Engineering the Future of Silk Materials through Advanced Manufacturing

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Engineering the future of silk materials through advanced manufacturing

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Abstract: Silk is a natural fiber renowned for its outstanding mechanical properties, which have enabled the manufacturing of ultra-light and ultra-strong textile. Recent advances in silk processing and manufacturing have underpinned a re-interpretation of silk from textile to technological material. In this review, we argue that silk materials – optimized by selective pressure to work in the environment at the biotic-abiotic interface – can be harnessed by human micro and nanomanufacturing technology to impart new functionalities and opportunities. We provide a critical overview of recent progresses in silk technology with emphasis on high-tech applications enabled by recent innovations in multi-level modifications, multi-scale manufacturing and multi-modal characterization of silk materials. These advances have enabled successful demonstrations of silk materials across several disciplines including tissue engineering, drug delivery, implantable medical devices, and bio-dissolvable/degradable devices.

1. Introduction

1.1. Structural proteins as technological materials

Structural biopolymers can be considered as the building block of life as they are produced by living matter to provide intra- and extra-cellular structural support and an interface with the abiotic world. Structural proteins, one of the most abundant and physiologically diverse structural biopolymers, are characterized by unique, repetitive amino acid sequences (e.g., GAGAGS for B. mori silk fibroin) that impart the formation of large, stable, 'crystalline' domains and promote self-assembly. The combination of crystalline structures with assembly in hierarchically organized materials, which often have the form of fibers, is evident in many structural proteins including collagens, keratins and silks, and represents their distinct feature. Structural proteins are commonly known through functional terms that are used to describe family of proteins with similar amino acid composition and characteristics. Their classification is still debated and sometimes departs from their common use in plain language. For example, the word 'collagen' covers a large family of proteins ubiquitous in the animal kingdom and that have also been found in fungi. Collagens are secreted from cells in the extracellular space and in connective tissues both in fibrillar and non-fibrillar form. The term 'keratin' is a broad term that was first used for all of the proteins extracted from skin and its modifications (e.g. hairs, horns, claws, beaks and hooves). Currently, the term 'keratin' covers all intermediate filament-forming proteins with specific physicochemical properties and produced in any vertebrate epithelia.[1] Similarly, 'silk' is a functional term used to describe a family of protein fibers spun (i.e. extracellularly made insoluble in filament form from an aqueous protein solution) by a number of arthropod lineages. A large body of research has centered on silk extracted from B. mori caterpillars and on dragline silk of spiders due to their outstanding mechanical properties. However, many insect species produce silk for a wide variety of purposes. It has then been proposed that the ability to produce silk has evolved multiple times among insects and that each origin has led to a novel silk 'lineage', which can be identified by grouping silks according to their gland of production, molecular structure, and phylogenetic distribution.

Silks have a rich history as materials for manufacturing. Before the 'micro' and 'nano' technological revolutions that have spanned for the last two centuries, structural proteins have been used mostly in their natural forms, i.e. fibers and tissues produced by animals, for millennia. In the last a few decades – given the advances in science and technology and the increasing thirst to find materials to interface with no harm to cells and to different tissues in the human bodies – silks have also found extensive application in biomedical fields, including wound healing, tissue engineering, regenerative medicine and drug delivery. Structural biopolymers possess in fact merits of non-toxicity, biodegradation, support of cells growth and differentiation, mechanical strength and biomimetism[2-4] that are difficult to find in synthetic materials and that have enable the use of silks in many 'biocompatible systems.[5, 6] Biomedical devices made of structural proteins have been fabricated using both materials in their natural form (e.g. silk fiber-made ligament grafts) or through the regeneration of 'raw' natural materials in suspensions of protein nanoaggregates that have then been processed in several materials formats, including hydrogels, films and nanofibers.[4, 7, 8]

More recently, further advances in structural proteins processing have set the foundations for their uses in edible and implantable photonics and electronics devices. Contrary to what seen before, this so-called 'third technological revolution' for structural biopolymers has been mostly driven by silk materials, and in particular by B. mori silk fibroin. The main reason beyond the reinvention of silks as technical material for optoelectronics originates from its polymorphism, which is defined as the possibility to obtain stable silk fibroin in several secondary and tertiary structures, ranging from random coils, to β-sheets and helices (e.g. 310 helix, α-helix and β-turn type II). Silk fibroin polymorphism facilitates the processing of silk

fibroin in crystalline and amorphous protein domains, which have different degree of solubility in water. Modulation of water solubility enabled the processing of the structural protein in multiple material formats using advanced fabrication techniques, ranging from electron-beam lithography to ink-jet printing. Water-based processing conditions has also allowed for the incorporation and stabilization of inorganic and organic molecules in silk fibroin materials, which have imparted unusual functions to silk materials as sensing, diagnostics and therapeutics platform. Another unique property of silk fibroin that has allowed its reinvention as material for optoelectronic is the thermal stability of the protein, which allows its processing well outside physiological environments. Thermal stability of silk is a direct consequence of its polymorphism as the protein undergoes an amorphous to crystalline (i.e. random coils to beta-sheets) transition when heated, with the formation of domains that are stable up to circa 170-190 ℃. Additionally, to the amorphous to crystalline transition of silk fibroin corresponds to an exclusion of non-structural water from silk materials, which yields a limited but significant thermoplasticity (i.e. thermal reflow) that has enabled the processing of silk materials through nanoimprinting and technological tools – such as lathe – generally restricted to synthetic materials.[9] Last, silk fibroin molecules assemble by forming inter- and intra-molecular hydrogen bonds – without the formation of covalent bonds. This unique assembly system may be directed to produce complex, hierarchical materials that exhibit a unique combination of mechanical, chemical and transport properties, in a controlled process that covers dimensions ranging from the nano- to the macro-scale, yielding materials that form integrated and adaptive materials and systems.

Thus, understanding the interplay between folding and assembly phenomena is the key to develop new manufacturing techniques that can shape structural proteins in unprecedented, technical, materials formats not found in nature. With this review, we aim at providing a deep and wide investigation of the fundamental properties and of the fabrication processing of silk materials (to which we will refer to as silk, for simplicity) together with a portfolio of new high technological applications to demonstrate how structural biopolymers, and silk in particular, can be considered a material platform with global impact in the biomedical, photonic and electronic fields.

1.2. Silk: Native Composition and Structure

Among structural proteins, silk is renowned for its outstanding mechanical properties,[10-12] inspiring the design of high-performing textile materials. Silk can be synthesized by several arthropods, including silkworms and spiders.[13] These arthropods have different spinning systems and produce silk in different ways for different purposes. While silkworms spin silk into cocoons - and in much less common cases, also into webs - to protect themselves from various threats and to regulate the environment such as to help conserving/blocking water and gases during the pupal stage,[14] spiders - a polymath with expertise spanning materials science, engineering mechanics, architecture and even camouflage - create a silk web to capture prey to feed themselves.[15, 16] Spiders can also produce and store silk proteins in glands, which allows them to spin fibers during their entire lifetime. Though nontrivial differences exist between the primary structure of silks produced by silkworms (fibroin) and spiders (spidroin), fiber spinning follows a similar mechanism, which involve the formation of an insoluble filament from an aqueous protein solution. Also, fibroin and spidroin share similar structure-properties relationships as well as the transition behaviors between conformational structures.[17, 18]

Generally, silk molecular structure consists of highly repetitive amino acid sequences staggered by non-repetitive regions. Repetitive regions form large and stable, crystalline β-sheet domains, while non-repetitive sequences fold in semi-amorphous structures, including helices and coils.[19-23] Depending on the relative amount and distribution of crystalline and amorphous regions, different silks possess unique properties. β-sheets domains are the distinct feature of

silk; these 'crystals' are formed by physical crosslinking of highly repetitive amino acid sequences, mainly alanine, glycine-alanine, or glycine-alanine-serine, and contribute to the high tensile strength and toughness of silk fibers.[10, 24, 25] Non-crystalline regions of silk are commonly made up of random coils, β-spirals and helical structures. These semi-amorphous regions provide silk with elasticity.[26-33] In addition to crystalline and semi-amorphous regions, non-repetitive regions are present at the amino- and carboxyl termini of the proteins.[34, 35] Although the impact of these termini on mechanical properties is not fully understood, it has been speculated that they might play roles in the storage and controlled assembly of silk proteins.

1.3. Silk: Bio-inspiration and beyond

In the past two decades, silk has undergone a technological revolution with a large body of research focused on engineering new material formats using the natural protein (extracted from the gland,[36, 37] or from silk fibers[38, 39]) as well as genetically engineered ones (especially, recombinant spider silk proteins[40-45]). Silk materials have been fabricated into various material formats for biomedical applications with successful demonstrations in tissue engineering,[46-51] drug stabilization and controlled delivery,[52-54] and implantable medical devices.[55-58] Furthermore, silk has also been widely used in flexible electronics,[59] optical and photonic devices[60, 61] owing to the great flexibility and optical properties. Recent research also demonstrates that silk can be utilized for preservation of perishable products by coating them with an ultrathin silk film.[52, 62-66]

With these advances in the reinterpretation of silk, we argue that although evolutionary pressure optimizes natural materials for specific functions in living systems, human can harness biopolymers with modern technologies (e.g., synthetic biology, chemical modifications, nanomanufacturing) to find new technological opportunities at the interface between the biotic and abiotic worlds. Driven by rational design and ingenuity, silk can be re-engineered from various perspectives (e.g., amino acid sequence, material format, chemical functionalization, hybridization with other materials, etc.), yielding an ad hoc structural biopolymer that can address technological challenges unattainable with synthetic materials.

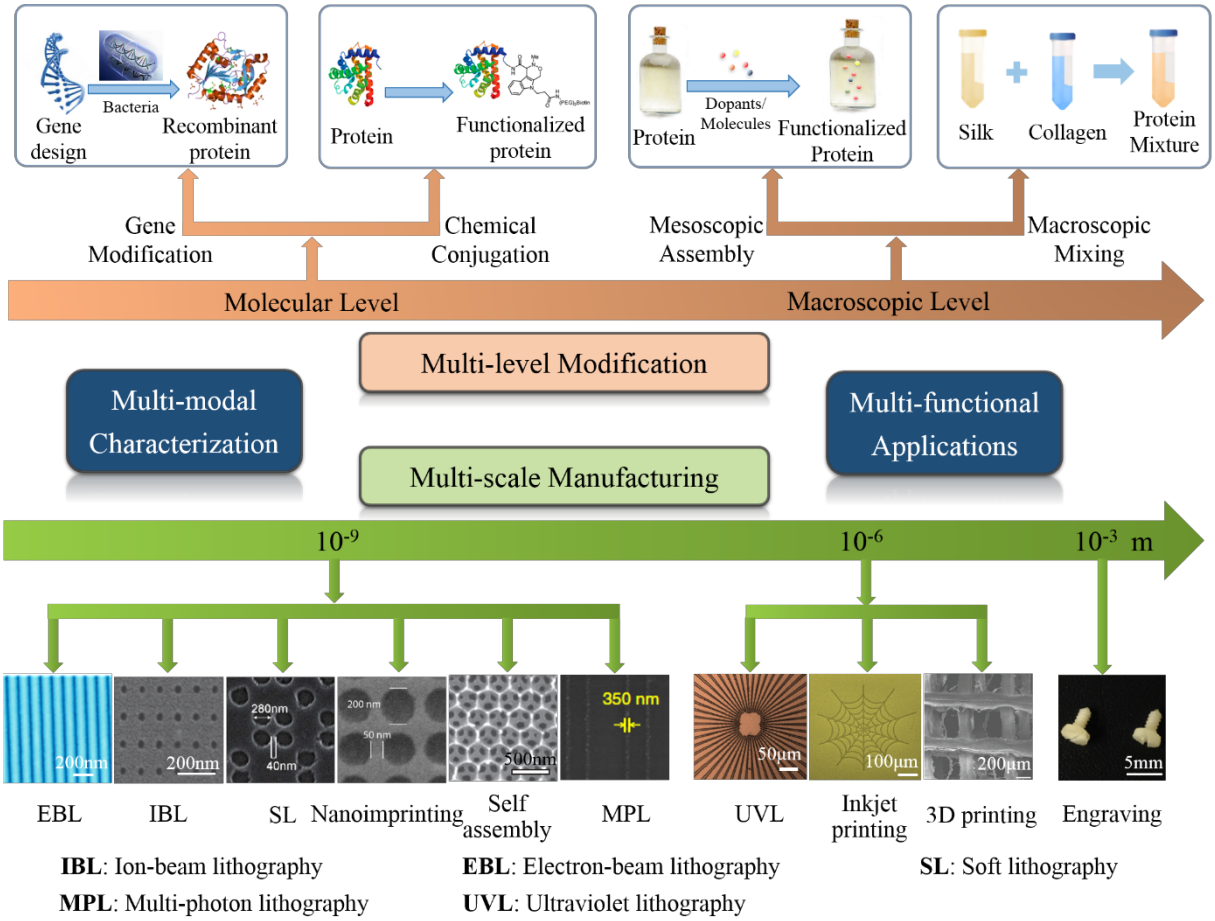

Figure 1. Schematic representation of the strategies used to modify, manufacture and characterize silk materials.[67-75]

In this review, we summarize the recent progress in characterizing silk-based micro/nanostructures to better understand silk polymorphism (**Section 2**), we cover recent multi-scale functionalization of silk proteins (**Section 3**), as well as innovative multi-scale manufacturing (**Section 4**) to impart features that are not readily available in the natural silk fibers and to fabricate a new class of multi-functional devices with unprecedented performances (**Section 5**) (**Figure 1**). In addition, a variety of silk-based biomanufacturing techniques will be compared, mainly from the perspective of spatial resolution, processing mode, processing conditions, and son on, as shown in **Table 1**. Lastly, we summarize the versatile applications of silk - with emphasis on available modification and manufacturing methods – in a flow chart of technology roadmap of "Revolutionary Silk Road" to provide a brief overview of recent developments in silk technology including precise bio-patterning, and controlled delivery, bio-optics and bio-photonics, flexible and degradable electronics, tissue engineering and medical implants, and further elaborate insights and outlooks to the future development of silk technologies (**Section 6**).

