HHS Public Access

Author manuscript

Expert Opin Drug Deliv. Author manuscript; available in PMC 2020 July 01.

Published in final edited form as:

Expert Opin Drug Deliv. 2019 July ; 16(7): 741–756. doi:10.1080/17425247.2019.1635116.

Extended release formulations using silk proteins for controlled delivery of therapeutics

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Abstract

Introduction—Silk is a promising biomaterial for controlled delivery of therapeutic molecules. Silk has a unique protein chemistry and structure that can be tuned to form different carrier formats. The protein has been studied for injectable or implantable sustained release depot systems for the targeted or localized delivery of drugs.

Areas covered—An overview of natural silk proteins for controlled delivery of therapeutics is provided, with a focus on the features of silk proteins that allow them to be useful tools for controlled delivery. Recent applications of natural silk proteins as controlled delivery systems are also summarized.

Expert opinion—The versatility of silk proteins makes them desirable biomaterials for a broad range of applications for controlled delivery of both small and large molecules. Further, the degradation profile leading to peptides and amino acids provides compatibility with pH-sensitive therapeutics like complex proteins in formulation and delivery. While silk sericin and spider silks are under study, silk fibroin extracted from silkworms (e.g., *Bombyx mori)* dominates pharmaceutical studies with silk. Silk fibroin can be formed into drug delivery tools for systemic or local injections, topical and transdermal applications, and implantation; depending on the target disease and therapeutic molecule. *In vitro* to *in vivo* correlations and scale-up needs are the next steps towards clinical applications.

Keywords

Silk; controlled release; drug delivery; nanoparticles; hydrogels; implants; microneedles

1. Introduction

The controlled delivery of therapeutics aims to extend the duration between doses and maintain constant therapeutic levels in plasma, tumors or local injection sites. Such systems also offer additional benefits, including reduced side effects, improved patient compliance for frequent or difficult applications and reduced cost of treatment with well designed controlled delivery systems [1]. The biomaterials utilized for controlled delivery need to be

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Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

cost effective, non-toxic and relatively simple to process with mild techniques in order to meet biocompatibility and regulatory demands. Organic solvents should be minimized, and release profiles should be adjustable in order to achieve clinically relevant therapeutic levels of the delivered therapeutics. Various polymers have been investigated for controlled therapeutic delivery; Including synthetic macromolecules like polyesters, polyorthoesters, polyphosphoesters, and polyanhydrides [2]. Available controlled release formulations currently on the market are mostly based on Food and Drug Administration (FDA) approved synthetic polymers such as polylactide-co-glycolide acid (PLGA) and polycaprolactone (PCL), while FDA approved natural polymers like albumin, alginate, gelatin, collagen, and silk fibroin are being investigated as alternatives, in part to avoid undesirable degradation products and formulation challenges associated with polyesters such as activity loss of peptide-protein structures[3–5].

Silk proteins are produced in fiber form by silkworms (e.g., *B. mori,* mulberry silk) or orb-weaving spiders (non-mulberry silk) and have been explored to understand their properties and potential as biomaterials. The first documentation of biomedical applications with silk are from 150 AD, where it was described as a suture material. In the 1500s, there are reports of sterilizing silk sutures in boiling oil, while the first sterile silk suture was officially introduced into clinics in 1869 [6]. The first attempt to reverse engineer silk cocoons and generate reprocessed silk was at the beginning of 20th century, while the first patent for the biomedical use of a regenerated silk appeared in the 1960s [7]. Lyophilized silk powders, silk films and gels have been patented as wound dressings, corneal coatings or blood vessels in the 1990s, while research and commercialization activities have increased in 2000s, especially in the USA [7].

Silk proteins, particularly the fibroin has been employed for drug delivery due to the biocompatible, biodegradable, self-assembling properties, mechanical strength and controllable structure [8, 9]. The source of silk is important due to considerations of supply, purity, physicochemical characteristics and biological responses. Spider silks are more diverse in composition; however they are difficult to obtain in reasonable quantities, therefore silkworm silks have been the focus of most studies[10]. Biosynthetic silk variants and copolymers have also been pursued for drug delivery [11, 12]. Silk proteins have desirable properties for controlled delivery due to chemistry, structure and biological impact. For example, silk fibroin is a high molecular weight amphiphilic protein that self-assembles into mechanically robust structures, can be processed in aqueous or solvent systems, provides a low water content environment, and is resistant to temperature, pH and organic solvents [13]. Further, these silks are considered biocompatible, can be tuned structurally (crystalline content) to control degradation rate based on enzymatic (not hydrolytic) digestion [14], offers stability to small drugs and complex proteins[15–17], and can be sterilized by different modes (e.g., gamma irradiation, ethylene oxide (EtO), hydrogen peroxide, autoclave)[18]. In addition, the silk can be formed into various delivery platforms including but not limited to nanoparticles, microparticles, macroparticles, hydrogels, implantable rods, foams, wafers and reservoirs [19–25]. Silk was first approved by FDA as a biomedical suture [26], has been approved for soft tissue reconstruction (Seri-scaffold −2008, Serica Technologies) and most recently received 510k clearance (Silk Voice - K180631 – 2018, Sofragen) as an injectable filler for vocal fold insufficiency [27].

In this review, we discuss applications of silk proteins for the controlled delivery of therapeutics. The details of the chemistry and structure of silk have been previously reported and will not be recapitulated here [8, 10, 28]. Thus, we will focus on the advantages of the unique features of silk proteins and how silk is being utilized for the controlled delivery of therapeutics.

2. Advantages of silk proteins for controlled delivery

Silk is a useful matrix for controlled delivery as the processing can be tailored for drug loading, release kinetics and stability by changing the process used in the formation and treatment of the material.

Silk consists of a fibroin protein heavy chain (~325 kDa) and light chain (~25 kDa) held together by a disulfide bone and encased in sericin proteins (20 kDa to 310 kDa) during fiber spinning by the silkworm [11]. The sericins have been implicated with inflammatory responses [29], they can be removed by boiling the silk fibers in alkaline solution [30]. In the absence of the sericin, silk fibroin causes minimal inflammatory reactions and essentially no immune response [10, 31]. Interestingly, sericin has also been reported to be minimally inflammatory in the absence of fibroin, suggesting the interaction of sericin with fibroin may related to inflammatory outcomes [32, 33].

The amphiphilic structure of the fibroin heavy chain consists of 12 hydrophobic “crystallizable” and 11 hydrophilic “amorphous” domains. The crystallizable domains provide control over drug release kinetics and the degradation profile of the silk formulations by manipulation of the crystalline content [1]. These same domains are also responsible for the self-assembly of the fibroin that leads to strong physical interactions and robust mechanical structures with the associated slow rate of degradation. The self-assembly of the heavy chain in aqueous solution without chemical additives is a key control point in the formulation of silk proteins [34, 35]. Modulating the degree of crystallinity, such as by water vapor annealing or exposure to methanol can also control the rate of silk degradation. The presence of crystalline domains interspersed with less crystalline domains is also responsible for the high mechanical strength and toughness of silk materials. Furthermore, the GAGAGS amino acid sequence in silkworm silk dominants the primary sequence as a key hydrophobic block, to enhance hydrophobic drug interactions to control loading and release kinetics [36, 37]. Due to the dominant hydrophobic nature of silk as mentioned above, hydrophobic therapeutic compounds usually interact better with silk and thus perform better in terms of sustained release [38]. Although the primary structures of silkworm and spider silk can be different (poly(GA) and poly(A) sequences tend to dominant for the mechanically robust silks as crystalline-forming regions), while providing similar hierarchical structures [39]. Chemical modification strategies of silk have also been reported, exploiting the non-crystalline domains and side chains of amino acids such as tyrosine, glutamic acid and others [10, 40].

