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Review

Silk as an innovative biomaterial for cancer therapy

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ABSTRACT

Silk has been used for centuries in the textile industry and as surgical sutures. In addition to its unique mechanical properties, silk possesses other properties, such as biocompatibility, biodegradability and ability to self-assemble, which make it an interesting material for biomedical applications. Although silk forms only fibers in nature, synthetic techniques can be used to control the processing of silk into different morphologies, such as scaffolds, films, hydrogels, microcapsules, and micro- and nanospheres. Moreover, the biotechnological production of silk proteins broadens the potential applications of silk. Synthetic silk genes have been designed. Genetic engineering enables modification of silk properties or the construction of a hybrid silk. Bioengineered hybrid silks consist of a silk sequence that self-assembles into the desired morphological structure and the sequence of a polypeptide that confers a function to the silk biomaterial. The functional domains can comprise binding sites for receptors, enzymes, drugs, metals or sugars, among others. Here, we review the current status of potential applications of silk biomaterials in the field of oncology with a focus on the generation of implantable, injectable and targeted drug delivery systems and the three-dimensional cancer models based on silk scaffolds for cancer research. However, the systems described could be applied in many biomedical fields.

1. Introduction

Silks are fibrous proteins produced by a variety of insects and spiders (Fig. 1). They provide structural roles in cocoon and web formation, nest building, and egg coating as lifelines.[1] The most extensively characterized silks are from the domesticated silkworm, *Bombyx mori*, and from spiders, *Nephila clavipes* and *Araneus diadematus*.[2]

The silkworm silk obtained from the cocoon of *B. mori* consists of two major fibroin proteins: light chain (~26 kDa) and heavy chain (~390 kDa). These core chains

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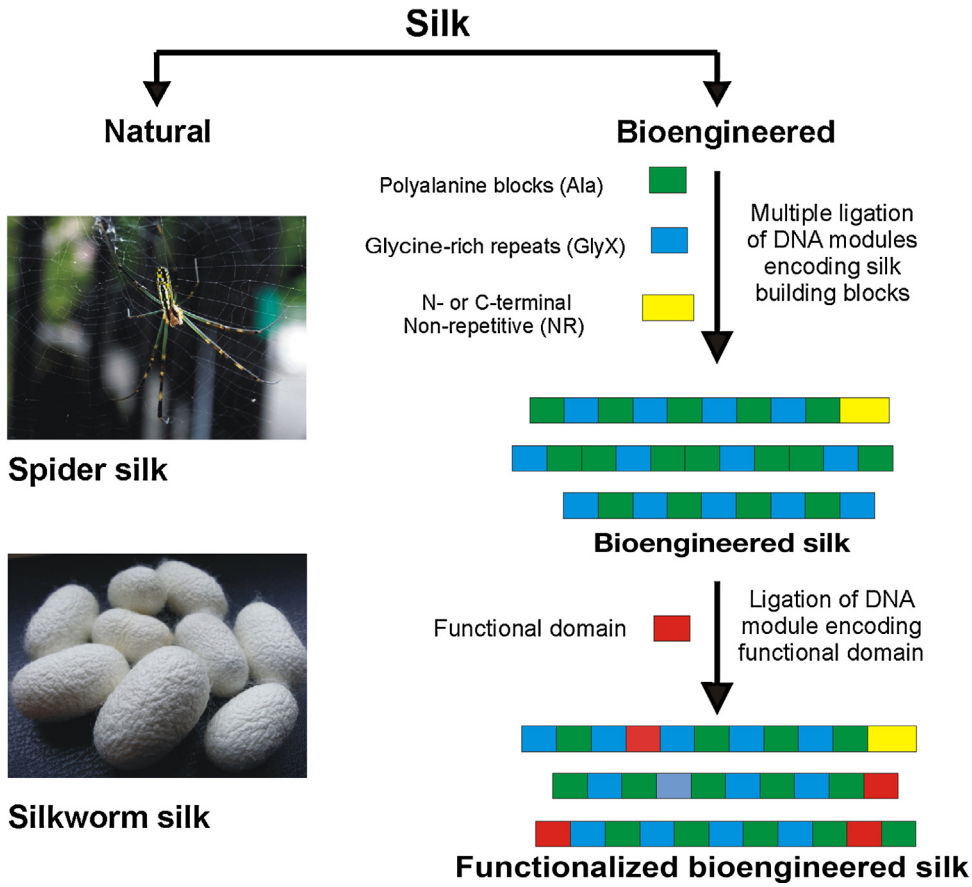

Fig. 1 – The origin of silk for biomedical application. The most intensively studied silks are silkworm silk and spider silks. Silks can be collected from nature or biotechnologically produced. Genetic engineering allows to design synthetic silk genes. Moreover, bioengineered silk can be modified by adding a sequence encoding peptide that confers a function to the silk biomaterial.

