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Transgenic and Diet-Enhanced Silk Production for Reinforced Biomaterials: A Metamaterial Perspective

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Abstract

Silk fibers, which are protein-based biopolymers produced by spiders and silkworms, are fascinating biomaterials that have been extensively studied for numerous biomedical applications. Silk fibers often have remarkable physical and biological properties that typical synthetic materials do not exhibit. These attributes have prompted a wide variety of silk research, including genetic engineering, biotechnological synthesis, and bioinspired fiber spinning, to produce silk proteins on a large scale and to further enhance their properties. In this review, we describe the basic properties of spider silk and silkworm silk and the important production methods for silk proteins. We discuss recent advances in reinforced silk using silkworm transgenesis and functional additive diets with a focus on biomedical applications. We also explain that reinforced silk has an analogy with metamaterials such that user-designed atypical responses can be engineered beyond what naturally occurring materials offer. These insights into reinforced silk can guide better engineering of superior synthetic biomaterials and lead to discoveries of unexplored biological and medical applications of silk.

Contents

1. INTRODUCTION

When spider silk and silkworm silk were first cultivated between 5000 and 2000 BCE, no one could have imagined that silk would evolve to become a highly useful biomaterial in numerous forms of fibers, coatings, particles, hydrogels, scaffolds, micro- and nanopatterns, and 3D-printed structures (1–87). Historically, silk fibers were used as sutures for ocular, neurological, and cosmetic surgery (88). Silk holds great potential for a variety of biomedical applications, including tissue engineering (8, 43, 53, 60–62, 65, 77, 78), drug/gene delivery (32, 37, 40, 42, 47, 51, 52, 75), wound healing (30, 36, 45, 46, 49, 74, 76, 89), bioimaging (39, 54, 72, 82, 86), biosensing (21, 58, 80, 84, 86), implantable devices (35, 45, 49, 50, 80), and antimicrobials (76, 80). Applications of silk have further been extended to the fields of electronics (7, 21, 58, 70, 72, 76, 82), optics (7, 11, 21, 24, 39, 70, 72), and energy harvesting (84), as already covered in numerous review articles. Such extensive utilizations of silk stem from its excellent physical and biological properties, rendering it an ideal biopolymer. Owing to the polymeric nature of silk proteins, silk can readily be processed and fabricated into different types of rigid or flexible structures with tunable physical and biological properties (1–87). Silk proteins can easily be combined with other materials [e.g., carbon nanotubes (CNTs), graphene, semiconductor nanoparticles, and metal nanoparticles] to enhance specific properties of interest (90–93). More importantly, silk has low immunogenicity, biocompatibility, and biodegradability (1, 7, 22). Silk proteins show minimal inflammatory and immune response in tissue microenvironments (12), and they are not only degradable in the body but also edible (15, 94, 95). The degradation rate can be controlled via different silk fabrication methods (94). Natural silk even has a passive radiative cooling function (18, 19). Overall, silk is a fascinating ancient biomaterial that is currently being exploited for a variety of biological and medical applications.

Aside from silkworms and spiders, silk proteins have been produced in several different ways (**Table 1**). Several alternative host systems (e.g., microorganisms, plants, and dairy animals) have been successfully exploited to efficiently produce spider silk proteins (24, 27, 29, 33, 36, 38, 44, 54, 80). Recently, silklike polypeptides have been produced by solid-phase and chemoenzymatic synthesis methods (25, 26, 63, 83). Several engineering technologies using regenerated or

Table 1 Review articles on silkworm- and spider-free production methods of silk proteins

Method of silk production	Title (year published)	Focus	Reference
Genetic engineering using host systems	Molecular Biology of Spider Silk (2000)	Fundamental understanding and manipulation of genes encoding spider silk proteins	24
	Spider Silk—Structure, Properties and Spinning (2006)	General properties of spider silk and regenerated silk production methods	27
	Biotechnological Production of Spider-Silk Proteins Enables New Applications (2007)	Production methods for major ampullate silk proteins	29
	Spider Silk Proteins: Recent Advances in Recombinant Production, Structure–Function Relationships and Biomedical Applications (2011)	General properties of spidroin domains and advances in recombinant production	36
	Recent Advances in Production of Recombinant Spider Silk Proteins (2012)	Advances in production of spider silk proteins using metabolic and cellular engineering	38
	Recombinant DNA Production of Spider Silk Proteins (2013)	Advances in recombinant spider silk production	44
	Fibrous Proteins: At the Crossroads of Genetic Engineering and Biotechnological Applications (2016)	Understanding of cloning, expression, and purification for repetitive fibrous protein production	54
	Engineering of Silk Proteins for Materials Applications (2019)	Advances in recombinant silk protein engineering for textile and biomedical applications	80
Biosynthesis	Synthetic Spider Silk: A Modular Fiber (2000)	Advances in spider silk protein production using genetic engineering and biosynthesis	25
	Spider Silks: Recombinant Synthesis, Assembly, Spinning, and Engineering of Synthetic Proteins (2004)	Advances in recombinant synthesis, assembly, spinning, and engineering of spider silk proteins	26
	Development of New Smart Materials and Spinning Systems Inspired by Natural Silks and Their Applications (2016)	Advances in general silk production methods	63
	Chemical Modification and Biosynthesis of Silk-Like Polymers (2019)	Advances in silk-based material production using chemical modification and chemoenzymatic synthesis methods	83

synthetic silk proteins have been used to spin and pull silk fibers in a similar or superior manner that maintains the mechanical properties of natural spider silk fibers (**Table 2**) (31, 48, 57, 64, 69, 79). Given this wealth of information, we focus primarily on silkworm-produced silk with properties reinforced using silkworm transgenesis and functional additive diets. Transgenic silkworms have been exploited to produce recombinant proteins of interest in a scalable manner (96–100). Even with considerable research on synthetic silk protein production, silkworm transgenesis using silkworms as hosts is still considered an economical and practical production platform. Obviously, the genetic engineering of silkworms can offer additional functionalities for unique biophysical and biochemical properties. Several functional additives have also been incorporated into silk via direct feeding or injection, taking advantage of silkworms' open circulatory system (101–105).

Table 2 Review articles on bioinspired spinning methods for silk fibers

Title (year published)	Focus	Reference
Electrospun Silk Biomaterial Scaffolds for Regenerative Medicine (2009)	Advances in electrospinning of silk for tissue engineering and regenerative medicine	31
Bioinspired Micro-/Nanostructure Fibers with a Water Collecting Property (2014)	Advances in wet spinning of spider silk and bioinspired fibers	48
Silk-Microfluidics for Advanced Biotechnological Applications: A Progressive Review (2016)	Advances in silk-based microfluidic devices for biomedical applications	57
Progress and Trends in Artificial Silk Spinning: A Systematic Review (2017)	Advances in artificial silk fiber spinning for biomedical applications	64
Recent Developments in Regenerated Silk Fiber (2017)	Advances in electrospinning with microfluidics for regenerated silk	69
Straining Flow Spinning of Artificial Silk Fibers: A Review (2018)	Fundamental understanding of straining flow spinning for silk-based artificial fiber production	79

In this review, we cover transgenic and diet-enhanced silk production with a focus on reinforced silk, given that many review articles about host cell–based silk synthesis, chemoenzymatic silk polymerization, and bioinspired silk spinning methods (24–27, 29, 31, 36, 38, 44, 48, 54, 57, 63, 64, 69, 79, 80, 83) are already available (**Tables 1** and **2**). To the best of our knowledge, there is no systematic review article on silkworm-based production of reinforced silk, although three recent reviews cover some aspects of silkworm transgenesis and nanomaterial feeding (72, 82, 106). We also cover research showing that reinforced silk is analogous to conventional metamaterials. Metamaterial research involves constructing materials to have user-designed atypical physical properties that often do not exist in nature (107–113).

