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Review

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Coatings and Films Made of Silk Proteins

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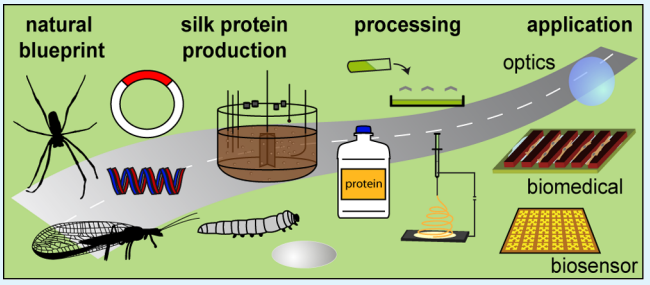
ABSTRACT: Silks are a class of proteinaceous materials produced by arthropods for various purposes. Spider dragline silk is known for its outstanding mechanical properties, and it shows high biocompatibility, good biodegradability, and a lack of immunogenicity and allergenicity. The silk produced by the mulberry silkworm *B. mori* has been used as a textile fiber and in medical devices for a long time. Here, recent progress in the processing of different silk materials into highly tailored isotropic and anisotropic coatings for biomedical applications such as tissue engineering, cell adhesion, and implant coatings as well as for optics and biosensors is reviewed.



KEYWORDS: *spider silk, silkworm silk, processing, biomedical application, biosensor, optics*

1. INTRODUCTION

Silks, like keratins and collagens,[1−3] are based on a class of structural proteins with highly repetitive amino acid sequences. The proteins are stored in a soluble state and are assembled into solid extracorporeal fibers when sheared or "spun". Humans have exploited silkworm silk (from *Bombyx mori*) for millennia, primarily for textiles. Silks produced by spiders have also been used for centuries, for example by Polynesians for fishing and by Romans and Greeks as wound dressing and sutures.[4,5] Because of the outstanding mechanical properties of spider silk compared to other synthetic and natural fibers,[6−10] their biocompatibility and good biodegradability and lack of immunogenicity and allergenicity, many more technical and biomedical applications are conceivable. Importantly, unlike in nature, spider silk proteins (either regenerated from silk fibers or recombinantly produced) can also be technically processed into nonfibrous morphologies. Here, we highlight recent work on the processing and applications of proteins derived from spider silk (e.g., *Araneus diadematus*, *Nephila clavipes*), mulberry silkworm (*B. mori*), and lacewing silk (e.g., *Chrysopa carnea*, *Mallada signata*, and *Chrysopa flava*) into β-sheet-rich coatings and films.

1.1. β-Crystalline Silks. Almost all arthropods can produce silk, each with a specific structural feature (e.g., helical, coiled-coil, β-sheet, etc.) tailored to specific purposes. This review focuses on β-crystalline silks, which are produced by larvae of mulberry silkworms (*B. mori*), lacewings (*Chrysopidae*), and orb weaving spiders (*Araneae*).

1.1.1. Silkworm Silk. *B. mori* silk has been well characterized, and there exist numerous reviews on its properties[6,11] and applications.[12−14] During metamorphosis, silkworms produce silk cocoons for protection. The silk fibers are composed of two silk fibroins (SF), the heavy chain (325 kDa) and the light chain (25 kDa), which are connected by a disulfide bond[15] and complexed by the small glycoprotein P25 (30 kDa).[16] The proteins have been thoroughly investigated and reviewed.[17−19] The main structural elements of this material are repeats of the GAGAGS motif, which forms antiparallel β-sheet structures because of intra- and intermolecular hydrogen bonding.[20] The fibers are coated with the glue-like glycoprotein sericin, which has to be removed (degumming)[21−23] prior to processing for use in medical applications because it can cause immunoreactions.[24−27] It is advantageous that silkworms can be reared in captivity, and the silk can be obtained in great quantities.

1.1.2. Lacewing Silk. To protect their eggs from predators, female lacewings lay their eggs on the ends of silk stalks attached to substrates such as the lower side of leaves. These fibers show unusual bending stiffness based on the structural features of the underlying silk proteins (see also section 1.1.4.).[28] The amino acid composition[29] and the cross-β-structure[30] of egg stalk silk were first described in the 1950s. Fifty years later, researchers from Tara Sutherland's group analyzed the lacewing silk of *M. signata* and identified two proteins: MalXB1 (109 kDa) and MalXB2 (67 kDa). MalXB1 is the main component of egg stalk silk and comprises a serine-, alanine- and glycine-rich tandem repeat.[31] In another lacewing species (*C. carnea*), at least five individual proteins were identified in the egg stalk silk dope.[32]

1.1.3. Spider Silk. Web spinning spiders (*Araneae*) are probably the best-evolved β-crystalline silk producers with the most specialized silk fibers. Female orb-weaving spiders, such as *Nephila clavipes* and the European garden spider *Araneus diadematus*, can produce up to seven different types of silk with task dependent properties.[33] There exist numerous reviews and

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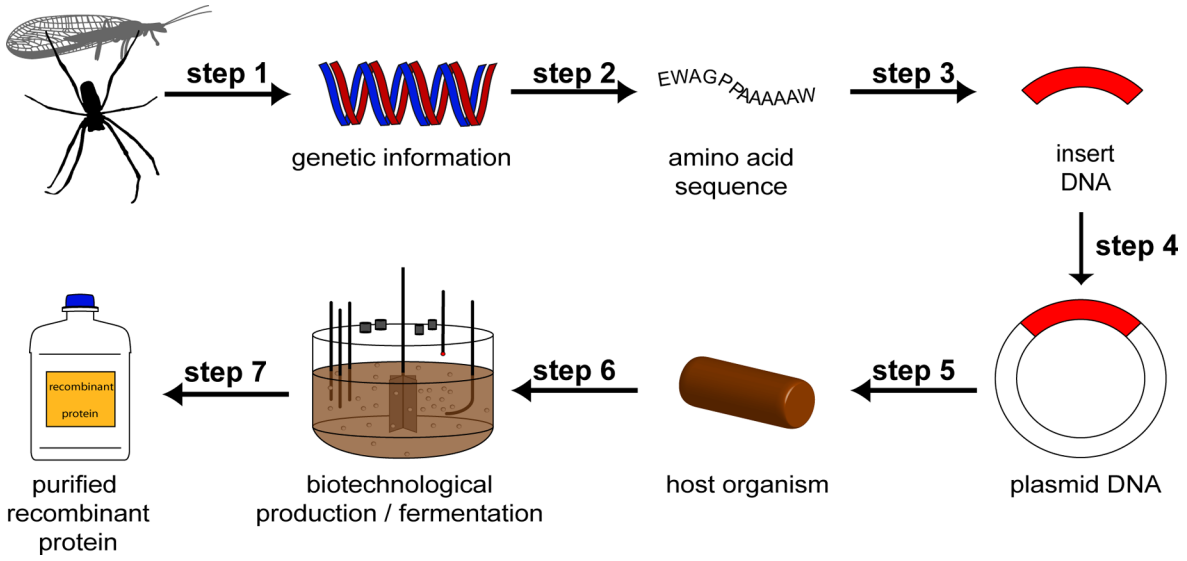

Figure 1. Scheme of recombinant silk production. Step 1, extracting genetic information; step 2, decoding the extracted DNA; step 3, reverse translation and gene engineering; step 4, ligation of insert DNA into the plasmid DNA; step 5, transfer of plasmid into host organism; step 6, fermentation; step 7, purification.