Another important feature of silk as a biomaterial for controlled delivery is the versatility of options for sterilization. The most widely used synthetic (PLGA) [41] or natural polymers (collagen) [42] in drug delivery are more limited in terms of options for common sterilization

methods due to their low thermal stability or degradation with gamma irradiation. Silk can be sterilized by autoclaving, gamma radiation and ethylene oxide [43]. In most cases it is also possible to filter sterilize silk solutions during preparation under aseptic conditions [19].

In Table 1, we compare silk proteins with commonly used synthetic and natural polymers in terms of critical features for drug delivery.

3. Applications of natural silk proteins for controlled delivery of therapeutics

Silk has been used as a biomaterial for a variety of applications for drug delivery via multiple formats[55]. Silk fibroin from silkworm cocoons (*B. mori*) is the most commonly used silk for controlled drug/protein delivery, while sericin and spider silk proteins have also been investigated. Injectable formats including nanoparticles, microparticles and hydrogels; implantable forms like films, wafers, foams, tubes, rods, reservoirs and transdermal systems like microneedles are among the silk-based delivery systems reported (Figure 1). Silk-based O/W/O micro-emulsions were used to encapsulate and control the delivery of oils and volatile compounds such as fragrances, also applicable to deliver hydrophobic therapeutic molecules [56]. Silk fibroin has also been used as a coating material to increase residence time and cell recognition [57]. Here we focus on recent developments in silk-based delivery systems and discuss the benefits for controlled delivery of therapeutics. This article does not focus on the material preparation techniques with silk, since we have previously published a protocol paper to summarize the fabrication methods for major silk carrier systems [55]. As most of the studies are focused on silk from *B. mori* cocoons, the silk term used will refer to mulberry silk unless otherwise is stated.

3.1 Silk-based particle systems

Microparticle and nanoparticle systems have been studied for controlled drug delivery due to their large surface area, enhanced permeability and targeting ability due to size and surface charges. Nanoparticles can penetrate through the physiological barriers and become incorporated into cells due to size, thus are important delivery systems for cancer treatment as they can passively target tumor sites due to enhanced permeability and retention (EPR)[58]. Microparticles are generally used as a subcutaneous, intramuscular or muchoadhesive drug depots, as well as passively targeted lung delivery systems due to size[59, 60].

The common methods to prepare micro/nanoparticles usually require toxic organic solvents and some techniques like spray drying can result in the degradation or denaturation of the drug [64]. In contrast, silk proteins can be formed into micro- or nano-particles without organic solvents, in part due to the self-assembly features of the protein, using physical methods like solution-enhanced dispersion [64], desolvation [24], self-aggregation [65] or micro injection pumps [66].

Several studies have reported encapsulated chemotherapeutics in silk nanoparticles to increase plasma retention time, increase cellular uptake, targeting to tumors via EPR, and to reduce application frequency and systemic toxicity [24, 66–71]. Curcumin-loaded silk fibroin nanoparticles were prepared to treat tumors with local sustained delivery; the nanoparticles

were cytotoxic to carcinogenic cells while not killing healthy cells, however *in vitro* release from was limited to 24 hours following burst release in the first 5 hours [68]. Silk nano- and micro-particles were used for oral delivery of curcumin and larger silk particles were successful in increasing plasma circulation up to 24 hours. As a result of avoiding first-pass metabolism due to the small size of the particles, curcumin $AUC_{0-\infty}$ was approximately 17 times that of the curcumin alone [69]. Cisplatin has also been entrapped in silk fibroin nanoparticles to achieve controlled release and enhanced cellular uptake, where the drug was released for 15 days and internalized by A549 lung cancer cells [66]. To increase lysosomal accumulation, pH dependent release of doxorubicin was investigated using silk fibroin nanoparticles, and release rates were pH 4.5 > > 6.0 > 7.4 which correlated with high lysosomal uptake and potentially low plasma concentrations [24]. Doxorubicin was also encapsulated in PEGylated (polyethylene glycol = PEG) silk nanoparticles to achieve pH-controlled release with a stealth design that allowed the particles to avoid the reticuloendothelial system and remain in circulation for a longer period of time [67]. Folate receptor targeted release of doxorubicin was investigated via folic acid conjugated silk fibroin nanoparticles [71]. Internalization of doxorubicin particles was observed in cervical cancer (HELA) cells and pH dependent drug release lasted for over 30 hours[71]. Doxorubicin was also formulated with spider silk from *N. clavipes* and pH-dependent extended release up to 15 days was achieved [72]. In addition to the release profile, these nanoparticles were functionalized to bind to Her2 positive cells and the particles showed no toxicity unless they were loaded with doxorubicin [72].

Silk microparticles have been used to increase residence time of drugs in a specific application area. The efficiency of silk fibroin microparticles for sustained release of small molecules into the articular cavity evaluated for fluorophore (Cy7) conjugated microparticles [73],. *In vitro* release of Cy7 lasted for over 7 days following intra-articular injection in rats; the fluorescence decay half-life increased significantly with microparticles compared to injection of silk-Cy7 solution (Figure 2A).

Aside from the examples above, silk has also been used in combination with different polymers, silk sericin protein from *Antheraea mylitta* cocoons was blended with pluronic F-127 and F-87 [74] and silk fibroin from *Bombyx mori* was blended with chitosan [75] for enhanced uptake of cancer therapeutics. Both studies were able to achieve sustained release, and cellular uptake of curcumin was increased with silk coating compared to uncoated curcumin and silk-chitosan coated curcumin. However, the production of these systems required addition of toxic organic solvents such as methanol, dioxane, dimethyl sulfoxide (DMSO) and N, N-dimethylformamide (DMF). A solution-enhanced dispersion method for nanoparticle preparation using supercritical CO_2 has been developed and did not require solvent additions [64].

Recent advancements in silk-based particle systems for controlled delivery of therapeutics, classified based on the silk source, formulation type and the application of the systems are summarized in Table 2.

3.2 Silk-based gel systems

Silk fibroin-based hydrogels have been developed for delivery systems due to their versatility, tunable properties for injection and smart gel designs for transformation by environmental stimuli [94, 95]. Vortexing, ultra-sonication, pH change, enzymes or organic solvents can be used to induce gelation of silk fibroin solution [96, 97]. The *in situ* formation of hydrogels is especially attractive as the pre-gel solution can be mixed with therapeutics and injected prior to enzyme or temperature induced gelation in the body, preserving the bioactivity of the entrapped therapeutic [98].