First, we summarize the unusual physical properties of silk and important silk production methods. Second, we explain the basic methods of silkworm transgenesis and direct feeding of biologically friendly nanomaterials as scalable silk production platforms. Third, we discuss recent biomedical uses of fluorescent silk, mechanically reinforced silk, reactive oxygen species (ROS)-generating silk, and artificial peptide–expressing silk. Finally, we present an outlook based on the current state of progress in silk research. We envision that an enhanced understanding of silkworm-based silk with superior physical and biological properties will allow us to explore new directions for the development of synthetic fibers and basic biomaterial research.

2. BASIC CHARACTERISTICS OF SILK PROTEINS AND PRODUCTION METHODS

2.1. Remarkable Mechanical Properties of Spider Silk Fibers

Although silk is often considered to refer only to cocoons spun by silkworms, spider silk fibers have inspired countless scientific studies aiming to better understand silk's unusual mechanical properties and eventually produce superior synthetic fibers (1, 3, 28, 114–119). Spiders have evolved the ability to produce as many as six or seven different types of silk fibers that vary in tensile strength and elasticity. Both dragline and flagelliform fibers have outstanding mechanical properties that permit the absorption of more energy prior to breaking than nearly any other common material (**Table 3**). In particular, spider dragline silk fibers exhibit a unique combination of low density, high tensile strength, and extreme extensibility, resulting in superior toughness (87, 119, 120). Despite decades of research, it is still an engineering challenge to simultaneously realize these three mechanical properties (i.e., density, tensile strength, and extensibility) in synthetic fibers. Note that recent advances in biomimetic spinning dopes have enabled some

Table 3 Mechanical properties of spider silk and other common materials (116, 117)

Material	Strength (N/m^2)	Elongation (%)	Energy to break (J/kg)
Dragline silk	4×10^9	35	4×10^5
Minor ampullate silk	1×10^9	5	3×10^4
Flagelliform silk	1×10^9	>200	4×10^5
Tubuliform silk	1×10^9	20	1×10^5
Aciniform silk	0.7×10^9	80	6×10^9
Kevlar	4×10^9	5	3×10^4
Rubber	1×10^6	600	8×10^4
Tendon	1×10^6	5	5×10^3

regenerated artificial silk fibers to outperform natural spider silk fibers in terms of toughness (89, 121). In addition, variations among different types of spider fibers are notable, as they encompass a fivefold range of tensile strength and a nearly 50-fold range of elongation (**Table 3**). In general, the superior mechanical properties of spider silk have motivated extensive research on silk.

2.2. Silk Proteins and Structures

The remarkable properties and characteristics of animal-produced silk are attributable to its constituent proteins and nanostructures. First, the nanostructures of silk fibers produced by silkworms and by spiders are very similar, as both are of core–shell type (2). Silkworms secrete silk fibroin synthesized in the silk glands through spigots of the spinnerets (i.e., silk-spinning organs). Subsequently, this insoluble protein crystallizes into nanofibrils in contact with the air while the nematic silk proteins are pulled out under shear stress and dehydration conditions; it is then assembled into fibroin filaments, with two filaments glued together with sericin into a silkworm cocoon (89, 115, 118, 122, 123). As a result, multiple parallel nanofibrils along the fiber axis form inside a single silk fiber. The diameter of silk fiber threads is dependent on the type and species of silkworm or spider (124). Typically, the silk fiber diameter of *Bombyx mori* silkworms, consisting of two core filaments coated with sericin, is approximately 20 μm. Spider dragline silk fibers have a diameter of 3–5 μm. The lustrous or silvery aspect of natural silk fibers can be explained by interactions of this exquisite nanostructure with light (18).

Second, silk fibroin produced by domestic silkworms (*B. mori*) consists of heavy-chain (molecular weight, ~350 kDa) and light-chain (molecular weight, ~25 kDa) proteins covalently linked by a disulfide bond at the C terminus of the two subunits (22, 124–126). The molecular weight of spidroin proteins produced by orb-weaving spiders (araneids) is in the range of 250–400 kDa (27, 34, 124, 127). The protein backbone (i.e., amide group) in the primary structure of fibroin and spidroin is composed of highly repetitive amino acid sequences. Both silkworm silk and spider dragline silk have the highly conserved and repetitive nature of protein sequences (2, 114). Their secondary structures of fibroin and spidroin include β-sheets, random coils, α-helices, and antiparallel β-sheets (123, 124). The high content of α-helices and random coils in silk fibers is associated with higher elongation at break and higher toughness modulus, as the α-helices and random coil conformation consist of easily movable chains (11, 128). Because animal-produced silk has the complexity of amino acid sequences and the reactive functional side groups of amino acids, it remains challenging to produce synthetic proteins that can mimic natural silk (23, 83, 114, 119, 124, 129). Overall, an improved understanding of the protein structures and functions can provide a foundation to custom-design unique synthetic spider silk with mechanical properties ideally suited for specific applications.

2.3. Silkworm- and Spider-Free Methods of Producing Silk Proteins

Silk proteins have been produced by transplantation of silk-making DNA into a variety of host systems, including microorganisms, plants, and even goats, and by enzymatic or chemical synthesis of polypeptides (**Table 1**). Specifically, the isolation of spider silk gene sequences enables production of recombinant spider silk proteins in several different host systems, including bacteria (130, 131), yeast (132), baculovirus/insect systems (133, 134), mammalian cells (135), transgenic plants (136), and transgenic animals (137, 138). Cloning and sequencing comparisons of complementary DNA encoding spider silk proteins successfully resulted in the identification of major ampullate spidroin 1, major ampullate spidroin 2, and flagelliform silk proteins of *Nephila clavipes* (139–141). The protein products of these genes are polymers consisting of highly repetitive blocks of amino acid sequence motifs, and specific sequence motifs are strongly correlated with the mechanical properties of spider silk fibers (116, 142). One of the most common hosts is bacteria (e.g., *Escherichia coli*), which can be scaled up to industrial production. Key challenges to this approach include the bacterium's limited expressible gene size and distinct codon usage as well as the removal of repetitive sequences (143). In addition, biosynthesis of silklike polypeptide materials using protein engineering and chemoenzymatic methods has been successfully demonstrated for industrial production on a large scale (25, 26, 63, 83). Note that after extraction of silk proteins, fiber spinning and pulling are required because of the insoluble characteristics of silk proteins (**Table 2**).