are coated with proteins called sericins that hold the fibroin fibers together and form the complex fibers of the cocoon case. Silk proteins have a modular structure containing large internal repetitive sequences flanked by shorter terminal domains (N- and C-terminals).[3] The repetitive motif is mainly composed of sequence of six amino acid residues $(Gly–Ala–Gly–Ala–Gly–Ser)_n$. It has amphiphilic characteristics, comprising hydrophilic, amorphous regions and hydrophobic, crystalline domains that form a beta-sheet secondary structure. The alanine-rich regions are responsible for the silk self-assembly properties and the mechanical stability of the biopolymer. Sericins are the source of immunogenic reaction; therefore, they are removed from the silk fibroin during the de-gumming process, in which silk cocoons are boiled in an alkaline solution. Numerous *in vitro* studies have shown that fibroins support attachment and proliferation of different cell types after prior sericin extraction.[3] Silk fibroin is a versatile biomaterial that can be formed into various structures, shapes, and dimensions.[4,5]

Spiders produce seven types of silk with various applications and properties.[6] Among them, dragline silk is the most extensively studied and the best characterized.[7] It is one of the strongest known natural materials. In silk from the *N. clavipes* spider, the dragline silk is made of two proteins: major ampullate spidroin 1 and 2, MaSp1 and MaSp2 (ADF3 and ADF4 for *A. diadematus*). Similar to silkworm silk, three regions can be distinguished in spidroins: (1) a non-repetitive N-terminal domain (of approximately 130 amino acids), (2) a dominant fragment consisting of repetitive motifs and (3) a non-repetitive C-terminal domain (of approximately 110 amino acids).[8] The N- and C-terminal domains are involved in the assembly and processing of silk fibers.[9,10] The amino acid composition of the repetitive peptide blocks consists mainly of glycine and alanine with a significant contribution of glutamic acid, proline and arginine to form four groups: (1) GPGXX, GPGGX and (2) GGX, which are responsible for elasticity of silk, (3) alanine (A) or glycine–alanine (GA) chains, which create β-sheet structures and are responsible for the strength of silk, and (4) a spacer sequence of unknown structural role.[7,11]

Silks are derived from nature or are produced in an expression system (Fig. 1). Silkworm silk is obtained from cocoons of domesticated silkworms. However, breeding spiders on a large scale is limited due to their cannibalistic nature. Moreover, they produce different types of silk simultaneously. Therefore, numerous studies have focused on the identification of the spidroin genes, recognition of the spidroin structures, and exploration of the process of silk assembly, which could finally result in the production of an artificial silk fiber. However, the

Table 1 – Bioengineered spider silk proteins.

Names	Amino acid sequence of the consensus motif	Origin	References
6x, 15x, MS1, 6mer, 15mer	$(SGRGGLGGQGAGAAAAAGGAGQGGYGGLGSQGT)_n$	MaSp1, *Nephila clavipes*	38–40,55,67,68
eADF3, (AQ)n	$(GPYGPGASAAAAAAGGYGPGSGQQGPGQQGPG-QQGPGQQGPGQQ)_n$	ADF3, *Araneus diadematus*	41,69
eADF4, C16	$(GSSAAAAAAAAASGPGGYGPENQGPSGPGGYGPGGP)_n$	ADF4, *Araneus diadematus*	35–37,41,42,69
eFLAG	$(GPGGX)_{36}(GGX)_{12}$GGTTIIEDLDITIDGADGPITISEELTI-$(GPGGAGGPY)_6(GPGGX)_{12}$	Flagelliform silk, *Nephila clavipes*	70
4Rep, 4RepCT	GGSGNSGIQGQGGYGGLGQGGYGQGAGSS$(A)_{12}$GGQ-GGQGQGGYGQGSGGS$(A)_{15}$GRGQGGYGQGSGGN$(A)_{15}$-GQGGQGGYGRQSQGAGS$(A)_{15}$GSGQGGYGGQGQGGYGQS	MaSp1, *Euprosthenops australis*	53,57,71
SELP	(GAGAGS)x(GXGVP)y	Silk fibroin, *Bombyx mori*	33,34,72

first step is to obtain a sufficient amount of spider silk protein. Several expression systems have been explored for recombinant spider silk production including bacteria, yeast, insect and mammalian cells, and transgenic plants and animals.[12] These systems differ from each other in terms of efficiency, cost, and the complexity of the process. There are two strategies for recombinant silk production. The first method is based on the expression of authentic cDNA fragments that encode fragments of silk proteins, whereas the second strategy uses synthetic silk genes. The last method involves the synthesis of short DNA modules that encode consensus motifs of particular spider silk spidroins. The DNA sequence is designed to enable their controlled ligation and insertion to the specific expression system.[12] The 3′ and 5′ ends of the modules are recognized by the restriction enzymes, which generate sticky ends. During ligation, the DNA sequence is doubled without retrieving the restriction sites. Hence, the synthetic genes of bioengineered spider silk proteins, based on natural silk fragments, can be obtained. The process described facilitates the ligation of numerous repetitive motifs (Fig. 1). Several different bioengineered spider silk proteins have been developed with the vast majority based on the consensus sequence of MaSp and Flag proteins originating from *N. clavipes* and *A. diadematus* spiders (Table 1).