Injectable silk gels are useful for localized delivery of chemotherapy drugs due to their ability to maintain high concentrations of drugs at the tumor site without the need for surgical implantation. As an example, sonication-induced silk gels were investigated for intratumoral delivery of chemotherapeutic drugs [19, 21]. Vincristine loaded gels sustained drug release up to 80 days and tumor growth was suppressed following intratumoral injection in a neuroblastoma-induced mouse model [19, 21]. Similarly, injectable silk nanofiber hydrogels were studied for intratumoral doxorubicin delivery [99], and doxorubicin release lasted over 8 weeks and the release kinetics were pH- and concentration dependent. Furthermore, the thixotropic structure of the gels allowed injectable formulations and significant antitumor response [99]. Injectable silk hydrogels were also useful for ocular drug delivery [100]. In an effort to reduce the injection frequency, bevacizumab-loaded silk fibroin hydrogels were compared to standard single injections of bevacizumab solution. Following intravitreal injection in rabbits, released bevacizumab concentrations from the hydrogels at day 90 were equivalent or greater than the released drug from the standard solution injection at day 30. Three months after the injection, hydrogel biodegradation was observed [100].

Subcutaneous injection of growth factor-loaded hyaluronic acid (HA)-based gels resulted in a localized angiogenic response [101]. These types of gels were ideal for injectable formulations, however they were not mechanically strong enough for orthopedic applications. One approach to overcome these limitations was to reinforce HA-silk hydrogels with electrospun silk mats [102]. In another study, acidic fibroin hydrogels (pH 3.8) for bone morphogenetic protein 2 (BMP-2) delivery was studied in rabbits for 12 weeks [92]. Here, polycaprolactone nanofiber tubes filled with sonication induced silk fibroin hydrogels (Figure 2B) were placed into the defect area of rat femoral segments and delivered BMP-2 with no inflammatory reactions. The hydrogel systems promoted bone remodeling and were completely degraded by the end of the study, 12 weeks after surgery [92].

Dual delivery systems combining hydrogels with nanoparticles, fiber mats or solid polymeric support systems were explored to enhance the mechanical properties of hydrogels, to design implantable systems or to use the hydrogels as a carrier platform [103–105]. Silk hydrogels loaded with silk nanoparticles were prepared to achieve dual drug delivery using fluorescein isothiocyanate (FITC) and rhodamine B [103]. The system showed no significant cytotoxicity against human mesenchymal stem cells and achieved rapid rhodamine B release from hydrogels and slow FITC release (over 55 hours) from the entrapped nanoparticles [103].

As an alternative approach, silk fibroin lyogels were prepared by lyophilization of hydrogels to increase release time and stability of an antibody, IgG [104]. IgG released for 38 days upon lyophilization of the hydrogels, while hydrogels without lyophilization released 10 days. Stability of the released antibody was also investigated and no significant physical or biological losses were observed [105].

Details for recent applications of silk fibroin gel systems are summarized in Table 3.

3.3 Solid silk formats

Silk fibroin based solid carrier systems have been used for local or transdermal delivery of both small molecules and proteins. A variety of carrier systems including silk films, wafers, reservoirs, discs foams and microneedles have been developed, allowing adjustments in release kinetics, mechanical strength and size of the delivery system (Table 4).

One of the earlier applications of silk fibroin was as a coating to extend the release of pharmaceuticals. Aqueous silk fibroin solution was used to coat theophylline tablets to achieve zero order release [113]. PEG/silk combinations (17/83 w/w) and 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC) cross-linked silk coatings formed film coatings and followed zero order release kinetics [113].

Silk fibroin films are commonly used as delivery systems due to their easy preparation and applications with tunable mechanical properties. In earlier studies, silk fibroin was blended with different polymers such as polyurethane, chitosan or alginate to prepare controlled release composite films. Chitosan/silk fibroin blend films were prepared by crosslinking with glutaraldehyde and several drugs (Table 4) were tested in terms of *in vitro* release kinetics under different pH conditions [114]. The highest drug release was observed at pH 2 due to swelling of the polymers. Different ratios of silk fibroin and polyurethane were tested to optimize film thickness, drug loading and release; heparin release correlated with film thickness and silk: polyurethane ratio [115]. Tetracycline hydrochloride was studied with silk/alginate-blended films to evaluate drug release and film transparency. Increased silk fibroin/alginate ratios resulted in decreased drug release and the system was suggested as a good controlled release platform for water-soluble drugs [116]. The mechanism of controlled release from silk films was addressed using fluorescein-iso-thio-cyanate (FITC)-labeled dextrans[117], as a function of silk molecular weight and film methanol treatment. Diffusion coefficients were smaller for higher molecular weight dextran and methanol-treated films. To understand the effects of silk processing, different silk degumming times (10 to 90 minutes) has been investigated [118]. Degumming was found to be a useful control point for silk molecular weight, viscosity and degradation. These earlier studies helped with understanding how to control the interactions between silk and target molecules in order to optimize formulations.

Silk films have been studied for focal delivery of antibiotics using microspheres, hydrogels, microsphere-hydrogel dual systems and silk coatings. Bacterial inhibition by f penicillin and ampicillin loaded films were evaluated; methanol treatment of the films did not degrade antibiotic activity and approximately half of the drug load was delivered within the first 24 hours of exposure [109]. Silk fibroin films have been used to entrap interferon gamma (IFN-γ) or interleukin-4 (IL-4) for macrophage polarization [119]. The crystalline (β-sheet) content of

the films was utilized to optimize solubility of the films and to adjust release rates. Insoluble films with high β-sheet content did not release the entrapped molecules, however they still polarized macrophages that adhered to the film surface. Soluble films with low crystalline structure released the contents in 24 hours, however the duration of release was extended up to 10 days by conjugating IFN- γ to the silk films [119].

Focal tumor therapy is also a major application for implantable silk-based delivery systems, including films, wafers and reservoirs [19, 20, 120–123]. Binding and release of the chemotherapeutic drugs vincristine and doxorubicin has been modulated in silk fibroin films [120]. In terms of drug binding no difference was found between low and high β-sheet (crystalline) content films. In contrast, binding was pH-dependent and optimum drug binding was observed at pH 6 [120]. Both drugs bound at higher loadings to carboxylated and sulfonated silk films than to unmodified silk films, however *in vitro* release from all films were similar and lasted about 28 days. Doxorubicin-loaded films were implanted in an orthotopic neuroblastoma mouse model and decreased tumor growth was superior to the control intravenous administration of the drug [121, 122]. To increase drug loading and optimize *in vitro* drug release, silk hydrogels, foams and wafers were also evaluated for intratumoral delivery of doxorubicin and vincristine [19, 123]. The duration of *in vitro* release from the silk wafers lasted longer than from the silk foams or hydrogels. Post-operative survival rates were less than 20 days following intravenous injections of vincristine or doxorubicin, while the animals treated with vincristine wafers and vincristine/doxorubicin combination foams survived for 2 and 6 months, respectively [19, 123]. Silk wafers were also utilized for etoposide delivery and achieved extended release up to 45 days and decreased tumor growth *in vivo* [20]. A reservoir system, silk rods, was designed to achieve therapeutic doses of drugs by entrapping high contents of powdered drug into the center hollow part of silk tubes, followed by sealing, the ends of the tubes via dip coating. The chemotherapeutic drug anastrozole was released from the system for 91 days with zero-order kinetics. The rods were implanted in rats for 6 months and an *in vitro – in vivo* pharmacokinetic correlation was found [22].