2.4. Bioinspired Spinning Methods for Silk Fibers

When silk proteins are produced by biological or chemical synthesis, spinning and pulling of reconstituted silk fibers are an important step in engineering the same level of mechanical and physical properties of natural spider dragline silk. Several bioinspired spinning methods for constructing artificial silk fibers have been successfully demonstrated by mimicking the natural spinning process, using solution-based silk proteins (31, 48, 57, 64, 69, 79). Silk fibers can be spun in a variety of methods, including electrospinning (31, 59, 144), wet spinning (26, 145), dry spinning (64, 146, 147), self-assembly (148, 149), and microfluidics (57, 150, 151) (**Table 2**). The resultant mechanical properties of the artificial silk fibers are dependent on spinning process conditions, including solvents, coagulants, draw ratios, and other parameters (e.g., pH, temperature, viscosity, voltage, polypeptide molecular weight, blending, metal ions) (64, 69). Typically, artificial silk fibers have mechanical properties comparable to those of silkworm silk fibers (64, 151). More importantly, recent advances in bioinspired spinning of silk fibers have enabled artificial silk to outperform natural spider silk fibers in terms of toughness (89, 121).

2.5. Silkworm Transgenesis

The obvious host for expressing silk is the silkworm (*B. mori*) itself, which can produce recombinant silk cocoons on a large scale. One of the advantages of silkworm transgenesis is that the engineering demands of artificial fiber spinning and pulling can be minimized as silkworms spin silk fibers. The first successful transformation of *B. mori* was accomplished using the *B. mori* actin 3 promoter (BmAc3) to drive expression of the transposase on a helper plasmid, pHA3PIG, together with a *piggyBac* vector encoding a BmAc3-controlled gene encoding enhanced green fluorescent protein (eGFP) (100). The successful transformation was clearly evidenced by whole-body fluorescence in the F1 generation. Subsequent studies explored the utility of alternate promoters for expression and detection of fluorescent protein markers, including the 3×P3 eye-specific promoter (152) and the silk fibroin light-chain promoter (153, 154). Specifically, the

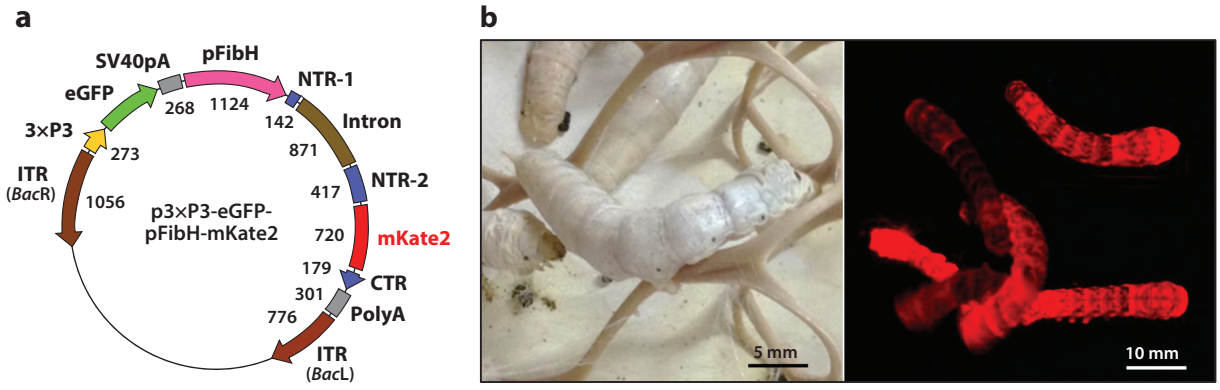

Figure 1

Representative genetic fusion of fluorescent proteins (e.g., mKate2) and silk (161). (*a*) Construction of transfer vector p3×P3-eGFP-pFibH-mKate2 for mKate2 silkworm transgenesis using a gene-splicing *piggyBac* transposase method. For hybridization of mKate2 and silk, the mKate2 gene is fused with N-terminal and C-terminal domains of pFibH. The nucleotide sequences of the pFibH NTR and CTR are derived from GenBank accession number AF226688. (*b*) (*left*) Photograph and (*right*) fluorescent image of mKate2 (transgenic) silkworms. Abbreviations: CTR, C-terminal region (179 bp); eGFP, enhanced green fluorescent protein; intron, first intron (871 bp); ITR, inverted repeat sequences of *piggyBac* arms; mKate2, monomeric far-red fluorescent protein (720 bp) derived from *Entacmaea quadricolor*; NTR-1, N-terminal region 1 (142 bp); NTR-2, N-terminal region 2 (417 bp); pFibH, fibroin heavy-chain promoter domain (1,124 bp); PolyA, poly(A) signal region (301 bp); 3×P3, 3×P3 promoter (273 bp); SV40, SV40 polyadenylation signal sequence (268 bp). Figure adapted from Reference 161 under a Creative Commons license (CC-BY-4.0).

genetic transformation of *B. mori* enables rapid functional characterization of silk gland promoters for silk protein production (**Figure 1**) (96). Silk gland–specific promoters exploit these glands for recombinant protein production and secretion in either tight or loose association with silk fibers, permitting noninvasive collection and simplifying downstream purification of recombinant protein products. The utility of such promoters for expression and detection of protein markers, including the silk fibrohexamerin gene promoter (155), the silk fibroin heavy-chain gene promoter (125), and the *sericin*1 gene promoter (156, 157), has been extensively studied. An experimental animal model has also been widely used for recombinant silk protein production (154). Another advantage is that transgenic silkworms can provide high-level expression and efficient secretion of several recombinant proteins (96–100). Silkworm transgenesis can be an economical means of producing recombinant human type III procollagen, human serum albumin, human acid fibroblast growth factor, and antibodies (153, 158, 159). Silkworms produce a large amount of silk protein (0.2–0.5 g per worm), and their speed of protein biosynthesis is 10^6-fold greater than that of mammalian cultured cells (160). Cost-effective breeding and relatively easy production scale-up also make transgenic silkworms a viable production system for biopharmaceuticals and other proteins, such as spider silk–based fibers. For example, the cost of silkworm rearing is less than five cents per larva, and it takes approximately 60 days to generate transgenic silkworms (160).

