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Delivering on the promise of recombinant silk-inspired proteins for drug delivery

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ABSTRACT

Effective drug delivery is essential for the success of a medical treatment. Polymeric drug delivery systems (DDSs) are preferred over systemic administration of drugs due to their protection capacity, directed release, and reduced side effects. Among the numerous polymer sources, silks and recombinant silks have drawn significant attention over the past decade as DDSs. Native silk is produced from a variety of organisms, which are then used as sources or guides of genetic material for heterologous expression or engineered designs. Recombinant silks bear the outstanding properties of natural silk, such as processability in aqueous solution, self-assembly, drug loading capacity, drug stabilization/protection, and degradability, while incorporating specific properties beneficial for their success as DDS, such as monodispersity and tailored physicochemical properties. Moreover, the on-demand inclusion of sequences that customize the DDS for the specific application enhances efficiency. Often, inclusion of a drug into a DDS is achieved by simple mixing or diffusion and stabilized by non-specific molecular interactions; however, these interactions can be improved by the incorporation of drug-binding peptide sequences. In this review we provide an overview of native sources for silks and silk sequences, as well as the design and formulation of recombinant silk biomaterials as drug delivery systems in a variety of formats, such as films, hydrogels, porous sponges, or particles.

Contents

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1. Introduction

Drugs are traditionally delivered via systemic administration for small molecule drugs, most commonly through the enteral route of oral administration, where the drug is primarily absorbed in the small intestine before ultimately dispersing throughout the body. This delivery route is not suitable for every drug, which is subject to the harsh gastrointestinal (GI) environment, a long transit time, and the first-pass effect, whereby the initial concentration of drug is reduced by metabolism in the liver [1]. Intravenous injection is the most common parenteral route of systemic drug delivery, which overcomes some of the challenges associated with oral administration by allowing a high concentration of drug to circumvent physiological barriers with the shortest transit time. These delivery routes can sometimes be ineffective at treating disease, as it is challenging to reach therapeutic concentrations at the target disease site while balancing the use of higher doses that can be harmful to healthy organs and tissues. For larger, more complex drugs such as peptides, proteins or nucleic acid, systemic delivery of the naked macromolecules can lead to premature degradation through native proteases and nucleases and may lead to immune responses. These larger molecules are also usually unable to cross the cell membrane for therapeutic action. To circumvent these limitations, drug delivery systems (DDSs) which can provide more targeted outcomes are necessary. DDSs can be formulated as locally implanted reservoirs where therapeutically-relevant concentrations can reach the disease site while minimizing side-effects [2]. Alternatively, DDSs can encapsulate drugs into nano- or micro-sized carriers that are programmed to target a diseased site and release drugs upon exposure to specific environmental signals or via cellular uptake. The drug release profile from DDSs can be adjusted to the specific need, while minimizing drug-related toxic side effects. In addition, DDSs should also provide protection of the drug to maximize stability and bioactivity [3,4] (see Fig. 1).

Polymers are suitable candidates for DDSs due to their physicochemical stability and tunable properties. However, synthetic polymers present challenges for DDS that can limit applicability. For example, they can exhibit a broad molecular weight distribution, sometimes can present issues of biocompatibility, are often processed in organic solvents and some degradation products can be inflammatory (e.g., some polyesters). The use of protein-based polymers avoids these limitations as they are usually monodisperse (if generated via bioengineering approaches), biocompatible, biodegradable, degrade enzymatically into amino acids, and can be processed under mild, aqueous conditions. Biopolymers also contain many reactive chemical handles which can be used for chemical conjugation to enhance targeting, stability and overall function. These characteristics make them attractive candidates for DDS. Within this context, silk protein has gained a lot of attention for its low immunogenicity and ability to be formulated into many material formats including hydrogels, films, sponges and particles. Silk-based DDS have demonstrated favorable bioactivities in delivery of chemotherapy agents for cancer treatments, promoting wound healing, sustained release of cytokines and morphogens, delivering antibiotics, and gene therapy, among others.

Silks have a unique combination of properties [5], and can be sourced from natural sources (silkworms) or by expression in heterological systems; mainly bacteria and yeast (although many other systems are available currently) [6]. The development of recombinant DNA technologies provides more consistent control of polymer features, and this approach has supported the combination of silk-like motifs with other therapeutically relevant protein sequences, providing tailor-made protein-based biomaterials for DDSs. In addition, recombinant polymers are homogeneous, with nearly monodisperse molecular weight and composition, and good batch-to-batch consistency. These materials are also biodegradable, often with no toxic side products, and the degradation rate can be tailored on demand, for example, via inclusion of protease-sensitive domains. Another advantage of these recombinant materials is that they allow the inclusion of cell- and/or tissue-targeting domains, increasing the specificity and selectivity of the DDS.

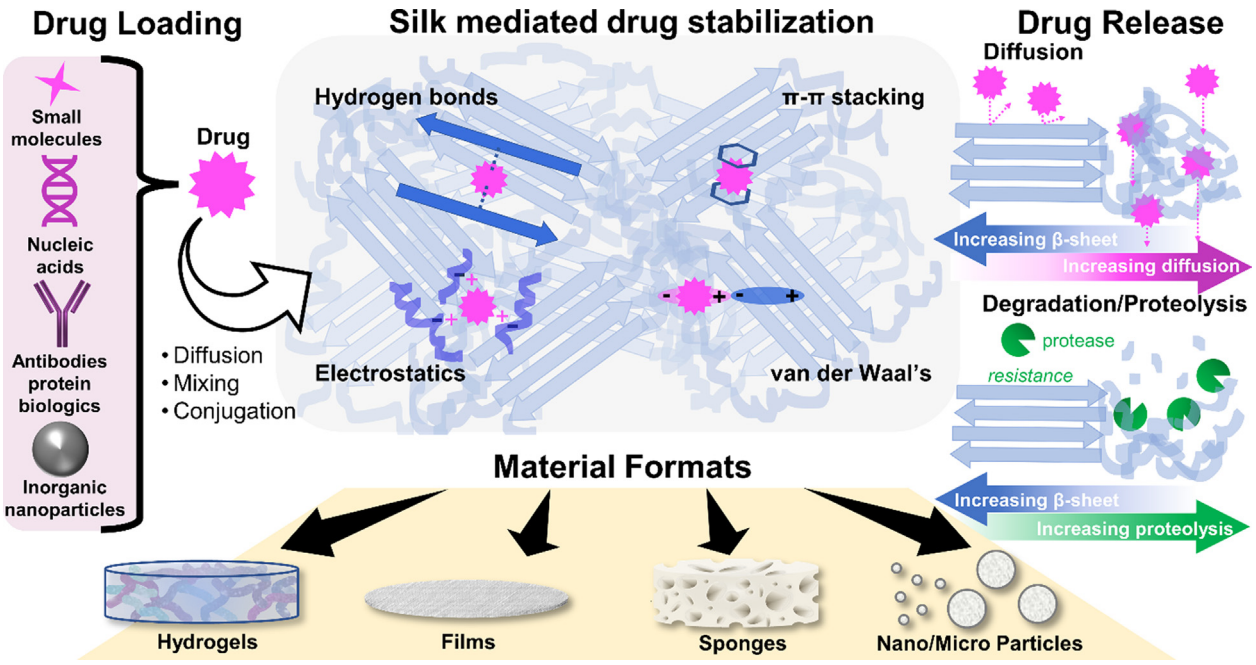

Figure 1. Silk-based drug delivery systems. Drugs such as small molecules, nucleic acids, protein biologics, and inorganic nanoparticles can be loaded into silk through diffusion, mixing, or chemical conjugation. The silk, which can be processed into various formats (hydrogels, films, sponges and particles) stabilize loaded drugs through hydrogen bonds, electrostatic interactions, pi-pi stacking, and van der Waals forces. The structural content of the silk can be tuned to control drug release through both diffusion and proteolytic degradation mechanisms, where higher beta sheet content creates resistance to both diffusion of the drug as well as protease activity.

In this review we discuss natural sources of silk, the structure and properties related to primary sequence and how these features have been utilized to guide the design of recombinant analogues. We then provide an overview of how these recombinant silks have been used as local implant DDSs in various formats, such as hydrogels, films, and sponges. We will also discuss silk particle-based DDSs for systemic delivery and how these have been tailored to accommodate drugs ranging from small molecules to large biomacromolecules, as well as inorganic nanoparticles. Lastly, we provide a perspective on the future outlook for silk and recombinant silk DDSs.

2. Natural silk sources and their recombinant analogues

Silks are protein-based fibers spun from various arthropods including silkworms, spiders, ants, bees, wasps, and lacewing insects [7]. These silks exhibit different mechanical properties depending on their required function. For example, orb weaving spiders produce seven types of silk from different glands for building web frames and draglines (major ampullate), supporting web frames (minor ampullate), catching prey (flagelliform), coating webs with sticky substrates (aggregate), wrapping prey (aciniform), attachment of silk to substrates (pyriform), and constructing egg sacs (tubuliform) [8]. The major ampullate dragline silks exhibit superior toughness, even to Kevlar [9]. The flagelliform silks, while three orders of magnitude lower in stiffness than dragline, also have high toughness due to the high extensibility for catching prey. The tubuliform silk in egg cases exhibit high strength but low elasticity, and unlike other silks can bend before breaking [10]. Aciniform silks are strong and also highly extensible (86 %) for wrapping prey, which makes them 50 % tougher than dragline [11]. Silkworm silks contain both fibroin, which makes up the core fiber and mechanics, and sericin, which acts as a sticky glue holding the fibers together [12]. The variety of mechanical properties of these natural materials provides an extensive tool box for the creation of various DDS. The mechanical stiffness of particulate DDS can influence circulation half-life, biodistribution, infiltration, and cell uptake [13]. Stiffness of implantable DDS has been shown to play a role in macrophage activation phenotypes [2].

The variety of mechanical properties is a result of the silk composition, protein structure, and primary sequence. The toughness of silkworm silks and major ampullate dragline silk is a result of repeats of alternating hydrophobic and hydrophilic domains, where the hydrophobic domains form antiparallel β-sheet crystals for strength, while the hydrophilic domains form amorphous regions for extensibility [14,15]. The domestic mulberry silkworm fibrion from *Bombyx mori,* contains GAGAGS motifs which make up the hydrophobic domains [15], while wild, non-mulberry silkworm fibroins and major ampullate dragline spidroins contain stronger interacting polyalanine motifs [14,16]. Silks from the order *Hymenoptera*, including bee, ant, and hornet silks, contain four small, highly alpha-helical, nonrepetitive silk proteins rich in alanine which assemble through coiled-coil motifs [17–20]. Egg stalk silks from lacewings have a unique cross-beta structure, which provides high stiffness but also high extensibility upon wetting or high humidity [21,22]. The mechanical properties of a silk-based DDS can be controlled via crystalline beta-sheet content, which in turn controls enzymatic degradation *in vivo* and drug release kinetics [23].