Larger foam systems, discs, were designed for vaginal or rectal delivery of HIV (human immunodeficiency virus) inhibitors 5P12-RANTES and griffithsin [124]. These proteins remained functional in the silk discs over 14 months even when stored at 50°C. Sustained release of griffithsin lasted for 4 weeks and the released protein was sufficient to inhibit HIV transmission based on their activity against CAP210 and PVO4 infection of TZM-bl cells. Ex-vivo studies showed that released 5P12-RANTES levels were sufficient for HIV inhibition in both blood and human colorectal tissue [125].

Electrospun silk fibers are usually designed for topical applications, including drug delivery. Silk/gelatin blend fibers were optimized using methylene blue. Bead formation on the fibers was induced to provide a depot and reduce burst release. A silk fibroin/gelatin ratio of 70/30 (w/w) resulted in homogeneous bead formation on the fibers and methylene blue release lasted for 36 hours from the fiber system [126]. Electrospun silk fibroin patches were also used to simplify storage and application of human platelet lysate for wound healing [127]. Release studies were evaluated by quantifying FITC-albumin release from fibers in the presence of protease XIV, and silk crystalline content was manipulated to control release kinetics. Silk

fibroin/FITC-albumin/ human platelet lysate fibers with >40% crystallinity released the dye for over 140 days [127]. Silk fibroin electrospun nanofibers were combined with silk nanoparticles for dual delivery of doxorubicin hydrochloride (in the fibers) and curcumin (in the nanoparticles) and the system was able to release the drugs for 40 hours [128]. An anal fistula plug for Crohn's disease treatment was studied with curcumin and 5-aminosalicylic-acid loaded into silk electrospun fibers on the surface of a silk plug [62]. The system showed no cytotoxicity with fibroblasts and both drugs released for about 10 days with a higher burst release for 5-aminosalicylic-acid than the curcumin [62].

As minimally invasive transdermal delivery systems, microneedles have been explored and silk showed significant success as a microneedle material with relatively simple fabrication methods like 3D printing or mold casting. Silk microneedles were prepared by casting silk solutions in polydimethylsiloxane (PDMS) molds [93]. Tetracycline and horseradish peroxidase (HRP) were loaded in the casting process as a small and large molecule. An *in vitro* gelatin hydrogel skin model was used to study the release kinetics and 48 hours of release was achieved and the released molecules had preserved bioactivity. The mechanical functions were also tested with mice to confirm skin penetration of the microneedles (Figure 2C) [93]. Swellable microneedles were also designed using 2-ethoxyethanol modified silk fibroin to enhance transdermal drug release [129]. These microneedles transformed into semi-solid hydrogels upon application to the skin. Transdermal delivery of FITC-dextran showed that higher swelling ratios correlated with higher transdermal release kinetics due to the larger pore sizes [129]. Silk microneedles have also been a focus for transdermal vaccine delivery. Vaccine coated silk microneedles were tested against influenza, *C. difficile* and *Shigella* on mice [130]. Microneedles were applied on mouse skin for 24 hours for initial dosing and a booster dose followed 2 weeks later, and successful vaccination was achieved against all three antigens [130]. Another approach was to design silk/poly(acrylic acid) (PAA) microneedles, where the PAA base rapidly dissolved following a brief application to deliver the initial vaccine dose, then methanol treated silk tips serve as vaccine depots in the skin for 2 weeks [63]. The immune response to the microneedles was significantly higher than when a single intradermal injection of the vaccine was used. The pharmaceutical industry has started investing in these types of silk-based microneedle systems; Vaxess, Inc., developed a silk microneedle platform called MIMIX™ for vaccine delivery that has successfully completed Phase II clinical studies [131].

4. Conclusion

Silk proteins are useful biomaterials for drug delivery as they are easily accessible, available in large quantities via the textile industry, relatively inexpensive as a biomaterial, biologically inert yet degradable via proteases, mechanically robust and versatile in fabrication. Control over concentration, molecular weight and crystallinity of the silk protein allows tunable mechanical properties and release kinetics with the delivery systems. Moreover simple fabrication methods under mild conditions (e.g., water, room temperature) provide further versatility related to retention of bioactive features of the therapeutics being delivered in the silk devices. Despite these favorable features of silk-based materials, there remain many challenges to address such as batch-to-batch variability, scale-up and achieving therapeutic dosing levels.

In this review, we discussed the use of silk proteins for the controlled delivery of therapeutic molecules with a focus on the advantages and versatility of silk-based delivery systems. In the upcoming Expert Opinion section, we offer an outlook on the potential challenges these systems might encounter and discuss the issues that require focus in terms of research in order to achieve clinical success with the silk-based delivery systems.

5. Expert opinion

Controlled delivery is important for the treatment of chronic diseases, to reduce bolus or burst toxicity, reduce dosing frequency and to minimize undesirable side effects, while providing therapeutic levels of the therapeutic in the target area. The requirements of the system depend on the physicochemical properties of the drug, duration of the treatment needed and the target area in the body. As a result, controlled delivery systems need to be optimized on a case by case basis. Silk is a protein biomaterial that can be tuned to form various carrier platforms depending on the needs of the drug and application route, while also allowing controlled release of the therapeutic and degradation rate of the delivery system. In the past two decades there has been increased research on the fundamentals of the relationship between silk structure and function. Various fabrication methods have been developed to meet different pharmaceutical needs and in the process, fine-tuning strategies or processes have been explored to achieve the desired release kinetics and mechanical properties.

Stability and bioactivity of the entrapped molecules were also investigated to ensure the released molecules retained therapeutic efficacy. Numerous studies showed that silk carriers have a stabilizing effect on both small molecules and proteins, allowing them to preserve bioactivity and structural integrity even at more extreme conditions like higher temperatures and humidity.

Although the silk structure is established, the interactions between silk and each therapeutic molecule should be considered individually in terms of binding, loading and release kinetics. Physicochemical properties like molecular weight and hydrophobicity play an important role in the release mechanisms. Furthermore, solubility of the therapeutic molecule has a significant influence on the process, where in general low water solubility reduces drug loading and high water solubility results in burst release. Achieving therapeutic levels of drug loading is a major challenge in the formulation of hydrophobic drugs, especially for systems that require dissolving the molecule in the silk matrix. Organic solvent incorporation and modification of silk proteins are among the approaches to improve drug loading. Another challenge in silk formulation is batch-to-batch differences in silk properties due to differences in silk source or slight changes in degumming or other processing procedures. Genetically engineered silk proteins eliminate these inconsistencies, in addition to the functional benefits that they possess by designing into the primary sequence. However, limitations of scale, costs, and regulatory issues remain as challenges for such designer proteins.