Table 1. Comparison of different manufacturing techniques for silk material processing

Technique	Critical feature size	Processing mode	Dimension [#]	Conformational transformation	Processing conditions	Ref.
IBL	~ 10 nm	Serial	2D, 2.5D	Yes	vacuum	[76]
EBL	~ 20 nm	Serial	2D, 2.5D	Yes	vacuum	[67, 77]
SL	~ 40 nm	Parallel	2D, 2.5D	No	vacuum	[58, 68]
Nanoimprinting	~ 50 nm	Parallel	2D, 2.5D	Yes	ambient *	[69, 78]
Self Assembly	~ 200 nm	Parallel	2D, 2.5D	No	ambient	[70, 79]
MPL	~ 350 nm	Serial	2D, 2.5D, 3D	Yes	ambient	[71]
UVL	~ 1.5 μm	Parallel	2D, 2.5D	Yes	ambient	[72, 80]
Inkjet Printing	~ 10 μm	Serial	2D, 2.5D	No	ambient	[73]
3D Printing	~ 50 μm	Serial	2D, 2.5D, 3D	No	ambient	[74, 81]
Engraving	~ 100 μm	Serial	2D, 2.5D, 3D	No	ambient	[75]

[#]: 2D: arbitrary patterns in x-y directions; 2.5D: greyscale patterns or repeating structures in the z direction; 3D: arbitrary geometries in both x-y and z directions.
*: Application of appropriate pressure and heat will facilitate the imprinting process.

2. Multi-modal Characterization

Understanding the structure-function relationship in silk materials requires a deep and wide investigation of protein domains as secondary and tertiary structures impart structural morphology and modulate material performance. At the molecular level, silk domains can be postulated by studying the protein IR spectrum. Amide bonds in silk protein possess characteristic absorption bands in the infrared spectrum, namely the amide vibrations (or bands). Among the nine amide band, Amide I (mostly due to the C=O stretching) and Amide II (due to the in plane NH bending and CN stretching vibration) bands are the most significant vibrational modes and are widely used to investigate secondary and tertiary structures of proteins. Measurement of the IR spectrum can be carried out using FTIR (Fourier transform infrared spectroscopy), which obtains the infrared absorption of a material using interferometric method. FTIR is a well-established and reliable characterization tool to study formation of protein domains.

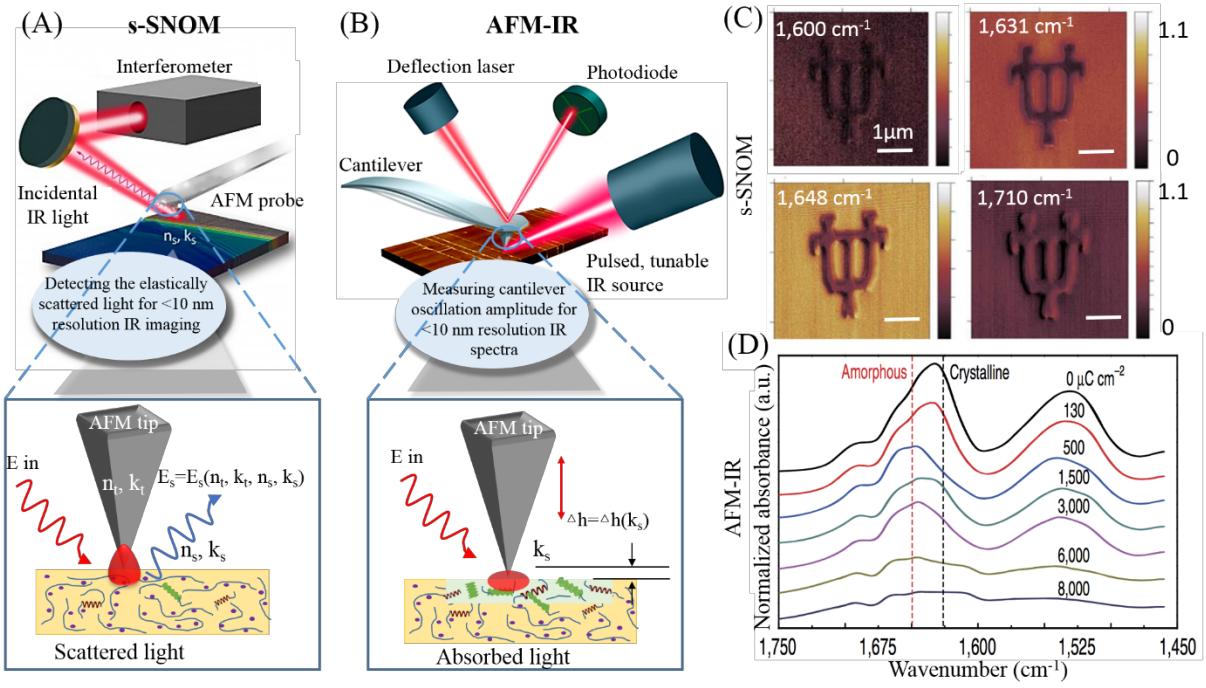

Figure 2. Multi-modal characterization using infrared scanning near-field optical microscopy (IR-SNOM). (A) Schematic of scattering type SNOM (s-SNOM). An IR beam is coupled to the sharp and conductive Atomic Force Microscopy (AFM) tip, allowing near-field interaction with sample in close proximity. The imaging capability of s-SNOM overcome diffraction limit of IR light source and provides nanometer resolution (B) The AFM-IR uses an AFM tip to detect the rapid thermal expansion of the sample surface due to the absorption of pulsed IR light. By sweeping the wavelength of the IR source, the absorption spectrum can be obtained. The s-SNOM and AFM-IR are successfully used to study the conformation transition of silk protein under the electron irradiation by both (C) nano-imaging and (D) nano-spectroscopy. Reproduced with permission. [77] Copyright 2016, Nature Communications, Anasys Instruments.

Advancements in silk nanoengineering pose significant challenges to the characterization of material properties at the nanoscale. Silk materials performances are modulated by the amount of β-sheet domains in the material.[82-84] Thus, investigating protein conformation at the nanoscale is fundamental to understand material behavior at the nanoscale. Conventional methods such as FTIR, scanning electron microscopy (SEM), and atomic force microscopy (AFM) are either limited by their spatial resolution (i.e. FTIR) or chemical sensitivity (i.e. SEM and AFM). In this context, IR scanning near-field optical microscopy (IR-SNOM) was successfully used to study the nanoscale characterization of silk conformation (**Figure 2**). Currently, SNOM has been widely applied in the characterization of a variety of solid state and polymer samples, including grapheme plasmonics,[85] phase transitions in correlated electron materials,[86] mineral polymorphs[87] and secondary structure analysis of single-protein complexes.[88] The multi-modal data acquisition of IR-SNOM allows simultaneous measurement of structural morphology, mechanical property and conformation of silk protein, significantly enhancing the capability to understand the structure-property correlation. The setup of an IR-SNOM is based on an AFM coupled with a tunable IR QCL laser. Two types of IR-SNOM are available for the study of silk protein. Scattering type SNOM (referred to as s-SNOM) offers direct near-field imaging of protein nanostructures.[89] IR beam scattered by the sharp and conductive AFM tip is collected by the IR detector and demodulated at the harmonics of the AFM tip. Through a pseudo-heterodyne detection scheme, infrared absorption of the sample can be obtained.[90, 91] On the other hand, thermal expansion based SNOM (referred to as AFM-IR) can be used to acquire the absorption spectrum of the sample at the nanoscale.[92-94] In this case, a pulsed laser is coupled to the AFM tip where the IR absorption of the sample causes the rapid expansion of the sample surface, which is sensed by the contact mode AFM. By sweeping through the wavelength of the IR source, the local absorption spectrum of the sample can be obtained. The characteristic peaks of the IR spectrum can then be correlated to the secondary structures of proteins.[95-97] Both tools are valuable to the study of the silk conformation and nanostructures, and have been used to investigate the electron irradiation regulated silk conformation transitions. For silk fibroin, the β-sheet structure corresponds to an IR absorption peak at ~1,622cm^{-1} – 1,637cm^{-1} whereas the amorphous state and the α-helix conformation correspond to an absorption peak at ~1,656cm^{-1} – 1,662cm^{-1}.[98] The results provide significant insight into the engineering of silk protein using electrons and thus proves the applicability of the SNOM technique on the studying of silk material.

3. Multi-level modification

Native silk materials including silk fibroin, sericin, and spider silk have attracted increasing attentions and have been widely used in various fields, due to their superior cytocompatibility and extraordinary mechanical properties. However, native silk materials may not meet some specific demands, such as enhanced strength (relative to native silk materials), photosensitivity, and therapeutic functions. Fortunately, the requirements including, but not limited to, the ones mentioned above can be implemented by multi-level modification of silk biomaterials thanks to the ease of functionalization. To be specific, the means of modification includes direct

feeding, gene modification, chemical conjugation, mesoscopic assembly, and macroscopic mixing, spanning from molecular level to macroscopic level. By selecting the appropriate method, new opportunities become available to attain new functions of silk biomaterials or silk-based devices, such as strength-enhanced silk fibers,[99-101] photosensitive silk photoresist,[72] silk screw with the function of drug sustained release,[102] and so on.

3.1. Feeding

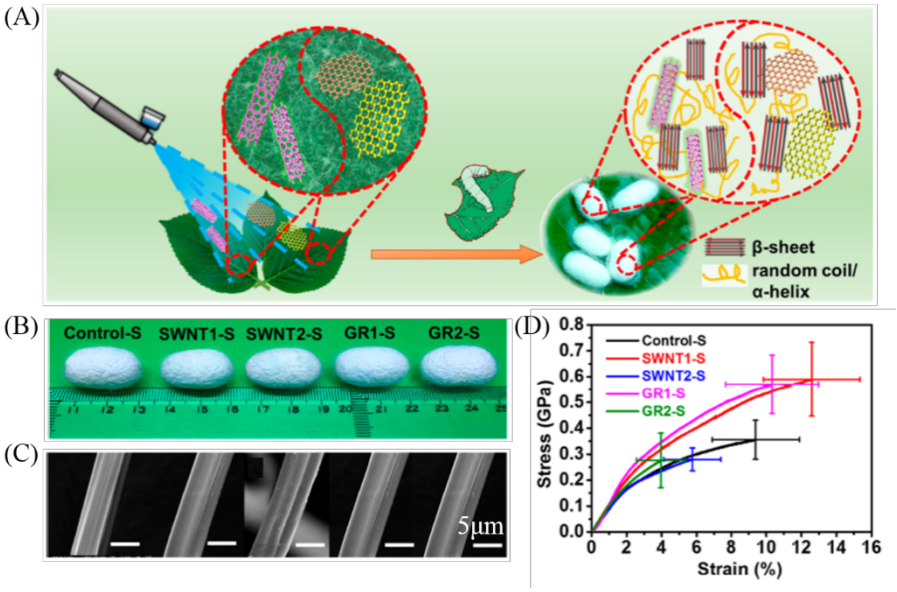

Figure 3. (A) Schematic of the process to feed silkworms with mulberry leaves spray-coated with single-walled carbon nanotube (SWNT) or graphene (GR) solutions. (B) Photograph and (C) SEM images of typical cocoons obtained from silkworms fed with five different diets. (D) Stress−strain curves of degummed silk fibers. Reproduced with permission.[103] Copyright 2016, American Chemical Society.

A highly productive and environmentally friendly method of modifying silk is feeding. For example, silkworms can be fed with specific diets to produce high-strength silk fibers. Carbon nanotubes and graphene are two kinds of carbon nano-materials receiving extensive attention due to their high tensile strength and stiffness, which motivate the development of methods for their use as reinforcement additives. Existing research indicates that by feeding Bombyx mori larval silkworms with single-walled carbon nanotubes (SWNTs) and graphene (GR), they can produce mechanically enhanced silk. More specifically, silkworms are fed with diets containing SWNTs with solution concentration of 0.2 and 1.0 wt % and GR at the concentration of 0.2 and 2.0 wt % to obtain silk fibers, which are denoted by SWNT1-S, SWNT2-S, GR1-S, and GR2-S, respectively. Among the four kinds of silk fibers, GR1-S shows the best mechanical properties with fracture strength. It is because the fed carbon nanomaterials are partially incorporated into the as-spun silk fibers, hindering the conformation transition of silk fibroin from random coil and α-helix to β-sheet, which may contribute to increased elongation at break and toughness modules (**Figure 3**).[103] The successful regeneration of SWNT or GR embedded silk fibers demonstrates the efficiency of functionalization of silk fibroin by in vivo feeding, which paves a new path for the large-scale production of silk fibers with superior properties. Furthermore, silkworms can also be fed with diets containing other non-toxic substances, such as dyes to produce fluorescent silk fibers.[104, 105]

3.2. Genetic Engineering

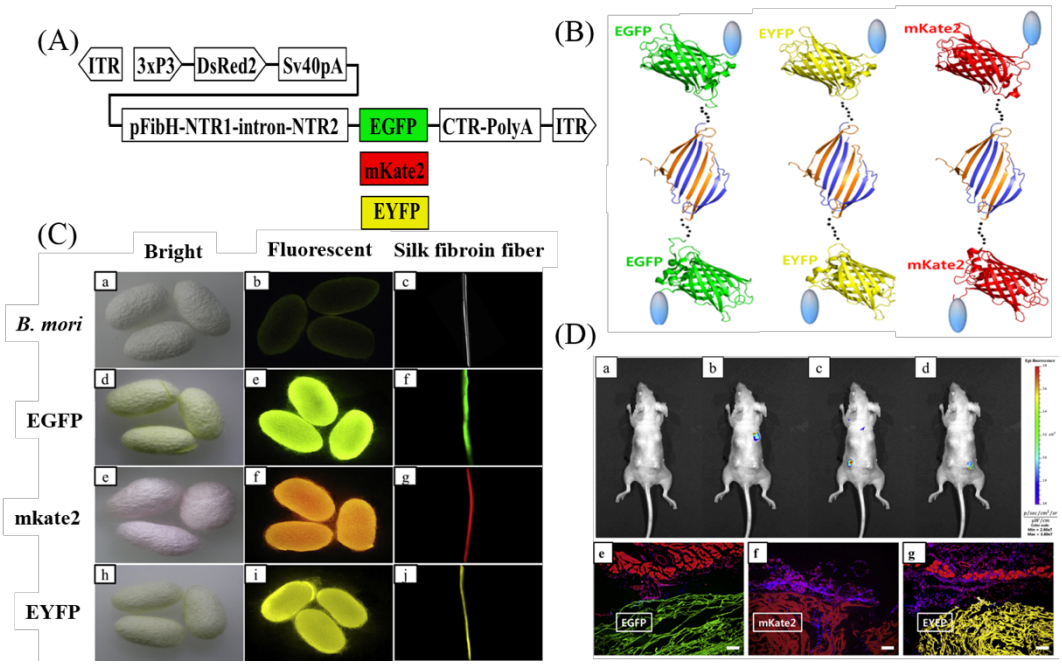

Figure 4. Fabrication of fluorescent silk for biological and medical applications. (A) Structures of transformation vectors for generating transgenic silkworms. ITR, inverted repeat sequences of piggyBac arms; 3xP3, 3xP3 promoter; DsRed2, Discosoma sp. red fluorescent protein 2; SV40, SV40 polyadenylation signal sequence; pFibH, promoter domain; NTR1, N-terminal region 1; intron, first intron; NTR2, N-terminal region 2; CTR, C-terminal region; PolyA, poly(A) signal region. EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; mKate2, monomeric far-red fluorescent protein. (B) The schematic diagram of the fluorescent silk fibroin, which was engineered by inserting a fluorescence protein, including EGFP (green), EYFP (yellow), and mKate2 (red), between the N-terminal domain and C-terminal region of fibroin. (C) Multicolored fluorescent cocoons and silk fibroin fibers. The fluorescent cocoons produced by transgenic silkworms had various fluorescent proteins including EGFP, mKate2 and EYFP. (D) Whole-body imaging of fluorescent silk fibroin sponges in the dorsal region of a nude mouse at 7 days. Reproduced with permission.[106] Copyright 2015, Elsevier Ltd.