In addition to mechanical properties, silks exhibit excellent biological properties that make them exceptional candidates for drug delivery applications. Spider silks as well as silkworm fibroin and sericin are individually well tolerated in *in vivo* applications; however, sericin must be removed from fibroin through a degumming process, as its use in conjunction with fibroin activates an adaptive immune response [24]. Fibroin alone is non-toxic, biocompatible and biodegradable [25], and degradation can be tuned based on the material morphology and processing conditions related to crystallinity [26]. Additionally, the use of silk fibroin and sericin individually have been shown to support cell proliferation and migration [27,28]. Non-mulberry silkworm fibroins contain integrin-binding fibronectin-like RGD motifs which further enhance proliferation and migration as they provide handles for cell attachment [29], where *Antheraea mylitta* and *Antheraea yamamai* contain 10 and 12 of these motifs, respectively [16]. Sericin's hydrophilic composition provides interesting antibacterial [30], antioxidant [31], anti-inflammatory [32], anticancer [33], and UV resistant properties [34].

In engineering materials for drug delivery, recombinant DNA technologies are utilized to modify silks with mutations to alter charge and drug transport kinetics [35,36], or to generate chemical conjugation handles [37,38]. The fusion of bioactive peptides can be performed to load certain drugs [39], improve drug retention [40], target particular cell types [41], tune material degradation [42], and enhance cellular infiltration [38,43–45]. *B. mori* silkworm silk has been harvested for textiles since the 4th millennium BC [46], thus silkworm farming, or sericulture, can produce large quantities of the material at a relatively low cost [47]. Due to this availability, recombinant engineering of silks have been pursued for more selective goals, such as transgenic silkworms to modify silk fibroin with other components directly [48], or bioactive fusions to native sericin though TALEN-mediated genome editing [49].

Unlike *B. mori* silkworms, spiders are territorial and cannot be farmed, thus unlike silkworm silk, large quantities of the protein are not readily available. Thus, recombinant DNA strategies have emerged to express major ampullate spidroins in other hosts such as *E. coli* [43], *Pichia pastoris* [50], goats [51], plants [52], and *B. mori* silkworms [53]. Recombinant analogues of dragline spidroin proteins from *Nephila clavipes* (MaSp1/1F9 and MaSp2/2E12) and *Euprosthenops australis* (MaSp1/4RepCT) have been generated by cloning sequences derived from the internal repetitive sequences of the respective spidroin (Table 1) [50,54]. Additionally, shorter length consensus repeats have been derived from *N. clavipes* (MaSp1 and MaSp2) and *A. diadematus* (ADF-3 and ADF-4) [43,55,56]. The number of repeats that can be expressed in *E. coli* is limited by protein solubility and stability of the repetitive, glycine rich gene composition [57]. Metabolically engineered *E. coli* with elevated glycyl-tRNA produced up to 96 repeats, a 284.8 kDa MaSp1, with yields around 0.5–0.75 g/L in high cell density cultivation. This recombinant silk was formed into fibers with comparable properties to native dragline silk fibers, where the toughness was twice that in the native fiber.

Repetitive, high molecular weight silks are difficult to produce heterologously in microbial hosts, while the expression of full length native silk proteins from Hymenopterans can be achieved as these are small nonrepetitive proteins [17–20]. For example, honey bee silk proteins (Amel1-4) were expressed in *E. coli* and recovered from inclusion bodies (0.2–2.5 g/L) [22]. However, research is limited in applying these materials to drug delivery.

Recombinant *B. mori* silk-like proteins have also been expressed heterologously. The predominant GAGAGS motif in conjunction with stimuli-responsive elastin-like polypeptides led to the development of recombinant silk-elastin-like polypeptides (SELPs) [58]. Recombinant sericin based on the 38 amino acid consensus repeat in the Ser1 gene (Table 1) of *B. mori* was expressed in *E. coli* [59], *Pichia pastoris* and cell free systems [60]. These recombinant sericins exhibited cell proliferative [61], cryoprotective [62], bactericidal [63], and antioxidant properties [64]. The 4mer repetitive sequence from the native sericin gene showed higher solubility than the synthetic consensus repeats [65]. For non-mulberry

Table 1
Silk sources and the repetitive motifs and their sequence alignments used in recombinant silk production.

Source	Protein	Recombinant Protein/ Motif	Repetitive Silk-like Sequence Alignments	Number of Repeats	Refs
Bombyx mori	Fibroin	Hydrophobic motif	GAGAGS	1, 2, 4 2 4 8	[68,69,70,71,42]
	Sericin	Ser1 Consensus	SSTGSSSNTDSNSNSVGSSTSGGSSTYGYSSNSRDGSV	2 4, 8, 12	[61,62,59]
		Sericin	STDLAGSSTSGGSSTYGYSSNSRDGSV SSTGSSSNTDASTDLTGSSTSGGSSTYGYSSSNRDGSV LATGSSSNTDASTTEE STTSAGSSTEGYS	1	[60,63,64]
		Ser4mer	SSTGSTSNTDSSSKSAGSRTSGGSSTYGYSSSHRGGSV SSTGSSSNTDSSTKNAGSSTSGGSSTYGYSSSHRGGSV SSTGSSSNTDSSTKSAGSSTSGGSSTYGYSSRHRGGRV SSTGSSSTTDASSNSVGSSTSGGSSTYGYSSNSRDGS	1	[65]
Antheraea pernyi	Fibroin	EAEFN	EEAAAAAAAAAAAAEETGRGDSPAAS	5, 10	[67]
Nephila clavipes	MaSp1	1F9	AGQGGYGGLGSQG AGRGGLGGQGAGAAAAAAAGGAGQGGLGGQG AGQGAGASAAAA GGAGQGGYGGLGSQG AGRGGLGGQGAGAVAAAAAGGAGQGGYGGLGSQG AGRGGQGAGA AAAAAGGAGQRGYGGLGNQG	9	[50]
		MaSp1 Consensus	SGRGGLGGQGAGAAAAAGGAGQGGYGGLGSQGT	6 15 32, 48, 64, 80, 96	[44,72–74,41,43,57]
		A block	GAGAAAAAGGAGTS	BA, BA_2, BA_3, BA_6	[75]
		B block	SQGGYGGLGSQGSGRGGLGGQTS		
	MaSp2	2E12	GPGGYGPGQQGPGAAAAASA GRGPGGYGPGQQGPGGSGAAAAAA SGPGGYGPGQQGPGGPGAAAAAAA GRGPGGYGPGQQGPGGSGAAAAAA GRGPGGYGPGQQGPGGPGAAAAAA	12	[50]
		MaSp2 Consensus	GPGGYGPGQQGPSGPGSAAAAAAAA GPGGYGPGQQTS	9 15	[40,55,40]
		EMS2	EGPGGYGPGQQGPSGPGSAAAAAAAA GPGGYGPGQQTS	15	[35]
Araneus diadematus	ADF-3	A block Q block	GPYGPGASAAAAAAGGYGPGSGQQ GPGQQGPGQQGPGQQGPGQQ	$(AQ)_{12}$, $(QAQ)_8$ $(AQ)_{12}$, $(AQ)_{24}$	[56,76]
	ADF-4	C block/eADF4(C16)	GSSAAAAAAAAASGPGGYGPENQGPSGPGGYGPGGP	16	[37,38,56,76–81]
		eADF4(κ16)	GSSAAAAAAAAASGPGGYGPKNQGPSGPGGYGPGGP		[36,37,80]
		eADF4(Ω16)	GSSAAAAAAAAASGPGGYGPQNQGPSGPGGYGPGGP		[80]
Euprosthenops australis	MaSp1	4RepCT	QGGYGGLGQGGYGQGAGSS AAAAAAAAAAAAGGQGGQGQGGYGQGSGGS AAAAAAAAAAAAAAAGRGQGGYGQGSGGN AAAAAAAAAAAAAAAGQGGQGGYGRQSQGA GS AAAAAAAAAAAAAAGSG QGGYGGQGQGGYGQSS	1	[45,54,82–88]
Latrodectus mactans	TuSp1	eTuSp1	FSSASSASAVGQVGYQIGLNAAQTLGISNAPAFADAVSQ AVRTVGVGASPFQYANAVSNAFGQLLGGQGILTQENAAG LASSVSSAISSAASSVAAQAASAAQSSAFAQSQAAAQAF SQAASRSASQSAAQAGSSSTSTTTTTSQAASQAASQSASSSXSA	1	[89]
Mallada signata	MalXB2	AS module	GSAGASSNGSSATASK GSAGATSNGSTAVASK GSAGASSGNSTASATK	8	[90]

fibroin, the increased stability of polyalanine rich domains makes the cocoon more difficult to process using LiBr extraction methods [66]. Inspired by *Antheraea pernyi*, recombinant silk $EAEFN_n$ (n = 5 or 10) was expressed in in *E. coli* to generate coatings to promote osteogenic adhesion and growth (Table 1) [67].

The specific application of these silks and their recombinant analogs in drug delivery will be discussed in more detail in the following sections.

3. Implantable devices

Silk in bulk format can be used as a depot for the delivery of drugs, biomolecules, growth factors, or DNA at a fast or slow sustained rate [91,92]. Silk provides for controllable DDS, while also providing stabilization and protection to the embedded or sequestered (bio)molecules, preserving bioactivity [93]. The first step when developing a DDS using silk is loading the bioactive molecules, which can be achieved by bulk mixing, diffusion, or chemical conjugation. Water-soluble drugs or biomolecules can be dissolved with the silk, and the mixture processed into the desired material format. Drug capture and stabilization in the silk matrix depends on the number and strength of the interactions, such as hydrophilic or hydrophobic interactions, electrostatic interactions, hydrogen bonding, pi-pi interactions, or Van der Walls forces [80,94–96]. When the drug-silk mixture is placed in an aqueous environment, drug is slowly released. The release of hydrophilic drugs is usually attributed to diffusion mechanisms through the amorphous domains of the silk carrier [97–99], whereas the crystalline domains act as physical barriers to diffusion which results in a more sustained release profile in some instances [96,100,101]. The influence of higher-order silk structures on drug binding and release has been extensively studied [94]. Hydrophobic drugs exhibit longer release profiles, usually explained by hydrophobic interactions between the drug and the silk matrix, along with the lower solubility in water.