articles summarizing the biology,[11,34,35] structure[36,37] and mechanical properties[6,38−40] of spider silk. Here, we focus on major ampullate (MA) silk, which is produced in the MA gland. This MA silk is used as the outer frame and radii of a spider's web and as its lifeline.[41] MA silk has a very high tensile strength and the highest toughness of all known natural as well as synthetic fibers.[3,6,38,42−44] MA silk is mainly composed of spidroins (**spid***er fib***roin**) and is divided into two classes (MaSp1 and MaSp2) according to its proline content. MaSp1 has a low proline content and MaSp2 is proline-rich. The physical and related mechanical properties, such as breaking strain, of MA silk are directly influenced by the proline content.[45] MA spidroins generally contain a repetitive core with individual amino acid motifs repeated up to 100 times accounting for over 90% of the sequences.[46] The core domain is flanked by nonrepetitive amino- and carboxy-terminal domains, which are highly conserved between different silks and between spider species. These terminal domains mediate the storage and assembly of the spider silk proteins.[47,48] MA silk fibers are coated with a very thin shell of glycosylated proteins, lipids, minor ampullate spidroins and other proteins.[49,50] However, these shell compounds are, in contrast to sericin from *B. mori*,[51] not immunogenic, making MA silk an interesting biomaterial for biomedical applications.

1.1.4. Comparison of β-Crystallinity in Silkworm, Lacewing, and Spider MA Silk. The β-sheet content of MA silk (11−46% *N. clavipes*,[20,52,53] 34−35% *A. diadematus*,[54] 46% *Nephila edulis*[55]) is similar to that of *B. mori* silk (40−55%).[20,52] Both silk materials form antiparallel β-sheets aligned along the thread axis. The β-sheets form crystalline-like regions embedded in an amorphous matrix, but also the presence of a so-called interphase was proposed. In contrast to *B. mori* and MA silk, Lacewing egg stalk silk shows an unusual β-sheet structure (content: 20−40%)[28,32] called cross-β structure, where β-strands are aligned perpendicular to the fiber axis.[30] The evidence for two different types of β-sheets in *N. clavipes* MA silk and *B. mori* silk was shown by hydrogen−deuterium exchange experiments. Crystalline β-sheets are D_2O-inaccessible and the β-sheets building the interphase are D_2O-accessible. The water-accessible interphase consists of weaker hydrogen bonded β-sheets. MA and *B. mori* silk differ in the fraction of the interphase which is significantly higher in case of MA silk (27 ± 3%) than in *B. mori* silk (8 ± 3%).[53] In the case of *B. mori* silk, crystalline as well as interphase β-sheets are likely formed by GAGAGS motifs. In MA silk, crystalline β-sheets are formed by polyalanine (A_n) regions, and AG and GGA blocks flanking the A_n regions are suggested to be present in β-sheets also.[52,53] The interphase probably contains GXG (X = Q, Y, L, R) motifs. A three-phase model was postulated where the crystalline regions are flanked by an interphase which is assumed to act as a transition zone between crystalline β-sheets and the surrounding amorphous phase.[53,56] The structural organization allows to describe the properties of spider silk using a hierarchical model.[57]

1.2. Natural vs Recombinant Silk Production. To exploit the manifold properties of silk, it can be advantageous to investigate the isolated underlying proteins. Silks can be harvested from their natural sources (i.e., for example of *B. mori* cocoons). The silk is degummed by boiling the cocoons in 0.02 M Na_2CO_3, and the degummed fibers are dissolved in strong chaotropic agents (e.g., 9.3 M LiBr), yielding soluble silk fibroins called regenerated silk fibroin (RSF). Because *B. mori* silk has been produced by sericulture (silk farming) for centuries, RSF has been available for investigations for decades.

In contrast, spiders cannot be farmed because they are typically territorial and cannibalistic[13,47−50] and produce silk of lower quality when held in captivity.[8,34,58] Although lacewings are bred commercially (they are used as pest control), the quantity of silk produced by each individual is too small for practical applications. In both cases, as an alternative to obtaining silk proteins from natural sources, recombinant strategies for producing silk proteins have been developed, but only a short overview is given here. For more detailed information on natural and synthetic spider silk genes, the reader is referred to Heidebrecht and Scheibel and references therein.[59] Different approaches for producing silk proteins in different host organisms have failed mainly because of the repetitive character of the gene sequences rich in guanine and

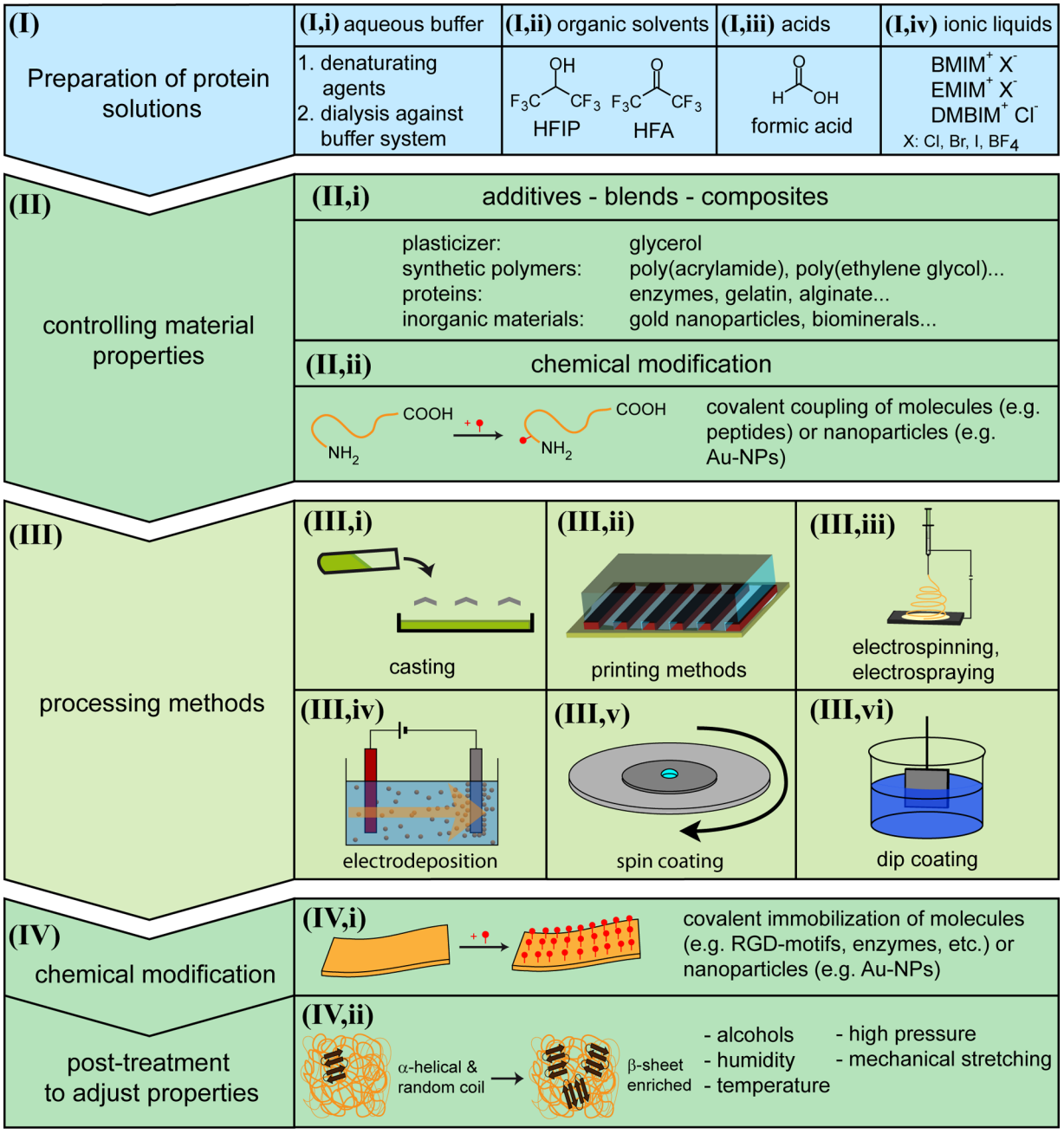

Figure 2. Schematic overview of silk processing. After (I) preparing the silk solutions, the materials properties can be controlled (II) before and (IV) after (III) further processing steps.