Besides feeding, genetic engineering is another useful tool for the modification of silk fibroin to attain new features.[107] Using this method, the sequence of silk protein can be carefully controlled by gene selection. Genetically modified silks can be directly produced from transgenic silkworms, which can be easily proliferated and retained once the silkworm strain is established.

Recently, fluorescent silk fibroin produced from transgenic silkworms has been reported. The silk protein is modified by inserting the transgene into the silkworm genome to acquire the desired properties. For example, multicolored fluorescent cocoons can be obtained by inserting fluorescence proteins, including enhanced green fluorescent protein (EGFP), enhanced yellow fluorescent protein (EYFP), and monomeric far-red fluorescent protein (mKate2), between the N-terminal domain and C-terminal region of fibroin. In addition, the fluorescent silk fibroin sponges are implanted subcutaneously in rats and all of them retain fluorescence 7 days later, which indicates long-term cytocompatibility of the fluorescent silk fibroin sponges. Therefore, the fluorescent silk fibroins can be utilized as a bio-imaging tool in clinic by conjugating with biomarkers (**Figure 4**).[106] Genetic engineering technique exemplifies a sustainable route to control naturally occurring proteins at the gene level, which is crucial for spider silk manufacturing as well. It opens up opportunities for large-scale production of recombinant spider silks to overcome the limitation of low yield and unstable quality of conventional spider silk-manufacturing process through spider farming.[108, 109]

3.3. Chemical Modification

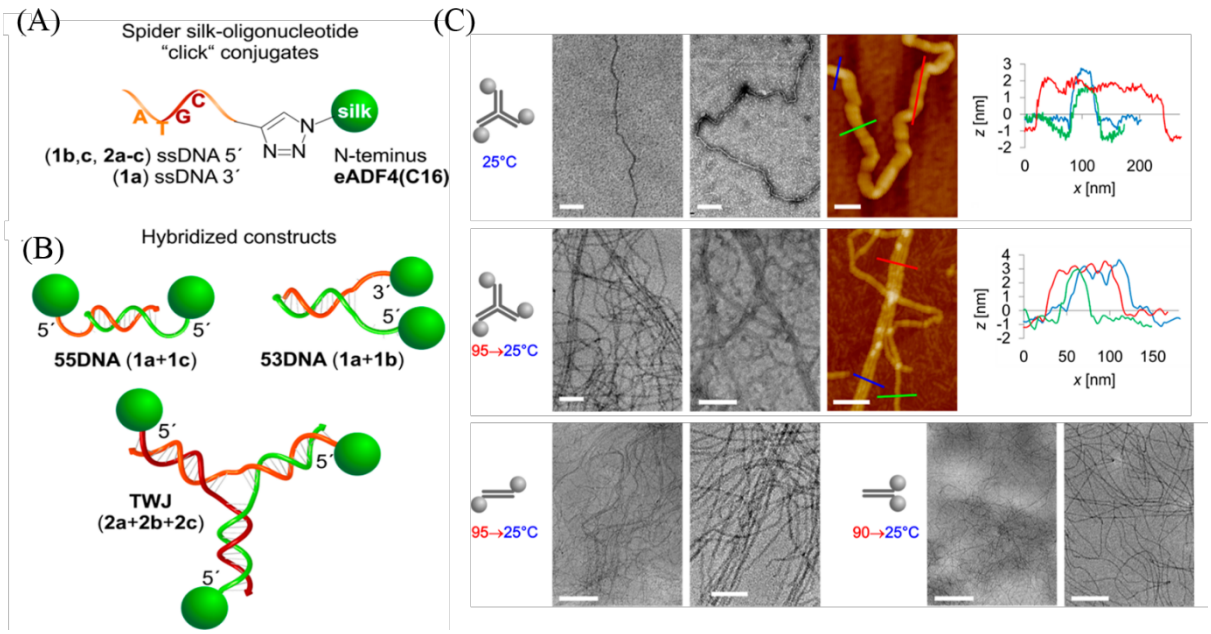

Figure 5. The assembly of fibril structures of DNA conjugated recombinant spider silk. (A) Spider silk oligonucleotide conjugates were prepared from azide modified eADF4(C16) and different 5′ - or 3′ -alkyne modified oligonucleotides. (B) The conjugated moieties were hybridized according to the designed complementarities of the oligonucleotides to arrange silk moieties in an antiparallel, parallel, and branched manner, respectively. (C) The assembly process controlled by the temperature gradient. Single three way junction (TWJ) fibrils were obtained under ambient temperature; TWJ ribbons and 55DNA ribbons are observed with a temperature gradient from 95 to 25℃ for 70min; no fibrils association was observed for 53DNA with the same temperature gradient.[110] Copyright 2014, American Chemical Society.

In addition to feeding and genetic engineering, which yields functional silk fibers directly by silkworms, chemical modification of the silk fibroin also plays an important role in the functionalization of silk materials. The modification can be achieved on the surface of silk materials by either physical adsorption or chemical immobilization.[111] For example, silk films conjugated by cyanuric chloride-activated poly(ethylene glycol) (PEG) shows increased hydrophobicity for anti-adhesions.[112] On the other hand, the modification can be achieved on the regenerated silk fibroin solution, which has better flexibility since the silk fibroin solution can be fabricated into diverse materials such as film, hydrogel, foam, etc. The amino acid side chains in silk protein can be conveniently conjugated with a variety of chemical groups in the aqueous environment. For example, the genetically engineered spider silk was conjugated with short oligonucleotides using "click" chemistry. The conjugated silk moieties could be arranged in a linear anti-parallel (55DNA), and parallel (53DNA) manner or in a branched three-way junction (TWJ) via the designed complementarity of the oligonucleotide moieties. More interestingly, hierarchical assembly of the DNA-spider silk conjugates into ribbons and rafts could be triggered by the temperature (**Figure 5**). Other types of functional molecules have also been successfully conjugated to the silk protein for a variety of applications such as photo-sensitization,[72] and cell attachment.[113] In particular, the biomedical applications of the chemically modified silk protein have been well documented and summarized in another review article.[114] The chemical modifications of the silk protein open up new opportunities in many areas that requires functions absent in the natural silk material.

3.4. Mesoscopic Assembly

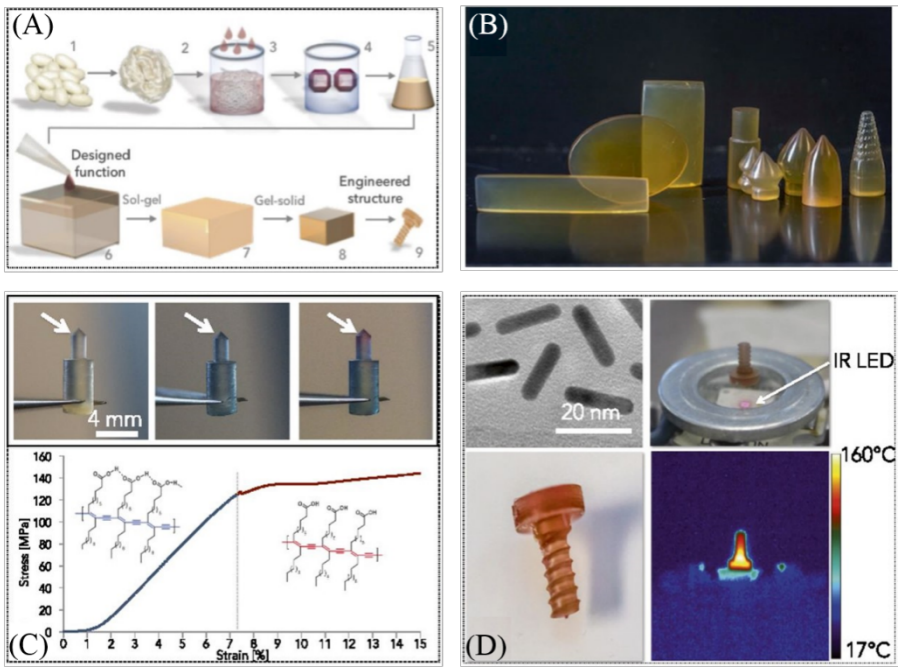

Figure 6. (A) The process of imparting designed functions to the engineered silk fibroin bulk materials. (B) Photograph of engineered 3D silk constructs. (C) Hybrid material consisting of silk fibroin and polydiacetylene (PDA) used for transducing internal strain by colorimetric changes. (D) Gold nanorods doped silk fibroin can be heated by a light matching the plasmonic resonance of the nanorods. Reproduced with permission.[102] Copyright 2017, National Academy of Sciences.

Silk fibroin is extracted from Bombyx mori silk cocoon through a degumming process which boiling silk cocoons in aqueous 0.02 M Na_2CO_3 solution, followed by solubilizing in 9.3 M LiBr solution, and then purified by dialysis for 48 hours. Doping with functional molecules is another way to functionalize silk fibroin. It is essentially a process of fixing functional component to the mesoscopic structure of silk material, which can be accomplished by adding desired dopants into silk solution to realize mesoscopic assembly. Silk fibroin can be doped with a variety of dopants, from inorganic components such as gold nanoparticles,[57] quantum dots,[115] to organic components such as enzymes,[73] polydiacetylene (PDA),[102] and so on.

By self-assembly, silk fibroin can form 3D bulk material, which can be easily pre-doped with water-soluble compounds to attain predesigned functions. Based on this technique, the 3D silk fibroin bulk material has been machined and polished into a variety of shapes (such as pins, screw, and so on) for surgical procedures by the researchers from Tufts University. By doping the 3D silk fibroin bulk material with different functional compounds, the fabricated devices show additional functions. For example, by adding PDA vesicles into silk fibroin, the subsequently created surgical pin will change color as it approaches its mechanical limits, giving the warning that it is about to fail. When silk fibroin is doped with gold nanorods, the fabricated functional screws can be heated by applying an infrared light, which enables the sustained release of doped bioactive agents, such as enzymes (**Figure 6**).[102] The ease of functionalizing silk bulk material by doping silk fibroin solution with other functional molecules such as carbohydrates, lipids, and nucleic acids, provides a compelling platform for the rapid design of sensing devices.

3.5. Macroscopic Mixing

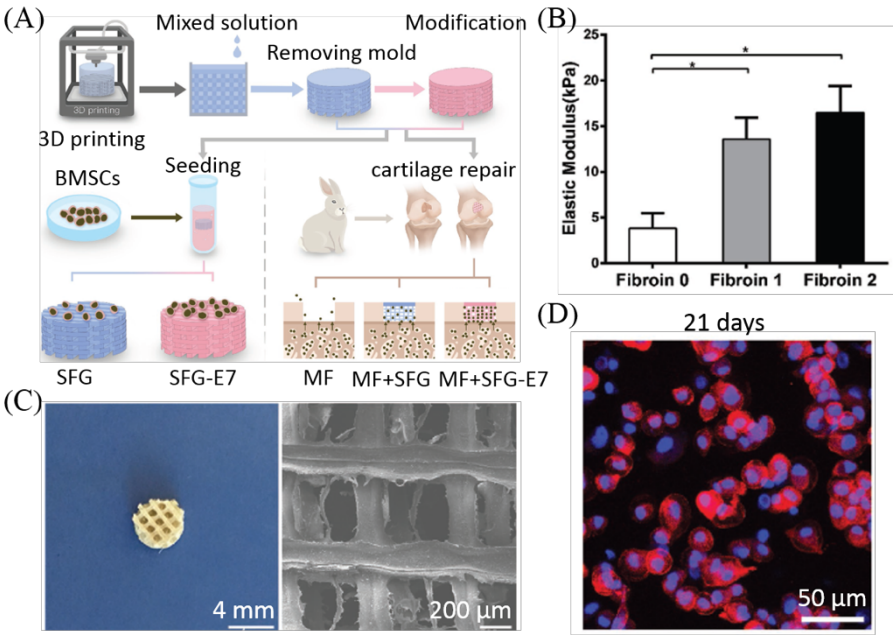

Figure 7. (A) Schematic illustration of silk-fibroin/gelation (SFG) scaffold fabrication process. (B) Elastic modulus of silk-fibroin/gelatin with different mass ratios: Fibroin 0 (0/3), Fibroin 1 (1/2), Fibroin 2 (2/1). (C) Photography and SEM images of the SFG scaffold. (D) The round shape of chondrogenic morphotype observed via Phalloidin/Hoechst assay after chondrogenic induction for 21 d with SFG scaffold. Reproduced with permission.[74] Copyright 2017, WILEY-VCH.

Tissue engineering has been an active field for a long time, due to its great promise for biological tissues/organs improvement or replacement. Silk materials offer an exciting opportunity for this application, for their excellent cytocompatibility, controllable biodegradability, and ease of functionalization. The functionalization of silk fibroin can be implemented in multi-level modification, such as gene modification, chemical conjugation, and mesoscopic assembly, as mentioned above. Actually, silk fibroin can also be functionalized by macroscopic mixing with other biopolymers such as chitosan or gelatin. The resulting composite materials owns adjustable properties such as mechanical and degradation characteristics, depending on the relative content of each types of materials.