Due to the inherent ability to self-assemble or to be chemically crosslinked, the silk proteins can be processed into numerous material formats [102], such as films [103], 3D-foams [104], hydro-

gels [105–107], or coatings [108] (among many others). An additional feature of recombinant silk materials, which exhibit many of the properties of natural silk [109] (tunable control of structure, chemical modifications, self-assembly, controllable mechanical properties, biocompatibility, biodegradability) is the possibility to alter the composition and introduce peptide ligands, epitopes, drug interacting sequences, and other protein-derived structures that extend their properties [110,111]. These features help recombinant silks to stand out over other natural or synthetic materials.

The ability to expand and tune the properties of silks, including enhancing interactions with drugs, directed delivery of the drug towards a specific cell type, inclusion of unnatural amino acids for chemical modification, or the tailored degradation of the silk drug carriers, among other features, makes recombinant silks a promising platform for the development of DDSs. The controlled customization of recombinant silk offers significant improvement over native silk, allowing the introduction of unique and multifunctional domains into the silk sequence. These modifications can refine the already outstanding properties of native silk, not only overcoming its limitations but also providing for new applications.

3.1. Hydrogels

Silk hydrogels are a useful format for drug delivery because they can mimic the extracellular matrix (ECM) in structure, to positively support 3D cell cultures [112]. Hydrogels can be formed out of silk solutions by physical [113–115] or chemical crosslinking [116–118], or by a combination of both approaches [119]. Physical crosslinking can be induced by a local increase in protein concentration, through the use of organic solvents [120], acidification, changes in the ionic concentration of the solution [121], or by mechanical forces, such as shear or ultrasound [122–124]. These inputs favor the formation of nano- and microcrystals composed of hydrogen bonded beta sheets among silk molecules. Chemical crosslinking takes advantage of the chemically reactive groups present in silk, such as the hydroxyl groups on tyrosines. Examples of chemicals for crosslinking include ammonium peroxodisulfate (APS) [125] and tris(2,20-bipyridyl) dichlororuthenium(II) [117], or the oxidizing enzyme horseradish peroxidase (HRP) in combination with H_2O_2 [126,127]. With this last methodology, tyrosine residues are oxidized with formation of di-tyrosine bonds. The advantage of using HRP to crosslink silk is that the reaction can be carried out under physiological conditions [128]. Moreover, chemical crosslinking modulates the mechanical properties [127]. Due to the mild conditions for HRP crosslinking, the inclusion of different drugs, bioactive molecules, and even living cells in the formulation can be achieved, the latter option leading to the development of live-cell DDS therapies using, for example, pluripotent stem cells or engineered T cells [129].

Hydrophilic drugs can be loaded into the recombinant silk hydrogels by different methods, such as direct loading, where the bioactive compound is mixed with the precursor solution prior to gelation, or introduced via diffusion post formation of the hydrogel. These strategies are not exclusive and can be combined to tailor the release profile of the drug. Hydrogels prepared from the recombinant spider silk eADF4(C16) were used to encapsulate biologically active compounds by direct loading and/or via diffusion in aqueous solvents [130]. Factors such as pore size influenced the release kinetics of model compounds. In the same work, recombinant silk hydrogel particles loaded with bioactive compounds in macroscopic silk hydrogels were used to influence the release profile of the bioactive compounds, extending them from days to weeks without affecting the bioactivity [130].

One of the problems with hydrophobic drugs relies on loading of the DDS. A novel gelation route was developed using eADF4 (κ16) and eADF4(Ω16) recombinant spider silks and aqueous-organic solvent mixtures for 3D printing [80]. The co-solubilization of poorly water-soluble 6-mercaptopurine with organic solvents increased the amount of drug loaded in the gels, while the use of potassium phosphate induced rapid gelation of the system, for 3D printable drug-loaded hydrogel inks. In another example, co-precipitation using organic solvents and oil was used to fabricate recombinant eTuSp1 tubuliform spidroin silk from black widow spider microgels for a DDS with enzymatically-driven drug release [131]. The use of organic solvents increased loading capacity of doxorubicin while reducing beta-sheet formation when compared to other methods. The higher alpha-helix and lower beta-sheet content resulted in a lower resistance to proteolytic degradation and thus, proteinase-triggered release of drugs carried in the system.

Composite materials are useful in drug delivery systems via synergistic properties to address limitations and complementarity with the individual components, such as to increase the drug-loading capacity of the DDS. *Antheraea assama* silk fibroin (AaSF) was functionalized with a recombinant spider silk fusion protein (GN-4RepCT) bearing a fibronectin cell-binding domain to develop wound healing scaffolds [88]. The presence of the recombinant spider silk enhanced wound healing in an *in vivo* murine burn model, with increased vascularization and re-epithelialization when compared to the control. Moreover, the composite material enhanced the expression of collagen type I and III [88]. In another example, the processability of recombinant spider silk eADF4(C16) and the loading and release capacity of mesoporous silica nanoparticles were combined to develop antimicrobial-loaded composite hydrogels with antimicrobial properties, with drug release extending over 15 days [132].

The use of a recombinant approach for these materials allows expansion of the inherent properties by genetic sequence-level inclusion of genetic fusions, to generate (bio)active peptides or other protein sequences with the silk protein chains upon expression. Bioengineered RGD-modified variants of eADF3 and eADF4 recombinant spidroins were prepared and tested for antimicrobial properties [133] to demonstrate microbes were now unable to form biofilms on recombinant silk films, hydrogel surfaces, or within hydrogels. The bacterial and fungal repellent performance of these recombinant spider silk materials was related to higher-order structural features (i.e., secondary and tertiary) responsible for the formation of hydrophobic patches. The RGD-modified spider silk repelled microbes but allowed mammalian cell adhesion and proliferation [133]. This unique combination of properties (antibacterial, antifungal and cell-adhesive) in a single recombinant silk opens the possibility for new wound-healing and DDS, where the load of bioactive substances is focused on the improvement of the healing process and not on the prevention of infections. Silk has also been combined with thermoresponsive proteins for DDS. The temperature-driven aggregation of these proteins favors the formation of beta-sheets among the silk domains via physical crosslinking to form stable hydrogels. A chimeric protein was prepared which contained repeat units of resilin with the carboxy-terminal domain of the major ampullate spidroin 1 (MaSp1) of *Nephila clavipes* [134]. The resulting copolymer was thermoresponsive in water, forming reversible hydrogels at low temperatures and irreversible hydrogels in response to an increase in solution temperature. The mechanical properties of the hydrogel were dependent on the ratio of resilin to silk and on the presence of different salts. Using Rhodamine B as a model compound for drug release, the release profile was pH-dependent, with a slower release rate correlated with lower pH [134]. The recombinant fusion of silk and elastin-like protein (SELP) has also been used as a DDS. A SELP bearing lysine residues for controlled delivery of GM-0111, a modified glycosaminoglycan product of hyaluronic

acid digestion and chemical sulfation, was developed and used to treat mucosal inflammatory diseases [71]. GM-0111 release from SELP was much slower compared with other thermally responsive polymers, which resulted in an increased bioaccumulation in the rectum. In a murine model, the SELP-GM0111 composite provided localized protection against radiation induced damage, reducing pain and animal mass loss.

3.2. Films

Film formats are a solid formulation for DDS. Where drug release is regulated by thickness, crystallinity, or layer-by-layer composites with other material [135–137]. Films can be applied directly as a device or used as a drug-reservoir coating with other devices [138]. The flexibility of films can be useful, such as for conformal coatings on irregular tissue surfaces to improve the consistent release of drug to the tissue, and this can be achieved by using plasticizers or the inherent properties of the silk [139,140]. Plasticizers can impact the mechanical properties of the film/coating as well as the release profile of the drug [12].

A water-based method was developed to fabricate transparent thin films of recombinant spider silk protein eADF4(C16), with and without plasticizer (glycerol or 2-pyrrolidone) [141]. The soft processing conditions allowed direct loading of the films with small bioactive drugs (paracetamol and tetracaine HCl), complex sugars (dextran), or model proteins (lysozyme and bovine serum albumin). Drug release was dependent on the strength of interactions with the recombinant silk and molecular weight, with a slower rate of release for the higher the molecular weight of the compound. By combining different layers and coatings of silk, the release profiles of the different compounds could be tuned or controlled. A recombinant silk based on the C-terminal domain of the *E. australis* (4RepCT) self-assembled into fibrillar nanometer-thick membranes with elasticity and strong mechanical properties, with the films permeable to small molecules and proteins [142]. Moreover, these nanomembranes contained a fibronectin-derived motif, which supported human keratinocyte attachment and proliferation. The combination of biocompatibility, cell attachment, and drug/protein permeability provided biomolecular communication between the cell layer and the drug reservoir, with potential applications in drug delivery systems, drug studies, surgical transplants, and wound healing.

Covalently linking drugs to the recombinant silks through different bonds further expands the properties of these materials, particularly with labile chemical bonds towards stimuli-responsive DDS. pH- and redox-sensitive systems were developed based on the polycationic eADF4(C16) and the polyanionic eADF4 (κ16) recombinant silk capable of triggering the release of drugs controlled by environmental conditions [37]. This mechanism was tested in different formats with 6-mercaptopurine, doxorubicin, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and para-dimethylaminobenzaldehyde (DMAB), to support the efficiency and versatility of the methodology. Recombinant 4RepCT spider silk was also engineered to incorporate the non-natural methionine analogue L-azidohomoalanine for functionalization using azide-alkyne cycloaddition [143]. The recombinant silk was simultaneously chemically functionalized with different fluorophores, demonstrating multiple and simultaneous modifications of the recombinant silks. When decorated with a broad spectrum antibiotic via a labile linker, the recombinant silk demonstrated prolonged antibiotic activity [143].