cytosine. A successful approach was the construction of synthetic sequences based on natural motifs (Figure 1, step 1−2) and adapting the gene sequences to the codon usage of different host systems like bacteria (e.g., *Escherichia coli*) or yeast (e.g., *Pichia pastoris*) (Figure 1, step 3−5).[60−63] A similar strategy was performed for a recombinant egg stalk protein (RESP).[32] The biotechnological production (Figure 1, step 6) yields silk proteins as primary material for further processing (Figure 1, step 7), but also allows the modification of proteins genetically. Synthetic analogues can be produced with targeted modifications to get desired material characteristics,[64] giving access to a broad range of applications.

2. PROCESSING OF SILK PROTEINS

The processing of silk proteins includes preparation of silk protein solutions (Figure 2 (I)), controlling materials properties (Figure 2 (II) & (IV)) and processing methods (Figure 2 (III)). Regenerated silk fibroin (RSF), recombinant egg stalk proteins (RESP) and recombinant spider silk proteins (RSSP) can be easily processed into different morphologies like nonwoven mats, films and coatings.

2.1. Preparation of Silk Solutions. At first, silk proteins are dissolved in a denaturating agent to prepare processable silk protein solutions (Figure 2 (I)). Such denaturating agents are chaotropic salts like lithium bromide (LiBr), lithium thiocyanate (LiSCN), guanidinium thiocyanate (GdmSCN), or guanidinium hydrochloride (GdmHCl). Chaotropic salts can easily be exchanged via dialysis (Figure 2 (I,i)). Alternatively,

Table 1. Overview of Synthetic Polymers, Biopolymers and Inorganic Materials for Silk-Based Composite Materials and Blends[a]

synthetic polymer		biopolymers		inorganic materials	
nonbiodegradable	biodegradable	proteins	polysaccharides	particles	biominerals
carbon nanotubes	poly(aspartic acid)	collagen	alginate	silver nanoparticles	calcium carbonate
nylon66	poly(ε-caprolactone)	enzymes	cellulose	gold nanoparticles	calcium phosphate
polyacrylamide	poly(ε-caprolactone-*co*-D,L-lactide)	fibroins	cellulose xanthate (viscose)	transition metal oxides and sulfides	silica
polyacrylonitrile	poly(carbonate-urethane)	gelatin	chitin		
polyallylamine	poly(lactic-*co*-glycolic acid)	green fluorescent protein	chitosan		
polyepoxide	poly(lactic acid)	growth factors	hyaluronic acid		
poly(ethylene glycol)	polyurethane	keratin			
polypyrrole		sericin			
polystyrene		spidroins			
poly(vinyl alcohol)					

[a]For details about silk composite materials, the reader is referred to Hardy et al.[70]

silk proteins can be dissolved in fluorinated organic solvents like hexafluoroisopropanol (HFIP) and hexafluoroacetone (HFA) (Figure 2 (I,ii)) or acids like formic acid (FA) (Figure 2 (I,iii)). Also, ionic liquids like 1-butyl-3-methylimidazolium chloride (BMIM Cl), 1-ethyl-3-methylimidazolium chloride (EMIM Cl), and 1-butyl-2,3-dimethylimidazolium chloride (DMBIM Cl) can act as denaturating agents (Figure 2 (I,iv)).[65,66]

2.2. Additives for Controlling Silk Properties. After preparing the initial silk protein solution, additional substances like plasticizers (e.g., glycerol[67−69]), polymers or proteins can be added before or during processing the silk materials (Figure 2 (II,i)). A broad range of material blends and composite materials containing silk proteins have been analyzed in the past, including synthetic polymers, biopolymers, and inorganic materials as additives. An overview on additives is given in Table 1. For details concerning composite materials based on silk the reader is referred to Hardy et al.[70]

2.3. Processing Methods of Silk and Silk Blends. Silk proteins and blended silk materials can be processed into films and coatings using various processing techniques (Figure 2 (III)) resulting in different morphologies. Films can be cast or printed e.g. using lithography[71] yielding 2D and 3D structured isotropic or anisotropic micro- or nanopatterned surfaces.[72] Thin coatings can easily be prepared by dip or spin coating.[73,74] Using spin coating, the silk proteins can self-assemble due to shear-forces as described for native SF.[75] Zeplin and co-workers used dip coating to modify the surface of breast implants using RSSP (for details see part 3.1).[76] Other methods for generating thin silk films are for example the Langmuir−Blodgett (LB) technique[77] and layer-by-layer (LbL) techniques.[78]

Nonwoven mats represent a completely different type of silk coating which can be produced e.g. by electrospinning[79,80] out of different solvents.[81−84] The silk protein solution or material blend is extruded from a syringe, and an electric field between the syringe and the collector plate accelerates the solution and the solvent evaporates. The resulting fibers can be deposited directly on any kind of substrate that is placed on the collector plate. Particles, too, can be deposited on substrates by electrophoretic deposition.[85−87]

Besides homogeneous films and coatings, materials with gradually changing properties, as found in biological systems (e.g., mussel byssus), are useful for various applications.[88] Fibroin/gelatin blends show a wide range of Young's moduli depending on the mixing ratio.[12,70] Gradient films were cast using glycerol-plasticized gelatin and 0−40% RSF, leading to a gradient material on a centimeter scale with a highly reproducible and smooth mechanical gradient with moduli from 160 to 550 MPa.[89]

2.3.1. Influence of Solvent and Post-Treatment on Silk Film Properties. Films can be cast from different solvents such as aqueous buffers, organic or ionic liquids (Figure 2 (III,i)) and obtained through simple solvent evaporation. The secondary structure of the silk proteins is dependent on the initial solvent and is, therefore, controllable. Fluorinated solvents induce α-helical structure in silk proteins. Films cast out of HFIP show a high amount of α-helical structures (RSF;[90] RSSP[91−93]), whereas films cast out of formic acid or water show higher β-sheet content.[90,94−97] RESP dissolved in HFA also has primarily α-helical structure, and therefore, films cast from these solutions have to be post-treated to yield more stable structures.[32] In case of RSSP, the initial solvent has no influence on the thermal stability of films made of a recombinantly produced engineered *A. diadematus* fibroin (eADF4(C16)[62]), where thermal decomposition starts around 270 °C. In contrast, the initial solvent showed a clear impact on the mechanical properties of the films.[98]