Articular cartilage injuries are frequently encountered in orthopedics clinic, but its repair remains a significant clinical problem for physicians and researchers, due to its limited ability to regenerate itself. The latest research puts forward a promising way for cartilage repair in situ, which utilizes scaffolds to recruit and retain an adequate quantity of BMSCs from subchondral bone. The mixture of silk fibroin and gelatin (SFG) is chosen as the building material for the scaffold, because the mechanical properties and degradation rate of the mixture can be optimized by tuning the combination ratio of silk fibroin and gelatin to perfectly match the newly formed cartilage. The SFG scaffold for cartilage repair is fabricated by 3D printing technology and the mass ratio of silk fibroin solution (6.9% w/v) and gelatin solution (6.9% w/v) is optimized to be 1:2. After 21 days of culturing with SFG scaffold, the round shape of chondrogenic morphotype is observed, which indicates a strong chondrogenic differentiation ability of the cultured bone marrow stem cells (BMSCs) (**Figure 7**).[74] Besides gelation, silk fibroin can also be mixed with other relevant components (e.g., collagen and chitosan) to tune other properties, such as elasticity and toughness.[116-118]

4. Multi-scale Manufacturing

Multi-level modification equips silk with a variety of additional excellent properties, to further expand the applications of silk, the manufacturing of silk-based architectures is necessary. Over

the last few decades, manufacturing has undergone tremendous development, numerous manufacturing techniques (mostly in semiconductor field) are emerging. The combination of manufacturing technology and biomaterials offers new methods to engineer biomaterials and biomanufacturing. Numerous research efforts have been invested in the biomanufacturing, inspired by the existing integrated circuit (IC) manufacturing and microelectromechanical system (MEMS) fabrication, multi-scale manufacturing for silk biomaterial has been developed, including electron-beam lithography (EBL),[67, 77] ion-beam lithography (IBL), soft lithography (SL),[58, 119] nano-imprinting lithography (NIL),[69, 78, 120, 121] self-assembly,[70, 79] scanning probe lithography (SPL),[122] multi-photon lithography (MPL),[71, 123] direct pattern transfer,[124] bio-inspired spinning,[125] covering from nanoscale to macroscale. Biomanufacturing, a highly interdisciplinary field, seeks to create novel bioarchitectures as functional devices and interfaces, and attempts to integrate inorganic and organic components for new properties and functions, which have the potential for a wide variety of biological research topics and medical applications.

4.1. EBL

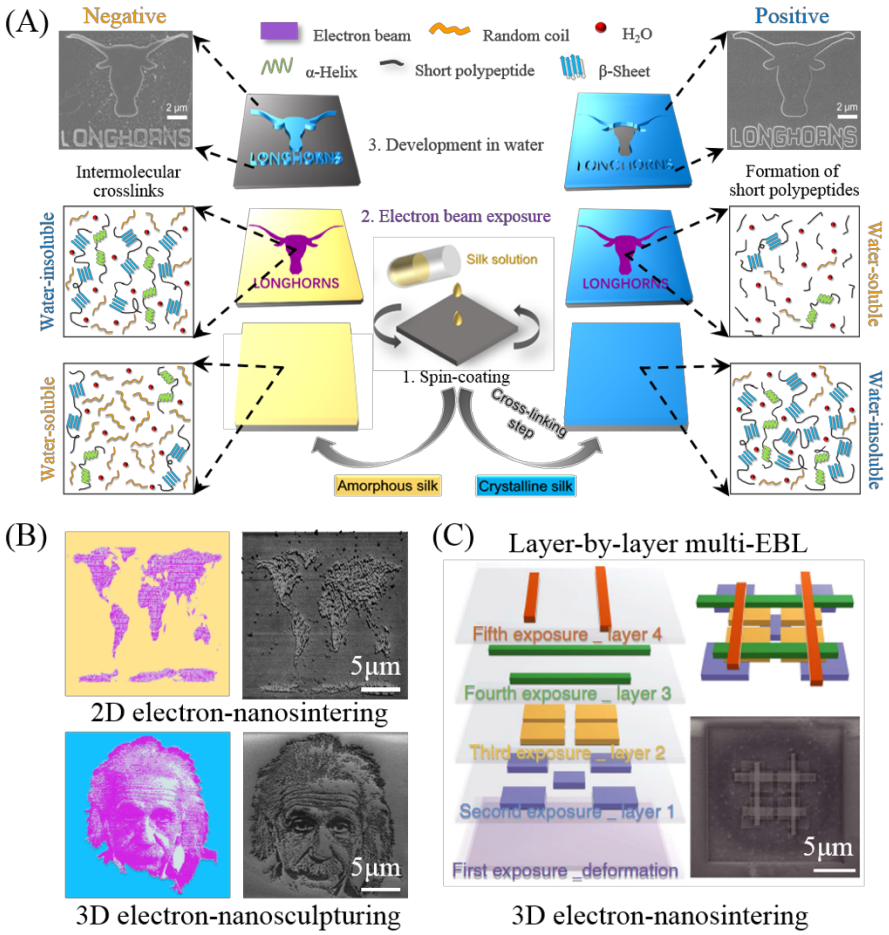

Figure 8. Silk conformational transitions regulated by electron irradiation and the 3D nanostructuring using EBL writing. (A) The silk fibroin conformation is modulated by the exposure to electron irradiation. (B) Both nanosintering and nanosculpturing can be realized by controlling the electron irradiation dosage and the initial fibroin conformation. (C) A layer-by-layer structure is realized by sequentially fabricating the nanostructures using EBL writing. Reproduced with permission.[77] Copyright 2016, Nature Communications.

The silk protein has shown high potential for being a functional material for a wide range of applications because of its compatibility with various manufacturing technologies that are used

in modern semiconductor industry,[84, 126] which considerably facilitates the engineering of silk protein on different length scales. In the nanometer size domain, the secondary structure of the regenerated silk fibroin dictates its solubility in water. The crystalline structure (β-sheet) is insoluble in water while the amorphous state is soluble. Interestingly, energy inputs such as electron irradiation can alter and even regulate the secondary structures (and therefore the solubility) of silk protein,[67, 77] suggesting a pathway of using silk as a resist material for lithographic processes such as EBL. Under the varying dosages of electron irradiation, the silk protein undergoes cycles of conformational transitions with progressive degradation. The amorphous silk protein is crystalized while the crystalline film can be de-crosslinked by the electron bombardment (**Figure 8A**). Due to the different electron penetration depth of silk protein in different conformations, the structure formation mechanism in crystalline silk is different from the amorphous silk. The fabricated structures form from top to bottom on the crystalline silk film (positive tone) while from bottom to top on the amorphous silk film (negative tone). Therefore, nanosculpturing and nanosintering can be achieved by using silk with different crystallinity (**Figure 8B**). Furthermore, by precisely controlling the dosage of electron irradiation, a layer-by-layer structure can be manufactured with high precision using this fabrication method (**Figure 8C**). Such versatility allows the construction of silk nanostructures with a rather simple procedure. Moreover, this manufacturing paradigm is bio-friendly since only water is involved in the storage and development step. The downside of this method is that the speed can be low due to the raster scanning nature of EBL writing.

4.2. IBL

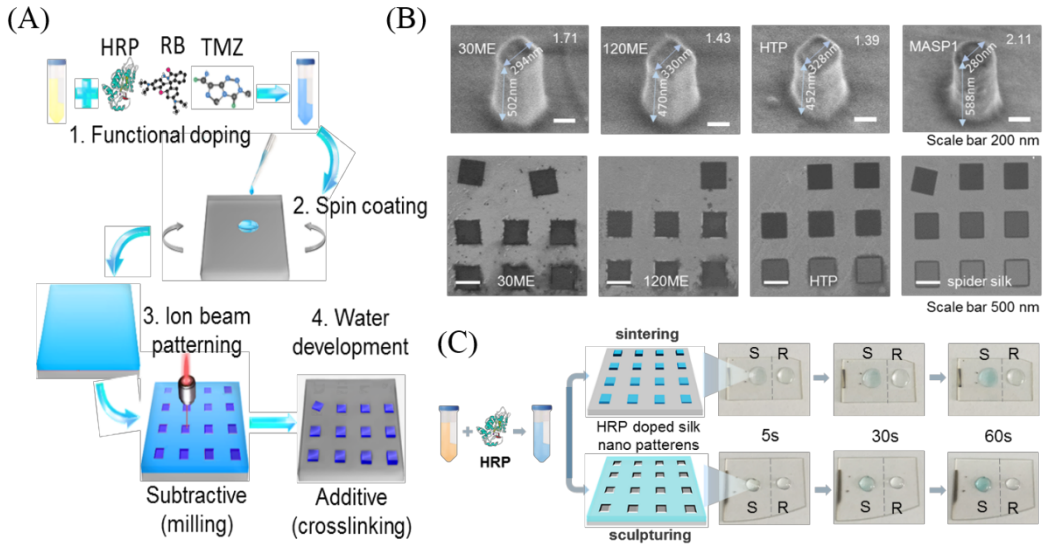

Figure 9. 3D bionanofabrication of recombinant spider silk using IBL. (A) The genetically engineered spider silk can be modified not only through genetic methods, but also easier processes such as doping. The spider silk film is then patterned using IBL. Both subtractive and additive manufacturing can be achieved. (B) Maximum structure aspect ratio of different materials fabricated by IBL (top), the influence of materials and dosage of IBL on fabrication sensitivity and contrast (bottom). (C) IBL has little adverse effect on the activities of enzyme embedded in spider silk. Reproduced with permission.[76] Accepted in Advanced Materials.

IBL is another widely used lithographic technique in semiconductor industry for defining nanoscale patterns, which can be also applied in biomanufacturing field. It is realized because the manipulation of silk secondary structures can also be accomplished by ion irradiation. The ions can etch off molecules on the surface of the silk film, as also observed in other types of IBL resist materials.[127-129] Meanwhile, the IBL writing has other effects on the silk film. The

ions that penetrate into the film carries energy that can crosslink the amorphous silk protein just underneath the etched areas. Once the ion irradiation dosage is high enough, the crosslinked structure anchors on the bottom of the sample and the patterns can stay on the substrate even after water development. Therefore, two complementary types of structures are formed at the same time during the fabrication process.

The precise nanostructuring on genetically engineered spider silk using IBL has recently been reported to create bio-functional patterns. It is accomplished by a three-step process, where functionalized spider silk solution is firstly spin coated into thin films, and then the designed nanoscale structures on the spider silk film are defined by an IBL writing tool. Finally, the defined structures emerge by a water development step. To further study the performance of IBL on silk proteins, different materials, including 30 min silk, 60 min silk, HTP silk, and Major Ampullate Spideroins 1 (MaSp1) have been patterned by IBL, the MaSp1 spider silk shows structures with the best aspect ratio. The effect of dosage of IBL on fabrication sensitivity and contrast has also been studied. In addition, the ELISA test indicates that the ion irradiation has little effect on the activities of enzyme doped in spider silk (**Figure 9**). The nano-structuring of silk protein ion beam lithography technique partially overcome the speed limitation of the EBL (although still not comparable to photolithography) since ions carry more energy than electrons and therefore needs less irradiation dosage to crosslink the protein. However, it means that the dosage of ion irradiation needs to be precisely controlled since the high energy input might decompose and even carbonize the silk protein.

4.3. Combination of EBL and IBL

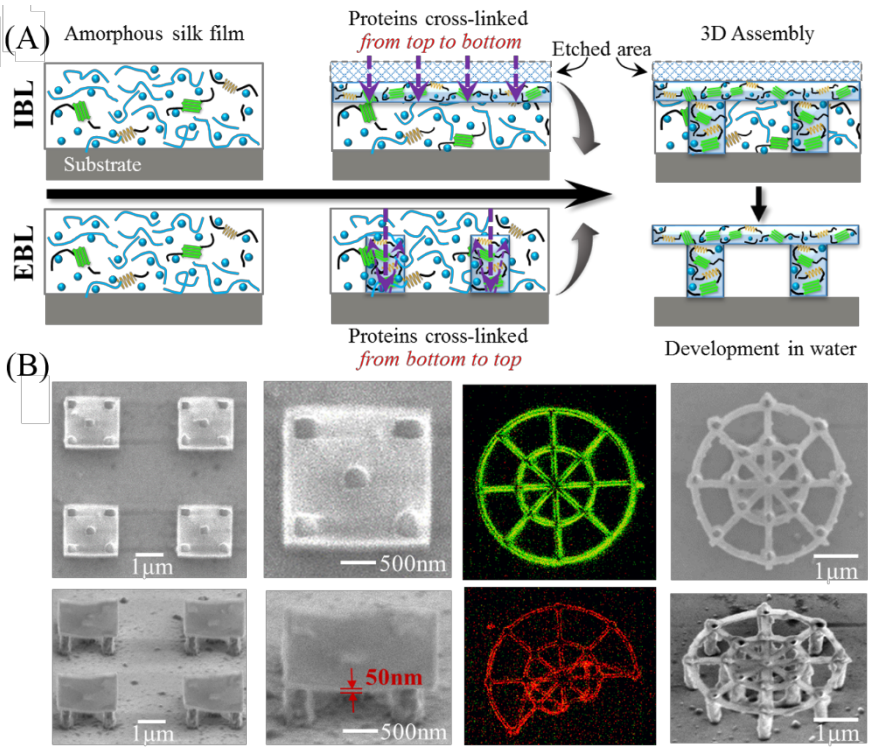

Figure 10. (A) The IBL creates nanostructures in the spider silk film from top to bottom, while the EBL behaves in the opposite way. It is mainly due to the penetration depth difference between ions and electrons. (B) Using the combination of IBL and EBL, complex 3D structures can be fabricated. The process can still maintain the activity of the dopants in the spider silk, as demonstrated by the nano-web doped with fluorescent dyes. Reproduced with permission.[76] Accepted in Advanced Materials.

Although both EBL and IBL can be used to create layered structures in silk protein, they are in fact pseudo-3D manufacturing techniques, because they cannot fabricate arbitrary 3D structures

individually. The current research indicates that EBL forms nanostructures from the bottom of the silk film to the top, while IBL behaves in the opposite way. This finding suggests an exciting pathway of creating complex and arbitrary 3D structures in silk by combining IBL and EBL. For example, nano-desks and biomimetic nano-webs are realized (**Figure 10**). The structures are accomplished with the following steps. Frist, the IBL writing creates the top part (that is, the desktop for the nano-desk and the web part of the nano-web) of the 3D architectures since the ions cross-links the silk protein from top to bottom. Meanwhile, the EBL writing creates the bottom structures (that is, the legs of the nano-desk and the anchor pillars of the nano-web) since the electrons can penetrate to the bottom of the silk film and starts to crosslink the silk protein from bottom to top. The capability of creating arbitrary 3D structures can be easily combined with the functionalization of the silk material by a simple mixing process. The technology is termed "Protein LEGO" by the author, highlighting the features of the fabricated nanostructures with shapes and functions on demand. This new bio-nanofabrication paradigm also provides a new alternative for engineering and immobilizing bio-components on hierarchical protein structures that could be constructed into functional nano-devices.