Another interesting strategy is to develop recombinant silk-based systems that are capable of recruiting specific substances in situ that tailor or direct biological behavior. For example, films were prepared from recombinant eADF4(C16) spidroin containing tag sequences derived from non-collagenous proteins in bone known to bind to collagen and to initiate mineralization [144]. Calcium phosphate deposition and mouse preosteoblast adhesion were directed by the presence of these peptides, demonstrating that these films could initiate mineralization and tailor cell-adhesion. Films were also prepared using a 15mer derived from the consensus repeat of the *Nephila clavipes* drag line silk protein engineered with a hydroxyapatite binding domain at either the N- or C-termini [145]. Functionalization of the recombinant spider silk did not affect the assembly or mechanical properties, or the growth and proliferation of human mesenchymal stem cells. The presence of the hydroxyapatite binding domain increased the content of crystalline hydroxyapatite and the osteoinductive properties up to 3-fold when compared to the control. A recombinant silver-binding peptide was engineered into 6 or 15 repetitions of the consensus repeat of *N. clavipes* dragline silk as a template for nucleation and growth of silver crystals was prepared and inhibited the growth of Gram-positive and Gram-negative bacteria [73].

3.3. Sponges

There are several methods to fabricate 3D porous sponge structures from silk. The most widely used methods are freeze–thaw treatments and salt leaching, followed by a beta-sheet forming process, such as autoclaving [146,147], immersion in ethanol/methanol [148,149], or water vapor annealing [150]. Silks in the form of sponges are attractive formats for drug delivery, as they offer a high surface-to-volume ratio [151], which makes them very efficient in the delivery of hydrophobic drugs. When using silk, the release profile can be tuned by varying the crystallinity. Another important characteristic that makes sponges attractive is their capillary action, which facilitates the permeation of the release media into the sponge, and thus drug delivery [102].

Self-assembling mucoadhesive sponges using recombinant spider silk based on the 4RepCT protein were prepared for gastrointestinal drug delivery [152]. The recombinant 4RepCT silk protein was fused to either a human galectin carbohydrate recognition domain (hGal3) that specifically binds the Galβ1-3GlcNAc and Galβ1-4GlcNAc mucin glycans or to a polylysine tail. The incorporation of hGal3 resulted in stronger mucoadhesive properties when compared to the polylysine functionalized version due to its more specific interactions with mucin.

4. Systemic delivery

Injectable silk-based DDSs have been used as a vehicle for the delivery of a variety of drugs, and most often for systemic delivery of chemotherapeutic agents. The majority of this research has been focused on the use of native silkworm material [153], however, recombinant silk proteins offer sequence-level control over material properties in a variety of formats, such as particles, spheres, and capsules.

DDSs, including nano-sized or micro-sized particles, spheres, and capsules, have been investigated due to their surface area, ease of systemic administration, and their ability to permeate cells and tumors [154]. Nanoparticles are generally defined by the range from 1 to 100 nm, while microparticles are 100 nm to 100 μm diameter [155]. Size is an important characteristic that affects drug delivery, with sub 100 nm particles showing increased uptake by mammalian cells depending on the particle shape and material composition [154]. While the size of a particle is important, several other properties govern the effectiveness of particle-based DDS, including size distribution, surface charge, biocompatibility, stability, and controllable drug release [154–156].

In addition to general physicochemical characteristics, particle-based DDSs must be tailored to the target disease to achieve ther-

apeutic benefits. For example, passive targeting mechanisms can sometimes be adequate; numerous cancer treatment strategies have taken advantage of the enhanced permeability and retention (EPR) effect, a process by which macromolecules or nanoparticles enter the tumor microenvironment through intercellular gaps in blood vessels and are retained due to pressure [157]. Active targeting mechanisms are being increasingly investigated to improve drug specificity and limit off-target effects, with a large number of biological ligands capable of binding to cell receptors and facilitating uptake [158]. These advances are summarized in a detailed review of nanoparticle delivery to tumors, where it was shown that only 0.7 % of the administered nanoparticle dose was successfully delivered to the target tumor [159]. DDSs must also be tailored to the drug itself, where properties like solubility, charge, toxicity, and degradation need to be considered. Recombinant silks offer a highly customizable DDS framework that can be tuned to meet these demands, as summarized in Fig. 2.

4.1. Small molecule delivery

Small molecules make up the largest portion of current therapeutic drugs and are generally defined as organic molecules with a molecular weight below 1,000 daltons or smaller for oral delivery routes [160,161]. The most widely studied small molecule drugs with respect to recombinant silk-based DDSs are chemotherapeutic agents [162]. These drugs make excellent candidates for encapsulation within a delivery system to reduce severe systemic side effects, which commonly include cardiovascular toxicity, pulmonary toxicity, nephrotoxicity, hepatotoxicity, and more recently severe cutaneous adverse reactions (SCARs) [163,164]. Recombinant silk, particularly spidroin-based materials, have been studied as DDSs for doxorubicin, etoposide, mitoxantrone, and 6-mercaptopurine [35,37,153]. Due to the severity of off-target effects from these drugs, a primary focus has been the control of drug loading and release [155].

The majority of recent studies involving recombinant silk as a chemotherapy platform have focused on the delivery of doxorubicin. Doxorubicin is a hydrophilic anthracycline drug with a positively charged primary amine at physiological pH. Doxorubicin binds to and inhibits topoisomerase I and II preventing the separation of the DNA double helix and inhibiting replication, and also intercalates in DNA base pairs which further inhibits the process of replication and DNA synthesis [165]. However, like many chemotherapeutic agents, doxorubicin has severe off target effects. The most concerning side effect is cardiotoxicity, with a substantial risk of cardiomyopathy correlated with a lifetime accumulation of doxorubicin [165]. To reduce this risk, researchers have focused on encapsulation and targeted delivery using recombinant silk-based systems.

Recombinant spider silk has been studied extensively as a DDS for the delivery of doxorubicin in cancer therapy, with a focus on recombinant MaSp1 and MaSp2 variants. The targeted delivery of doxorubicin to human epidermal growth factor receptor 2 (HER2)-positive breast cancer cell lines using recombinant MaSp1 (MS1) microspheres was demonstrated [41]. Fusion of a Her2-binding peptide, denoted either H2.1 or H2.2, to the N-terminus

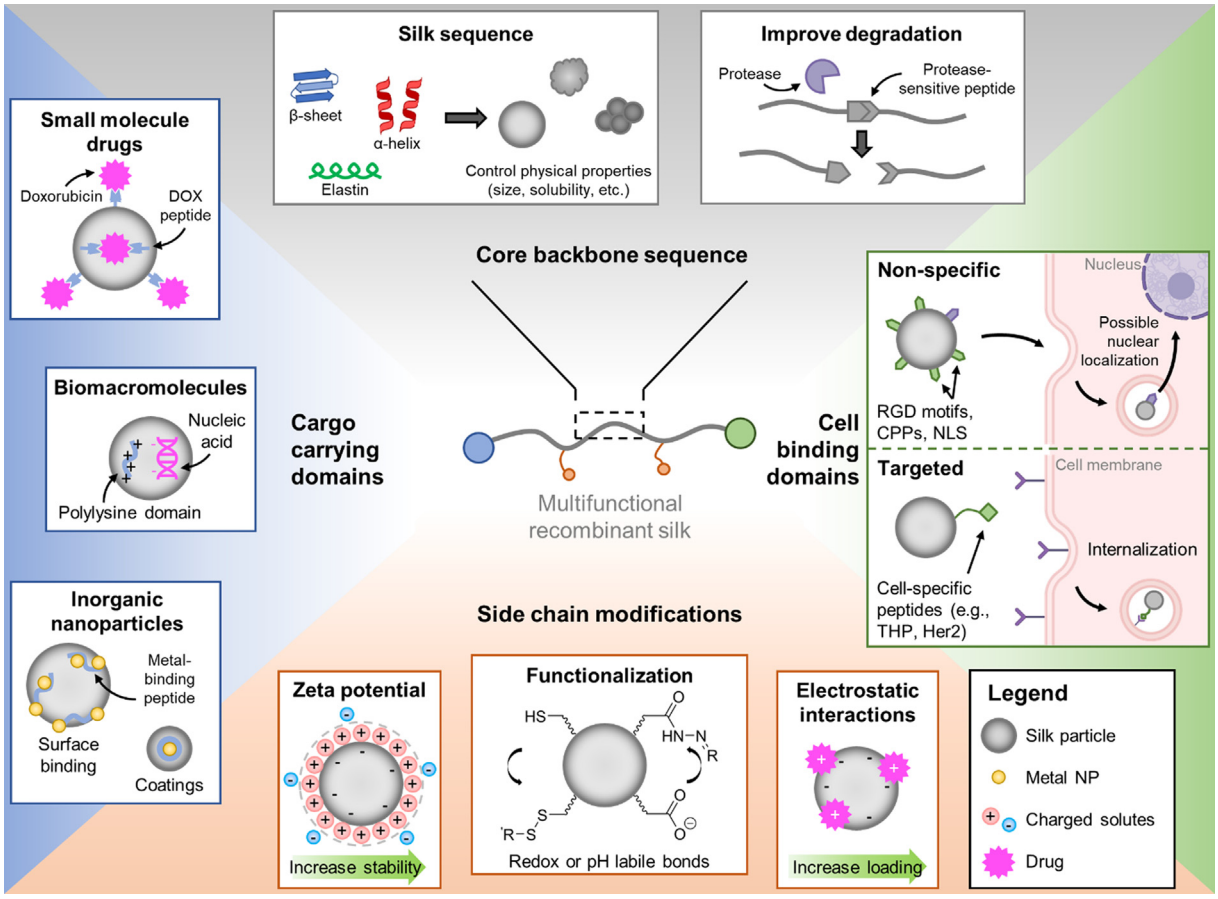

Figure 2. Overview of sequence modifications used for particle-based systemic drug delivery. Modifications are grouped as: 1) cargo carrying domains which interact specifically with a therapeutic agent, 2) side chain modifications through incorporation of additional amino acid residues, 3) cell binding domains to interact with various cell types in a non-specific or targeted manner, and 4) the core backbone sequence made up of one or more types of silk or elastin motifs. Abbreviations: RGD (tripeptide Arg-Gly-Asp), CPP (cell penetrating peptide), NLS (nuclear localization sequence), THP (tumor homing peptide), NP (nanoparticle). Created in part with BioRender.com.

of the MS1 sequence increased particle binding to Her2-positive cells *in vitro* when compared to the control MS1. When loaded with doxorubicin, H2.1- and H2.2-functionalized silk reduced cancer cell viability by up to 40 % over the non-functionalized silk. Follow on studies investigated the role of the silk component in this DDS, comparing the particle size, morphology, zeta-potential, drug loading efficiency, and efficacy when the MS1 sequence was exchanged for a sequence derived from MaSp2 (MS2) [55,166]. Particles produced with the MS2 variants (fused with H2.1, H2.2, or non-functionalized) were smaller, more spherical, and less prone to aggregation than the MS1 counterparts, but displayed lower drug-loading efficiency. Blending of the two functionalized silks resulted in particles with superior intermediate properties and a decrease in non-specific toxicity, highlighting the importance of silk identity to physicochemical and therapeutic properties [166].