Bioengineering of the silk sequence provides control over silk properties, such as the protein sequence, molecular weight, and ratio of hydrophilic and hydrophobic blocks.[13] These modifications can significantly change the characteristics of the silk, which may affect the silk–cell and/or silk–drug interactions. Moreover, a great advantage of genetic engineering is the possibility of introducing different silk modifications at the DNA level. Thus, spider silk proteins modified by genetic engineering can be designed to display new features alongside native properties. The hybrid silk protein would combine the sequence encoding the bioengineered spider silk, which is responsible for the biomaterial structure, with the sequence encoding polypeptides, which act as functional groups.[5,14] Such silk hybrids avoid post-translational chemical modification steps for biomaterial decoration. This characteristic is of great importance because the chemicals used for functionalization can cause potential cytotoxicity. The functionalization of spider silk proteins with peptides possessing binding or enzymatic properties is a promising method to develop novel biomaterials.

Silk has been known and used for centuries in the textile industry and as a suture material. The unique mechanical properties of silk together with its biocompatibility, biodegradability, lack of immunogenicity and compatibility with common sterilization techniques make it a perfect biomaterial for a wide range of biomedical applications including cancer therapy.

2. Silkworm silk as an anticancer drug carrier

The vast majority of anticancer drugs are poorly soluble in water; hence, a biomaterial carrier that can bind and release these drugs would improve drug bioavailability and contribute to better therapy outcomes. For drug delivery purposes, silk fibroin has been processed into films,[15] hydrogels,[16] coatings,[17] capsules and micro- and nanoparticles[18–23] (Fig. 2). These systems can be organized into two groups: silk-based drug delivery systems for local, intratumoral application and systemic delivery vehicles for intravenous injection (Table 2).

2.1. *Intratumoral delivery systems*

B. mori silk fibroin films, rods and hydrogels have been fabricated and applied for intratumoral treatment.[15,16,24,25] Silk films were stabilized with water vapor for different amounts of time to produce different amounts of crystallinity. The anticancer drug doxorubicin was loaded into films using two methods: (i) mixing the silk fibroin solution with doxorubicin prior to film casting and (ii) soaking a prepared film in the doxorubicin solution. The films showed sustained doxorubicin release over 30 days and toxicity toward breast cancer cells *in vitro*. Films without the drug showed no adverse effects to the tissues when implanted into mice. Stabilized, doxorubicin-loaded films had a better anti-tumor response and lower systemic toxicity in a mice breast cancer model than an equivalent dose of doxorubicin administered intravenously.[15] In another study, doxorubicin-loaded silk films were used as a post-tumor resection treatment of neuroblastoma in an orthotopic xenograft injection mouse model.[24] The authors showed that doxorubicin-loaded films applied to the residual tumor bed after tumor resection hindered the tumor re-growth compared with an equivalent dose of free doxorubicin.[24] Hence, the application of films that slowly release chemotherapeutics after tumor resection can extend the margins of tumor treatment and increase protection against tumor re-growth.