4.4. Self-Assembly

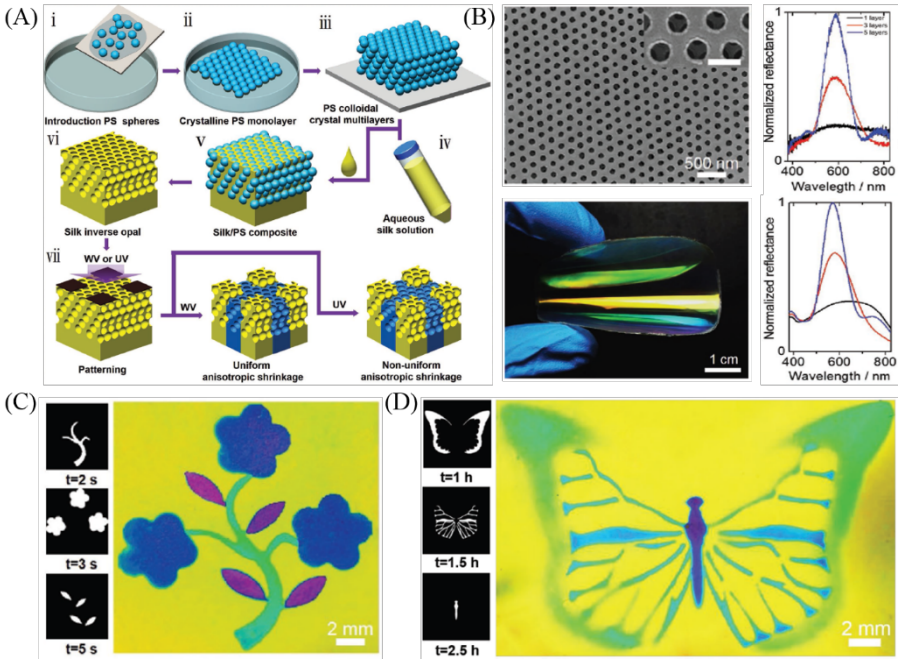

Figure 11. (A) Fabrication steps for large-scale patterned silk inverse opal. (B) SEM images of silk inverse opals (SIOs) templated from the colloidal crystals composed of polystyrene spheres with diameter of 210 nm and photograph of a 50 μm thick bent SIO film with initial lattice constants Λ = 300 nm. Measured (upper right) and simulated (lower right) reflectance spectra of SIOs (Λ = 300 nm) with different layers. (C) A floral pattern on the SIO created by selectively exposing part of SIO to water vapor for different times. (D) A butterfly pattern on SIO produced by exposing masked SIO to ultraviolet (UV) for different times. Reproduced with permission.[79] Copyright 2017, WILEY-VCH.

3D photonic crystals are artificially designed periodic structures that have attracted a lot of interest due to their potential applications in optics and sensors.[70] Silk fibroin is regarded as a promising candidate for optical applications due to its excellent optical transparency and mechanical robustness. The versatile micro-fabrication technology is obviously unsuitable for the implementation of such 3D periodic structures. Thanks to the fact that as a naturally occurring materials, silk fibroin can be an inspiring template for material design and synthesis, which offers an efficient way to accomplish 3D photonic crystals via self-assembly method.

The self-assembly of silk fibroin is regulated by controlling over the dynamics of water evaporation, which further affects the crystalline state of the silk fibroin.

An amorphous silk-based large-scale inverse opal has been fabricated by infiltrating the silk solution into polystyrene template and drying. [79] The polystyrene template is generated by self-assembly of polystyrene spheres to form large-scale crystalline monolayers at the water/air interface, and the monolayers are then transferred onto a hydrophilic substrate, layer-by-layer stacking to form a 3D polystyrene sphere array. Furthermore, the nanoscale lattice of the fabricated silk inverse opals (SIOs) can be tuned by water-vapor exposure or ultraviolet (UV) radiation exposure (**Figure 11**). 3D photonic crystals composed entirely of silk fibroin looks more appealing because of its cytocompatibility and ease of biological functionalization, which paves the way for unattainable device applications of traditional 3D photonic crystals,[130] such as implantable optical sensors. Moreover, self-assembly can be also utilized to form other intriguing structures such as silk-based 3D coffee stains, which can be developed further into all-protein lasers by integrating silk fibroin with fluorescent proteins.[131]

4.5. MPL

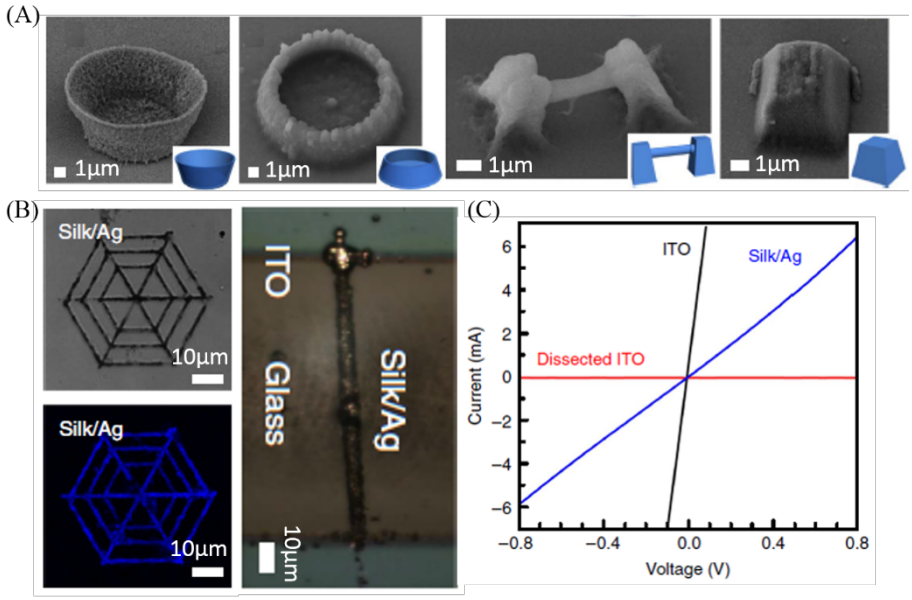

Figure 12. (A) SEM images of all-silk-based 3D micro/nanosculptures. (B) Confocal microscopic images of a silk/Ag composite 'microcobweb' (left) and metallographic optical microscopy image of a femtosecond laser direct writing (FsLDW) -fabricated silk/Ag composite microwire between two indium tin oxide (ITO) electrodes (right). (C) Current–voltage curves of the as-fabricated silk/Ag composite microwire. Reproduced with permission.[71] Copyright 2015, Springer Nature.

As described above, silk fibroin can be precisely patterned into desired micro/nanostructure in two and "pseudo" three dimensions (2D & pseudo-3D) by electron beam or IBL, which is accomplished through electron/ion-induced structural transitions of silk protein. However, electron/ion beam-based manufacturing techniques are fairly complex for the implementation of arbitrary 3D nano/microstructures. As a cytocompatible material, silk fibroin is a promising candidate for biological scaffold, which demands a method to create customized 3D structures.

Femtosecond laser direct writing (FsLDW) is a laser-based precise 3D micro/nanofabrication method based on two-photon polymerization.[132, 133] It has been demonstrated as a noncontact, maskless and fast technology for true 3D manufacturing.[134] Aqueous multiphoton lithography of various silk fibroin-based inks using FsLDW has been reported. In the experiments, silk fibroin is mixed with proper methylene blue (as a photosensitizer) is FsLDW-crosslinked into arbitrary fine 3D micro/nanostructures with remarkable mechanical characteristics to maintain the complex structures. In addition, a silk/Ag composite microwire

is also written out by FsLDW multiphoton lithography and exhibits good electrical properties, which provides a simple method for the fabrication of multi-functional metal/biomaterial-based electronic micro/nanodevices used for electrical bioengineering (**Figure 12**).[71] The aqueous FsLDW MPL paves the way for the development of silk based 3D micro/nanoprocessing techniques, which will make silk a suitable candidate for a wider range of applications, such as micro/nano-level tissue engineering and bio-sensing.

4.6. 2D Inkjet printing

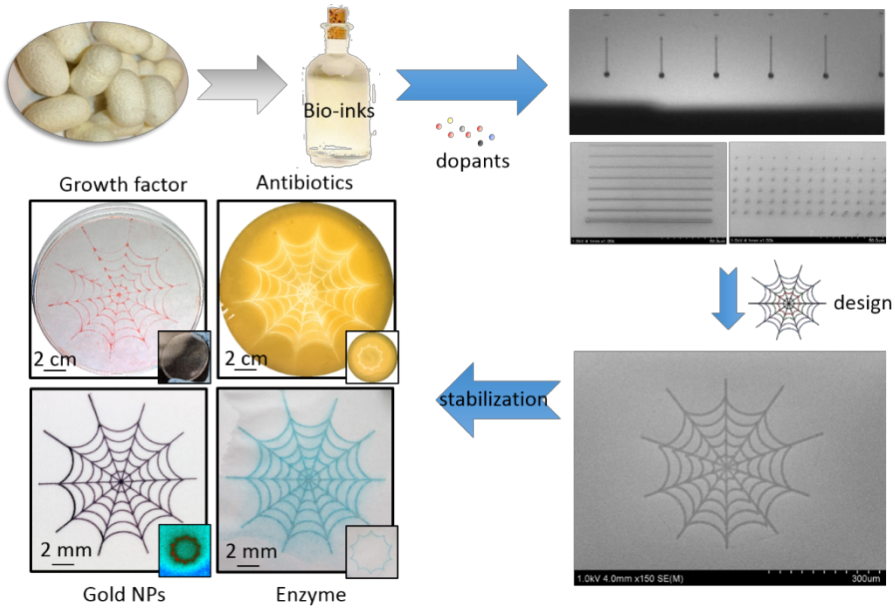

Figure 13. Silk fibroin solution doped with functional molecules (such as growth factor, antibiotics, gold nanoparticles and enzyme) is patterned by inkjet printing. Reproduced with permission.[73] Copyright 2015, Wiley-VCH.

The above mentioned manufacturing techniques including EBL, IBL or MPL, often offer high fabrication precision but slow fabrication speed. Therefore, they may not be suitable in some cases where high accuracy is not necessary but large-scale fabrication is important. Furthermore, silk fibroin usually needs to be spin-coated or cast into solid films and has to experience a structural transformation when it is patterned by EBL, IBL or MPL, which adds the complexity of silk-based device's fabrication. On the other hand, a piezoelectric-based inkjet printing process can be utilized to directly print aqueous silk fibroin solution into desired patterns in a large-scale without causing any structural transitions in silk fibroin.

Inkjet printing is a simple, mature and versatile technique which has great potential in biomanufacturing field, due to widespread availability and the ease of usage. Silk fibroin solution doped with functional molecules, such as growth factor, antibiotics, gold nanoparticles and enzyme, can be used as the functional inks.[73] The as-prepared silk inks are then loaded into printer cartridges and inkjet printed into spider web patterns on numerous surfaces for use in sensing, therapeutics, and regenerative medicine, and the thickness of the printed spider web patterns can be easily customized by controlling the printed drops of silk inks (**Figure 13**). In addition, the uncrosslinked silk fibroin film can also be "positively" (relative to silk fibroin solution printed patterns) patterned through inkjet printing technique by using deionized water as the printable inks. The combination of functional silk inks and inkjet printing technique provides a compelling platform for the rapid design of sensors and assays.

4.7. 3D Bio-printing

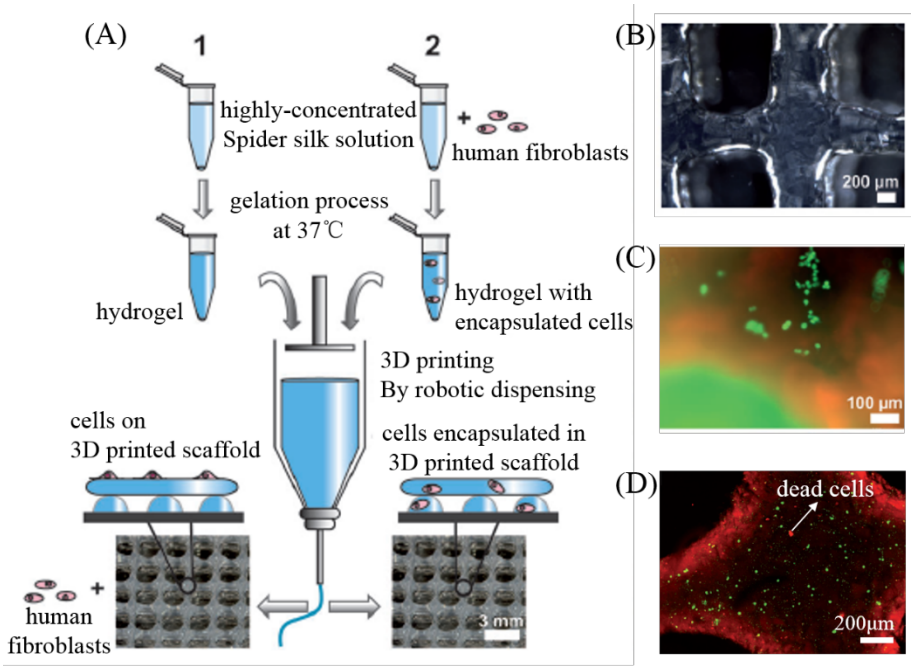

Figure 14. 3D bio-fabrication of cell loaded spider silk hydrogel. (A) the schematic process of creating the 3D spider silk scaffold, including the cell loading, gelation, and printing through dispensing devices. (B) The as fabricated spider silk hydrogel mesh with 8 layers. The fluorescent images of the human fibroblasts cultured on the 2-layer spider silk scaffold after (C) 24 hr and (D) 48 hr shows that the cells attached well to the scaffold and were able to proliferate within hydrogel.[81] Copyright 2015, Wiley-VCH.

The 3D printing of functional silk based bio-structures has also been investigated as an important material platform for tissue engineering and scaffolding. The silk based "bioinks" are generally functionalized by mixing with other organic and inorganic components, such as metallic ions, drugs, and antibody, to obtain features such as self-curing, therapeutic, and antibacterial effects, which can be tailored according to the requirements of the intended applications. Even larger dopants such as cells can be included in the silk "bioinks" to directly print 3D scaffolds. As shown in **Figure 14**, the concentrated spider silk solution can be loaded with cells in the solution phase and solidify through a mild gelation process.[81] The resulting cell-loaded "bioink" can then be printed with an extrusion based dispensing device into arbitrary 3D structures. The cells within the resulting spider silk scaffolds are able to adhere and proliferate for over at least a week. Since the spider silk hydrogel is formed by the physical crosslinking of the protein chains, the structure integrity can be maintained without post-treatment after the fabrication while ensuring sufficient diffusion of nutrients, oxygen, and waste products during the cell cultivation. Additionally, although the 3D printed silk structures are usually on the macroscale, the mesoscale structures and the nanoscale structures can also be controlled by using mechanical stress and dopants. For example, the pore size of the silk hydrogel in the nanoscale can be controlled by the inclusion sacrificial nanoparticles with varying sizes.[135] The hierarchically organized silk scaffolds serves can be used as physical cues to direct the stem cell differentiation.[136] The 3D bio-printing also has high potential for large-scale manufacturing since the printing speed can be improved through parallelization. Therefore, the biomaterial based 3D scaffolds serves as an indispensable tool for tissue engineering, regenerative medicine and medical implants.