The efficacy of H2.1-functionalized MS1 silk was tested *in vivo* in a murine Her2-positive breast cancer model [167]. The findings were similar to the *in vitro* studies performed with human Her2-positive cell lines; the H2.1MS1 silk particles loaded with doxorubicin had a selective cytotoxic effect on Her2 positive mouse cancer cells, and significantly reduced tumor volume over 20 days in a dose-dependent manner. Additionally, there appeared to be significantly lower off-target cytotoxicity with this DDS compared to unencapsulated doxorubicin, as the weight of mice was unaffected by the loaded MS1 sphere treatment. In a separate study, the unloaded MS1 spheres showed no toxicity when administered in a healthy mouse model [168]. Although particle biodistribution was affected by the presence of the H2.1 targeting peptide, with significantly more particles observed in organs compared to the non-functionalized MS1 spheres, histopathological examination of the organs indicated no morphological changes. The combination of these two studies is a significant advance in the application of recombinant silks for cancer treatment and could pave the way for early clinical trials in the future.

A consistent challenge for many DDSs, including those formulated with recombinant silks, is to control drug release until it has reached its target. To address this issue, an MS2 silk was bioengineered to contain a doxorubicin-binding peptide (DOXMS2) to enhance drug encapsulation and reduce leakage in the circulatory system [40]. By blending the DOXMS2 with the H2.1MS1 silk described previously [166], selective delivery of the particles to Her2-positive cancer cells was demonstrated *in vitro*. Furthermore, the DOXMS2 improved initial loading of doxorubicin by 12 % and reduced release by 23 %, 28 %, and 30 % (pH 4.5, pH 6, and pH 7.4, respectively). Another strategy to control drug binding and release is to manipulate electrostatic interactions between the carrier and the drug [169]. This strategy was recently employed to study the binding and release of positively charged doxorubicin [35]. The researchers modified the sequence of MS2, introducing one glutamic acid residue to each repeating unit to obtain negatively charged EMS2 silk with a lower isoelectric point (pI = 3.15 vs 5.27). The loading efficiency of doxorubicin into EMS2 particles was unchanged compared to MS2, however the EMS2 particles released doxorubicin at a faster rate with a dependence on pH. Using silk spheres consisting of recombinant tubuliform spidroin (eTuSp1) from the *Latrodectus mactans* (black widow) spider, the positive charge of doxorubicin was again utilized to control drug release [89]. Histidine residues were introduced into the silk sequence to achieve a pI of 4.8, resulting in a positively charged molecule at lysosomal pH (~4.5). The spheres displayed tight control over release in a pH-dependent manner, with 15 % and 20 % cumulative release at pH 7.4 and 6.5, respectively, and greater than 70 % cumulative release at pH 4.5.

In addition to electrostatic interactions, another attractive strategy to control drug binding and release is the direct covalent attachment of the drug to the carrier. There are several examples of this strategy being applied to elastin-like proteins [170,171], however only one instance of this for silk-based systemic delivery of small molecule drugs [37]. In this study, doxorubicin was chemically conjugated to the surface of silk particles from eADF4(C16), an engineered polyanionic spidroin from *Araneus diadematus* (European garden spider), using a pH-labile hydrazine linker. The conjugated particles showed little drug release for 48 hours at pH 7.4; when the pH was reduced to 4 to trigger drug release, 100 % cumulative release was achieved over the next 24 hours. *In vitro* experiments using hydrazine-linked fluorescent dyes indicated that release was triggered within 16 hours of internalization.

One group of alternative materials that has been studied for small molecule delivery are SELPs. There are several examples of nanoparticles developed using SELPs without drug encapsulation; varied ratios of *B. mori* silk fibroin motifs to elastin motifs to achieve spherical micellar-like particles [68], and the effects of various charged and polar guest residues within elastin blocks to form mucoadhesive nanoparticles [70]. The encapsulation of doxorubicin within SELP nanoparticles was also reported [69]. The SELPs delivered a cytotoxic dose of doxorubicin to HeLa cells *in vitro*, with one formulation (S4E8Y, with a 1:2 ratio of silk blocks to elastin blocks) performing better than free doxorubicin alone. To improve doxorubicin release from self-assembling SELP nanoparticles, a matrix metalloproteinase-responsive peptide (MMP) was encoded within the SELP sequence [42]. Insertion of the hydrophilic MMP sequence into the elastin region did not impair particle assembly and showed a correlation between extent of assembly and distance of the insertion site from the hydrophobic silk. Similarly, the release of doxorubicin from the particles was faster when the MMP sequence was further from the hydrophobic silk blocks. However, incubation with matrix metalloproteinase-9 (MMP-9) had no effect on doxorubicin release in any conditions tested, with the conclusion that doxorubicin release was driven entirely by water penetration and dissolution of doxorubicin from SELP assemblies. Although matrix metalloproteinase-dependent release studies have shown promising results in other DDSs [172], it seems further study is required to translate this efficacy to SELP formulations.

Several studies have demonstrated the flexibility of recombinant silks as a drug carrier by testing other small molecule formulations. Of note, recombinant spider silks have been studied for their efficacy in the encapsulation and delivery of three other chemotherapeutics, etoposide, mitoxantrone, and 6-mercaptopurine. Similar to doxorubicin and other chemotherapeutic agents, a major function of these drugs is to inhibit DNA synthesis and replication to prevent proliferation of cancer cells, with additional mechanisms of action relating to cell metabolism, cytokine secretion, and apoptosis [173–175]. Due to their systemic toxicity, these therapeutics can similarly benefit from encapsulation and controlled release. Microspheres composed of either MS1 or MS2 recombinant spider silk were analyzed for their efficacy in loading and release of mitoxantrone and etoposide [176]. The MS2 spheres were superior for the loading and release of both mitoxantrone and etoposide, correlated with a lower hydrophobicity and greater negative zeta-potential. To further investigate this relationship, a microsphere DDS was developed using the recombinant silk EMS2, a negatively-charged variant of MS2 containing glutamic acid residues with an even greater negative zeta-potential [35]. Loading efficiency of both neutral etoposide and positively charged mitoxantrone was higher in the EMS2 spheres than the unmodified MS2. Drug release was pH-dependent for both drugs regardless of silk composition; however, mitoxantrone exhibited a fast burst release from EMS2 that limited its potential application, while etoposide exhibited sustained release from EMS2 spheres up to 100 % of the encapsulated drug (at pH 4.5). In the case of 6-mercaptopurine, the triggered release of the drug

when coupled to cysteine-tagged eADF4(κ16) particles was demonstrated using a redox-sensitive disulfide bond [37]. Release of 6-mercaptopurine was rapidly induced over 30 minutes in 5 mM glutathione (GSH), mimicking the intracellular redox environment. However, *in vitro* release of a similarly coupled fluorophore was more persistent, only observed after 8 hours within HeLa cells.

Another class of small molecule drugs for drug delivery are antibiotics. With the rise of multidrug-resistant bacterial infections, new strategies have become necessary to combat these pathogens. Nanomaterials, such as core–shell nanoparticles, micelles, and liposomes, have been studied extensively as a potential strategy to deliver antibiotics systemically and with targeted efficacy to overwhelm drug-resistant bacteria [177–181]. Recombinant silk DDSs are an intriguing platform for the targeted delivery of antibiotics due to the ease with which peptides can be incorporated, either for targeting or for direct antimicrobial activity; however, there has only been limited study on this approach to date. One example is the smart delivery of vancomycin using infection-responsive MS2 nanospheres [182]. Vancomycin is currently used in the treatment of serious infections that have been unresponsive to other antibiotics, such as methicillin-resistant *Staphylococcus aureus* [183]. To combat this pathogen, functionalized MS2 nanospheres with a thrombin-sensitive peptide (TSP) were prepared and studied, leveraging studies that indicate an increase in thrombin-like activity during *S. aureus* infections due to its secretion of staphylocoagulase [184,185]. *In vitro* analysis of vancomycin-loaded TSP-conjugated particles incubated with *S. aureus* exudate showed a significant increase in drug release compared to the unconjugated MS2 particles (85 % vs 15 % cumulative release, respectively). *In vivo* analysis of the drug-loaded, TSP-conjugated particles in a murine septic arthritis model demonstrated improved bacterial clearing. Although this application was restricted to the chemical conjugation of TSP, these infection-responsive peptides could be included directly in the genetic sequence of recombinant silk proteins in future studies.

4.2. Biomacromolecule delivery

While small molecule drugs have historically played a large role in disease treatment, there are many so-called "undruggable" diseases that have not responded to these treatments [186,187]. Biomacromolecules such as nucleic acids and proteins are an alternative class of therapeutics that have increasingly become the focus of studies in the treatment of these diseases, due in large part to rapid advances in genetic engineering. Nucleic acids can be utilized in a variety of ways; as plasmid DNA (pDNA) and messenger RNA (mRNA) to express genes locally as part of therapeutic protein replacement strategies [188,189], small interfering RNA (siRNA) and micro RNA (miRNA) to knock down harmful protein expression [190–192], as well as aptamers that can provide highly specific targeting capabilities [193,194]. Peptide- and protein-based therapeutics can interact directly with target cells, providing specific cellular functions or regulating biological processes, and have already grown into a large global market [195–197]. However, like small molecule drugs, biomacromolecular therapeutics come with their own challenges for drug delivery. Factors such as high molecular weight and charge can prevent these molecules from permeating the cell membrane, and they are highly susceptible to enzymatic cleavage by endonucleases or proteases [23,198]. As such, DDSs can be used to mitigate these challenges by providing a protective effect and facilitating transport across the membrane, and recombinant silks offer some attractive properties in this regard.