Silk is known to be biodegradable due to protease enzymes, but the biodegradation of silk in the body depends on many variables such as the degree of crystallinity, formulation type and

the application site in the body. As part of the drug development process, in vivo studies are essential to evaluate the application of a specific silk delivery system and the pharmacokinetics of the therapeutic molecule. Considering enzymatic degradation, blood flow or pH conditions of the application site, establishing *in vitro-in vivo* correlations is critical. *In vivo* release kinetics, pharmacokinetic profiles, efficacy of the systems as well as toxicity should be evaluated in order to support the clinical relevance of the systems. As the formulations get well-defined, the focus of the research shifts to animal studies and most of the formulation ideas highlighted in this review have already been supported with *in vivo* studies. Silk can induce a mild inflammatory response *in vivo* over 1 to 3 weeks, which is beneficial to increase the degradation of delivery systems with longer clearance time. Based on reports of successful *in vivo* studies regarding silk-based delivery systems, an increase in clinical studies is expected. One of the first successful silk delivery platforms, MIMIX™ microneedles, has completed Phase II clinical studies successfully as a transdermal vaccine delivery system.

In the coming years silk-based delivery systems are anticipated in clinical use with the increased industrial interest and investments. This is especially the case for implant systems such as reservoirs, films and wafers due to their promise for focal treatment of tumors, as well as microneedle systems for controlled transdermal delivery of therapeutics.

Acknowledgments

Funding

This work was supported by grants from NIH (R01NS094218, U01EB014976).

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

LIST OF ABBREVIATIONS

BMP-2	Bone Morphogenetic Protein 2
BSA	Bovine Serum Albumin
Cy-7	Cyanine 7
DMF	N, N-dimethylformamide
DMSO	Dimethyl Sulfoxide
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EPR	Enhanced Permeability and Retention
EtO	Ethylene Oxide
FDA	Food and Drug Administration

FITC	Fluorescein Isothiocyanate
HA	Hyaluronic acid
HER-2	Human Epidermal Growth Factor Receptor 2
HIV	Human Immunodeficiency Virus
HPMA	[N-(2- hydroxypropyl)methacrylamide]
HRP	Horseradish Peroxidase
IFN-γ	Interferon Gamma
IGF-I	Insulin-like Growth Factor
IgG-1	Immunoglobin G-1
IL-4	Interleukin 4
NGF	Nerve Growth Factor
PAA	Poly(acrylic acid)
PCL	Polycaprolactone
PDMS	Polydimethylsiloxane
PEG	Polyethylene Glycol
PEI	Polyethylenimine
PLGA	Poly(lactic-co-glycolic acid)
PVA	Polyvinyl Alcohol
Rh-BSA	Rhodamine-labeled Bovine Serum Albumin
SEM	Scanning Electron Microscope
VEGF	Vascular Endothelial Growth Factor

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Article highlights

- Silk is a suitable candidate for controlled delivery of therapeutics due to controllable degradation and release kinetics, biocompatibility, all aqueous processing to maintain bioactive features of the therapeutics, and compatibility with sterilization.
- Silk has the ability to form various delivery systems, which can be applied via injections, implantation or transdermal routes.
- There is a focus on silk nanoparticles for the delivery of chemotherapeutics in order to reduce application frequency and systemic toxicity by increasing release duration, plasma circulation time and accumulation in the target area.
- Silk hydrogels are being used as injectable sustained release depots as well as implant systems in combination with a solid support material.
- Implantable solid silk platforms such as films, wafers, foams and reservoirs have been studied for focal delivery of chemotherapeutic molecules.
- Silk microneedles are promising transdermal delivery systems with easy fabrication techniques, controllable release, mechanical strength and successful skin penetration.

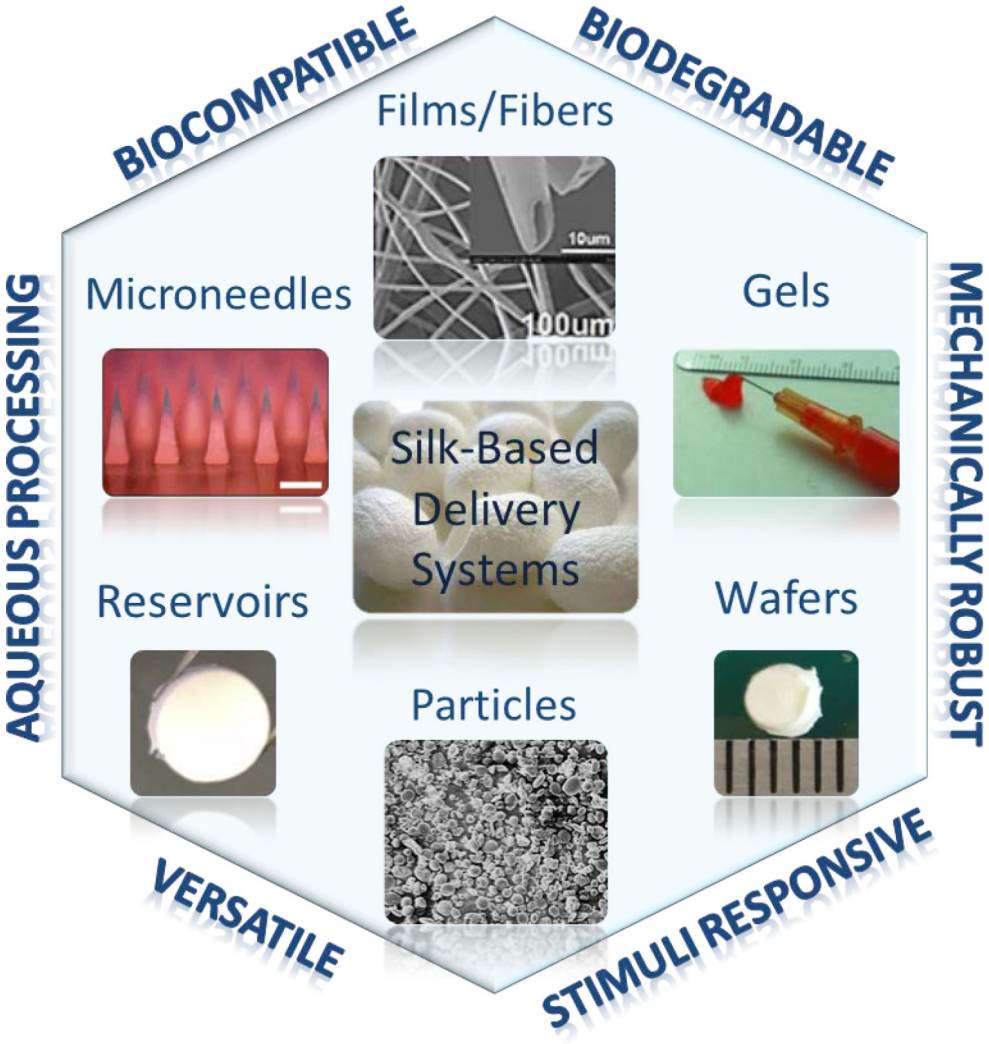

Figure 1.
Silk-based systems used for controlled delivery of therapeutics. Images were reproduced with permission from the cited articles for gels [61], fibers [62] and microneedles [63].