2.6. Direct Feeding of Artificial Additives

Directly feeding silkworms or spiders artificial additives can be a practical way to produce reinforced silk fibers (102, 103, 162–166). In this approach, silkworms can easily produce silk containing functional additive nanomaterials. This is possible in part because silkworms have an open circulatory system. All of their organs float in hemolymph, which is a combination of lymph and blood cells that surrounds all tissues (**Figure 2*a***) (101, 167). In other words, the unique anatomy of silkworms is highly useful for producing functional silk by direct feeding methods (oral exposure

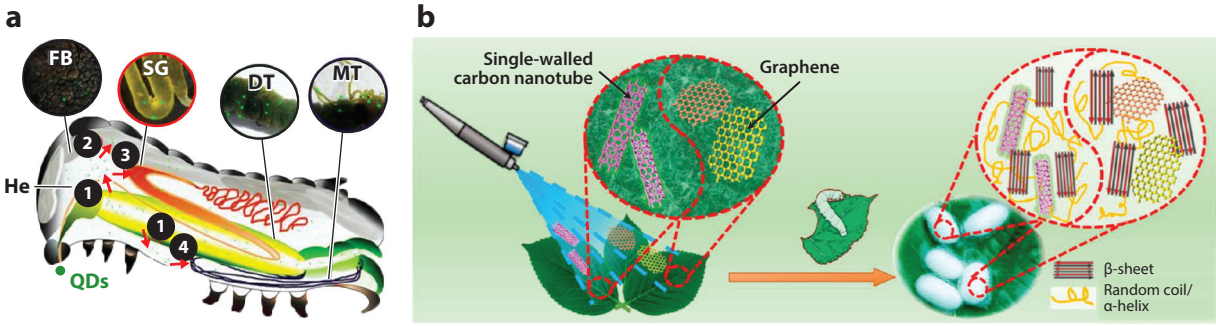

Figure 2

Diet-enhanced functionalization of nanomaterials with silk using the open circulatory system of silkworms. (*a*) Transfer of quantum dots (QDs) (e.g., sizes of 2.8–3.6 nm). After being consumed orally, (❶) QDs immediately pass through the digestive tract (DT) membrane barrier and gather in hemolymph (He) and blood cells. Eventually, QDs move to the floating tissues in He, including (❷) the fat body (FB), (❸) the silk gland (SG), and (❹) the Malpighian tubule (MT) (101). (*b*) Incorporation of functional additives into silk by feeding silkworms with mulberry leaves spray-coated with nanomaterials of interest (e.g., single-walled carbon nanotubes, graphene) (102). Panel *a* adapted from Reference 101 under a Creative Commons license (CC-BY-4.0). Panel *b* adapted with permission from *Nano Letters*. Copyright 2016, American Chemical Society.

and intake) of nanomaterials (**Figure 2*b***). Nanoscale additives can diffuse out of the alimentary canal into the hemolymph and then into the glands and other tissues. Specifically, if nanomaterials are injected orally, they are absorbed by the digestive tract, pass through the digestive tract membrane barrier, and are circulated into the hemolymph and blood cells. They are transported concomitantly to the floating tissue compartments in the hemolymph, such as the silk glands, fat body, and Malpighian tubule. Several different functional nanomaterials (e.g., dye molecules, graphene, CNTs, titanium dioxide, and quantum dots) have been successfully incorporated into silk via direct feeding or injection, resulting in enhanced mechanical, thermal, electrical, and optical properties (101–105).

3. BIOMEDICAL APPLICATIONS OF REINFORCED SILK

3.1. Fluorescent Silk

The production of fluorescent silk genetically hybridized with fluorescent proteins is a common example of silkworm transgenesis (168, 169). A transformation gene vector is constructed by genetically encoding a fluorescent protein [e.g., eGFP, DsRed (derived from *Discosoma* spp.), Kusabira Orange, mKate2, or enhanced yellow fluorescent protein] as a color gene into the silkworm genome via the gene-splicing *piggyBac* transposase method (168–171). Upon optical excitation using a common light source (e.g., light-emitting diodes), strong fluorescent emission intensity from fluorescent silk can be detected that corresponds to the fluorescent proteins in silk (**Figure 3*a*,*b***). For widespread use in fabrics and textiles, it is important to maintain the mechanical strength of the transgenic fluorescent silk. In some cases, the mechanical properties were slightly diminished compared with those of nontransgenic silk (e.g., in the commercial race C146 × J137) (168). This side effect can be attributed to the presence of fluorescent proteins fused with fibroin heavy-chain N- and C-terminal domains, which may disturb silk crystallization. Nevertheless, transgenic fluorescent silk cocoons can be reeled and woven into fabrics by an automatic reeling machine without loss of fluorescence (168).

Silk fibroin transgenically hybridized with fluorescent proteins can be processed and regenerated into various forms with nano- and microstructures for applications in optics, electronics,