One way that recombinant silk can be used to deliver nucleic acids is by using polycationic blocks to interact with and mask the negatively charged DNA. Poly(L-lysine) blocks can be encoded in the recombinant sequence to provide a positive charge at physiological pH, as demonstrated [199]. Six repeats of the MaSp1 consensus repeat sequence were fused to either 15, 30, or 45 consecutive lysine residues on the C-terminus. When complexed with pDNA by coincubation, the lysine-containing silks formed particles in the range of 250–500 nm. These particles were noncytotoxic (with the exception of silks containing the 45-lysine block, which reduced cell viability by ~ 15 %) to human embryonic kidney (HEK) cells and were capable of transfecting them with green fluorescent protein (GFP)-expressing pDNA with limited efficiency when the particles were deposited onto a silk film. Inclusion of multiple cell-binding RGD motifs at the N-terminus further increased transfection efficiency while simultaneously reducing the average particle diameter to 186 nm in solution [200]. Replacement of the RGD motifs with a dimeric ppTG1 peptide, a lysine-rich cell penetrating peptide (CPP) that enhances translocation, improved transfection efficiency further to a level comparable to the commercial transfection reagent Lipofectamine 2000 [201]. To increase the therapeutic relevance of silk-pDNA complexes, tumor-homing peptides (THPs) can be fused to the sequence as well. Incorporation of the F3 or CGKRK THPs enabled the targeted transfection of MDA-MB-435 and MDA-MB-231 cells both *in vitro* and *in vivo* in a murine xenograft model [202]. Building on this foundational work, the functionality of MaSp1-polylysine nanoparticles was expanded by investigating their efficacy for delivering pDNA to human mesenchymal stem cells (hMSCs) [203]. By fusing the nuclear localization sequence of the large tumor antigen of the Simian virus 40 (SV_{40}) and a dimerized hMSC high affinity binding peptide (HAB) to MaSp1 silk, transfection of hMSCs with GFP-expressing pDNA at 50 % of the efficiency of Lipofectamine 2000 was demonstrated.

Therapeutic protein and peptide delivery has largely been focused on native silk fibroin strategies. However, there have been some notable advances in the use of recombinant silks, which benefit from the ease with which peptide sequences can be added directly to the silk sequence. The protective benefits of encapsulating an active protein or peptide are clear, as demonstrated utilizing eADF4(C16) spider silk to encapsulate β-galactosidase [204]. Using an aqueous emulsion in silicon oil, capsules ranging from 1 to 30 μm in diameter were formed that contained the active enzyme and resisted protease degradation. Although this study did not focus on therapeutic drug delivery, demonstrating the proof of concept was an important step in biomacromolecule delivery. eADF4(C16) silk was utilized as a DDS to protect the ovalbumin epitope from degradation and deliver it to antigen-presenting dendritic cells as a part of a novel vaccination strategy [78]. The $OVA_{257\text{-}264}$ peptide (SIINFEKL), which is routinely used in vaccine studies, was fused to eADF4(C16) via a cleavable linker (cathepsin B or cathepsin S), and these recombinant proteins were processed into microparticles. The particles were preferentially taken up by dendritic cells *in vitro* and did not affect their viability or induce a proinflammatory response. When fused to the core silk sequence via cathepsin S, the particles induced strong cytotoxic T-cell proliferation both *in vitro* and *in vivo* using a murine model. When coupled with the stability of the particle suspensions and the simplicity of manufacturing, these findings present a promising new DDS for peptide vaccination using recombinant spider silks.

In addition to spider silks, recombinant silk sericin has been used effectively for the delivery of protein-based therapeutics. Ulcerative colitis was treated using an oral DDS containing recombinant human lactoferrin (rhLF) [205]. Lactoferrin is an iron-binding glycoprotein with antibacterial properties that plays an essential role in the human immune system, and has been studied as a potential therapeutic for the prevention and treatment of inflammatory bowel diseases [206]. To generate rhLF-functionalized silk, researchers hybridized previously developed

recombinant silkworm strain (D9L-rhLF) [207] with another silkworm strain (cottony cocoon) that produces larger sericin-rich cocoons with a greater yield of rhLF. Nanospheres prepared from this recombinant sericin provided a protective environment for the rhLF, with 75 % remaining after 24 hours of incubation in simulated gastric fluid and 42 % remaining after a similar incubation with artificial intestinal fluids. Uptake and bioavailability of the rhLF nanospheres was significantly higher than free rhLF both *in vitro* and *in vivo* (murine model), and oral administration of the nanospheres reduced dextran sulfate sodium-induced colitis in the mice.

4.3. Inorganic therapeutics

The earliest scientific description of inorganic nanoparticles was by Michael Faraday in 1857 as the result of a reduction of gold chloride, making these one of the longest-studied nanomaterials [208]. As of 2019, there were 25 inorganic nanomedicines on the market for the treatment of diseases like cancer, diabetes, bacterial infections, anemia, dental caries, and many more [209]. In addition, inorganic nanoparticles are uniquely beneficial for diagnostic and theranostic applications due to their optical, electronic, and thermal properties, in addition to the benefits shared by organic nanoparticle systems [210,211]. To facilitate the binding of targeting moieties, increase biocompatibility, and to prevent aggregation, inorganic nanoparticles can be coated by organic materials such as silk. Although the benefits of recombinant DNA technology are clear with regard to the facile fusion of metal binding domains and targeting peptides, few studies have been conducted using recombinant silks as the field continues to develop.

Magnetic iron oxide nanoparticles (IONPs) are one such DDS that have been investigated in combination with recombinant silk materials. IONPs are one of the most studied inorganic nanomedicines, with applications as contrast agents in techniques such as magnetic resonance imaging (MRI) and magnetic particle imaging (MPI) [210,212–214], and as therapeutic agents themselves for hyperthermia-based treatments or magnetically guided drug delivery [215,216]. Bioengineered spider silks have been used in conjunction with IONPs; the recombinant silks MS1, MS2, and EMS2 were investigated for their ability to form microspheres in the presence of IONPs [217]. The IONPs did not interfere with sphere formation, and although IONPs were associated with each of the three silks investigated the EMS2 variants were able to bind significantly more of the material. The IONPs were localized to the surface of the silk spheres and exhibited good magnetic properties, although the magnetic properties were not investigated for therapeutic applications such as hyperthermia. Instead, the authors investigated the composite spheres for the loading of doxorubicin and found that the presence of IONPs in the EMS2/IONP composites enhanced drug loading efficiency more than twofold while also slowing the drug release kinetics at a pH above 4.5. Building on this foundational work, bioengineered spider silks with metal-binding and targeting moieties were pursued [218]. MS1 silks were functionalized with an Fe1 adhesion peptide, which enhanced the binding of negatively charged IONPs, and this MS1Fe1 silk was blended with the previously described H2.1MS1 silk to provide targeting to Her2-positive cancer cells. Doxorubicin loading in the composite particles was enhanced by the presence of IONPs (1.3-fold increase) with a similar pH-dependent release profile as observed for the EMS2/IONP composites. *In vitro* cytotoxicity analysis with Her2-positive SKBR3 cells showed that the IONP blended spheres containing doxorubicin reduced cell viability slightly more than those without IONPs. However, the most striking results were seen with the use of a magnetic field to stimulate hyperthermia, which resulted in an almost 3-fold increase in cells in the late phase of apoptosis.

Several other inorganic nanoparticles have been investigated in conjunction with recombinant silks, including selenium, gold, and manganese carbonate, although their therapeutic applications are less developed than the previous IONP examples. Selenium nanoparticles (SeNPs) are of interest due to their antibacterial properties coupled with a low cytotoxicity for human cells, and have been explored as a new type of antibiotic to combat MDR pathogens [4–6]. SeNPs were coated with positively charged eADF4(κ16) recombinant spider silk and investigated for antibacterial properties [219]. The positively charged composite particles showed strong antibacterial effects by reduction in colony forming units on agar plates, with a minimum bactericidal concentration (MBC) against *E. coli* of 8 ± 1 μg/mL. The cytotoxicity of these particles was evaluated using Balb/3T3 mouse embryo fibroblasts and HaCaT human skin keratinocytes and were found to be noncytotoxic up to concentrations of 31 μg/mL. Although these findings are promising, further study is required to investigate the therapeutic use of these particles as a DDS. Gold nanoparticles (AuNPs) share many properties with IONPs, and have a unique property known as localized surface plasmon resonance (LSPR) that can generate heat when exposed to resonant frequency photons and be used as a form of photothermal therapy [211]. Coating of Ni-NTA functionalized AuNPs with recombinant SELPs with an N-terminal His-tag was investigated [220]. These composite nanoparticles displayed thermally reversible aggregation when heated to 60 °C, which also shifted the plasmon resonance, however the goal of this study was not therapeutic in nature. Manganese carbonate nanoparticles have also been explored as potential photothermal therapy agents and MRI contrast agents [221,222]. Although recombinant eADF4 silk has recently been shown to enhance manganese carbonate mineralization, there has been no direct investigation of these composites for therapeutic drug delivery [223].

5. Engineering silkworms for recombinant protein expression

Currently, there is an increasing demand for therapeutic proteins with reduced costs. With the development of advanced tools for genetic manipulation, the silkworm silk gland can be engineered as a cost-effective bioreactor for the expression of recombinant proteins along with the synthesis of the native silk proteins [224]. *Bombyx mori* is one of the most attractive choices for the production of recombinant proteins due to the high protein production, low feed costs, short generation time, rich genetic resource and insight, and the feasibility of post-translational modifications. The first use of *B. mori* as a bioreactor used a baculovirus as the transfection vector for the expression of α-interferon [225].

Recombinant proteins can be co-expressed in the silk gland [207,226,227], or fused to a single chimeric silk molecule [228], with *piggyBac* as one of the most common vectors used for transfection [229,230]. Fertilized B. mori silkworm eggs are transfected with modified versions of the *piggyBac* vector containing the recombinant protein gene together with a helper plasmid carrying the *piggyBac* transposase gene. Due to the stochastic insertion of the recombinant gene, successful insertion in the transgenic silkworm is confirmed in the next generation using reporter genes, such as the DsRed, which induces the expression of a red fluorescent protein in silkwormś eyes. After successful transfection a recombinant protein can be continuously produced after a transgenic silkworm strain is established [224]. Recombinant protein can either be co-expressed or fused to the silk backbone sequence. When co-expressed, the bioactive compound remains embedded in the silk matrix and is released due to diffusion, degradation, or proteolysis of the matrix, a characteristic also controllable via fusion of a matrix metalloprotease with the fibroin structure [231,232]. When expressed as fusion proteins, they are inherently

a structural part of the matrix so long as the fused domains do not interfere with the hierarchical assembly of silk fibers (Fig. 3).