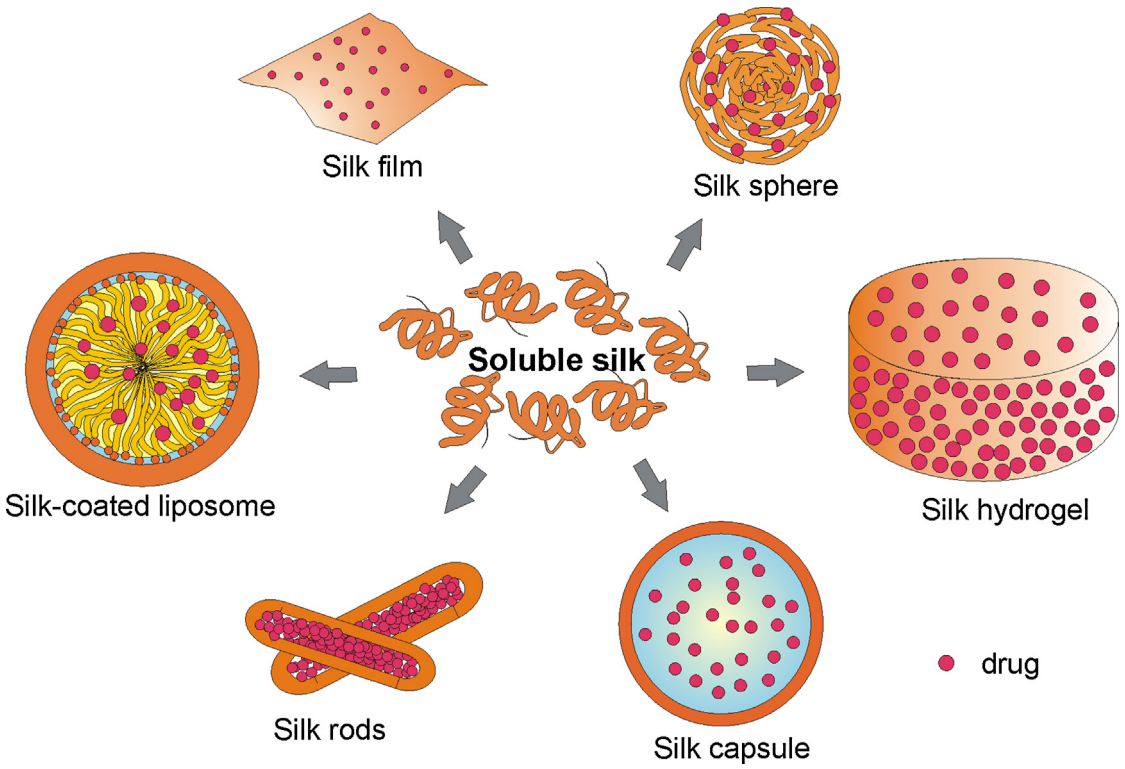

Fig. 2 – Silk-based biomaterials for chemotherapeutic delivery.

In comparison with drug-loaded films, hydrogels present larger capacity reservoirs of a therapeutic agent and hence provide a more prolonged drug release. Silk fibroin hydrogels were loaded with doxorubicin and were shown to improve the safety and efficacy of the treatment in human breast cancer xenografts compared with systemic doxorubicin injections.[16] The doxorubicin-loaded hydrogels were produced under mild, aqueous conditions by the self-assembly of a silk fibroin solution (6%, 4% and 2%) pre-mixed with doxorubicin. The hydrogels showed sustained doxorubicin release over 4 weeks without swelling. The drug loading capacity and release rate could be modified by the concentration of silk fibroin in the gelation process. Drug-loaded hydrogels injected into tumor-bearing mice reduced the tumor growth and metastatic spread.[16]

Silk fibroin hydrogels and lyogels (lyophilized hydrogels) were used to encapsulate and release monoclonal antibodies.[26,27] The antibodies showed sustained release over 38 days and maintained their biological activity. The antibody release was governed by hydrophobic interactions and hydration resistance, which could be controlled by the beta-sheet content in the silk lyogel.[26] Although the study utilized a model antibody, this work can serve as a proof of concept for the encapsulation of anticancer monoclonal antibodies, such as Trastuzumab or Cetuximab.

Silk rods are another effective approach for long-term sustained, locoregional delivery of a poorly water-soluble anticancer drug.[25] Anastrozole was encapsulated in a powder form inside the silk rods. The rods were manufactured using a unique film-spinning technology with simultaneous drug entrapment. During the release process, hydration and swelling occurred simultaneously with gradual diffusion of the encapsulated drug through the silk film. The drug was released from the film surface in a nearly zero-order release profile. Moreover, the release could be additionally enhanced by proteolytic degradation of the biopolymer. Gradual release of the drug over 28 days was detected in the blood of rats that were treated with injected rods.[25] By controlling the rod dimensions, the drug dose can be controlled.

Paclitaxel was encapsulated into nanospheres due to the self-assembly of the silk triggered by hydrophobic interactions between the drug and the hydrophobic regions of silk fibroin.[20] The particles, approximately 130 nm in diameter, were taken up by human gastric cells *in vitro*. In an *in vivo* study, locally injected paclitaxel-loaded particles showed significantly higher antitumor efficacy than an equivalent dose of intravenously administered paclitaxel.[20] In another study, paclitaxel-loaded silk fibroin nanoparticles were formed by the self-assembly process of an ethanol-paclitaxel solution and silk fibroin.[28] The drug loading efficiency and drug release kinetics were controlled by the concentration of the silk fibroin. Sustained release of the drug was observed, depending on the conditions, for up to 9 days or 2 weeks.[28]

2.2. *Intravenous delivery vehicles*

The carriers for systemic cancer treatment have to overcome the challenges of reaching the tumor site and accumulating in the tumor microenvironment. In general, intravenously administered therapeutic particles must be small enough to penetrate through capillaries. However, most of the reported anticancer drug carriers take advantage of the enhanced

Table 2 – Silk-based drug delivery systems for cancer therapy.

Silk biomaterial	Preparatiom method	Drug/route of administration	Target	Cell line/*in vivo* model	References
B. mori silk film	Film casting with water vapor annealing	Doxorubicin/intratumoral	Breast cancer	MDA-MB-231/orthotopic adrenal tumor xenograft in mice	15,24
B. mori silk hydrogel	Self-assembly mediated by sonication	Doxorubicin/intratumoral	Breast cancer	MDA-MB-231, MCF-7/tumor xenograft in mice	16
B. mori silk particle	Self-assembly with paclitaxel solution in ethanol	Paclitaxel/intratumoral	Gastric cancer	BCG-823/tumor xenograft in mice	20
B. mori silk particle	Desolvation method with acetone	Doxorubicin	Breast cancer	MCF-7/n/a	18
B. mori silk-albumin particle	Desolvation with acetone and crosslinking with glutaraldehyde	Metotrexate	Breast cancer	MDA-MB-231/n/a	21
B. mori silk fibroin particle and silk fibroin-chitosan particles	The microdot capillary method, lyophilization, methanol treatment	Curcumin	Breast cancer, melanoma	MCF-7, MDA-MB-453/n/a	22
B. mori silk film coated liposome	Lyophilization of silk fibroin with emodin-loaded liposomes and methanol treatment	Emodin	Breast cancer, melanoma	MCF-7, MDA-MB-453, BT-474/n/a	17,30
Bioengineered silk fibroin–elastin-like particle	Self-assembly with hydrophobic doxorubicin	Doxorubicin	Cervical cancer	HeLa/n/a	34
Bioengineered functionalized spider silk sphere	Salting-out with potassium phosphate	Doxorubicin	Her2/neu overexpressing ovarian and breast cancer	SKOV3, SKBR3/n/a	40
Bioengineered spider silk and DNA complexes	Self- assembly with DNA	Plasmid DNA/intravenous	Breast cancer, melanoma	MDA-MB-231, MDA-MB-435/tumor xenograft in mice	38,39

