the diffusive characteristics of these 2D (such as films) and 3D structures (such as particles) is based on modulating features including pore sizes, crosslinking as well as degradation. All of these changes directly influence transport properties of the material both in terms of storage and release. Aqueous-based silk fibroin films promote long term adenosine release from adenosine kinase deficient embryonic stem cells [170] and site specific drug delivery in brain astroglial cells [171]. These films stabilize glucose oxidase, lipase and horseradish peroxidase for over 10 months, allowing them to be stored at 37 °C without significant loss in enzyme activity, thus performing well as glucose and bio-photosensors [172]. Silk–polyurethane–heparin composites effectively demonstrated as long-term sustained release system of heparin for anti-thrombogenicity [52]. Silk–hydroxypropylmethyl cellulose (HPMC)–polyethylene glycol (PEG) blended films present effective vehicles for transmucosal delivery [173] and silk/chitosan blends (cross-linked with glutaraldehyde) may be employed for controlled release of various low molecular weight drugs like ampicillin trihydrate, dichlofenac sodium, salicylic acid and theophylline [174]. Silk fibroin based-nerve conduits discussed above can also be used for growth factor delivery to promote peripheral nerve repair and vascular grafting [175,176].

Particulate architectures of silk fibroin (500 nm to 2 μm in diameter) have also been demonstrated to be vehicles for controlled release. The loading of drugs in nano- or microparticles is carried out by adsorption or covalent coupling during the fabrication or pre-fabrication process [177]. This process regulates drug stability and provides the biological potency and release time [178,179]. Administration is performed intramuscularly, subcutaneously, orally or intra-nasally to release the drug by reaching the target site [180]. The rich content of serine and glycine in silk protein sericin provides a higher content of hydroxyl groups allowing easy cross-linking and co-polymerization of drugs [181]. Composite silk sericin nano-and microparticles of methacrylate are used for the delivery of drugs and genes [182] as well as the immobilization of chemotherapeutic agents like L-asparginase [183]. Composites with polyethylene glycol (PEG) may be considered for transmucosal drug delivery [184]. Hope-sericin matrices also act as drug delivery systems [145], wherein the release behavior of sericin matrix is controllable by enzymatic degradation [185]. *A. mylitta* microenvironments can further be utilized to investigate growth factors, signaling peptides and anticancer drugs [154]. The micro-modeled system of *A. mylitta* silk fibroin improves fibroblast-biomaterial interaction and prolongs the release of drugs when incorporated within calcium alginate beads [181]. Hydrogel-based controlled delivery may employ blending of polymers such as chitosan [174], or polyacrylamide [186] with silk to improve sustained release [187].

In combination with scaffold technologies discussed above, the sustained release from porous 3D silk fibroin matrices includes delivery of growth factors [146] such as bone morphogenetic protein-2 (BMP-2) to induce osteogenic differentiation both *in vitro* and *in vivo* [188], insulin-like growth factor I (IGF-I) for cartilage repair [189], VEGF [8] and BMP-2 to reconstruct irregular bony cavities [190], adenosine for refractory epilepsy treatments [182,191] and monoclonal antibodies for therapeutic purposes [192]. Silk fibroin-derived polypeptides (FDPs) could potentially have a significant impact on cell delivery for tissue engineering and bone regenerative therapy [193].

6. Conclusions and future directions

In summary, we have discussed a number of perspectives of this amazing natural biopolymer as they pertain to specific applications in the bioengineering arena. The unique structure, biocompatibility, diversity in morphologies, options for genetic engineering, ease of sterilization, thermal stability, surface chemistry for facile chemical modifications and controllable degradation, all make it extremely promising for many clinical and biomedical functions. A variety of physical and chemical avenues for the adaptation of silks for different applications have been outlined (Fig. 3). With the advancement of engineering strategies including new processing techniques capable of spanning length scales, new and innovative silk protein-based constructs are being developed. These include recent exciting advances in implantable optics, photonics and electronics [194–196]. There is still the need to address some critical challenges including developing advanced micro- and nanofabrication strategies, controlled biodegradation and surface engineering. Biodegradation rates of silk materials can be better engineered to match the re-growth of new tissue. Surface engineering strategies need to be optimized to lower the graft rejection of silk-based constructs in clinical applications. The structural differences among silks obtained from different natural sources make this immune-specific modulation of surfaces a challenge. Hence, universal modification approaches are greatly desirable. Challenges in our understanding of tissue micro-environments, batch-to-batch variation of this natural polymer and the lack of suitable quantitative and qualitative *in vivo* analyses are some of the issues that presently restrict the direct clinical trials of numerous

promising silk-based products. The development of viable strategies for the long-term *in vivo* evaluation of modified and/or un-modified silk protein micro-architectures is paramount in translating this promising biomaterial to clinical and bedside products.

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