Furthermore, surface hydrophobicity can be controlled by casting conditions as shown in the following experiments with films made of RSSP. eADF4(C16) films with a thickness of 9−11 μm were cast out of aqueous buffer (10 mM NH_4HCO_3), HFIP, and FA on poly(tetrafluoroethylene) (PTFE; Teflon), polystyrene (PS) and glass at 30% relative humidity and 20 °C. The films showed different surface hydrophobicities depending on the hydrophobicity of the substrate used to cast the films on. After post-treatment with methanol, the surface hydrophobicity was analyzed by contact angle measurements at the film−air interface. Films cast on hydrophilic glass substrates were more hydrophobic at that surface in comparison to films cast on hydrophobic PS and PTFE substrates (Figure 3A). A structural model of microphase separation of silk proteins based on the amphiphilic nature of the silk protein was generated, in which nonhydrophilic polyalanine stretches are arranged into packed β-sheet crystallites causing water exclusion, and hydrophilic

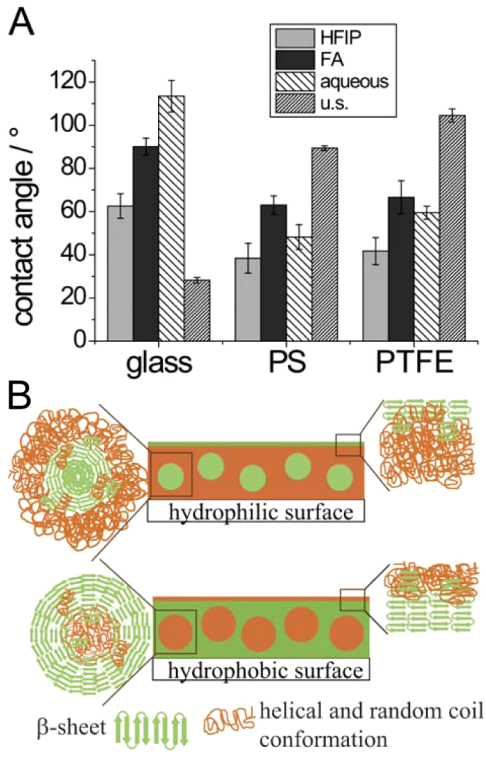

Figure 3. (A) Surface hydrophobicity of eADF4(C16) films dependent on the substrate and the solvent used, determined by water contact angle measurements. Uncoated substrates (u.s.) were measured as reference. (B) Influence of the template on the secondary structure of eADF4(C16). Reproduced with permission from ref 99. Copyright 2012 The Royal Society of Chemistry.

glycine-rich motifs remain in unstructured or helical conformations (Figure 3B).[99] On hydrophilic templates like glass, hydrophilic silk regions are oriented toward the substrate, and the hydrophobic polyalanine stretches are organized into micellar-like structures or oriented away from the hydrophilic bulk to the silk-air interface, inducing a hydrophobic film surface. On hydrophobic templates, e.g., PTFE, the hydrophobic silk regions are oriented toward the substrate, and the hydrophilic blocks are organized into micellar-like structures or oriented away from the hydrophobic bulk to the silk-air interface.[99] Microphase separation is a common effect of block copolymers, and RSF can also be described as multiblock polymer composed of crystallizable and uncrystallizable blocks. β-sheet crystallization is therefore spatially limited by microphase separation of the two different blocks.[100] Furthermore, Cebe et al. performed fast scanning chip calorimetry with RSF and reported the first reversible melting of β-sheet crystals similar to the behavior of lamellar crystals composed of synthetic polymers.[101]

In the case of RSF, Lawrence and co-workers demonstrated the influence of RSF film hydration on material properties depending on the processing technique. Methanol treated RSF films showed a less-ordered secondary structure arrangement than water annealed RSF films. The methanol treated films had a higher water absorbing capacity and reached higher oxygen permeability rates.[102]

2.3.2. Processing Techniques for Adopting Different Surface Topographies. It is possible to produce structured micro- and nanopatterned silk protein surfaces by common micro- and nanopatterning methods. For example, RSF microstructures have been assembled by rapid transfer-based micropatterning and dry etching[103] and RSSP microstructures by solvent-assisted microcontact molding and capillary transfer lithography.[104] Micropatterned films made of RSF were cast on poly(dimethylsiloxane) PDMS replica molds to transfer surface structures of patterned surfaces to silk films,[105,106] and micropatterned films made of RSSP and RESP have also been processed for controllable cell adhesion, cell growth and cell orientation (for details, see section 3.1.1).[9] The RSSP/RESP patterns were made using photolithographically produced silicon templates to generate a microstructured PDMS negative which was placed on a smooth cast silk protein film (RSSP or RESP). Then, the second silk solution was soaked into the molds by capillary forces. After evaporation of the solvent, the PDMS stamp was removed yielding a patterned silk film.[9]

2.3.3. Influencing Silk Film Properties Using Chemical Modifications. Various coupling reactions can be used for chemical modification (Figure 2 (II,ii) & (IV,i)) of silk proteins depending on their amino acid composition i.e. the number and type of functional groups. In the case of *B. mori* SF, the most abundant reactive amino acid residues of the heavy chain are threonine, serine, tyrosine, aspartic acid and glutamic acid. Common coupling reactions used for chemical modification of these amino acids are cyanuric chloride-activated coupling, carbiodiimide coupling, and reaction with glutaraldehyde. Further, amino acids can be modified by arginine masking and sulfation and azo-modification of tyrosine. For details about these chemical modifications the reader is referred to Murphy and Kaplan and references therein.[107]

Huemmerich and co-workers modified films made of the RSSP eADF4(C16) by carbodiimide activation of the carboxy groups of glutamate residues and the carboxy-terminus (overall 17 reactive sites) for reaction with amines in solution. The coupling of fluorophores and of the enzyme β-galactosidase was successfully shown with RSSP.[92] For biomedical applications cell binding properties can be influenced by cell binding peptides like RGD-motifs which were coupled by thiol chemistry to RSSP (eADF4(C16)) films and by carbodiimide coupling to RSF films to improve cell adhesion (see section 3.1.1.).[107,108] In the case of RSSPs, like eADF4(C16) which do not have cysteine residues in their sequence, the protein can be genetically modified during production (see chapter 1.2), for example, with a cysteine-containing tag at the amino-terminus. The eADF4(C16)^{ntagCys}-film surfaces expose the thiol groups of the cysteines, allowing controlled and specific modification with reagents containing a maleimido function.[74] Maleimido-fluorescein, biotin maleimide, RGD-motifs, β-galactosidase and monomaleimido-nanogold (d = 1.4 nm) could be successfully coupled to the silk film surface.[74] It was also possible to covalently attach sulfonic acid groups to tyrosine of RSF using diazonium coupling. The negatively charged and hydrophilic sulfonic acid groups can selectively promote pyrrole absorption to sulfonic acid modified RSF films, yielding conductive polypyrrole patterns on silk surfaces by printing or stamping inks made of sulfonic acid-modified RSF on RSF films. Pyrrole adheres selectively to the acid modified RSF and sets up conductive structures out of polypyrrole after polymerization.[109]

2.4. Post-Treatment. Post-treatment of processed silk proteins (Figure 2 (IV,ii)) can be used to increase the β-sheet content of a silk material resulting in more stable and water insoluble protein materials.[80,110,111] The structural change from

α-helical and random coil structures to β-sheets and can be induced by temperature,[112] alcohols (e.g., methanol, ethanol, 2-propanol),[92,93,95,111] humidity and water vapor,[113] high pressure,[114] mechanical stretching,[115] and cosmotropic salt solutions (e.g., 1 M potassium phosphate).[93,116] Anisotropic materials can also be produced by post-treatment leading to alignment of the underlying structural elements. Both types of processing influence the mechanical properties of the resulting materials.[13,20,117]

3. APPLICATIONS OF SILK FILMS, COATINGS, AND NONWOVEN MATS

3.1. Tissue Engineering and Medical Applications. Silk shows interesting features such as biodegradability and biocompatibility (reviewed in Leal-Egaña and Scheibel[14]),[118,119] and nothing has been reported concerning allergies against pure silk materials, probably based on low or no inflammatory responses when in contact with animals and humans.[119] Nonwoven mats have been applied as wound dressings, coatings, or scaffolds,[120,121] and silk films have been investigated as coatings and wound dressings as well as drug delivery systems.[122]

B. mori silk has been reported to cause allergic reactions and immune responses when using so-called "virgin silk" (silk directly obtained from the silkworm), whereas removing sericin from the silk fibers (i.e., degumming) yields a material that causes no allergic reaction and/or immune response.[24−27,123−127] MA spider silk is not coated with glue, therefore, MA silk causes no allergic reactions/immune response like virgin silk. In the case of lacewing egg stalk silk, it is currently not known if it causes allergic reactions and/or immune responses because not all the proteins have been identified and tested yet.