permeability and retention (EPR) effect occurring in the tumor tissue. The particle accumulation within a tumor is enhanced by the tumor hypervascularization due to abnormally permeable blood vessels and the lack of lymphatic drainage. The "leaky" tumor vasculature retains the drug carriers and enables prolonged, selective drug release to the tumor microenvironment.[29]

A silk fibroin-doxorubicin formulation was tested in the form of nanoparticles.[18] Nanospheres were formed using an acetone-based desolvation process, resulting in uniform, approximately 100 nm in diameter, negatively charged silk nanoparticles. The particles were able to enter the cancer cells *in vitro* and were found in lysosomes. The lysosomal fate of the absorbed drug carriers enhanced the release of doxorubicin from the particles, which was shown to be pH dependent.[18] The doxorubicin entrapped in the particles was significantly more effective against MCF-7 breast cancer cells compared with free doxorubicin and was able to overcome the drug resistance in the antracycline-resistant MCF-7 cell line.[18]

In study by Gupta et al., silk fibroin nanoparticles were compared to nanoparticles obtained from silk fibroin-chitosan blends.[22] Both particle types were formed using a microdot capillary method involving the entrapment of an anticancer agent, curcumin. The nanoparticles (both less than 100 nm in diameter) released the drug over 9 days and demonstrated efficacy against Her2/neu-overexpressing breast cancer cells. The silk-fibroin nanoparticles outperformed the particles made of the silk-chitosan blend, showing that silk fibroin itself is an excellent carrier of an anticancer drug.[22]

Silk fibroin blended with albumin was successfully applied to load and release a poorly water-soluble anticancer drug, methotrexate.[21] Spherical particles ranging from 140 to 300 nm in diameter were formed by mixing in acetone and subsequent crosslinking with glutaraldehyde to improve the stability. The particles were taken up by cells *in vitro* and selectively killed MDA-MB-231 cancer cells.[21]

Silk fibroin was utilized as a coating for liposomes loaded with a hydrophobic anticancer drug, emodin.[17,30] The drug was initially encapsulated in 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) phosphatidylcholine liposomes and then coated with the silk fibroin polymer by freezing, subsequent lyophilization and methanol treatment. The coated particles were 316.6 ± 43.0 nm in diameter. The silk fibroin coating decreased the emodin release rate, resulting in a

sustained emodin release profile and enhanced uptake of the drug-loaded liposomes, which significantly improved the efficacy against Her2/*neu*-overexpressing breast cancer cells.[17,30]

3. Bioengineered silk as an anticancer drug carrier

In addition to anticancer drug delivery systems based on regenerated silk fibroin, a number of studies have been performed to develop drug delivery vehicles from recombinant silk proteins. Depending on their origin, the bioengineered silk proteins can be divided into two groups: (i) proteins derived from the natural silk fibroin of *B. mori* and (ii) proteins from spider silks.

3.1. *Bioengineered silkworm silk-based particles*

B. mori silk-based copolymers contain multiple repeats of the GAGAGS motive from the silk fibroin coupled with the GVGVP motive derived from the elastin sequence (silk elastin-like proteins, SELPs).[31] SELPs were processed into hydrogels for adenoviral gene delivery in head and neck tumor therapy.[32] Adenoviruses embedded into the silk–elastin hydrogel were injected into the tumors and showed 10-fold higher transduction efficiency, which provided a proof of concept for an effective anticancer gene therapy using a gene encoding thymidine kinase in conjunction with ganciclovir.[33] In another study, the silk–elastin-like copolymer was utilized as a drug delivery vehicle for tumor treatment.[34] A hydrophobic form of doxorubicin was encapsulated into the protein copolymers containing silk and elastin domains at ratios of 1:8, 1:4 and 1:2 by a self-assembly process. The formation of the nanoparticles was triggered by hydrophobic interactions between the drug and the hydrophobic silk blocks. The nanoparticles were taken up by the HeLa cells and then released the doxorubicin, which was observed in the nuclei of the cells. The dox-loaded nanoparticles showed higher cytotoxicity than an equivalent of the free drug, likely due to sustained release caused by enzymatic degradation of the copolymeric nanoparticles inside of the cells.[34]

3.2. *Bioengineered spider silk-based particles*

Drug delivery vehicles made of bioengineered spider silk have been mostly explored in terms of particle formation, characterization and loading/release potential tested by employing model drugs.[35–37] Only a few studies were directly related to cancer treatment[38–40] (reviewed in Section 3.2.1). However, the overall characteristics of silk spheres provide a great insight into their overall potential for biomedical applications including cancer treatment.