A recombinant protein comprising the basic fibroblast growth factor (bFGF)-binding P7 peptide was introduced at the C-terminus of the fibroin light chain [233]. A transgenic variant expressed the recombinant version in 26 % of the light chains, exhibiting high bFGF binding affinity. Human epidermal growth factor was fused to a truncated version of the *B. mori* fibroin heavy chain [234]. The transgenic silkworm cocoon silk significantly increased human fibroblast cell proliferation with no apparent cytotoxicity, demonstrating potential as wound dressing material. The use of recombinant proteins permits the incorporation of other proteins, to expand the bioactivity of these chimeric silks. To increase the biocompatibility of silk, and using transgenic silkworms as the recombinant factory, partial sequences of collagen or fibronectin were inserted into the fibroin light chain [47]. The recombinant silks possessed higher cell-adhesive activity when compared to the unmodified silk.

Sericin is the other major component of *B. mori* silk and is also amenable to being engineered using recombinant techniques. *B. mori* silkworms were engineered to co-express acidic fibroblast growth factor (FGF1) together with sericin [235]. Degumming of the silk fiber resulted in FGF1-loaded sericin which formed porous hydrogels upon cooling. The FGF1-loaded sericin was injectable and exhibited relevant mechanical properties while helping retain the bioactivity of the FGF1. The sustained release of FGF1 promoted cell proliferation in the sericin hydrogels, and based on the absence of inflammatory responses, demonstrated biocompatibility and no immunogenicity. Following a similar approach, a sericin hydrogel loaded with recombinant human platelet-derived growth factor (PDGF-BB) was developed [228]. Upon degumming the silk fibers, the PDGF-BB-loaded sericin formed hydrogels with similar mechanical properties to chemically crosslinked sericin, with a 13.1 % content of the growth factor. Sericin hydrogels slowly released the PDGF-BB while retaining its bioactivity for more than 42 days, and was biocompatible, exhibiting no immunogenicity (*in vitro* and *in vivo*) and supporting the differentiation of osteoblastic and human mesenchymal stem cells. A strategy to deliver recombinant human lactoferrin (rhLF) using transgenic silkworms was also developed [207,236]. The genetically engineered silkworms co-expressed rhLF that was secreted together with sericin and incorporated into the silk fibers. Hydrogels prepared from this rhLF-silk sericin blend were biocompatible and biodegradable, and this DDS demonstrated a protective effect on the rhLF in the gastrointestinal tract for efficient delivery in mice [236].

6. Future outlook

Polymer-based DDSs have been attracting research interest due to their controllable properties. However, the drawbacks of synthetic polymers (broad molecular weight distribution, issues of biocompatibility, use of organic solvents, and inflammatory degradation products) have directed attention towards other types of materials. As discussed in this review, natural polymers such as silk and recombinant silk-like proteins have beneficial properties that can address these shortcomings. In addition to the examples documented here, summarized in Table 2, there are several areas that warrant further investigation. Lesser studied silks such as those from the order Hymenoptera (ants, bees, and wasps) offer some interesting properties, such as a high mechanical strength (sometimes surpassing *B. mori* silk fibroin) and a non-repetitive alpha-helical structure [17–20]. Several of these silks have also been demonstrated to be highly expressed at full length in *E. coli* at much greater titers than many recombinant silkworm or spider silks, which are often challenging to express due to their repetitive sequence. Exploration of these alternative silks can expand the portfolio of potential materials for DDS, and may help to address challenging designs. The incorporation of multiple functional domains into one DDS is another exciting candidate for further research. Several recent studies have demonstrated the benefits of this multifunctionality, and with the continual advancements seen in genetic engineering and modeling we can better predict complex interactions and optimize them for drug delivery.

Although the use of recombinant silk materials for DDSs shows great promise, challenges remain that hinder translation to clinical trials and beyond. No recombinant silk DDS has yet been clinically approved. As is often the case with recombinant proteins, the scalability of silk materials is currently limited and often exacerbated by complications with heterologous expression of repetitive sequences. For *B. mori* silks, the direct engineering of silkworms

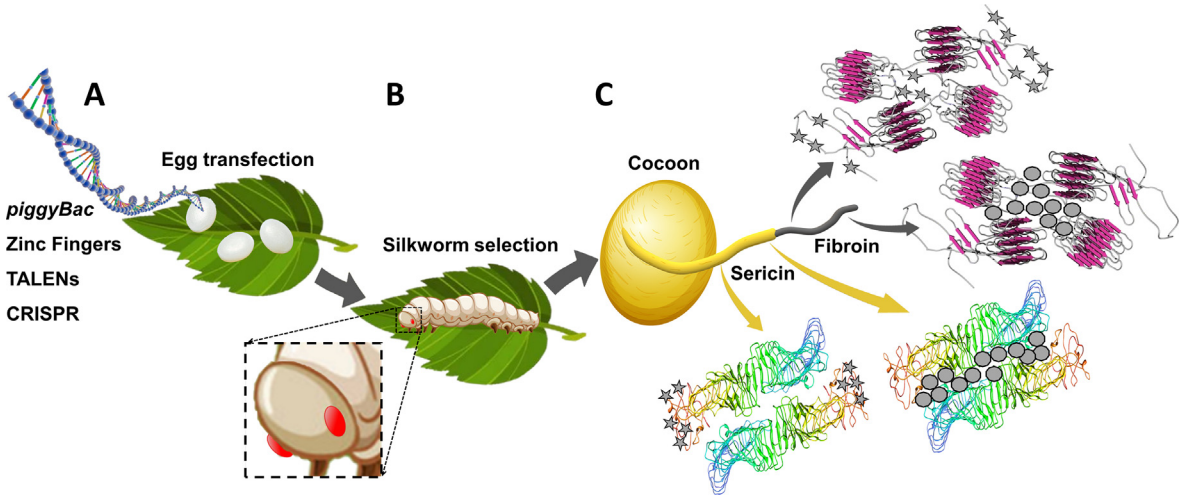

Figure 3. Schematic of the silkworm-based strategy for recombinant protein coexpression. A) Foreign genes are inserted into fertilized eggs of B. mori silkworms using recombinant vectors, the most common of which is the piggyBac vector, or different recombinant techniques such as Zinc Fingers, TALENs or CRIPSR. B) Transgenic worms are selected in the following generation using reporter gene expression, such as fluorescent proteins expressed in the eyes. C) Recombinant proteins can be either co-expressed (grey octagons) or fused to the backbone of both sericin and fibroin (grey stars). When co-expressed, recombinant protein remains embedded in the silk matrix, while if expressed as a fusion protein they are part of the matrix itself. In both cases, sericin and fibroin can be then processed into the desired DDS.

Table 2
Examples of recombinant silks fused to small peptides/factors, recombinant silk copolymers, recombinant silk composites and chemically functionalized recombinant silks used as DDSs.

Small peptides and factors	Sequence	Silk Type	Application	Refs
N-terminal Histidine tag	10xHis	SELP	SELP binding to Ni-NTA AuNP for nanoparticle photothermal therapy or MRI contrast agents	[220]
Histidine residues	10xHis	Recombinant tubuliform spidroin (eTuSp1)	Controlled, pH-dependent release of doxorubicin	[89]
Positively or negatively charged C-terminal tag	8xLys or 8xGlu	eADF4(c16) silk from *A. diadematus* fibroin	Enhanced carbonate nanoparticle mineralization for DDS	[223]
Iron binding peptides - Fe1 - Fe2	KSLSRHDHIHHH SVSVGMKPSPRP	MaSp1 protein of *N. clavipes*	Enhanced inorganic nanoparticle adhesion	[218]
$OVA_{257-264}$ peptideCleavable linkers: - Cathepsin B - Cathepsin S	SIINFEKL GFLG PMGLP	eADF4(C16) from *A. diadematus* fibroin	Microparticle encapsulation of bioactive enzymes for protection, with antigen-specific delivery fused via a cleavable linker	[78]
Poly-lysine + integrin binding domain	30xLys + RGD	MaSp1 protein of *N. clavipes*	Nanoparticle DNA Polyplexes Human embryonic kidney (HEK) cell transfection with GFP	[200]
Poly-lysine + Dimeric ppTG1 cell penetrating peptide	30xLys + (GLFKALLKLLKSLWKLLLKATS)2	MaSp1 protein of *N. clavipes*	Nanoparticle DNA polyplexes for HEK cell transfection with GFP, with efficiency similar to Lipofectamine 2000	[201]
Poly-lysine + Tumor-homing peptides	30xLys + KDEPQRRSARLSAKPAPPKPEPKPKKAPAKK or (CGKRK)n, n=1 or 2	MaSp1 protein of *N. clavipes*	Nanoparticle DNA polyplexes for transfection of MDA-MB-435 and MDA-MB-231 cells (*in vitro* and *in vivo*- murine model)	[202]
Poly-lysine + SV40 nuclear localization sequence + hMSC high affinity binding peptide + Cell-penetrating peptide	LysX15 + PKKKRKV + SGHQLLLNKMPNGGGS + PLSSIFSRIGDP	MaSp1 protein of *N. clavipes*	Nanoparticle DNA polyplexes with 50 % transfection efficiency compared to Lipofectamine 2000	[203]
Her2-binding peptide	MYWGDSHWLQYWYETS	MaSp1 protein of *N. clavipes*	Microsphere targeted delivery of doxorubicin to (HER2)-positive breast cancer cell *In vivo* model: - Unloaded - Loaded	[41] [168167]
Her2-binding peptide	MYWGDSHWLQYWYETS	MaSp1 and MaSp2 protein of *N. clavipes*	Microsphere targeted delivery of doxorubicin to (HER2)-positive breast cancer cell. Blend of both recombinant silks results in superior intermediate properties and decreased non-specific interactions.	[55,166]
Her2-binding peptide + Doxorubicin-binding peptide (DOXMS2)	MYWGDSHWLQYWYETS + MASLWSPWYGGSWTS	MaSp2 protein of *N. clavipes*	Microsphere targeted delivery of doxorubicin to (HER2)-positive breast cancer cell; increased doxorubicin loading and sustained release	[40]
Matrix metalloproteinase-responsive peptide (MMP)	GPQFIGQ	SELPs: 815 K, 815 K-RS1, 815 K-RS2 and 815 K-RS5	Improved release of doxorubicin - with MMP - without MMP	[4269]
Fibronectin cell binding motif	TGRGDSPA	Recombinant silk based on the C-terminal domain of *E. australis*: 4RepCT	Blended with *A. assama* silk fibroin for wound healing in third-degree burn (rat model)	[88]
Fibronectin cell binding motif	TGRGDSPA	Recombinant *A. diadematus* silk fibroin: eADF4(C16), eADF4 (C16)-RGD,eADF4(C32NR4), $eADF3(AQ)_{12}$ and eADF4(Ω16) variant. Recombinant B. mori fibroin	Preparation of hydrogels, flat and patterned films, with antimicrobial properties and improved mammalian cell binding properties	[133]