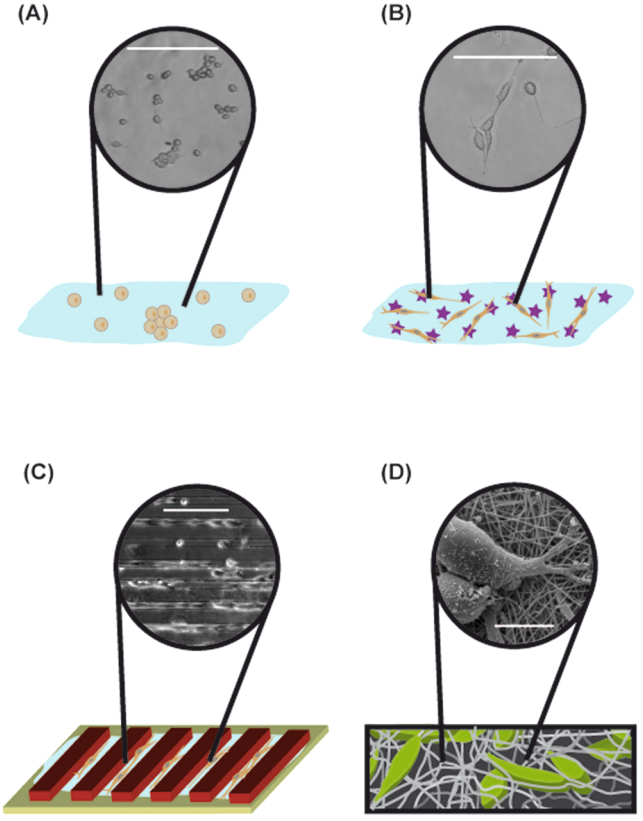

Figure 4. Cell adhesion on different silk morphologies and on modified silk films. (A) Cells were seeded on flat RSSP (eADF4(C16) films cast from HFIP). Low cell adhesion and round-shaped cell morphologies of BALB/3T3 fibroblasts are detected. (B) RGD-modified eADF4(C16) films show improved cell adhesion and cell spreading. The surface topography can be altered (C) with patterning or (D) by creating nonwoven meshes. Both approaches have a large impact on cell adhesion and orientation.[9,106,120,139,146,151,152] Scale bars: (A−C) 200 μm; (D) 10 μm.

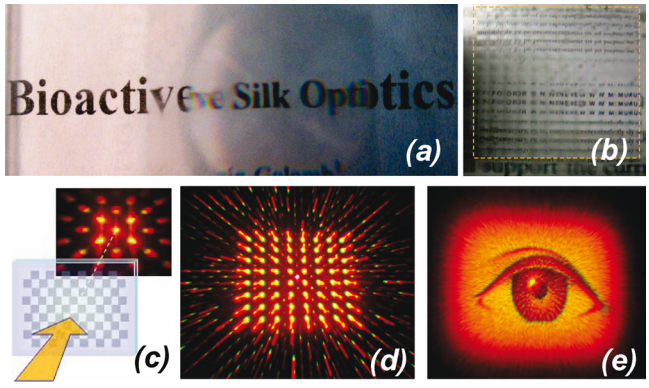

Figure 5. Optical elements made of silk. (a) silk lens, (b) 12 × 12 silk lens array, (c) scheme showing the approach for generating images, (d, e) different projected patterns obtained from propagation of a white light laser source through 2D, 64 phase level diffraction patterns. The images are taken in the far field at a distance of 10 cm from the silk optical element. (Masters from Digital Optics Inc., Tessera Corporation). Reproduced with permission from ref 168. Copyright 2008 American Chemical Society.

The properties of silk films (hydrophobicity, water-contact-angle, secondary structure, etc.) differ because of the protein used, the solvent, template surface, post-treatment, and film thickness as mentioned above,[98,99,113,128,129] with dramatic impact on cell organization, adhesion, and proliferation when used as a scaffold. Cell interactions with surfaces or other cells are mainly mediated by the integrin protein family.[130] Binding motifs recognized by integrins are present in proteins of the extracellular matrix (ECM) (e.g., laminin, collagen, fibronectin).[130−132] Therefore, it is also of great importance how such ECM proteins interact with a "technical" surface (such as in silk scaffolds). Below we will give an overview of general aspects of cell−silk surface interactions followed by a more detailed description of recent results (from the last 10 years) concerning putative medical applications of silk scaffolds.

3.1.1. Cell−silk Surface Interactions and Silk Surface Modifications for Improved Cell Binding. Generally, weak cell attachment has been detected on RSF, RSSP, and RESP films (Figure 4A), such as for osteoblast-like cells SaOs-2 on RSF[133] or BALB/3T3 fibroblasts on eADF4(C16) RSSP and RESP films.[9,108,120] Cells on these silk films are round and form cell aggregates (i.e., cell−cell interactions instead of cell−matrix interactions).[108,120,134] SaOs-2 cells up-regulated the production of ECM proteins (100%) such as collagen type *I*-α or alkaline phosphatase on RSF films, whereas fibroblasts up-regulated collagen-I on RSSP (eADF4(C16)) films as a result of weak attachment.[120,135] Weak cell attachment can be partly explained by the absence of cell recognition motifs.[136] Furthermore, RSF and RSSP (eADF4(C16)) are negatively charged proteins under cell culture conditions, which is also not conducive for cell attachment because cell surfaces are also negatively charged.[137,138] In contrast, films made of the

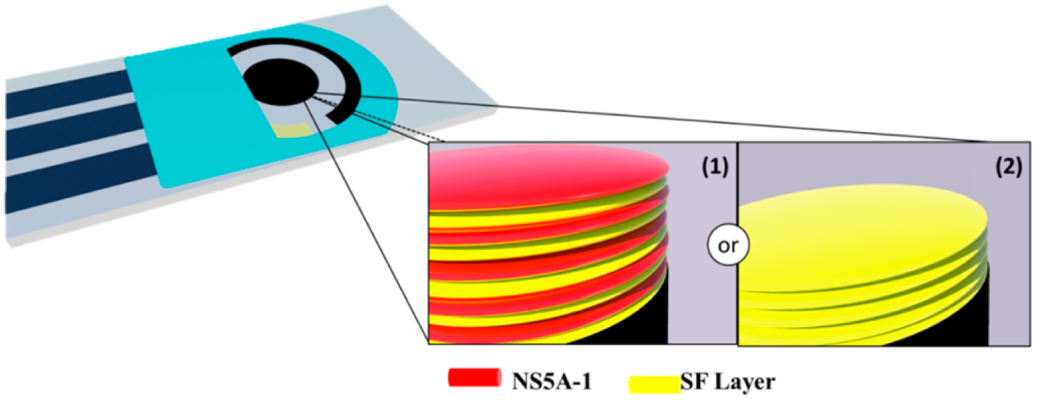

Figure 6. Schematic representation of five-layer LbL film of SF/NS5A-1 (1) and RSF (2) assembled onto carbon screen-printed electrodes. Reproduced with permission from ref 176.