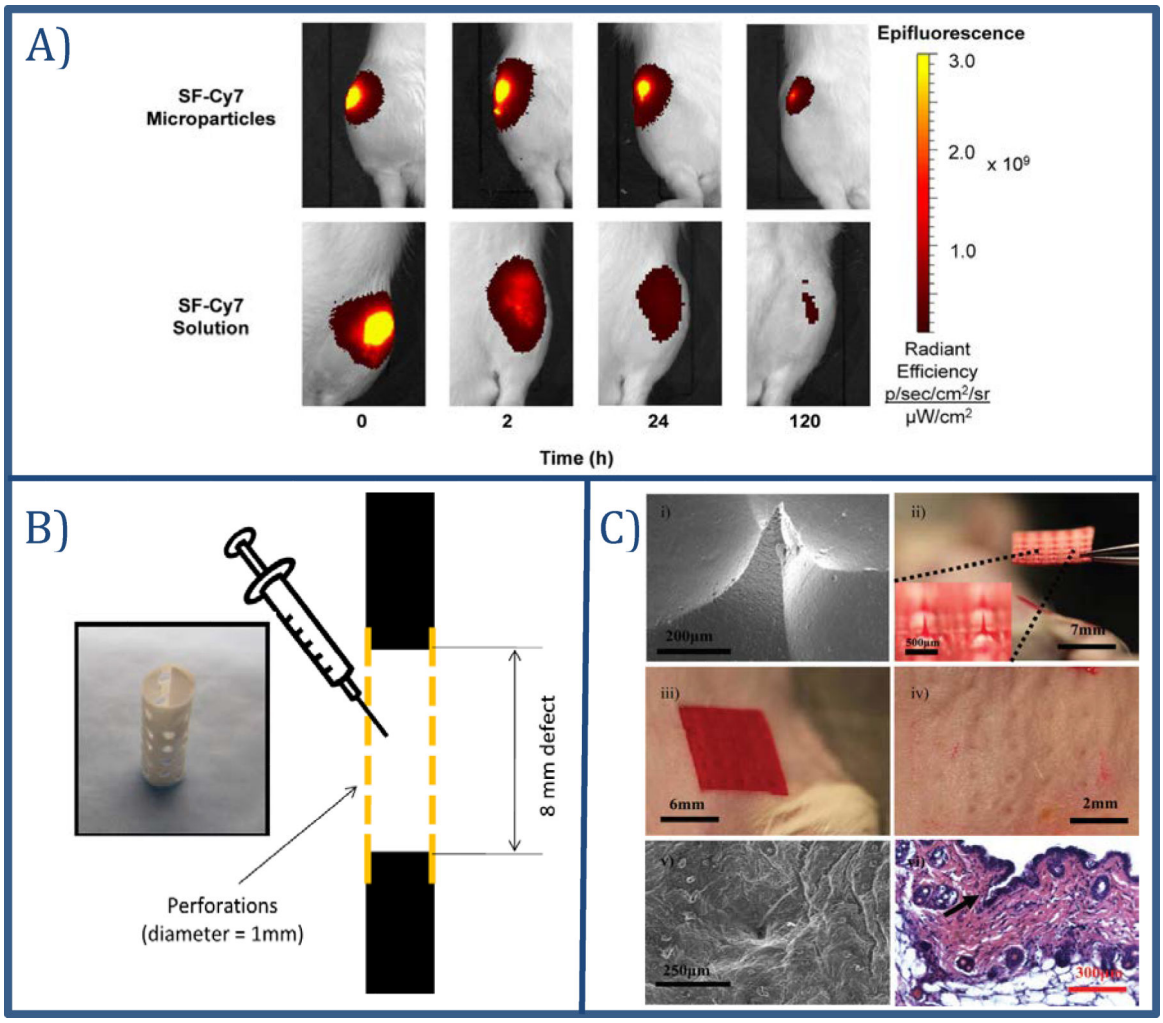

Figure 2.

Different applications of silk fibroin for controlled delivery of therapeutics.

A) *In vivo* image of rat knees following intra-articular injections of silk fibroin-Cy7 (SF-Cy7) particles or SF-Cy7 solution. Images of microparticle injected knees display stronger and more persistent fluorescence intensity 5 days after injections. Images reproduced with permission from [73]. B) Perforated electrospun PCL nanofiber mesh tube placed around the defected bone and pre-gelled silk hydrogel with or without BMP-2 injected into the defect to promote bone modeling. Image reproduced with permission from [92]. C) Silk microneedles for controlled drug delivery. i) SEM image of a silk microneedles, ii) picture of silk microneedle patch, iii) silk microneedles applied to mouse skin, iv) mouse skin after removal of the patch, v) SEM image of penetrated skin after removal of the patch, vi) histology showing breach of epidermis around the indentation site. Image reproduced with permission from [93].

Table 1.

Natural and Synthetic Polymers for Controlled Delivery of Therapeutics

Polymer	Synthetic/Natural	FDA approved	Biodegradability Biocompatible	Stimuli responsive	Sterilization	Challenges
Albumin[44]	Natural	Yes	Yes/Yes	Thermoresponsive	-Sterile filtration	Various side effects (allergic reactions, immunological response)
Alginate [45]	Natural	Yes	Yes/Yes	Thermoresponsive pH responsive	-Sterile filtration -Heat treatment	Limited long-term stability in physiological conditions
Chitosan [46]	Natural	No	Yes/Yes	Thermoresponsive pH responsive	-Ozonation -Ultraviolet	Poor solubility at physiological pH Batch to batch variation
Collagen [42]	Natural	Yes	Yes/Yes	No	- Chloroform - Ethylene oxide - Gamma rad.	Poor mechanical strength
Dextrins [47]	Natural	Yes	Yes/Yes	No	- Ethylene oxide - Gamma rad.	Microbial contamination, Uncontrolled rates of hydration Decrease in viscosity during storage
Gelatin [48, 49]	Natural	Yes	Yes/Yes	Thermoresponsive pH responsive	-Steam heat -Sterile filtration - Gamma rad.	Poor mechanical properties
HA [50]	Natural	Yes	Yes/Yes	No	-Heat treatment -Sterile filtration	Accumulation in liver Low accumulation in tumor sites
HPMA [51]	Synthetic	Yes	No/Yes	No	-Heat treatment	Non biodegradable Elimination via kidney
PCL [52]	Synthetic	Yes	Yes/Yes	No	-Ultraviolet	Low melting point
PEG [51, 53]	Synthetic	Yes	No/Yes	Thermoresponsive	-Sterile filtration	Accumulation in the body Low peptide/protein conjugation
PEI [54]	Synthetic	No	No/No	No	-Ethylene oxide -Gamma rad. -Steam heat	Cytotoxicity
PLGA [4, 41]	Synthetic	Yes	Yes/Yes	No	- Plasma - Ethylene oxide - Gamma rad.	Low solubility High rate of degradation Acidic degradation products Challenge of biosynthesis Protein aggregation and instability Burst release of large molecules
Silk Fibroin **Silk Sericin** [19, 43]	Natural	Yes	Yes/Yes	pH responsive Thermoresponsive Enzyme activity Physical stimuli	-Steam heat -Sterile filtration -Gamma rad.	Batch to batch variation

*HA: Hyaluronic acid, HPMA: [N-(2- hydroxypropyl)methacrylamide], PEG: polyethylene glycol, PEI: Polyethylenimine, PLGA: poly lactic-co-glycolic acid, PCL: Polycaprolactone

Table 2.