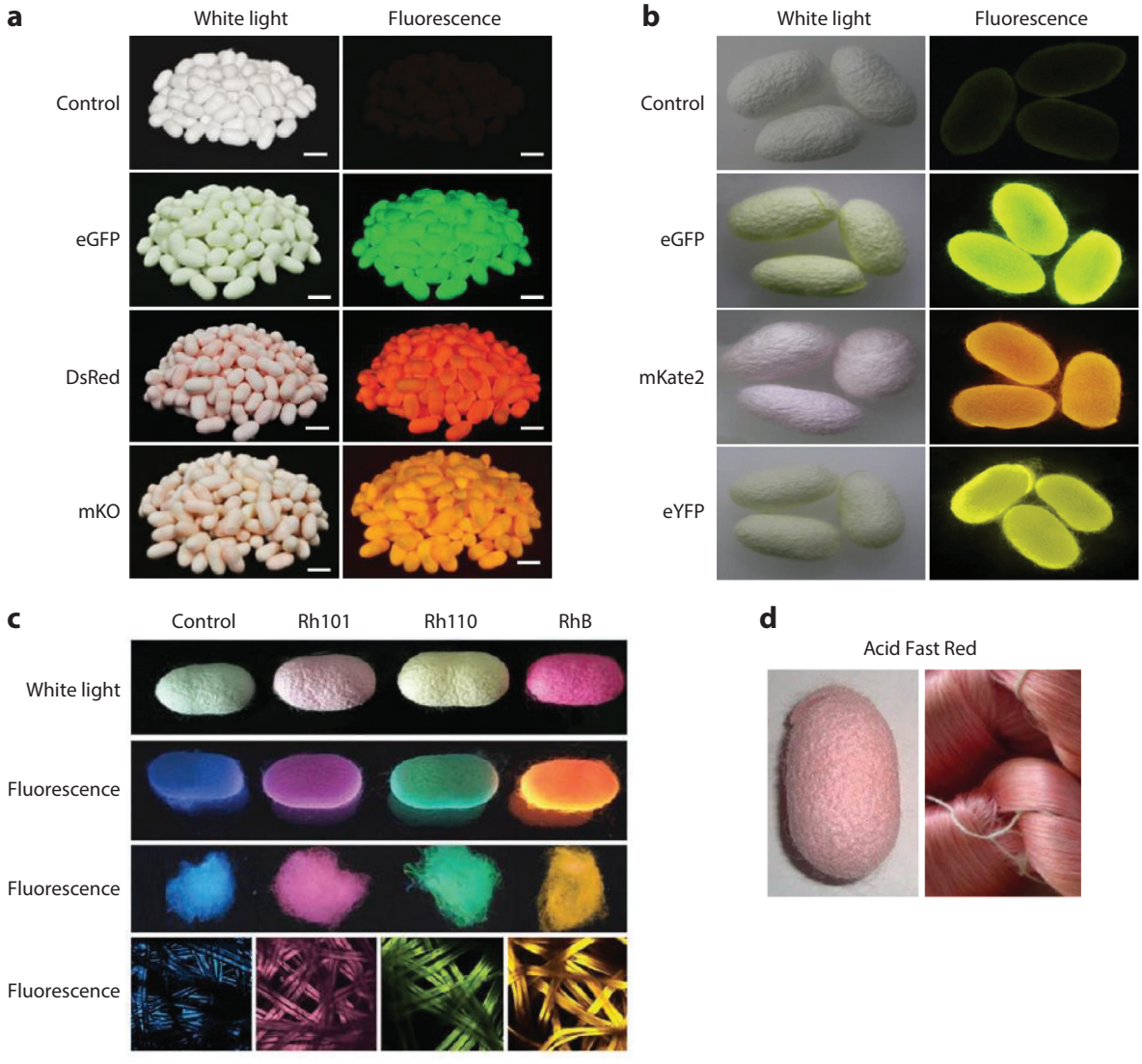

Figure 3

Fluorescent colored silk fibers and cocoons produced from transgenic silkworms and functional additive–fed silkworms. (*a*,*b*) Fluorescent proteins (eGFP, DsRed, mKO, mKate2, and eYFP) are fused into silk (from *Bombyx mori*) by the *piggyBac* transposase method (168, 169). Scale bars in panel *a*: 20 mm. (*c*,*d*) Rhodamine dyes (sulforhodamine 101, RhB, Rh101, Rh110, and Rh116) and azo dyes (Brilliant Yellow, Congo Red, Acid Orange G, Acid Orange II, Mordant Black 17, Direct Acid Fast Red, Sudan III) are directly fed to silkworms orally (104, 167). Abbreviations: DsRed, protein derived from *Discosoma* spp.; eGFP, enhanced green fluorescent protein; eYFP, enhanced yellow fluorescent protein; mKate2, monomeric far-red fluorescent protein derived from *Entacmaea quadricolor*; mKO, Kusabira Orange. Panel *a* adapted with permission from *Advanced Functional Materials*. Copyright 2013, Wiley. Panel *b* adapted with permission from *Biomaterials*. Copyright 2015, Elsevier. Panel *c* adapted with permission from *Advanced Materials*. Copyright 2011, Wiley. Panel *d* adapted with permission from *ACS Sustainable Chemistry and Engineering*. Copyright 2014, American Chemical Society.

optoelectronics, and medicine, as it is transparent, mechanically stable, edible, biocompatible, and implantable in the human body (2, 7, 69). For example, regenerated fluorescent silk fibroin was used for surgical inspection by enabling localization of gastrointestinal fistula lesions (169). Regenerated solutions and particles of fluorescent silk fibroin were also applied for bioimaging (169). Through the use of an eGFP fluorescent silk fibroin solution, an inspection of esophageal perforations was performed, permitting intraoperative surgical field imaging in an animal model. Note that the conventional fibroin processing methods, which include boiling, are inappropriate for the process of regenerating fluorescent silk because fluorescent proteins are highly susceptible to denaturation from high temperature and pH values (5, 172, 173). Silk fibroin fused with fluorescent proteins must be processed at low temperatures (≤60°C) in order to avoid heat-induced denaturation of the fluorescent proteins.

Another way to produce fluorescent silk is to utilize direct feeding methods that rely on in vivo uptake of fluorescent dye molecules into the silk glands (**Figure 3*c*,*d***) (104, 167). Typically, fluorescent colored silk can be achieved using a modified diet of mulberry leaves containing dye molecules. Several different dyes have been successfully used, including rhodamine dyes (i.e., sulforhodamine 101, RhB, Rh101, Rh110, and Rh116) (104) and azo dyes (i.e., Brilliant Yellow, Congo Red, Acid Orange G, Acid Orange II, Mordant Black 17, Direct Acid Fast Red, and Sudan III) (167), all of which are common in the textile industry. Again, it is important to ensure the mechanical strength of dye-fed fluorescent silk. The typical average tensile strength and strain of colored silk range from 406 to 454 MPa and from 23.7% to 26.5%, respectively, which are comparable to those of typical white silk (approximately 455 MPa and 27.1%, respectively) (11, 104). The combination of reliable mechanical properties and high biocompatibility allowed the construction of scaffolds made of intrinsically RhB dye–fed silk, demonstrating the feasibility of visualizing human colon fibroblast (CCD-112CoN) and lung cancer cell (A549) lines (104). This result indicates that the addition of fluorescent molecular dyes did not affect the crystallinity of silk fibers, relatively maintaining the mechanical properties of natural silk.

For fluorescent dye molecules to be successfully integrated with silk, hydrophilicity and hydrophobicity must be balanced, which can be estimated by the partition coefficient (i.e., measure of hydrophobicity) of the dye molecules (104, 167). The dye's partition coefficient determines its preferential association with either sericin or fibroin in silkworm glands and, ultimately, with silk fibers. For example, dyes with partition coefficients of 0–1.5 (e.g., Rh110, Rh116, Acid Orange II, Mordant Black 17, and Direct Acid Fast Red) are well expressed in silk fibers. Hydrophilic dyes with negative partition coefficients (e.g., fluorescein sodium, sulforhodamine 101, Brilliant Yellow, Congo Red, and Acid Orange G), as well as hydrophobic dyes with extremely high partition coefficients (e.g., Sudan III), are not well expressed. Acridine Orange, with a partition coefficient of 1.8, is also not colored in silk fibers. Because Acridine Orange's nonamphiphilic structure does not allow tuning of hydrophobicity upon the formation of dimers (174), molecules of this dye cannot penetrate hydrophilic sericin and thus are retained in silk gland cells. Although Rh101 and RhB are hydrophobic dyes with high partition coefficients greater than 2, they accumulate in high concentrations in silk fibers. This finding might be attributed to the formation of dimers that can result in more efficient transfer of dye molecules into silk glands (175–177). In other words, hydrophobic dyes with good water solubility are promising candidates for producing fluorescent silk.

Another factor that affects the absorption of dye molecules into silk is molecular weight. Higher molecular weights limit the absorption of dyes into the silkworm's biochemical pathways. Dyes accumulate in the peritoneal membrane of the alimentary canal. Thus, molecular weight lower than 400 g/mol is essential for effective transport of dyes into the biochemical pathways of the silkworm body and in the production of naturally dyed silk fibers (167). In addition, the solubility

of dyes in hemolymph and the differential permeation through the linings of various types of tissue are likely to control the transfer of dye molecules to the silk glands.