The bioengineered spider silk eADF4(C16) based on the ADF4 spidroin of the *A. diadematus* spider was designed[41] and self-assembled into spherical particles.[42,43] The process of sphere assembly was mild, biocompatible and triggered by >400 mM potassium phosphate.[42] The particles remained chemically stable even when exposed for 3 days to denaturants such as 8 M urea and 4 M guanidine chloride.[23] By controlling the processing conditions, such as protein concentration and mixing speed, nanometric spheres could be obtained (as small as 250 nm).[43] The properties of the eADF4(C16) spheres were comprehensively studied, showing colloidal stability, negative surface charge, narrow size distribution, smooth surfaces, and high beta-sheet structure content.[36,37,42] A group of 12 low molecular weight model drugs were employed to test the drug loading and release of eADF4(C16) spheres. Among them, the highest loading efficiency was observed for positively charged drugs, indicating a role of electrostatic interactions in the drug loading process.[36] Lysozyme, a macromolecular model drug was also successfully loaded into the spheres due to the electrostatic interactions between negatively charged silk particles and the positively charged lysozyme.[37]

To further improve the drug loading and release kinetics, the eADF4(C16) particles were subjected to crosslinking by ammonium persulfate (APS) and 100 μM tris(2,2′-bipyridyl)dichlororuthenium(II) (Rubpy), which led to tyrosine bond formation between silk protein chains.[35] The study found that the co-precipitation of the model drug with the silk protein increases the loading efficiency compared with diffusion-driven drug loading, whereas crosslinking hinders loading and reduces the release of the model drug from the spheres.[35] Moreover, the amphiphilic eADF4(C16) protein was employed to encapsulate β-carotene,[44] a hydrophobic, water insoluble model drug. This study provided an important proof that bioengineered silks can potentially encapsulate water-insoluble substances.

The bioengineered eADF4(C16) spider silk was employed to form hollow microcapsules.[45–47] The capsules were formed at an oil/water interface and were shown to encapsulate a β-galactosidase.[47] The drug was protected against proteolysis. The capsules provided an enclosed reaction environment with a semi-permeable membrane with an average molecular weight cut-off of 27 kDa.[45] Hence, it was permeable for the enzyme substrates and reaction products while shielding against the proteolytic enzymes. These features suggest the great potential of silk microcapsules as protective enzyme reaction containers for various medical applications.

The bioengineered silk eADF4(C16) showed distinctive properties as a polymer for drug delivery. Although the profiles of sustained release of model drugs are promising, the potential of eADF4(C16) formulations with anti-cancer therapeutics still needs to be investigated.

3.2.1. *Functionalization of the bioengineered spider silk*

The construction process of the bioengineered silks provides a very simple method for polymer functionalization. Using genetic engineering, a DNA sequence encoding a polypeptide or protein domain can be added to the DNA sequence of silk. The obtained bioengineered silk protein comprises structural (silk) and functional parts in one molecule. The additional domains can display different functions such as binding sites for receptors, enzymes, growth factors, metal or sugars. A few studies have explored the application of functionalized bioengineered silk for cancer therapy.[38–40]

Selective targeting of tumor cells is very desirable for cancer treatment strategies. Numata et al. developed a spider silk-based system that can be used as a carrier for tumor-specific gene delivery.[38,39] For a new transfection system,

a bioengineered 6-mer based on MaSp1 of *N. clavipes* was designed. The 6-mer silk was functionalized with two peptides: (i) poly(L-lysine) that interacts with pDNA through electrostatic interactions and (ii) tumor-homing peptide (THP) that is responsible for targeting the tumor cells. Two THPs were examined: (i) F3 peptide that recognizes a nucleolin (a molecule which is presented on the surface of tumor angiogenic endothelial cells and cancer cells)[48] and (ii) CGKRK peptide that binds to neovascular endothelial cells and tumor cells.[49] The functionalized 6-mer and plasmid DNA (pDNA) formed ionic complexes of nanoscale diameters (150–200 nm). Both THPs significantly enhanced the transfection of MDAMB231 and MDAMB435 cancer cells compared with the transfection of MCF10A breast epithelial cells.[39] In another approach, silk proteins were functionalized by the addition of a F3 peptide or a Lyp1 peptide, which binds to the tumor lymphatics in certain tumors.[38] The pDNA complexes of the silk-poly(L-lysine) containing additional F3 peptides showed significantly lower cytotoxicity toward MCF10A breast epithelial cells compared with pDNA complexes of the silk-poly(L-lysine)-Lyp1 block-copolymer. Moreover, the silk-based ionic pDNA complexes with higher contents (25 mol%) of THP demonstrated better transfection efficiency in comparison with complexes that contained a lower amount of THP (12 mol%).[38] To further improve the system, the MaSp1 monomer was used instead of the silk 6-mer, which resulted in the generation of smaller pDNA complexes (90 nm in diameter). The complexes of pDNA and silk monomer-15L-lysines/F3 showed enhanced transfection efficiency compared with the complexes of pDNA and silk chimera 6-mer/30L-lysines/F3.[38] Accordingly, the THP-functionalized silk proteins can potentially serve as a new platform for nonviral tumor-specific gene delivery.