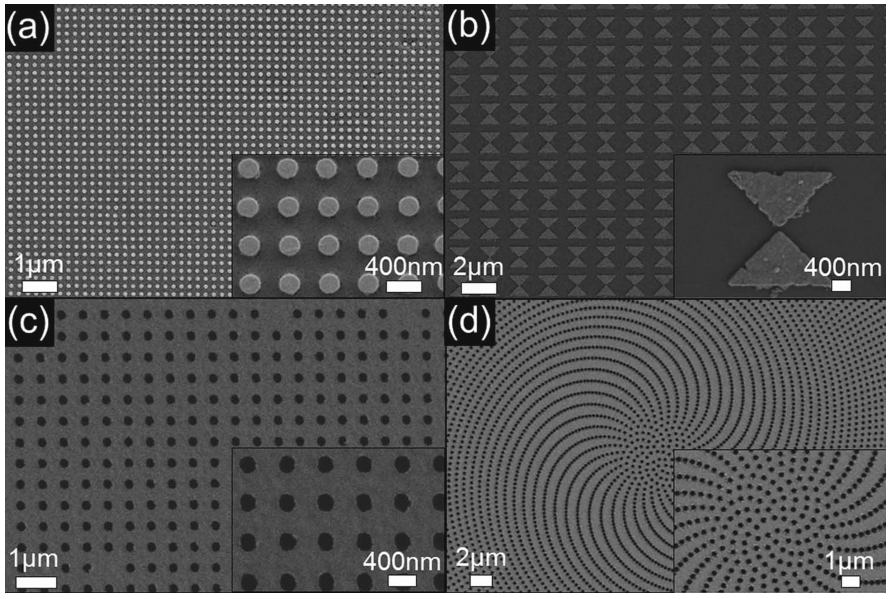

Figure 7. SEM images of transfer imprinted plasmonic nanodot arrays on doped silk fibroin films: (a) Periodic pattern of a = 400 nm and (b) bow tie structures with gaps of 40 nm. SEM images of transfer imprinted plasmonic nanohole arrays on doped silk fibrion films: (c) Periodic pattern of a = 600 nm and (d) α_2 spiral array with a_{ave} = 308 nm. Reproduced with permission from ref 182. Copyright 2012 Wiley–VCH.

positively charged RSSP (4RepCT) allowed cell attachment similar to control plates.[139] But on RESP (N[AS]$_8$C) films, also consisting of a positively charged protein, cell adhesion is quite low and the few cells detected on these films show a round morphology.[9]

One strategy to improve poor cell–silk surface interactions is to modify silk surfaces with recognition motifs (i.e., peptides) like the integrin binding motif RGD from fibronectin (Figure 4B).[22,108,133,140,141] Silk proteins can be modified genetically (in case of recombinant versions) or coupled chemically in all cases with RGD motifs. Here, a few examples will highlight the usability of such experimental set-ups. RGD-modified silk surfaces showed improved cell adhesion properties, and this effect could be detected for films made of negatively as well as for positively charged silk proteins.[108,142] In this context, researchers have created different blended RSF films with a synthetic RGD-containing spidroin for the analysis of osteoblastic differentiation,[143] analyzing the adhesion, proliferation and differentiation of an osteoblast precursor cell line (MC3T3-E1) on blended films (different RGD content accompanied by a different crystallinity). Increased crystallinity stabilizes the film and results in increased numbers of adherent cells, but no dependency of cellular differentiation was noted due to the β-sheet content. A ratio of 90:10 RSF to RGD-spidroin was optimal for the increased film stability and cell attachment. Another strategy to improve cell binding can be the coupling of macromolecules from the ECM (e.g., glycopolymers) to silk films.[144,145] RSSP films (e.g., eADF4(C16)) modified this way showed improved cell adhesion, and the morphology of the cells changed from round (unmodified films) to spread (modified films).[144]

Other peptides can be also suitable for increasing cell interactions with scaffolds and can lead to bone regeneration. Foo et al.[146] for example, modified the surface of RSSP 15mer films and electrospun fibers (nonwoven meshes) with the cell recognition motif RGD or a R5 peptide (a silicification inducing domain), which could be beneficial in the field of bone regeneration. Silicification could be only detected in the presence of the R5 peptide. Mieszawska and co-workers[152,153] were interested in silk-silica composite films and the behavior of human mesenchymal stem cells (hMSCs) seeded thereon. The RSF solution and silica solution (containing silica particles of

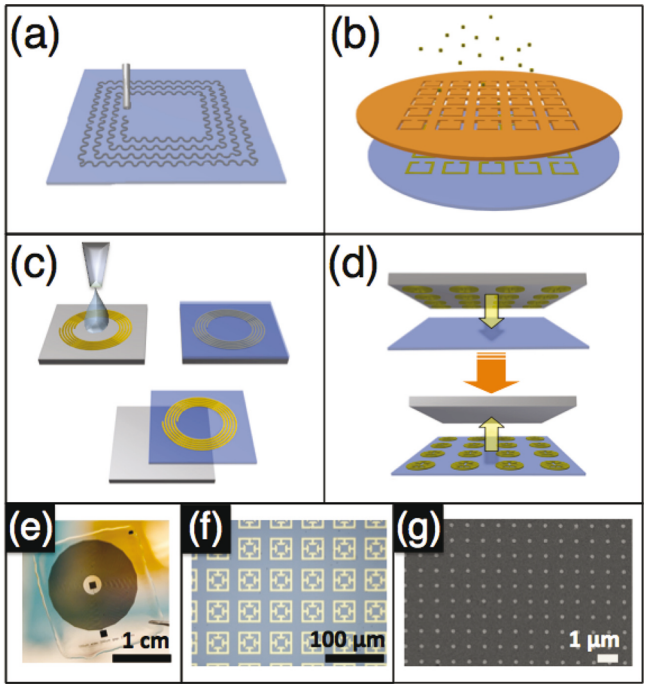

Figure 8. Schematics of fabrication processes for passive silk sensors. (a) Inkjet printing of functional components directly on the silk substrate. (b) Shadow-mask transfer. c) Casting-lift-off process. Functional components are fabricated directly on silanized silicon wafers. Silk is cast directly onto the silicon substrate, and the functional components are transferred onto the silk surface after drying under ambient conditions. (d) Direct transfer. Functionalized surfaces are applied to the silk substrate along with heat and pressure. Removal of the original substrate leaves the functional components on the surface of the silk substrate. (e) Example of a GHz resonant coil on silk, fabricated using the silk transfer applied micropatterning process (STAMP). (f) THz resonant silk metamaterial array, fabricated via shadow mask deposition. (g) Au nanoparticle array on silk, fabricated using direct transfer. Reproduced with permission from ref 184. Copyright 2012 Wiley–VCH.