Fibronectin cell binding motif	TGRGDSPA	Recombinant silk based on the C-terminal domain of *E. australis*: 4RepCT	Nanometer-thick membranes with outstanding elasticity Permeable to small molecules (Rhodamine B, Texas Red-Dextran) and proteins (FITC-BSA) Allows biocomunication between cell layer and drug reservoir	[142]
Non-collagenous proteins from bone known to bind to collagen and to initiate mineralization	Osteo-tag: GLRSKSKKFRRPDIQYPDATDEDITSHM SialoN-tag: DSSEENGNGDSSEEEEEEETS SialoC-tag: EDESDEEEEEEEEE	Recombinant eADF4(C16) from A. diadematus fibroin	Enhanced calcium phosphate deposition	[144]
Hydroxyapatite binding domain (VTK peptide)	VTKHLNQISQSY	15mer derived from the consensus repeat of N. clavipes dragline silk protein	N' and C' terminal fusion of the VTK peptide Enhanced biomineralization *in vitro*	[145]
Silver binding peptides	NPSSLFRYLPSD WSWRSPTPHVVT	6 or 15 repetitions of the consensus repeat of *N. clavipes* dragline silk	Inhibited growth of Gram positive and Gram negative bacteria	[73]
-Human galectin-3 carbohydrate recognition domain (hGal3)	PLIVPYNLPLPGGVVPRMLITILGTVKPNANRIALDFQRGNDVAFHFNPRFNENNRR VIVCNTKLDNNWGREERQSVFPFESGKPFKIQVLVEPDHFKVAVNDAHLLQYNHR VKKLNEISKLGISGDIDLTSASYTMI	Recombinant spider silk 4RepCT from E. australis	Increased muchoadhesive properties	[152]
-Cell-adhesive REDV peptide -Matrix metalloprotease cleavable peptide -VEGF-mimicking peptide (QK)	-REDV -GPQG↓IWGQ -KLTWQELYQLKYKGI	Composite of B. mori fibroin inspired recombinant silk and fibroin extracted from B. mori	Implantable hydrogels for accelerated cell infiltration and Increased blood vessel formation in an *in vivo* rat model	
-Basic fibroblast growth factor (Bfgf)-binding P7 peptide -PreScission Protease recognition site (PP)	PLLQATLGGGS LEVLFQGP	Transgenic B. mori silkworm fibroin fusion protein	Recombinant B. mori silk fibroin with the ability to specifically find bFGF growth factor bFGF fused at the C-terminus of fibroin light chain	[233]
Human epidermal growth factor (hEGF1)	NSDSECPLSHDGYCLHDGVCMYIEALDKYACNCVVGYIGERCQYRDLKWWELR	Transgenic B. mori silkworm fibroin fusion protein	hEGF-functionalized truncated cocoon fibroin heavy chain with increased cell proliferation for wound dressing applications	[234]
Partial collagen sequence Partial fibronectin sequence	[GERGDLGPQGIAGQRGVV(GER)3GAS]8GPPGPCCGGG[TGRGDSPAS]8	Transgenic B. mori fibroin light-chain fusion protein	Films with increased mammalian cell-adhesive properties	[47]
Acidic fibroblast growth factor (FGF1)	MAAGSITTLPALPEDGGSGAFPPGHFKDPKRLYCKNGGFFLRIHPDGRVDGVR EKSDPHIKLQLQAEERGVVSIKGVCANRYLAMKEDGRLLASKCVTDECFFFER LESNNYNTYRSRKYTSWYVALKRTGQYKLGSKTGPGQKAILFLPMSAKS	Co-expression with B. mori sericin	Self-gelling and injectable sericin hydrogels loaded with FGF1 with increased cell proliferation and the absence of immunogenicity Sustained release of FGF1	[235]
Human platelet-derived growth factor (PDGF-BB)	MNRCWALFLSLCCYLRLVSAEGDPIPEELYEMLSDHSIRSFDDLQRLLHGDPG EEDGAELDLNMTRSHSGGELESLARGRRSLGSLTIAEPAMIAECKTRTEVFEIS RRLIDRTNANFLV WPPCVEVQRCSGCCNNRNVQCRPTQVQLRPVQVRKIEIVRK KPIFKKATVTLEDHLACKCETVAAARPVTRSPGGSQEQRAKTPQTRVTIRTVRV RRPPKGKHRKFKHTHDKTALKETLGA	Co-expression with B. mori sericin	Temperature-induce gelation of sericin with a 13.1 % w/w content of PDGF-BB Sustained release of PDFG-BB for over 30 days Increased cell proliferation	[228]
Recombinant human lactoferrin (rhLF)	MKLVFLVLLFLGALGLCLAGRRRRSVQWCAVSQPEATKCFQWQRNMRRVRGPP VSCIKRDSPIQCIQAIAENRADAVTLDGGFIYEAGLAPYKLRPVAAEVYGTER QPRTHYYAVAVVKKGGSFQLNELQGLKSCHTGLRRTAGWNVPIGTLRPFLNWTG PPEPIEAAVARFFSASCVPGADKGQFPNLCRLCAGTGENKCAFSSQEPYFSYSG AFKCLRDGAGDVAFIRESTVFEDLSDEAERDEYELLCPDNTRKPVDKFKDCHL ARVPSHAVVARSVNGKEDAIWNLLRQAQEKFGKDKSPKFQLFGSPSGQKDLLFK DSAIGFSRVPPRIDSGLYLGSGYFTAIQN LRK - GenBank: M93150.1) with 6 × His in the C-terminal	Co-expression with B. mori sericin	Hydrogels for sustained release and gastrointestinal delivery of rhLF in miceProtection against degradation of the rhLF	[207,236]

Recombinant Protein Copolymers

Comonomer	Sequence	Silk Monomer		Refs
N-terminal domain (exon 1) in native resilin (fruit fly Drosophila melanogaster resilin gene CG15920)	(GGRPSDSYGAPGGGN)^{18}	Carboxyl-terminal (CT) domain of major ampullate spidroin 1 (MaSp1) of the spider N. clavipes (NcCT)	Hydrogels with a pH-dependent release of Rhodamine B	[134]
Elastin-like (SELP-415K)	[(GVGVP)4GKGVP (GVGVP)^{11}]	Recombinant B. mori silk	Sustained gastrointestinal delivery of GM-0111 for mucosal inflammatory diseases (murine model)	[71]

Composites

Non-silk Component	Silk Type	Application	Refs
Glycerol or 2-pyrrolidone	Recombinant spider silk protein eADF4(C16) from A. diadematus fibroin	DDS for small bioactive drugs (paracetamol and tetracaine HCl), complex sugars (dextran), or model proteins (lysozyme and BSA)	[141]
Mesoporous silica nanoparticles	Recombinant spider silk protein eADF4(C16) from A. diadematus fibroin	Over 15 days release of antimicrobial peptide	[132]

Chemical Functionalization

Conjugate	Bond Chemistry	Silk Type	Application	Refs
DTNB (5,5′-dithiobis-2-nitrobenzoic acid)	Disulfide bonds	Polyanionic recombinant silk protein from A. diadematus fibroin eADF4(C16),	Redox-responsive Release DDS	[37]
DMAB (para-dimethylaminobenzaldehyde)	Carboxyl groups via and hydrazine linker	the polycationic variant eADF4(κ16) as well as the cysteine-bearing variants of both proteins	pH-responsive carrier DDS	
Different fluorophores and the antibiotic Levofloxacin	Click-chemistry	Recombinant spider silk 4RepCT with nonnatural methionine analogue L-Aha (4RepCT^{3Aha})	Multifunctional derivatization of recombinant silk with click-chemistry pH-labile linker for controlled drug-release	[143]
Thrombin-sensitive peptide (TSP)	GFDPRGFPAGG	MaSp2 protein of N. clavipes	Vancomycin loaded particles Increase in thrombin-like activity during *S. aureus* infections due to its secretion of staphylocoagulase Improved bacterial clearing in a murine model	[182]

Delivery of Drugs / Model Compounds

Drug / Model drug	Silk Type	Application	Refs
Lysozyme, Bovine serum albumin (BSA), Horseradish peroxidase (HRP)	Recombinant spider silk fibroin eADF4(C16) from A. diadematus fibroin	Direct or diffusion mediated loading of hydrogels and particles	[130]
6-mercaptopurine, FITC	recombinant spider silk eADF4(κ16) and eADF4(Ω16) from A. diadematus fibroin	Organic co-solvent incorporation of hydrophobic drugs in hydrogels and 3D printable systems	[80]
Doxorubicin and Rhodamine B	Recombinant eTuSp1 tubuliform spidroin from black widow spider	1,1,1,3,3,3-Hexafluoro-2-propanol + Oil mixture Proteinase-enhanced release behaviors *In vivo* studies with Hela cells	[131]
Mitoxantrone and etoposide	MaSp1 and MaSp2 protein of N. clavipes	MaSp2 spheres more dispersed, smaller, with solid core, higher beta-sheet structure content, Superior loading capacity of the MaSp2 variant MaSp1 were a better choice for doxorubicin DDS	[176]
Mitoxantrone and etoposide	MaSp2 protein of N. clavipes	Negatively-charged variant of MaSp2 Increased loading capacity Burst release of mitoxantrone Sustained release of etoposide	[35]

may be a viable solution to scale-up, however this comes with its own challenges such as low genetic stability and low expression of transgenic products.

Genetic engineering advances are primed to improve therapeutic outcomes of delivered drugs while minimizing off-target effects. The different silk types, each with specific characteristics, in combination with on-demand tunable properties of recombinant silks, offers a wide pool of materials and properties for DDS designs. The inclusion of specific disease-targeted peptides, as well as on demand drug release from the DDS, can help to customize the DDS to increase efficiency. With emerging personalized therapies, recombinant systems are expected to become a key to success. In total, with silk proteins or domains from these proteins as core building blocks, the versatility and impact of genetically modified silks for DDS offers immense potential in the future as additional fundamental and translational studies are pursued. In addition, with growing awareness towards animal welfare, the opportunity to generate recombinant silks towards DDS goals should also increase in importance.

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Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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