4.8. 4D Manufacturing

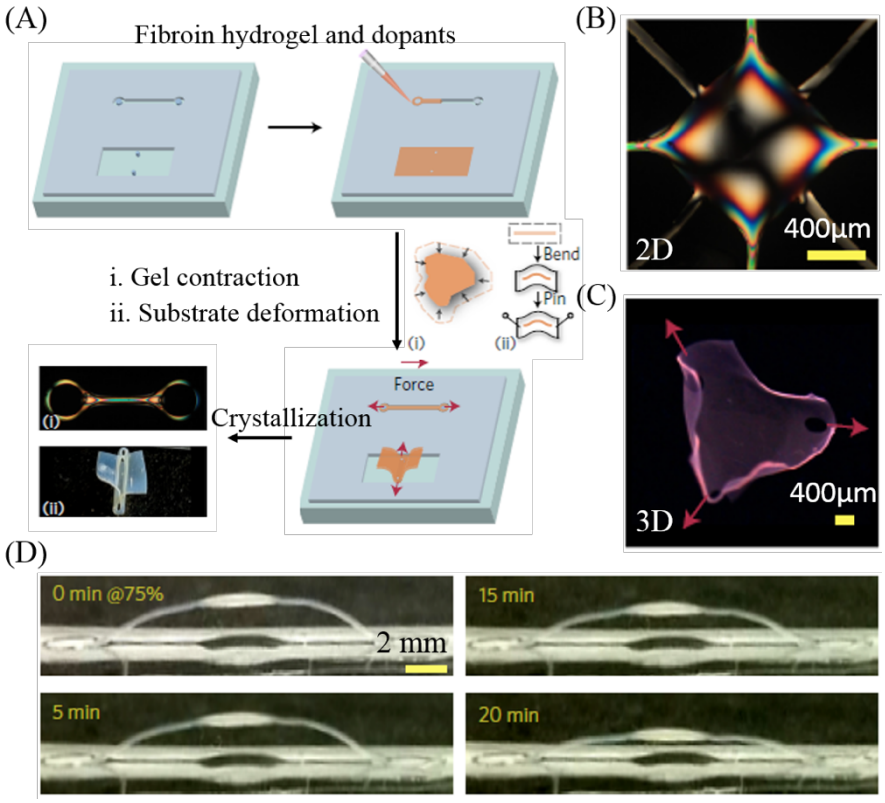

Figure 15. The directed assembly of bio-inspired silk fibroin hierarchical structures. (A) The hierarchical silk fibroin structure can be manufactured through a combination of top-down lithography and self-assembly process. The application of mechanical force directs the alignment of the silk micro-fibrils. (B) The alignment of the silk micro-fibrils is visualized using a polarization microscopy. (C) 3D structures can be achieved by controlling the folding behavior of the silk structures upon hydration. (D) The structural evolution of the fabricated silk fibroin structure in high humidity environment. Reproduced with permission.[137] Copyright 2017, Nature Publishing Group.

In nature, the living organisms are always consisted of materials that are organized hierarchically. Their structures are formed from evolved processes that governs directed self-assembly. Materials such as extracellular matrix (ECM), nacre, bone and fibers, possess heterogeneous and hierarchical structures that span multiple length scales.[138-142] Many of the structural components (especially in plants) exhibit shape-morphing capability due to the alignment of cellulose fibrils upon the change of humidity. Inspired from the natural material engineering process, a combination of top-down manufacturing (lithography) and self-assembly process using silk fibroin has been investigated,[137] where the resulting structures can change their shape in a controlled manner upon hydration. The shape manufacturing of the shape morphing structures is referred to as 4D printing in many published works.[143] The proposed method utilizes a predesigned macrostructure to orient and constraint the formation of porous fibrillary silk fibroin hydrogel networks (**Figure 15**). The resulting hierarchical structures with the anisotropic micro-fibers orient along the direction of applied mechanical stress, which can be visualized using a polarization microscopy. Such micro-arrangement of the silk fibroin allows the bulk material to have anisotropic mechanical properties and transport characteristics, assisting the accomplishment of biomimetic functions. This technique is readily adaptable to create structures that are environmentally responsive (that is, humidity responsive), taking advantage of the water absorption of silk fibroin. The swelling behavior of the aligned nanofibrils can be predicted and therefore used for designing the transformation of the fabricated structures upon the change of humidity. The anisotropic material properties can also be achieved by doping isotropic materials with components that are naturally anisotropic (e.g.

carbon nantube) where the printing process automatically aligns the doped components. The mild processing conditions of this technique allows the facile integration of bio-active components in the silk solution, opening up opportunities for even broader spectrum of functionalities.

4.9. Fiber Spinning

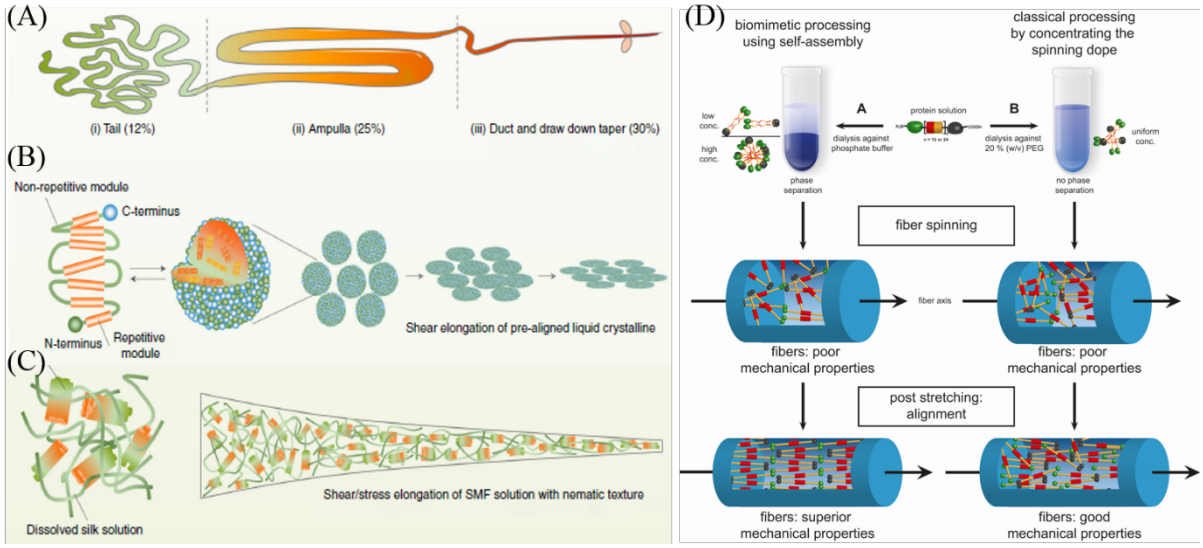

Figure 16. Bio-inspired spinning of regenerated silk and spider silk fiber. (A) The schematic of a silkworm spinning gland is divided into three parts. The structures of the silk protein evolve as it goes through each section of the gland. (B) Schematic model of the natural silk fiber assembly mechanism occurring along the spinning apparatus. The scheme is adapted from ref.[144] , with permission from Elsevier. The silk proteins are synthesized in the tail and are transferred to ampulla with increased concentration. In this region, the silk proteins are assembled to micelle-like configurations with anisotropic liquid-crystalline properties. Finally, silk fiber formation occurs under shear stress and dehydration conditions during pulling out the nematic silk proteins from the spigot. (C) Schematic of the SMF evolutionary process during spinning. The SMFs are aligned in the spinning jet (or fiber) axis direction under the shear/stress elongation.[145] Copyright 2017, Nature Communications. (D) The schematic comparison between the fiber production process of using the biomimetic self-assembly and the classical processing by concentrating the spinning dope.[146] Reproduced with permission. Copyright 2015, Wiley-VCH.

Silk fibers produced by silkworms and spiders possess exceptional mechanical properties that exceeds many synthetic polymers.[84, 126] Numerous efforts have been devoted to mimicking the fiber production process by the insects using regenerated silk solutions.[32, 147, 148] However, the as-spun regenerated silk fibers (RSF) are usually brittle and requires complex post-treatment (e.g. dehydration and crystallization processes) to yield useful fibers.[148, 149] The key to the high performance RSF lies not only in the spinning process but also the preparation of the spinning dope. The natural silk spinning dope is a high concentration (~25 wt %) solution where the silk proteins are assembled into micelle-like configuration with liquid-crystalline properties.[18, 144, 150] This liquid crystallinity allows better alignment of the molecules due to shear stress and dehydration. In a recent work, a method to prepare a high concentration and liquid crystalline silk spinning dope is proposed (**Figure 16A-C**).[145] Natural silk fibers are gradually and partially dissolved in hexafluoroisopropanol (HFIP), yielding a highly viscous and uniform solution that contains suspended microfibrils with diameters of 5 – 10 μm. This nematic liquid-crystal like solution is then used as the spinning dope for the following extrusion process in an ambient air condition. The resultant RSF retains the hierarchical structures of the natural silk fibers and possesses mechanical properties that are comparable to the spider silk fiber, without the need of post-treatment. The RSF can be further functionalized by mixing with carbon

nanotubes to act as humidity and temperature sensors. Additionally, polymorphic RSF is also created with pre-designed 3D structures, providing a new protocol to fabricated complex structures beyond mimicking natural fiber construction.

Compared to the fibers produced by silkworms, the dragline fibers produced by spiders have even higher mechanical toughness. The artificial spinning of the spider silk fibers has also been of high research interests for decades, and significant progress has been made to improve the mechanical properties of the spun fibers. As shown in **Figure 16D**,[146] the recombinant spider silk is used for producing artificially spun fibers. The recent success in increasing the fiber toughness lies in a biomimetic process. Before the fiber spinning, the spinning dope is concentrated through a dialysis process where the spideroin proteins tend to self-assemble. The resulting high concentration spinning dope plays a crucial role in the fiber formation stage. The toughness of the fibers spun using the biomimetic process is slightly higher than even the natural fibers, and much higher than those spun using the classical spinning dope. In addition, the as-spun fibers are post-stretched such that the molecules align along the direction of stress. It is similar to the natural spinning process where the spider post-stretches the spun fibers using their hind legs as soon as the fiber leaves the spinneret. Such alignment significantly increased orientation of the β-sheet crystals and thus the overall toughness of the fibers.

5. Multi-functional Applications

The silk is a favorable candidate material in many areas due to its intrinsic properties, such as superior mechanical strength, cytocompatibility, biodegradability, optical transparency when casted into films, and the facile inclusion of functional dopants. In particular, the mechanical strength of the silk protein ensures its capability to maintain structural integrity as a functional material. Furthermore, with the multi-level modification (from genetic engineering to simple mixing) and multi-scale manufacturing (from nanoscale to macroscale), the silk protein can be fabricated into various structures with complex functionalities beyond the capability of natural silk fibers. The modification of the silk protein alone mainly improves the chemical and biological functions while the manufacturing processes help to shape the silk into structurally supporting geometries. However, the combination of the modification and the manufacturing of silk has enabled functions that are not limited by the mere sum of these two components. One example is the silk scaffold where both the surface properties and the 3D micro-structure of the material are essential for the cell attachment and proliferation. This combinational complexity opens up immense opportunities for applications of silk in areas such as optics, transient devices, drug delivery and tissue engineering.

5.1. Bio-optical Devices

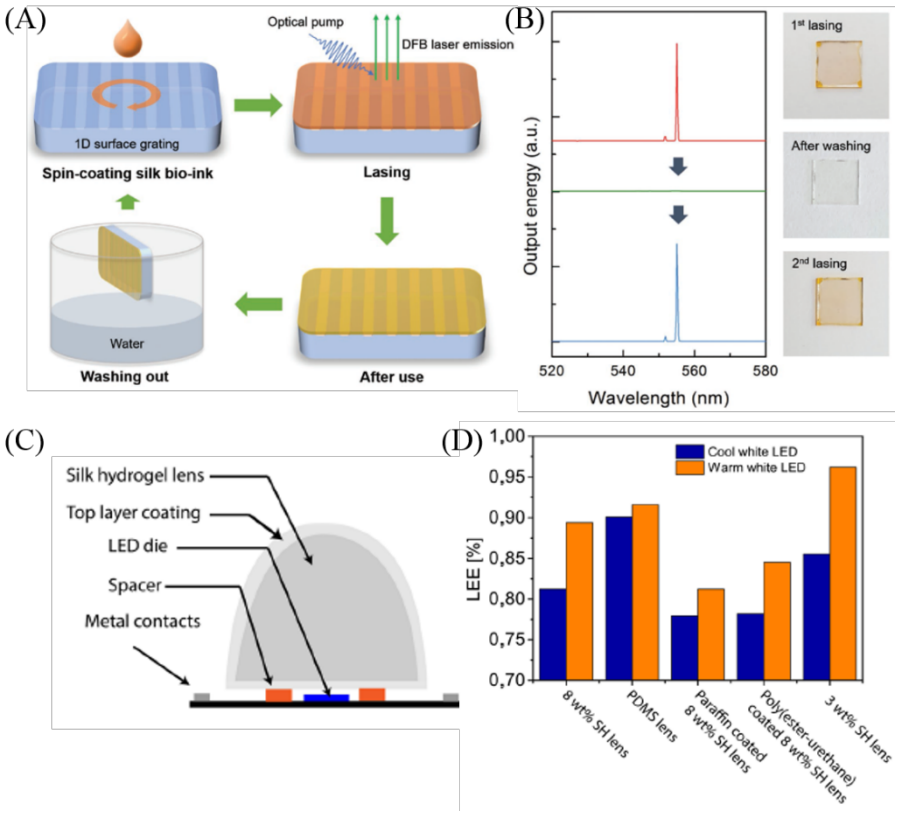

Figure 17. (A) Working principle of the silk-based physically transient distributed feedback (DFB) laser. (B) Lasing spectra from the DFB laser.[151] Copyright 2016, WILEY-VCH. (C) Schematic of silk hydrogel used in light-emitting diodes (LEDs). (D) Light extraction efficiency (LEE) of different lens on cool and warm white LEDs.[152] Copyright 2017, Scientific Reports.