Functionalized bioengineered silks can serve as specific drug carriers. For targeted cancer therapy, the hybrid composed of the bioengineered silk MS1 (15-mer based on the MaSp1 protein from *N. clavipes*) and Her2 binding peptides, H2.1 or H2.2, was designed.[40] Her2 is a molecule overexpressed in approximately 20–30% of invasive breast carcinomas.[50] Receptor binding peptides were fused to the silk sequence without any additional peptide linker. The N-terminal functionalization of silk resulted in more efficient targeting to Her2-overexpressing cells (SKOV3, SKBR3) compared with C-terminal fusion. The binding motifs did not impede the self-assembly of spider silk, and silk spheres were formed. Moreover, the H2.1 and H2.2 domains were exposed on the surface of the spheres because sphere binding to the cells overexpressing Her2 and their subsequent internalization into target cells were observed. Interestingly, the signal of the fluorescently labeled internalized silk spheres decreased over time, suggesting degradation of the silk particles within the cells. The silk spheres were loaded with chemotherapeutic doxorubicin (Dox) with high incorporation efficiency. The Dox release profile was pH-dependent — the factor accelerating the release process was low pH. The Dox-loaded functionalized spheres induced a significantly higher reduction of the viability of Her2-overexpressing cells compared with control spheres (without functionalization). Moreover, their cytotoxicity toward Her2-positive cancer cells was significantly higher compared with the cells without Her2 receptors.[40] Accordingly, the system based on functionalized silk that specifically targets tumors can increase the therapeutic index of chemotherapeutic agents against tumor cells, simultaneously reducing the toxicity in normal tissues.

Although a large number of the genetically functionalized silks are not directly related to application in the field of oncology, they may be potentially useful for studying the biology of cancer or for use in the treatment of cancer. The simplest genetic modification of silk is the introduction of a single amino acid. A bioengineered eADF4(C16) spider silk was modified by adding a cysteine residue.[52] The chemically specific side chain of cysteine, which comprises a thiol group, enables the covalent coupling of molecules such as peptides, drugs, dyes, biotin or enzymes.[52] Jansson et al. genetically functionalized 4RepCT silk with several larger affinity domains, such as the biotin-binding domain M4, the albumin-binding domain ABD, and the IgG-binding domains Z and C.[53] Similar to the cysteine introduction, the application of affinity domains allows for the immobilization of various drugs, growth factors or active enzymes on silk biomaterials. Silk spheres, films, hydrogels, or scaffolds decorated with active compounds may be used for cancer therapy.

To study the biology of cancer, *in vitro* three-dimensional (3D) cancer models are very useful tools. Several 3D cancer models built on silk scaffolds have been described (reviewed in Section 4). There are several examples of functionalized bioengineered silks that may serve as matrices for scaffold preparation with enhanced cell adhesion, growth, differentiation and migration. The most common modification is the functionalization of silk sequences with the cell recognition peptide RGD (Arg-Gly-Asp).[54–56] The RGD motif originates from sequences of fibronectin and vitronectin, which are extracellular matrix (ECM) proteins that act as integrin ligands and support cell growth. A film made of genetically designed hybrid RGD-silk showed improved cell adhesion compared with a film made of chemically modified silk.[54] In addition to the RGD motif, other cell binding peptides found in natural ECM proteins, such as the peptide IKVAV (present in the laminin α1 chain) and YIGSR (found in β1 chain), were genetically fused to 4RepCT silk.[57] The obtained hybrid proteins were processed into films, foams or fibers. Four different human primary cell types (fibroblasts, keratinocytes, endothelial cells and Schwann cells) adhered significantly better to RGD-modified films. Moreover, Schwann cells had improved adhesion on matrices containing the IKVAV peptide.[57] These scaffolds, in which a defined mix of cells would be recruited by selected cell adhesion motifs and growth factors, would be desirable in 3D cancer models.

4. Three-dimensional cancer models on the silk scaffold

Silk fibroin has been extensively studied as a biomaterial for tissue engineering[3]; however, its potential as a matrix for engineering a cancer model is still not well explored. Three-dimensional cancer models are a bridge between *in vitro* two-dimensional (2D) cell cultures and animal studies.[58] Two-dimensional cell cultures do not provide the essential critical environmental context of cancer. They lack the proper cell–cell

Table 3 – Three dimensional (3D) cancer models build on silk scaffold.