3.2. Mechanically Enhanced Silk

While several transgenic microorganisms, plants, and animals have been successfully used to produce spider silk proteins, none of these hosts were less expensive than spiders to scale up or naturally equipped to spin silk fibers. One approach that overcomes these limitations is to use silkworms as surrogate hosts for spider silk production. Indeed, silkworms are preferred as a host system for producing recombinant spider silk fibers because they are naturally equipped to spin silk fibers. The relationship between spider silk protein structures and mechanical functions is relatively well understood (142), allowing us to design artificial fibers for specific biomedical applications. Specifically, silkworm transgenesis for spider silk production can be efficiently performed using *piggyBac* vectors (100, 178, 179) or CRISPR/Cas9 direct gene replacement (180, 181). For example, recombinant protein production can be targeted to silk glands with tissue-specific promoters (153, 155, 182–184). Natural and customized spider silk fibers have been produced by assembling DNA sequences encoding synthetic spider silk proteins with mixed motifs for expression in transgenic silkworms (127, 181, 185, 186). The more recent introduction of CRISPR/Cas9 has led to direct replacement of the silkworm's endogenous fibroin genes with synthetic spider silk genes; the properties of the resulting silk fibers closely mimic those of native spider silk fibers (180).

Direct feeding of silkworms or spiders with functional nanomaterials in order to produce reinforced silk has been demonstrated with carbon-containing materials (e.g., graphene and CNTs) (102, 162–164), titanium dioxide (103), metal nanoparticles (e.g., silver and copper) (165), and ion precursors (e.g., Ca^{2+} and PO_4^{3-}) (166). **Table 4** summarizes the mechanical properties of silk fibers from silkworms or spiders fed various artificial diets containing nanomaterials. One of the underlying mechanisms is that such nanomaterials can act as knots in fibers, resulting in cross-linked networks with crystallites, lower crystallinity, and higher elongation at break (187). Typically, artificial diets were generally fed to silkworms and spiders by spraying aqueous solutions containing reinforcing nanomaterials onto normal diets. Control groups of silkworms or spiders were routinely fed normal diets (e.g., mulberry leaves). Importantly, simple feeding with such functional nanomaterials resulted in improved mechanical properties of the silk fibers, with stronger fracture strength and higher elongation at break, that did not strongly depend on silkworm or spider strain, type of mulberry leaf, or rearing environment.

Although a functional nanomaterial diet can be relatively well incorporated into silk fibers, there are several caveats. First, the key parameter for successful integration of a nanomaterial with the silk glands is the size of the nanomaterial, because diffusion of large-sized nanoparticles is significantly limited by the silkworm's open circulatory system, which typically requires sizes smaller than 30 nm (101, 188). Second, the presence of nanocarbon materials (e.g., graphene and CNTs) in the silk matrix may hinder the transformation of α-helices and random coils into β-sheet structures, resulting in noncovalent interactions between nanocarbon materials and silk fibroin through physical adsorption (102). Third, excessive nanomaterials in silk can reduce the mechanical strength of silk fibers, because the nanomaterial-conjugated nanofibrils may aggregate and act as defects instead of link points (102, 103). Fourth, silver nanoparticles are toxic and should be used with caution (189, 190). They can affect silkworms' metabolic cycle, signal transduction, apoptosis, and ion transport, weakening metabolic function and increasing energy storage and utilization (191). Silver nanoparticles can also degrade the ability of silkworms to withstand oxidative stress, interfere with programmed cell death, and attenuate the expression of detoxification proteins.

Table 4 Mechanical properties of silk produced from silkworms and spiders fed with functional nanomaterials

Silk type	Group (reinforcing additives)	Tensile strength (MPa)	Elongation (%)	Toughness modulus (MPa)	Young's modulus (MPa)	Reference
Steatoda sp. (spider)	Control	534.7 ± 222.1	102 ± 30	534.7 ± 222.1	3,200 ± 800	162
	SWCNT-1 (CoMoCAT)	3,907.2 ± 874.1	60 ± 28	1,144 ± 555.3	37,900 ± 4,400	
Pholcus opilionoides (spider)	Control	1,726.6 ± 565.3	39 ± 7	476.4 ± 257.8	15,100 ± 6,400	
	SWCNT-2 (electric arc discharged)	5,393.5 ± 1,202.4	75 ± 9	2,143.6 ± 684.6	47,800 ± 18,000	
Holocnemus pluchei (spider)	Control	825.2 ± 182.9	26 ± 6	101.8 ± 9.6	9,200 ± 1,500	
	Graphene	1,245.6 ± 559.4	43 ± 24	254.7 ± 164.3	13,000 ± 6,500	
Bombyx mori (silkworm)	Control	360 ± 70	9.39 ± 2.29	22.7 ± 4.3	No data	102
	SWCNT1 (0.2 wt%)	590 ± 140	12.59 ± 2.75	48.24 ± 7.80	No data	
	SWCNT2 (1.0 wt%)	280 ± 40	5.76 ± 1.65	10.33 ± 0.65	No data	
	Graphene1 (0.2 wt%)	570 ± 110	10.33 ± 2.62	38.04 ± 5.38	No data	
	Graphene2 (2.0 wt%)	280 ± 110	3.95 ± 1.39	6.4 ± 0.84	No data	
	Control	600 ± 60	12.67 ± 2.68	47.79 ± 7.09	No data	163
	SWCNT1@LGS (purified)	880 ± 70	12.43 ± 1.45	68.30 ± 8.05	No data	
	SWCNT2@LGS (unpurified)	690 ± 120	14.73 ± 3.00	64.00 ± 22.77	No data	
	MWCNT1@LGS (purified)	1,070 ± 160	16.80 ± 2.17	113.23 ± 23.78	No data	
	MWCNT2@LGS (unpurified)	780 ± 80	14.18 ± 3.29	66.70 ± 20.31	No data	
	Control	440 ± 100	20.64 ± 11.9	12.05 ± 1.28	No data	166
	Low Ca^{2+}, PO_4^{3-}	490 ± 50	13.01 ± 4.37	23.17 ± 2.07	No data	
	High Ca^{2+}, PO_4^{3-}	620 ± 140	17.42 ± 9.05	22.55 ± 4.34	No data	
	Control	800 ± 300	17.5 ± 0.3	No data	140 ± 50	164
	CNT	1,690 ± 300	24.0 ± 0.3	No data	380 ± 50	
	Control	279 ± 108	20.8 ± 3.4	No data	7,000 ± 1,500	165
	Cu NPs	365 ± 103	21.2 ± 5.2	No data	8,000 ± 2,600	
	Ag NPs	331 ± 129	21.1 ± 3.9	No data	7,700 ± 2,900	
	Control	393 ± 36	15.2 ± 1.6	No data	No data	103
	TiO_2 (1%)	548 ± 33	16.7 ± 0.8	No data	No data	

Abbreviations: CNT, carbon nanotube; LGS, lignosulfonate; MWCNT, multiwalled carbon nanotube; NPs, nanoparticles; SWCNT, single-walled carbon nanotube; TiO_2, titanium dioxide.