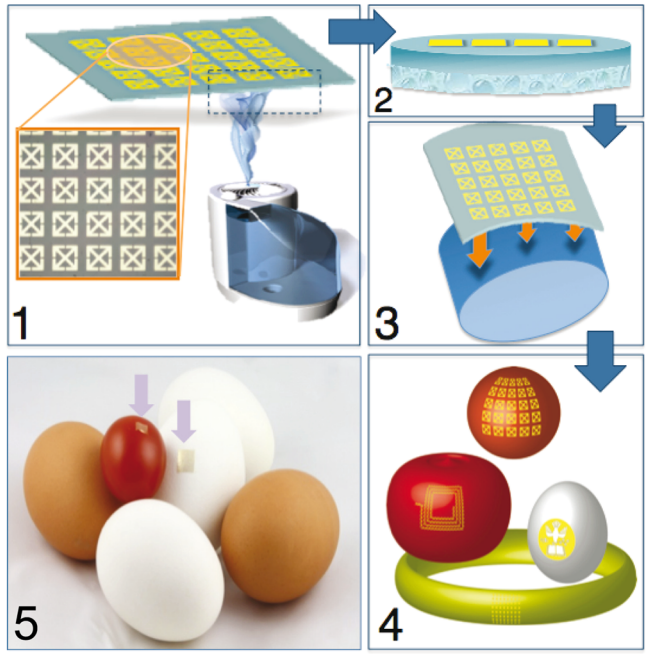

Figure 9. Rapid transfer of silk antennas onto curved substrates. (1) Water vapor is applied to the back of noncrystalline functionalized silk films, yielding (2) a functionalized film of which the back surface of the film has been partially molten. (3) The quasi-molten surface is conformally applied to arbitrary surfaces, yielding (4, 5) functional sensors thereon. Reproduced with permission from ref 184. Copyright 2012 Wiley–VCH.

24 nm, 500 nm, or 2 μm in diameter) were cast together on a cell culture plate and post-treated with methanol, yielding good proliferation rates of hMSCs as well as high cell densities. Osteogenic markers were up-regulated, and an increased formation of collagen/calcium phosphate was detected in the ECM, indicating osteoinductive properties of RSF/silica films.[147,148]

Cell attachment and proliferation, the polarity, morphology and cytoskeleton reorganization of bound cells, can be influenced by cell recognition motifs and the topography of a silk film's surface.[149,150] On flat films made of RSSP (eADF4(C16)) or RESP (N[AS]$_8$C), BALB/3T3 fibroblast adhesion was very weak.[9,108] BALB/3T3 fibroblasts as well as C2C12 myoblasts seeded on patterned films of eADF4(C16)/N[AS]$_8$C, with eADF4(C16) being the bottom layer, adhered in the grooves and proliferated better than on films of each individual protein. The cells aligned within the grooves and myoblasts even started to form myotubes, which is an important first step toward skeletal muscle regeneration (Figure 4C). Surprisingly, RGD-modified RSSP (eADF4(C16)) films as a ground layer did not result in significantly increased cell binding.[9]

An alternative to changing surface topography of silk films is the production of silk nonwoven meshes as a coating of substrates (Figure 4D). Nonwoven meshes with fiber diameters of 10–70 μm of RSSP (4RepCT) did not show improved cell adhesion and proliferation compared to smooth films.[139] However, hybrid matrices made of a bottom film layer on which a nonwoven mesh (diameter of fibers: 10–70 μm) was applied showed improved cell adhesion compared to smooth films or nonwoven meshes alone.[139] Cells like the vascular cell line HAECs or HCASMCs as well as endothelian cell line PIEC grew better on RSF nonwoven meshes (fiber diameter: 1016 nm[151] and 377 nm ±77 nm[152]) than on smooth RSF films.[151,152] Similar results were obtained by seeding BALB/3T3 fibroblasts on RSSP (eADF4(C16)) nonwoven meshes.[120] Nonwoven meshes with different fiber diameters (between 150 and 680 nm) were produced, and fibroblasts seeded thereon. The adhesion and proliferation rate of the cells increased with increasing fiber diameter likely due to the relation of cytoskeleton organization and space between the fibers of the mesh.

Patterned RSF films of different widths and depths were also used to orient mesenchymal stem cells (MSC) for compact bone regeneration.[106] One pattern in particular (3500 nm width/500 nm depth) induced osteogenic differentiation, a robust cell alignment and also ECM production similar to native cortical bone.

Neural stem cells were also cultured on films made of the positively charged RSSP (4RepCT). Cell adhesion and proliferation was comparable to the positive control (cell culture plates specifically treated for nerve cell cultivation).[153] Patterned films of negatively charged RSF with electrodes incorporated for axon alignment and outgrowth stimulation yielded varying results.[154] Stimulating the cells and monitoring over 3–5 days showed that the neural stem cells (P19) were

growing, orientating along the grooves, and their axons aligned and grew out. However, after several days, the cells started to detach from the RSF surfaces. Not only RSF films but also fibers were used for nerve regeneration, which are not discussed further here. However, RSF fibers with a quite small diameter (400 nm) are more favorable to the development and maturation of neurons.[105]

3.1.2. Implants Made of or Coated with Silk. Animals with implants coated with silk generally show low levels of inflammation markers like cytokines.[76,119,155] Zeplin and co-workers coated silicone breast implants with RSSP with the aim of reducing the risk of capsular fibrosis.[76] Fibroblasts, which are critically involved in fibrosis, show decreased proliferation rates on spider silk coated silicone implants in comparison to uncoated ones. Primary human monocytes involved in fibrosis showed a significantly reduced differentiation into CD68-positive macrophages (histiocytes). Upon implantation into subcutaneous pockets of Spraque-Dawley rats, the coated implants were well-tolerated, and no wound healing disorders were detectable. Importantly, no liver granulomas and alterations of lymph nodes were detected, excluding the presence of infections or epitopic inflammation. After explantation, histological examinations of the uncoated implants showed periprosthetic tissue rich in fibroblasts and histiocytes organized in multiple cell layers, whereas silk-coated implants were surrounded by significantly fewer cells organized in only two layers. Additionally, the expression levels of several other fibrosis/inflammatory markers were significantly reduced.

For specific biomedical applications, enzymes and proteins can be coupled to silk films. Several specific proteins can be used for bone or dentin regeneration and some selected examples are explained in detail. The bone morphogenetic protein 2 (BMP-2) stimulates osteogenesis and was covalently bound to RSF films. BMP-2 silk films were more efficient in inducing osteogenic differentiation of bMSCs (bone mesenchymal stem cells) than free BMP-2 or a control silk film without BMP-2.[156] The dentin matrix protein 1 (DMP-1) is involved in nucleation and orients crystallization of hydroxyapatite within teeth, which is a prerequisite for their remarkable toughness and hardness.[157] Recently, RSSP/DMP-1 hybrids were analyzed versus plain RSSP films concerning biomineralization, i.e., the growth of hydroxyapatite crystals on the film surfaces. Films containing the DMP-1 protein were biomineralized, whereas films without DMP-1 did not induce biomineralization. Biomineralized RSF scaffolds were also used as bone grafts to repair canine inferior mandibular border defects.[158] The combination of bMSCs and apatite silk scaffolds led to fully repaired mandible defects in large animals, whereas the scaffold or bMSC alone showed incomplete bone repair 6 months after implantation. Furthermore, RSF can be processed directly into bone screws for fixation devices.[159] For better bone ingrowth into these implants or healing regulation, the RSF screws could be easily modified with BMP-2 protein or antibiotics to prevent infections.