To meet the present sustainable development strategy, great demand for biodegradable and environmentally friendly optical devices stimulates interest in biomaterials research. Due to the excellent optical and eco-friendly properties, silk has gained an increasing prominent role in bio-optical devices.

The first example to be presented is a physically transient distributed feedback (DFB) laser using sodium fluorescein dye-doped silk fibroin. The DFB laser is achieved by spin coating sodium fluorescein-doped silk fibroin on the fused silica substrate with a 1D surface grating whose geometrical parameters selected as 60 nm height, 370 nm pitch size, and 60% duty cycle. The fabricated DFB laser displays a single mode lasing at the wavelength of 555 nm upon pulsed optical pumping. By washing away the coated silk fibroin, the lasing peak disappears. After spin-coating the silk bio-ink, it appears again (**Figure 17A & Figure 17B**).[151] In addition to films, silk can be also processed into other forms such as hydrogels. **Figure 17C** shows the use of silk hydrogels as a lens material for light-emitting diodes (LEDs). The silk hydrogels can be fabricated into crater- or dome-type lenses to attain desired spatial intensity profile. The optical efficiency of the fabricated silk hydrogel lenses are evaluated by light extraction efficiency (LEE). When silk concentration in the hydrogel is adjusted to 3 wt%, the lenses show LEE over 0.95 on a warm white LED (**Figure 17D**).[152] In general, bio-optical devices have attracted more and more attentions, apart from the aforementioned examples, other relevant research results such as enhanced green fluorescent/silk fibroin-based DFB lasers also exhibit good performance.[153]

5.2. Physically Transient Optics

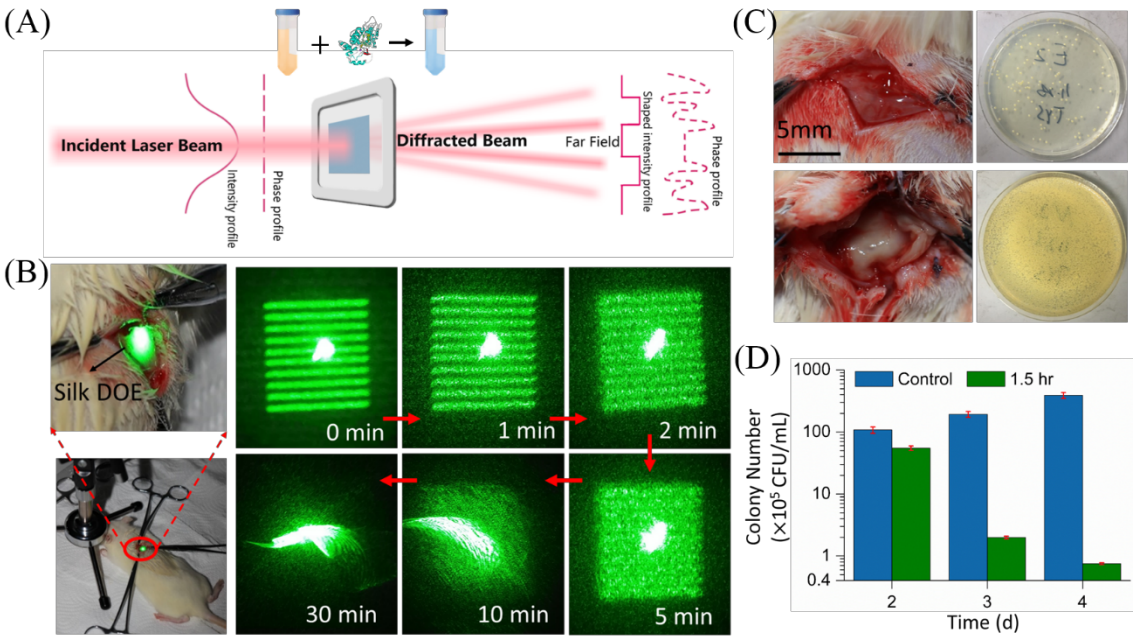

Figure 18. (A) Schematic of the working principle of the silk-based diffractive optical element (silk DOE). (B) Penicillin-loaded silk DOE is used as an antibacterial skin patch and real-time drug release can be read out by monitoring the reflected diffractive patterns. (C) Visual examination and (D) bacterial colony culture results indicate the effectiveness of the therapy. Reproduced with permission.[58] Copyright 2017, Wiley-VCH.

In addition to be used as a substrate or structural layer in transient electronic devices, silk fibroin can be also used in transient optics. Silk film is highly transparent to the visible light, mechanically robust,[68] and can accurately replicate the microstructures from other inorganic molds pre-patterned with desired surface features using a "cast-and-peel" soft lithography technique, all of which make it suitable for a wide range of optical devices. Furthermore, the controllable biodegradation rate of silk-based device offers a variety of sensing applications based on its transient behavior. The ability of maintaining the activity of embedded bioactive dopants makes silk fibroin widely used in medical field.

Recently, silk fibroin films processed into diffractive optical elements (DOEs) have been used as a multi-functional sensing platform, based on the fact that the diffraction pattern of the DOE is highly sensitive to its surrounding environments and its structural integrity. A set of bioactive DOEs micro-fabricated using functionalized silk fibroin films have been tailored for hydration sensing, biologically enabled data concealment, therapeutic treatment, and in vitro and in vivo drug release monitoring upon degradation.[58] The antibiotics of penicillin-loaded silk DOE acted as an antibacterial skin patch and is applied to the skin of Sprague-Dawley rats at a site that has been wounded and infected with Staphylococcus aureus. The release of the embedded penicillin molecules can be monitored by the decrement of the integrity of the reflective diffraction patterns in real-time. The visual examination and experimental result of bacterial colony culture both demonstrate the effectiveness of the therapy realized by penicillin-loaded silk DOE (**Figure 18**). The silk fibroin based optical devices features high sensitivity in sensing application, ease of functionalization, and controllable biodegradability, which are favorable properties for physically transient optics.

5.3. Implantable Medical Devices

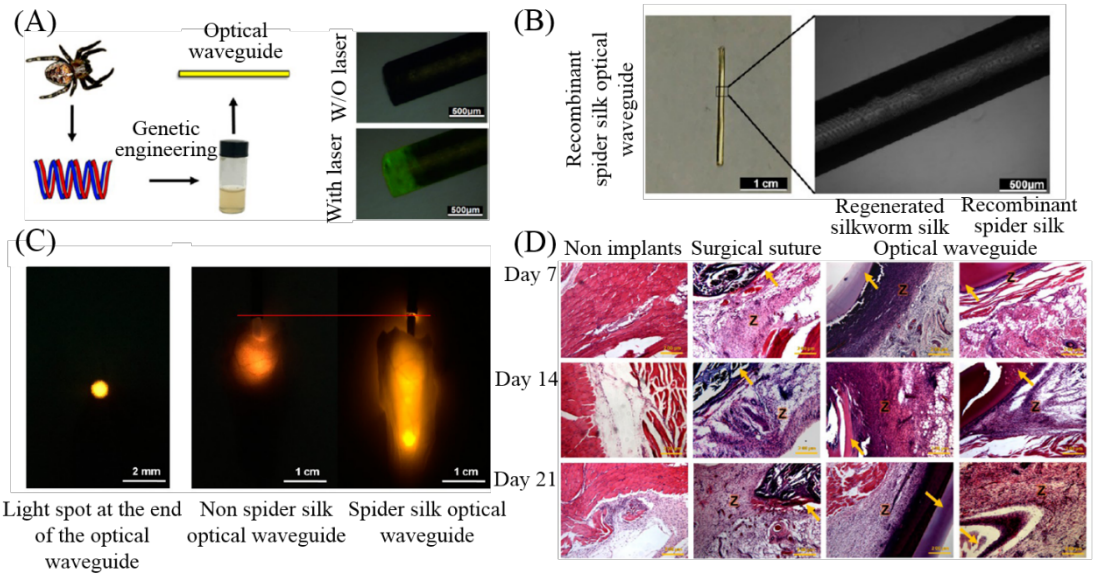

Figure 19. Simplified fabrication process (A) and optical images (B) of the recombinant spider silk optical waveguide. (C) Light spot at the end of recombinant spider silk optical waveguide passing through muscle (left panel) and light penetration length with or without a recombinant spider silk optical waveguide inserted into muscle (right panel). (D) H&E stain images of rats subcutaneously implanted with surgical sutures, regenerated silkworm silk and recombinant spider silk optical waveguides at 7, 14, and 21 days after implantation.[154] Copyright 2017, American Chemical Society.

Nowadays, optical devices have attracted extensive attentions for their great potential in biomedical applications from diagnostics to therapeutics. Various types of implantable optical devices such as optical waveguides, optical fibers and microprism arrays have been fabricated using bioabsorbable polymer for their cytocompatibility and biodegradability.

Recombinant spider silk has rapidly emerged as promising building material for biomedical optical devices due to its distinctive optical and biological properties, such as high optical transparency, excellent cytocompatibility and biodegradability. Currently, recombinant spider silk-based optical waveguide has been reported (**Figure 19**).[154] In virtue of the homogeneous smooth surface, high refractive index, and low optical loss, the as-reported recombinant spider silk optical waveguide can deliver light to deep biological tissue with a light penetration length of 3 cm, which indicates the high light guide efficiency. In addition, the recombinant spider silk optical waveguide has also been subcutaneously implanted in rat to evaluate its in vivo cytocompatibility. H&E stain results show that the recombinant spider silk optical waveguide has low inflammatory response to tissue, which indicates its good cytocompatibility. This newly developed optical waveguide paves the way to light-based diagnostics and therapeutics. Besides of recombinant spider silk, implantable optical waveguides fabricated by bioabsorbable polymer such as poly(D,L-lactide-co-glycolide) (PLGA) and poly(L-lactic acid) (PLA) have also been reported and exhibit great effectiveness for inducing photochemical processes in deep tissue, which also stimulates significant development in photomedicine.[155]

5.4. Drug Delivery

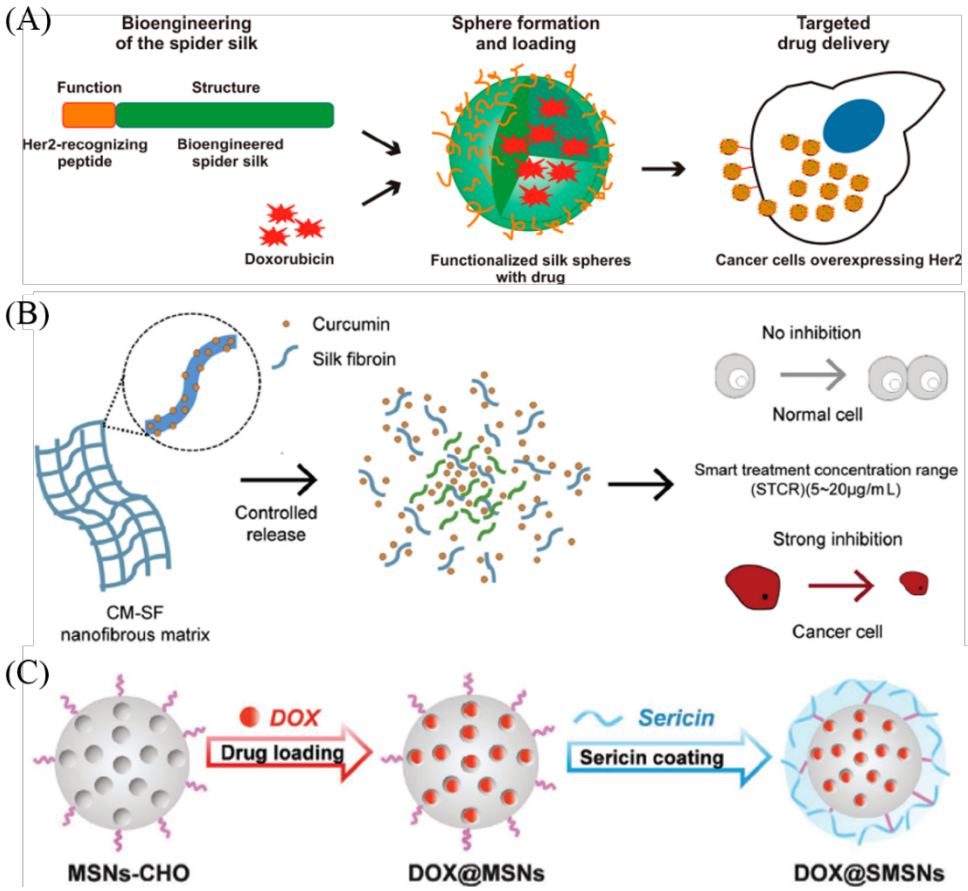

Figure 20. (A) Schematic representation of doxorubicin (DOX)-loaded bioengineered spider silk spheres for targeted cancer therapy.[156] Copyright 2014, American Chemical Society. (B) Illustration of curcumin-loaded silk fibroin nanofibrous matrix for smart cancer treatment.[157] Copyright 2016, Elsevier Ltd. (C) Synthesis procedure of DOX-loaded sericin-coated mesoporous silica nanoparticles (MSNs). [158] Copyright 2016, WILEY-VCH.

Chemotherapy is a very common cancer treatment which uses drugs to kill cancer cells. However, currently used chemotherapeutic drugs usually have adverse side effects on normal cells, which greatly limits their use. Therefore, an important challenge in cancer treatment is to find a controlled targeted drug delivery strategy to deliver the anticancer agents directly to tumor cells while sparing normal cells. Drug delivery nanosystems such as protein-based nanoparticles or nanofibrous have been proven as a feasible way to overcome the above mentioned problems in chemotherapy of cancer.

The chemical and physical characteristics of bioengineered spider silk make it especially suitable for spheres-based drug delivery. Functionalized bioengineered spider silk spheres loaded with doxorubicin (DOX) has the ability to serve as an effective carrier for targeted cancer therapy. In vitro DOX release experiment indicates the nanosystem can target breast cancer specifically (**Figure 20A**).[156] **Figure 20B** shows silk fibroin nanofibrous-based drug delivery nanosystem for cancer treatment. The silk fibroin nanofibrous with controllable fibre diameter loaded with curcumin displays a superior anticancer potential in colorectal cancer cells at a concentration of greater than 5 μg/mL.[157] In addition to bioengineered spider silk spheres and silk fibroin nanofibrous, sericin nanoparticles can also serve as a targeted drug delivery platform. Sericin is extracted using the following process: Bombyx mori cocoons are boiled for 30 min in aqueous 0.02 M Na_2CO_3 (Sigma-Aldrich, USA). The obtained solution is dialyzed for 48 hours in deionized water and centrifuged at 8000 rpm for 30 minutes. The supernatant is collected and lyophilized to obtain sericin powder. Sericin is coated onto mesoporous silica nanoparticles (MSNs) by self-assembly, serving as a functional shell to improve MSNs' capping efficiency and drug release. The DOX-loaded sericin-coated MSNs delivery system

prevents the premature leakage of encapsulated DOX in extracellular environment and transports DOX to the designated location (**Figure 20C**).[158] These protein-based drug delivery systems exhibit remarkable effects in cancer treatment, but the concept of targeted drug delivery is not limited to the treatment of cancer, it has much broader applications.