Silk-based nanoparticle and microparticle systems for controlled delivery of therapeutics

Silk source	Formulation	Outcome/ Benefits	Released Agent	Ref.
B. mori, Fibroin	Microparticles	Sustained release over several weeks Increase in *in vivo* joint residence time	Cy-7	73
		Extended release (up to 25 days) No burst release with methanol treatment Longer release with methanol treatment than NaCl treatment Enzyme activity retained during preparation	HRP	76
	Microparticle coating	Delayed degradation of PLGA microparticles (up to 30 days) Sustained protein release as diffusion barrier	HRP, Rh-BSA	77
	Nanoparticles	Preparation by super critical CO_2 – no organic solvent Sustained release (2 days)	Indomethacin	64
		Stimulus responsive pH-dependent release, pH 4.5>>pH 6.0>pH 7.4 (up to 6 days) Enhanced endocytic uptake and lysosomal accumulation	Doxorubicin	24
		Silk- albumin conjugates Sustained release: 85% release over 12 days High drug encapsulation and loading efficiency Cytocompatible	Methotrexate	65
		Sustained release (up to 3 days) Increased curcumin bioavailability Cytotoxic to carcinogenic cells Not toxic to healthy cells	Curcumin	68
		Dual drug loading Drug release for 7 days Suppression of cancer cell growth	Paclitaxel, Doxorubicin	70
		Simple nanoparticle manufacturing Sustained release	Doxorubicin	78
		Silk fibroin (shell) and polyvinyl alcohol (PVA, core) Controlled release for 72h Control of drug release achieved by alternating PVA/silk ratios and by applying ultrasound	Doxorubicin	79
		Drug release over 15 days Internalization in cancer cells Low toxicity in mouse fibroblasts	Cisplatin	66
		Carrier-in-carrier delivery system Silk/curcumin nanoparticles loaded in extracellular vesicles Release of drug for up to 100h Improved curcumin bioavailability	Curcumin	80
		Improved cyto- and hemo compatibility of the drugs High encapsulation efficiency Sustained release up to 56 hours	Celecoxib, Curcumin	81

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Silk source	Formulation	Outcome/ Benefits	Released Agent	Ref.
		Enhanced cellular uptake and prolonged retention time *In vitro* drug release up to 200 hours *In vivo* biocompatibility Accumulation and extended retention time in retina following intravitreal injection	FITC-BSA	82
		In vivo transdermal delivery of fluorescent dyes	Rhodamine B (fluorescent dye)	83
	Particles	Release based on charge interactions Prolonged release with positively charged molecules (up to 14 days)	Alcian blue, rhodamine B, crystal violet	84
		High encapsulation efficiency Release up to 7 weeks Bioactivity preserved during \ release	Salicylic acid, propranolol hydrochloride, IGF-I	85
		Targeted and pH-responsive, folic acid-modified particles Controlled release over 32 days Higher drug release at low pH with enhanced cell internalization	Doxorubicin	71
B. mori and *A. mylitta* Fibroin	Nanoparticles	Accumulation of nanoparticles in cytoplasm of carcinoma cells Sustained release up to 3 weeks	VEGF	86
B .mori PEGylated silk	Nanoparticles	Stealth design for increased clearance time High encapsulation efficiency (>93%) pH-dependent release over 14 days Cytotoxic to breast cancer cells	Doxorubicin	67
	Nanoparticles Microparticles	2 days release, longer plasma exposure Improved curcumin bioavailability Reduced burst release	Curcumin	69
B .mori Sericin	Nanoparticles	2 days release No toxicity from nanoparticle carrier Lower doxorubicin systemic toxicity Stable nanoparticles	Doxorubicin	87
A. mylitta Fibroin	Nanoparticles	Folate-conjugated nanoparticles allow targeted delivery Sustained release up to 21 days with enhanced release at acidic pH	Doxorubicin	88
A. pernyi Fibroin	Nanoparticles	Sustained release up to 23 days pH-dependent release	Doxorubicin	89
	Nanoparticles	Self-assembled nanoparticles using cations (Na^{+}, Ca^{2+}, Ce^{3+}) Sustained/pH-dependent release up to 11 days	Doxorubicin	90
A. pernyi Sericin	Microspheres	Microspheres are made of silk sericin and hydroxyapatite Sustained/pH dependent release of the drug up to 120 hours	Doxorubicin	91
Spider silk *N. clavipes*	Nanoparticles	Her2 targeted Enhanced targeted binding pH dependent drug release (up to 15 days) Non toxic nanoparticle carriers Higher cytotoxicity for doxorubicin-loaded nanoparticles	Doxorubicin	72

Table 3.

Silk-based gel systems for controlled delivery of therapeutics

Silk source	Formulation	Outcome/ Benefits	Released Agents	Ref.
B. mori, Fibroin	Lyogels & Hydrogels	Controlled release of IgG1 Longer release with lyogels compared to hydrogels (38 days) Antibody maintained activity	IgG1	104
		Controlled release of IgG1 (26 days) Higher release with hydrated gel (lowest density of silk) Antibody oxidation levels decreased with addition of methionine	IgG1	105
	Gels	Sustained release (25 days) Dual combination of vincristine and doxorubicin Intratumoral injections→ significant decrease in tumor growth Higher intratumoral drug concentration than IV administration Reduced systemic exposure	Vincristine Doxorubicin	19
		Sustained intratumoral delivery via multiple injections (80 days) Diffusion distance halved, growth inflexion time doubled	Vincristine	21
		Intratumoral injections→ significant decrease in tumor growth Slower tumor growth compared to foam format	Vincristine Doxorubicin	106
	Hydrogels	Controlled release (30 days) Mixed with polyacrylamide to yield semi-interpenetrating gels Hydrogel with better mechanical properties Maximum release achieved with 70:30 of silk/polyacrylamide ratio	Trypan blue FITC-inulin	107
		Controlled release and degradation (up to 42 days) *In vivo* degradation Improved mechanical properties by embedding silk fiber mat in hyaluronic acid network Sufficient mechanical strength for fastening to tissues *in vivo*	Dexamethasone Hydrocortisone 6a-methylprednisolone Cortisone, Prednisolone Prednisone VEGF, FITC-dextran	108
		Stabilization of antibiotics Focal delivery of antibiotics Sustained release (up to 4 days from the hydrogels) *In vivo* ampicillin efficacy in a murine infected-wound model	Penicillin Ampicillin Gentamicin Cafazolin Rifampicin Eryhtromycin Tetracycline	109
		Drug release over 8 weeks pH- and concentration-dependent release kinetics Thixotropic hydrogel structure → injectable Significant antitumor response *in vivo*	Doxorubicin	99
		Doxorubicin release over 4 weeks Intratumoral injection – Reduced tumor growth Minimized systemic side effects	Doxorubicin	61
		Curcumin-loaded cationic nanoparticles in silk fibroin hydrogel Deeper skin penetration in mouse with psoriasis *In vitro* sustained release for over 72 hours with no burst	Curcumin	110
		Controlled release (28 days) High crystallinity and high drug loading	Risperidone	111