For peripheral nerve repair Gu and co-workers fabricated a chitosan/RSF based scaffold.[55] For creation of a Schwann cell (SCs)-derived coating they were seeded on RSF/chitosan conduits for 14 days. By implanting the SC-derived ECM chitosan/RSF scaffold into rats the scaffold supported axonal outgrowth at an early regenerative stage and nerve regeneration. Although the new composite scaffold was not better than the acellular scaffold, it provided several advantages like pathogen-free production.

For application of silk scaffolds or coatings of implants in vivo, sometimes antibiotics or an antibacterial surface are required. Therefore, silver ions, known to be antimicrobial, were bound through a silver binding peptide to RSSP to yield antimicrobial silk surfaces.[160] In another series of experiments, antibiotics were loaded into RSF films which were incubated with *E. coli* and *Staphylococcus aureus*.[161] The antibiotic-loaded RSF films suppressed bacterial growth completely. RSSP films coupled with antimicrobial peptides also showed antimicrobial properties in tests with *E. coli* and *S. aureus*, whereas the simultaneously tested cell line (SaOs-2) was not affected.[162]

3.2. Silk Optics and Biosensors. Silk films and coatings can also be used in optical devices or biosensors and various applications mainly using *B. mori* fibroin have been reported, and some of them are highlighted herein. The oxygen permeability of RSF membranes is similar to that of hydrogel membranes which are used for soft contact lenses. Due to its biocompatibility, optical properties (e.g., high transparency) and oxygen permeability, RSF membranes are applicable as contact lens material.[163−165] Silk materials can also be processed into morphologies (e.g., by nanoimprinting, casting, spinning) useful in optical, photonic, electronic, and optoelectronic applications.[166] By nano- and micropatterning of optically transparent biocompatible RSF films, 3D diffraction patterns with a high fidelity were obtained.[167] Lawrence and co-workers used RSF to produce highly tailored structures and morphologies for optical devices (Figure 5).[168]

Curved rodlike optical waveguides have been produced by direct ink writing using RSF "ink" containing 28−38% RSF, followed by methanol post-treatment showing controlled structure and composition.[169] Photoactivation of drugs in silk structures is also possible, because silk biomaterials provide a perfect matrix for stabilizing enzymes because of their thermal, chemical, and mechanical robustness. Different approaches for immobilizing enzymes in silk protein materials were successful, conserving enzyme activity over months to years e.g. for their use in biosensors.[170−172] Lu and co-workers blended glucose oxidase (GOx), horseradish peroxidase (HRP) or lipase with RSF and cast the blended material into films.[173] Demura[171] and Asakura and co-workers[174] immobilized glucose oxidase (GOx) for biosensing of glucose. HRP or lipase immobilized in RSF films were used for the determination of hydrogen peroxide and uric acid in a flow injection system.[173] Tao and co-workers used a microstructured multifunctional RSF optical element for simultaneous drug delivery and feedback response.[175]

Antigens as disease detecting biosensors were also immobilized on RSF membranes.[176,177] The peptide NS5A-1 (PPLLESWKDPDYVPPWHG) derived from the hepatitis C virus (HCV) was immobilized on RSF films coating carbon screen-printed electrodes using a layer-by-layer technique (Figure 6).[176] Although plain RSF films showed no significant response, the immunosensor made of SF/NS5A-1 LbL-films showed a signal in presence of the anti-HCV (1 μg/mL), thus establishing a highly sensitive immunosensor.[176]

Other types of sustainable sensors made of silk are photonic crystals.[178,179] Three-dimensional photonic crystals were fabricated by pouring RSF solution over a mask of a close packed self-assembled colloidal crystal of poly(methyl methacrylate) (PMMA) spheres. After a drying step, PMMA is dissolved and the silk inverse opal (SIO) is obtained. The color can be controlled by changing the size of the PMMA spheres and by filling the voids with liquids like acetone. Because of their intrinsic structural color and biocompatibility, these silk

materials are suitable for microscale implantable biosensing and targeted therapeutics.[178,179] Further work generated silk-protein based hybrid photonic-plasmonic crystals (HPPC), incorporating a 3D SIO and a 2D plasmonic crystal formed on top of the SIO to combine the properties of these two structures (2D SIO & 3D pseudophotonic band gap) making it suitable as a multispectral refractive index sensor.[180] Diao et al. produced SIOs with bistructural colors at UV and visible, UV and IR, and visible and IR wavelengths. The SIOs showed a linear relation between humidity and the wavelength of the reflected light, which gives rise to optical humidity sensors.[181]

Lin and co-workers demonstrated a direct transfer of subwavelength plasmonic nanostructures on bioactive RSF films, overcoming problems of integrating plasmonic metallic nanostructures into biopolymeric ones.[182] A direct transfer nanofabrication technique (based on nanotransfer printing) was used for the fabrication of large-scale metallic nanoparticles (plasmonic nanodots) and perforated metallic films (plasmonic nanoholes) on RSF films, obtaining a high fidelity sequential transfer of plasmonic nanoparticles, optical bow tie nanoantennas, and nanohole arrays with periodic and nonperiodic geometries, preserving the functionality of the imprinted biopolymer. Some examples of the structures obtained are shown in Figure 7.

Silk films also can be used to produce curvilinear electronics for different applications like biointegrated electronics for diagnosing, treating disease, or improving brain/machine interfaces;[183] adhesive and edible food sensors;[184] and to attach graphene-based biosensors onto biomaterials like tooth enamel as fully biointerfaced nanosensors.[185] Silk-based conformal, adhesive, and edible food sensors made of RSF described by Tao et al. are also a good example for such sensors.[184] Different methods can be used to produce/transfer micro- and nanopatterns onto silk substrates, and Figure 8a–d shows the most common methods used by Tao and coworkers. Examples of the fabricated structures are GHz resonators, THz metamaterials and nanopatterned Au-nanoparticle plasmonic arrays on silk (Figure 8e–g).

To attach the structures to surfaces for biosensing applications, the noncrystalline (not post-treated) carrier RSF film is exposed to water vapor to be softened. The adhesive thin layer of silk acts as "glue" and can be used to adhere the antenna to the target surface without damaging the antennas. The attachment process is shown in Figure 9.

An interesting application of this technology is the monitoring of the fruit ripening process. The resonant frequency of a banana's surface was measuered over 9 days while ripening, showing an initial resonance at day 0 of 36.1 MHz, which increased constantly to higher frequencies (up to 42.6 MHz at day 9) during ripening.

4. CONCLUSION AND FUTURE PERSPECTIVES

Silks are proteinaceous materials with a long history of use by humans. In recent decades, the structure and composition of several silk proteins of different animals have been characterized, and biotechnological protein production strategies give rise to high yields of pure proteins. The silk protein materials are biocompatible and biodegradable, but are not immunogenic, allergenic, or toxic. There are various possibilities to control the properties of silk materials, and though various processing methods (e.g., casting, printing, electrospinning, dip coating, etc.) result in a broad range of structures (e.g., films, nonwoven mats, coatings, etc.). The properties of the fabricated materials, like material stability, surface hydrophobicity, oxygen permeability and optical properties, vary depending on the silk protein used, the processing method and the processing conditions (e.g., solvents, additives, post-treatments). Cell binding properties can be improved by modifying the surface topographies and introducing cell binding motifs. Other proteins and enzymes can be coupled to or immobilized in silk materials by chemical and genetic modification or blending. Enzymes, for instance, show high long-time stability when incorporated in silk materials. Understanding the structure–function relationship of silk proteins allows the design of sustainable and more complex and highly tailored silk-based structures like photonic nanostructures, silk inverse opals (SIO), LbL films, curvilinear electronics, optoelectronics, and biosensors.

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Notes

The authors declare no competing financial interest.

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