5.5. Tissue Engineering

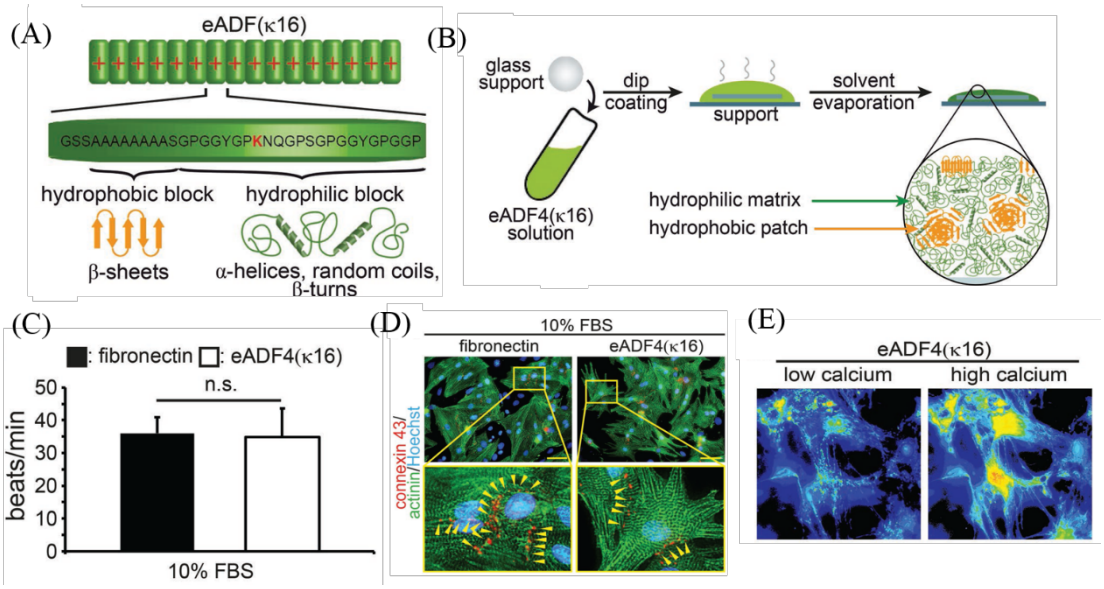

Figure 21. (A) Design of engineered Araneus diadematus fibroin 4 (κ16) (eADF4(κ16)). (B) Processing of eADF4(κ16) into films. (C) Quantitative analysis of contractile activity of cardiomyocytes on eADF4(κ16) films and fibronectin films. (D) Ratiometric intracellular calcium imaging of ardiomyocytes seeded on matrix stimulated with 10% fetal bovine serum (FBS) and then stained for connexin 43 (red), sarcomeric-α-actinin (green) and DNA (Hoechst 33342, nuclei, blue). (E) Representative images of local calcium minima and peak calcium concentration for cardiomyocytes cultured on eADF4(κ16) films.[159] Copyright 2017, WILEY-VCH.

The loss or failure of an organ or tissue is getting more and more frequent, which has become a devastating and costly problem in human health care. Tissue engineering, as a new field, aiming to regenerate damaged tissues or creat new tissues by combining cells from the body with biomaterials has emerged.

Genetically engineered spider silk protein, due to its high mechanical strength, excellent cytocompatibility, noncytotoxic properties, and precisely controlled quality, is regarded as a suitable material for tissue engineering. **Figure 21** illustrates the application of genetically engineered spider silk in cardiac tissue engineering. In this example, the engineered positively charged spider silk protein, Araneus diadematus fibroin 4 (κ16) (eADF4(κ16)), is selected as the building material. Experimental results demonstrate that eADF4(κ16) films with polycationic surfaces are suitable substrates for cell adhesion of cardiomyocytes, endothelial cells, fibroblasts, and smooth muscle cells, which are the most important cell types in cardiac tissue engineering. In addition, analysis of contractile activity of cardiomyocytes cultured on eADF4(κ16) films shows proper contractility of cardiomyocytes and exhibits no arrhythmia. Moreover, immunofluorescence analyses and calcium homeostasis changes reveal that cardiomyocytes grown on eADF4(κ16) films can well-establish cell-to-cell communication electric coupling.[159] Moreover, easy-to-prepare natural silk fibroin is also excellently suitable for tissue engineering because of its similar mechnical and biological properties as genetically engineered spider silk protein.[160, 161]

6. Conclusion and Outlook

After millions of years of evolution, silk fibers have achieved a perfect balance between strength and toughness in a light-weighted and flexible fashion, serving as the critical structural material for the survival of silkworms and spiders. Such mechanical properties have attracted many scientific and commercial efforts to replicate silk fibers for applications beyond their natural function, although challenges still remain to fabricate fibers with comparable mechanical performance. Nevertheless, as a sustainable material, silk - among many other natural biopolymers - holds great promise in a broad range of scientific, environmental and economic applications that benefit both the people and the planet.

Compared with the long history and wide applications of silk fibroin materials produced by silkworms - because of the cultivation of Bombyx moray silkworms for the industry and being amenable to large-scale manufacturing - the progress on scientific research and commercialization of spider silk relatively fall behind mainly due to difficulties in harvesting large amounts of materials. The territoriality and the cannibalism of the spiders make them difficult to farm and therefore the mass production of spider silk has been impractical. The advancement of biochemical synthesis holds promise on producing increasing volumes of high-quality silk biopolymers with desired mechanical properties and/or biological functionalities after optimizations via a vast amount of explorations.

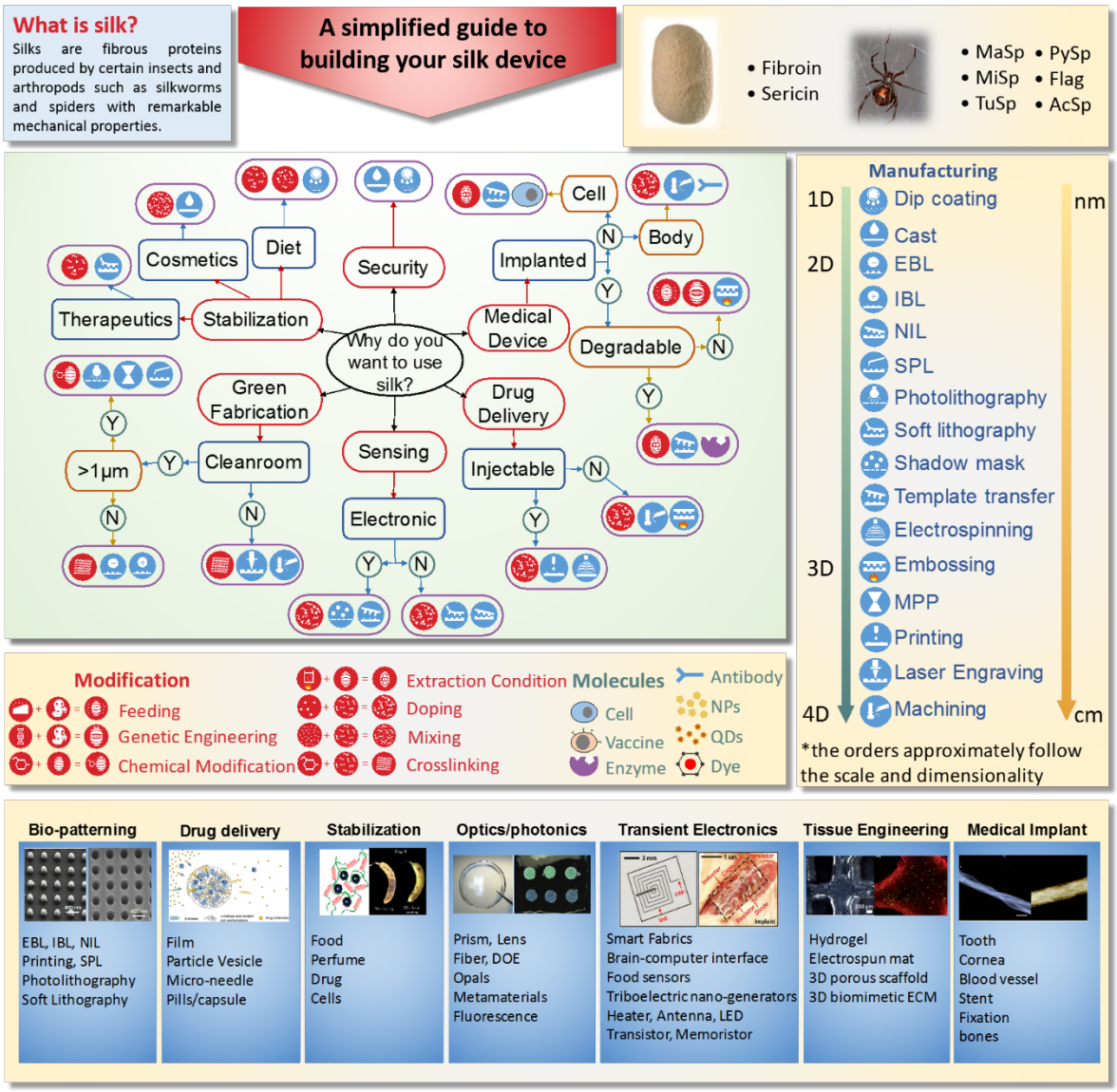

Figure 22. The technological roadmap of "Revolutionary Silk Road". The roadmap serves as a simplified guide that connects the applications of the silk fibroin with the appropriate manufacturing and modification technologies in a systematic manner. The roadmap starts at the center of the figure with an objective of the

application, followed by the requirements that needs to be addressed by the manufacturing and modifications of the silk fibroin. The final recommended combinations (the roadmap only listed the most common ones) is summarized in the outmost layer of the figure.[62, 67, 70, 81, 82, 162-167]

Silk was used as a suturing material over a century ago was an early attempt for its entry in biomedical applications. Since then, many research efforts on silk have followed along this direction thanks to its robust mechanical properties, outstanding cytocompatibility, the controllable degradation, and mild aqueous environmental conditions during silk processing. The control of materials properties can be implemented through the control of water content during processing. The modification and functionalization are also achievable at multiple levels ranging from genetic engineering and chemical modification to mesoscale assembly and macroscale mixing. Additionally, silk proteins can be readily formed into a variety of material formats ranging from gels,[152, 168-170] strands,[171] sponges[172-174] and blocks,[102, 175, 176] through to foams[177-179] and films.[47, 180] It offers unlimited opportunities on the creation of multi-functional, hierarchical and heterogeneous structures and devices at multiple scales over orders of magnitudes - ranging from nm to mm and above - with shape and function on demand. We summarize a technological roadmap of "Revolutionary Silk Road" to provide a brief overview of recent developments in silk technology with emphasis on (non-textile) applications including precise bio-patterning, and controlled delivery, bio-optics and bio-photonics, flexible and degradable electronics, tissue engineering and medical implants, enabled by advances in the material modification and structure/device manufacturing (**Figure 22**). The roadmap serves as a reference that matches applications with the appropriate technology. For example, if a green fabrication process in a cleanroom environment is needed, and required feature size are relatively large, then photolithography, multiphoton lithography, or scanning probe lithography can be applied to the silk protein with the appropriate chemical modification. Similarly, if an implanted solid device is needed, then the silk bulk material doped with the appropriate therapeutic agents can be manufactured through machining. The chart serves as a simplified guide for connecting the application with the appropriate manufacturing and modification technologies in a systematic manner.

While decent progress has been made on re-empowering silk with more new functionalities while maintaining its advantageous intrinsic properties, the comprehension of certain aspects (such as structure-property relationships and assembly mechanism of natural or engineered silks) of the biological paradigms are still incomplete. Understanding the fundamental biomaterial behavior will indeed continue to move us forward and facilitate bioinspired technology development in many important aspects. For example, silk has been proven as an effective carrier material for enhanced thermos-stability of both antibiotics and vaccines, which could potentially help to save millions of lives each year in developing countries where equipment and procedures used in transport, storage and handling of vaccines - i.e. cold chains - are not readily available. Economically, the revolution of silk could potentially convert the entire silk industry from a currently labor-intensive textile industry with main products on silk yarns, fabrics, garments and carpets into a high-tech and high-value-added one with ground-breaking applications in tissue engineering, regenerative medicine, medical implants, advanced manufacturing and information technology. Recently, silkworms were sent to the outer space for the investigation of their spinning, cocooning and transformation in microgravity and to assess the impact of space radiation on structure and function of as-spun fibers. The future opportunities offered by silk - an ancient material on earth - are only limited by our imaginations.

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Author Photograph(s) ((40 mm broad, 50 mm high, gray scale))

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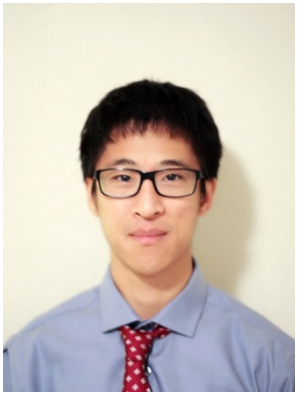

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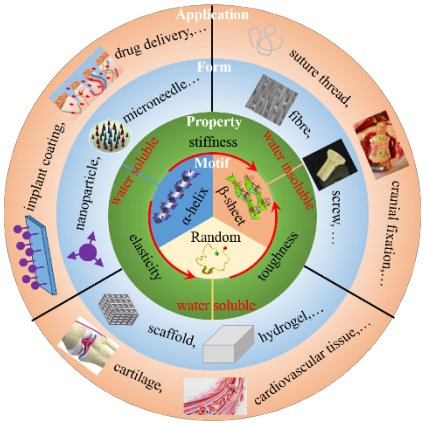
Keyword

Zhitao Zhou, Shaoqing Zhang, Yunteng Cao, Benedetto Marelli, Xiaoxia Xia*, Tiger H. Tao**

Title

Engineering the future of silk materials through advanced manufacturing



Silk is undergoing a revolution with a large amount of research interests originated from replicating/mimicking the properties of natural silk materials gradually shifted to achieving advantageous and/or non-existing performances compared to natural ones via appropriate modifications (from the molecular to macroscopic level) and innovative manufacturing (from 1D to 4D and from nanometers to millimeter and above).