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Silk source	Formulation	Outcome/ Benefits	Released Agents	Ref.
		Sustained ocular delivery - release over 90 days Intravitreal injection – biodegradation starts after 3 months Drug available in vitreous 90 days after injection	Bevacizumab	100
		Extended release over 5 weeks Acid-modified silk hydrogels prevented burst release	Chemokine CXCL12	112
		Acidic fibroin hydrogels (pH 3.8) – polycaprolactone nanofibers Local bone delivery – promoted bone remodeling	Bone morphogenetic protein 2	92
		Silk nanoparticle loaded silk hydrogels – Dual drug delivery	FITC and Rhodamine B	103

Table 4.

Solid silk formats for controlled delivery of therapeutics

Silk source	Formulation	Outcome / Benefits	Released Agents	Ref.
***B. mori*, Fibroin**	Disk	Local delivery of HIV inhibitors (rectal & vaginal) Sustained release for over 4 weeks Higher microbicide stabilization during storage (up to 14 months)	HIV inhibitors: Griffithsin Griffithsin-C37 5P12-RANTES 5P12-RANTES-C37	124
		Sustained released up to 31 days for both IgG and 5P12-RANTES Preserved stability of antibody HIV inhibition in both blood and human colorectal tissue	IgG 5P12-RANTES	125
	Fiber mat	Silk – gelatin fiber mats Controlled release for 36h	Methylene Blue	126
		Controlled delivery (up to 3 months) Local delivery of human platelet lysate for wound healing Improved storage and handling of human platelet lysate	Human platelet lysate FITC-albumin	127
		Silk membrane attached on anal fistula plug For Crohn's disease treatment Controlled dual drug release (up to 10 days)	Curcumin 5-aminosalicylic acid	62
		Silk electrospun nanofibers combined with silk nanoparticles Dual drug delivery Sustained release for 40 hours	Doxorubicin HCl Curcumin	128
	Film	Sustained release (up to 28 days) Higher efficacy with intratumoral application of films when compared to intravenous application of drugs	Doxorubicin Crizotinib	122
		Sustained release (up to 14 days)	Vincristine, Doxorubicin	120
		Sustained release of cytokines for macrophage polarization Release up to 10 days	IFN-γ. IL-4	119
		Transdermal delivery Chitosan-silk fibroin cross-linked films pH dependent release (pH2>pH 5.5> pH 7.2) over 10h Released drug: salicylic acid>theophylline>diclofenac sodium>amoxicillin	Theophyllin Diclofenac Sodium Amoxicillin Salicylic acid	114
		Silk/gelatin blend film for wound healing In vitro release up to 48 hours *In vivo* rat model → faster healing in 7 days PEG-modified film allowed better uptake results	Ciproflaxin	132
		Stabilization of antibiotics Implant systems for focal delivery of antibiotics Sustained release (up to 5 days from the films)	Penicillin Ampicillin Gentamicin Cafazolin Rifampicin Eryhtromycin Tetracycline	109

Silk source	Formulation	Outcome / Benefits	Released Agents	Ref.
		Designed to be used as a nerve conduit in peripheral nerve defect NGF release for over 3 weeks PC12 cells proliferation and maturation	Nerve growth factor	133
		Heparin releasing composite material against thrombosis Silk fibroin and polyurethane films Controlled release up to 24 hours	Heparin	115
		Controlled release for 29 hours Diffusion model for *in vitro* release	FITC-dextran	117
		Controlled delivery up to 30 days Effects of silk degumming time on drug release was investigated	Azoalbumin Reactive-red 120, Rifampicin Indigo carmine	118
		Alginate/silk fibroin blend films Controlled release for over 30h Higher release rate with higher alginate ratio	Tetracycline	116
		Controlled release up to 30 days Intratumoral application → slower tumor growth	Doxorubicin	121
		Sustained release without a burst with silk/gelatin/glycerin films Release up to 350 minutes using films Release up to 24h using spray-dried microparticles	Naproxen	134
	Coating	Sustained release up to 40 days with layer by layer silk coating	Rhodamine B Evans Blue Azoalbumin	135
		Multi layer silk coating for vascular stent Reduced platelet adhesion Promotion of human aortic endothelial cell proliferation	Heparin Paclitaxel Clopidogrel	136
		Layer by layer heparin-silk nanofilms Controlled release up to 7 days Higher efficacy against cancer cells with higher beta sheet ratio	Epirubicin Hydrochloride	137
		Silk used as the film coating material on a tablet 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide (EDC) and Polyethylene glycol (PEG) blends Sustained release following zero order kinetics Enhanced release with EDC blend	Theophyllin	113
	Foams	Intratumoral implantation → decreased tumor growth Sustained release up to 48 days release	Vincristine	123
		Sustained release Dual combination of vincristine and doxorubicin Intratumoral injections→ significant decrease in tumor growth Reduced systemic exposure	Vincristine Doxorubicin	19
	Microneedles	Controlled release up to 48h Mild drug encapsulation method 10-fold reduction in bacterial density	Horseradish peroxidase (HRP) Tetracycline	93
		Swellable silk fibroin microneedles for trandermal delivery Better release kinetics over non-swellable microneedles	FITC-dextran	129

Silk source	Formulation	Outcome / Benefits	Released Agents	Ref.
		Release up to 95 hours/ high mechanical strength		
		Microneedles using polydimethylsiloxane (PDMS) mold PEG diacrylate and sucrose as needle matrix Release kinetics controlled by sucrose content Up to 144h release	Rhodamine Indocyanine green Doxorubicin	138
		Vaccine coated on silk microneedles Transdermal application on mice (24hours) Booster immunization 2 weeks after initial dose In vivo immune response >28 days	Vaccination against: Influenza *C. difficile* *Shigella*	130
	Reservoirs	Silk rod reservoirs – implant systems for cancer therapy Sustained release up to 91 days following zero order kinetics	Anastrozole	22
		Sustained release up to 14 days following zero order kinetics Implant system for local delivery of adenosine for epilepsy	Adenosine	139
		Sustained release up to 30 days Entrapment of drug powder allows high drug loading Intratumoral application → decrease in tumor growth	Cisplatin	140
	Wafer	Sustained release up to 45 days Intratumoral application → decrease in tumor growth Tumor cell necrosis adjacent to wafers	Etoposide	20
		Sustained release up to 7 weeks Intratumoral application → decrease in tumor growth Survival up to 60 days	Vincristine	123
Spider Silk eADF4(C16)	Films	Mono- and multi-layer films→ prolonged release (90 days) Release correlated with molecular weight of the drug	Paracetamol FITC-dextran FITC-BSA	141