3.3. Reactive Oxygen Species–Generating Silk

Functional silk with disinfecting properties has received considerable attention for a variety of antimicrobial applications (76, 80). Typical approaches to producing it rely on treatment (i.e., simple attachment or coating) of organic or inorganic nanomaterials (e.g., titanium dioxide, zinc oxide, CNTs, silver/gold nanoparticles, natural dyes) (192–194) to generate ROS upon light illumination. In this respect, silkworm transgenesis can provide a more effective hybridization alternative by genetically encoding phototoxic fluorescent proteins into silk proteins (161, 195, 196). Integration

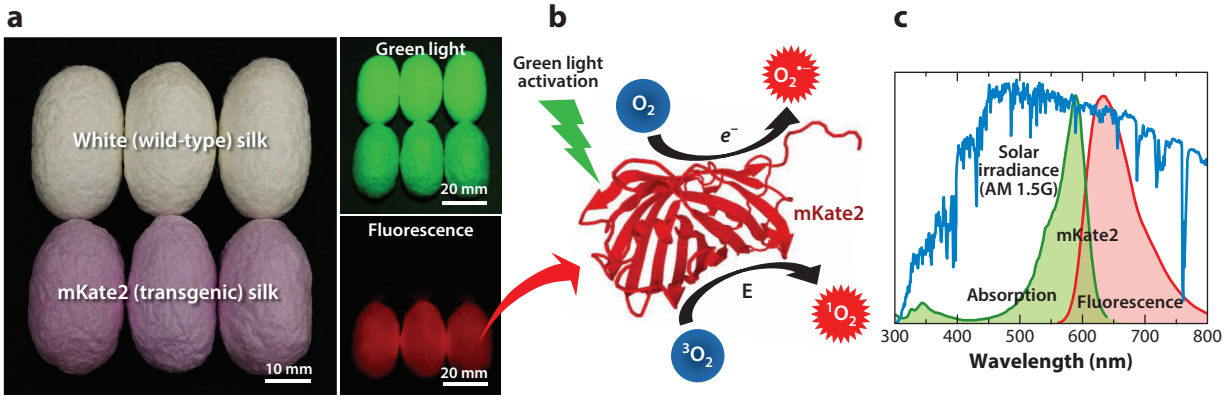

Figure 4

Reactive oxygen species (ROS)-generating silk with genetic hybridization of phototoxic fluorescent proteins. Genetically encoded hybridization of a far-red fluorescent protein (mKate2) and silk can act as plasmonic photocatalysis-like photosensitization, in particular for disinfection (161). (*a*) Photograph of white (wild-type) and mKate2 (transgenic) silk cocoons and fluorescent image of mKate2 silk cocoons. (*b*) Schematic illustration of ROS-generating mKate2 silk under green light activation. Superoxide ($O_2^{\bullet-}$) and singlet oxygen ($^1O_2$) are generated by mechanisms of electron (e^-) transfer and energy (E) transfer, respectively. (*c*) Green light belongs to the peak wavelength range of the solar spectrum. Figure adapted from Reference 161 under a Creative Commons license (CC-BY-4.0).

of phototoxic fluorescent proteins such as GFP, KillerRed, SuperNova, TurboGFP, and mKate2 is another way to generate and release ROS upon light excitation (197–200). The phototoxic action of red fluorescent proteins (e.g., KillerRed, SuperNova, and mKate2) is known to originate from a cleftlike opening filled with water molecules, allowing for enhanced generation and release of ROS (201, 202). The exact types of ROS vary among different red fluorescent protein variants, depending on the type of photoreaction and the concentration of local molecular oxygen (i.e., electron acceptor) (197). Superoxide ($O_2^{\bullet-}$) and other ROS, such as hydroxyl radical ($-OH^{\bullet}$) and hydrogen peroxide (H_2O_2), are generated via the type I photoreaction by electron transfer to and from the substrate in the excited triplet state (T_1). The most common electron acceptor is molecular oxygen [$O_2(^3\Sigma_g^-)$] (i.e., $^3O_2$). The resultant $O_2^{\bullet-}$ can further interact with its surroundings to produce other reactive oxygenated products (e.g., $-OH^{\bullet}$ and H_2O_2). In contrast, singlet oxygen ($^1O_2$) [i.e., $O_2(^1\Delta_g)$] is generated by energy transfer from T_1 to $^3O_2$ in a type II photoreaction (197).

mKate2, a phototoxic far-red fluorescent protein, has been genetically hybridized with silk using the *piggyBac* method (**Figure 4**) (161). Specifically, in order to combine mKate2 and silk protein, the mKate2 gene was fused with N-terminal and C-terminal domains of the fibroin heavy-chain promoter (pFibH), giving rise to a p3×P3-eGFP-pFibH-mKate2 transformation vector. Its antimicrobial activity was demonstrated by the inactivation of *E. coli* (DH5α) bacteria and the photodegradation of dye molecules (i.e., methylene blue) (161). Under visible (green) light illumination, the mKate2 silk generated two types of ROS, $O_2^{\bullet-}$ and $^1O_2$, by type I and II photoreactions, respectively. The ROS generated by mKate2 silk significantly inactivated the bacteria and photodegraded the dye. This result supports the idea that phototoxic fluorescent protein–expressing silk can offer an alternative means of generating ROS that is comparable to visible light–driven plasmonic photocatalysis. Note also that ROS generation and photoelectric conversion are two sides of the same coin in terms of redox reactions and electrochemistry, as photoinduced electrons are closely related to photoelectric conversion. Thus, silk fused with phototoxic fluorescent proteins can have basic current–voltage characteristics that can be exploited for biological photosensors and energy-harvesting devices (203–206).

3.4. Antimicrobial Peptide– and Noncanonical Peptide–Expressing Silk

Silkworm transgenesis has been expanded to incorporate antimicrobial peptides into silk proteins. Antimicrobial peptide–expressing silk is a viable way to impart a disinfection functionality to silk. A transgenic silkworm carrying a recombinant *B. mori* fibroin light chain fused to GFP was successfully hybridized with antimicrobial peptide cecropin B (*CEC* B) by use of a gene-targeting technique (i.e., homologous recombination) (195). Strong antimicrobial activity of *CEC* B–expressing silk cocoons was demonstrated by counting the number of *E. coli* colonies. Different transgenic silkworms were able to express *CEC* B or Moricin (*MOR*) antimicrobial peptides as a result of *piggyBac*-mediated germline transformation (i.e., *pFibH-CEC* B1/3×P3DsRed and *pFibH-MOR*/3×P3DsRed constructs) (196). Transgenic silk fused with *CEC* B or *MOR* antimicrobial peptides also inhibited the growth of *E. coli*. Furthermore, a silk yarn maintained antibacterial activity against *E. coli*. Importantly, these results indicate that the silk fibers retained active *CEC* B and *MOR* antimicrobial molecules after the degumming process.

The inactivation mechanisms of antimicrobial peptides are generally explained by two major actions: direct killing and immune modulation (**Figure 5**). Antimicrobial peptides bind to the bacterial membrane through electrostatic interactions, either to disrupt the membrane or to enter the bacterium in order to inhibit intracellular functions. Some antimicrobial peptides also modulate host immunity by recruiting or activating immunocytes or by influencing Toll-like receptor recognition of microbial products and nucleic acids that are released upon tissue damage (207, 208).

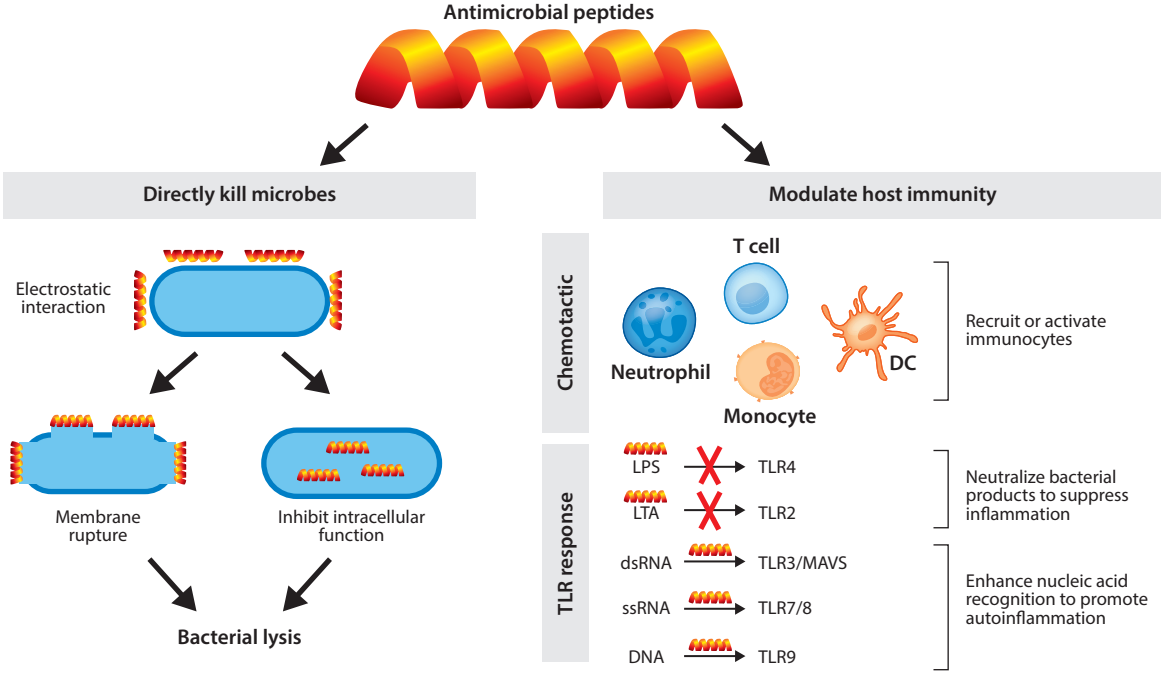

Figure 5

Antimicrobial functionalization of silk with genetic hybridization of peptides. Two antimicrobial actions involving antimicrobial peptides are direct killing and immune modulation (208). Abbreviations: DC, dendritic cell; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MAVS, mitochondrial antiviral signaling protein; TLR, Toll-like receptor. Figure adapted with permission from *Current Biology*. Copyright 2016, Elsevier.

More interestingly, silkworm transgenesis has recently been used to express noncanonical peptides and amino acids that do not exist in nature. Azidophenylalanine-expressing silk was obtained from a genetically engineered silkworm (*B. mori*) by constructing a *piggyBac* plasmid vector (209). Azido groups in living systems play an important role in bioorthogonal chemistry, in which a chemical reaction occurs without interfering with natural biochemical processes (210, 211). In order to screen azido derivative azidophenylalanine-recognizing variants, a pool of *B. mori* phenylalanyl–transfer RNA synthetase (BmPheRS) variant genes was introduced into *E. coli* cells (209). Selected BmPheRS variants were then examined for possible adverse effects in *B. mori* cultured cells. Finally, only safe variants were expressed in the silk glands of transgenic *B. mori* larvae to produce azido-functionalized silk. The feasibility of bioorthogonal chemistry using azido-functionalized silk was successfully demonstrated in a click modification experiment (209), while maintaining the basic mechanical properties for use in other general applications.

4. OUTLOOK AND CONCLUSION

Silkworm engineering via transgenesis and diet-enhanced methods can produce silk with reinforced or superior material properties that often do not exist in naturally occurring materials. This idea has an analogy to conventional metamaterials. Research on metamaterials and metastructures involves the construction of materials to offer user-designed atypical responses that are often absent in nature. Optical metamaterials are often realized through control of their electric and magnetic properties, and are not limited to negative refractive index and invisibility cloaks (107, 109, 110, 113). On one hand, mechanical metamaterials take advantage of artificially engineered structures to determine mechanical properties, relatively independently of material compositions (108, 111, 112, 212, 213). On the other hand, conventional metamaterials are not intended for biomedical applications, and their construction relies primarily on exotic materials and on complex nanofabrication and nanomanufacturing. Often, metamaterials not only are limited by the material toxicity and biocompatibility but also are not amenable to scalable, economical, and eco-friendly production. For example, typical nanomanufacturing processes for nanoproducts require a large amount of toxic raw materials and fossil fuels and are often difficult to apply in sustainable mass production (214, 215). In this respect, the concept of reinforced or superior silk can be extended to a type of metamaterial. In other words, reinforced silk produced by transgenic silkworms and silkworms fed with special diets could be considered biomedical metamaterials that are designed for direct use in biological and medical applications.

Reinforced or superior silk (metamaterial-like silk) produced by transgenic silkworms and functional diet additives can serve as an ab initio foundation for new opportunities. Biofactory and bioreactor strategies will be very useful for producing such silk in a scalable and eco-friendly manner, given that there is always a need for continuous and scalable production of nanobiomaterials. Incorporating the superior biocompatibility and bioresorbability of natural silk, such silk can be directly used in biological and medical applications. For example, silk integrated with desirable and enhanced functionalities can be used in wound dressings with monitoring or sensing features; tissue engineering scaffolds with antibacterial, anticoagulant, or anti-inflammatory features; vaccine manufacturing; photodynamic therapy; and bioenergy harvesting. In particular, antimicrobial peptides or ROS-generating fluorescent silk will offer exploitable and scalable photocatalyst-like biomaterials, potentially ruling out hazards associated with foreign semiconductor nanomaterials. Because interactions between light and fluorescent proteins are often understood on the basis of quantum mechanics (216–219), fluorescent protein–expressing silk could provide an alternative model system for studying quantum biology and quantum biophotonics. Silkworm transgenesis and diet-enhanced silk production could help guide synthetic biology approaches to enable the

production of superior (metamaterial-like silk) synthetic silk. Overall, silk produced by silkworms and spiders has been used for more than 5,000 years and is currently reemerging as an exceptional biomaterial platform for fundamental research and biomedical applications.

DISCLOSURE STATEMENT

J.W.L. and Y.L.K. are inventors named in two patent applications related to some of the research described in this review, filed with the US Patent and Trademark Office by the Purdue Research Foundation (application numbers 15874869 and 15874864) in January 2018. M.J.F. is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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