Cancer model	Silk	Scaffold fabrication method	References
Mammary adenocarcinoma	*Antheraea mylitta*	Lyophilization	61
Squamous cell carcinoma	*Bombyx mori*	Cryogenic electrospinning	62
Osteosarcoma	*Bombyx mori*	Lyophilization	60
Hepatocarcinoma	*Antheraea mylitta*	Lyophilization	63
Breast cancer metastasis to bone	*Bombyx mori*	Salt leaching	64
Breast cancer	*Bombyx mori*	Salt leaching	66

and cell–extracellular matrix interactions. The cell morphology, adhesion and proliferation properties are modified. In contrast, three-dimensional *in vitro* cell cultures reconstruct an environment much more similar to that observed *in vivo*. Cells grown in three dimensions generate complex multicellular structures. The metabolism and expression profiles of cells cultured in 3D significantly differ from those grown in 2D conditions. In addition to tissue regeneration applications, the 3D cultures allow for the *in vitro* reconstruction of biologically relevant disease models. A 3D *in vitro* cancer model may be useful for studying cancer biology and for evaluating cellular responses to the pharmaceutical compounds in drug discovery applications.[59]

The common morphological structure of silk made for 3D cell culture is a porous scaffold. These scaffolds can be prepared using lyophilization or salt leaching methods. The first method involves lyophilization of the water-based or HFIP (hexafluoroisopropanol)-based silk solution. Pores are formed during lyophilization. The second method uses salt crystals of a defined-size. Salt is poured into the silk solution, which induces gelation of the silk. Subsequently, the salt is dissolved in water, leaving pores in the non-soluble silk scaffold. Silk scaffolds can be easily modified in terms of their size, porosity, pore-size, mechanical strength and degradation time.[3] A few cancer models constructed based on silk matrices have been described, including hepatocarcinoma, osteosarcoma, squamous cell carcinoma and mammary adenocarcinoma models (Table 3).[60–64]

Tan et al. prepared a porous silk scaffold made of silkworm silk using lyophilization.[60] Osteosarcoma cells (143.98.2) were seeded on scaffolds to analyze the angiogenic growth factors and proliferation markers in comparison with the secretion profile of cells grown under 2D and mouse xenograft conditions. The expression levels of proliferation markers, such as cyclin B1, E2F1, and Ki-67, were lower in the 3D model compared with 2D culture. The levels of HIF-1 alpha, VEGF-A and VEGF receptors in cancer cells grown on 3D silk scaffolds more closely resembled those in serve combined immunodeficiency (SCID) mouse xenograft tumors than the levels in 2D cultured cells. The analysis of angiogenic factors, including bFGF and IL-8, indicated that their expression was strongly dependent on the conditions of the cell culture, *i.e.*, their expression was higher in 2D cultured cells and lower in 3D culture and SCID xenograft tumors. In general, the secretion profile of proteins from cells cultured in 2D *in vitro* conditions significantly differed from that observed *in vivo*. Because cancer cells grown on silk scaffolds had an expression pattern similar to cancer *in vivo*, this was proposed to be a good *in vitro* cancer model.[60]

A silk extracted from the glands of *Antheraea mylitta* silkworms was used to produce scaffolds by lyophilization.[61] This silk was proven to be more suitable for cell attachment than the commonly used *B. mori* fibroin due to the natural presence of the cell binding sequence RGD.[61] The scaffolds were seeded with MDA-MB-231 breast cancer cells or LNCaP prostate cancer cells. Good cell viability and proliferation in the long-term culture (up to 8 weeks) was observed. A higher matrix metalloproteinase-9 activity, which may indicate a more invasive potential, was found in cells cultured in 3D *A. mylitta* scaffolds compared with 2D cultures.[61]

A cryogenic electrospinning method was used to fabricate *B. mori* silk scaffolds that closely resembled tumor ECM.[62] For comparison, the tumor ECM was first evaluated by microscopy analysis of decellularized tumor samples. Interestingly, the fibrous structure of silk scaffold resembled the morphology of tumor ECM. HN12 cells were seeded on the silk scaffold, and their proliferation rate and sensitivity to paclitaxel treatment were measured. Consistent with other studies, the cells grown on 3D silk scaffolds had a lower percentage of proliferating cells compared with those grown on 2D cultures, as measured by the expression of the Ki-67 proliferation marker. Moreover, the number of proliferating cells was much closer to that observed in tumor xenografts. HN12 cells grown on the scaffolds were much more resistant to paclitaxel than those grown on 2D cultures, and they retained their proliferative properties even at high doses of paclitaxel.[62]

The morphology, proliferation and chemoresistance of hepatocarcinoma cells (HepG2) modified to overexpress hyaluronan binding protein 1 (HABP1) were analyzed using a 3D hepatocarcinoma model constructed on a silk scaffold.[63] A silk solution was directly extracted from the *A. mylitta* silkworm glands. Scaffolds were obtained by lyophilization, and they were seeded with HABP1-modified (HepR21) and non-modified HepG2 cells. The HepR21 cells formed multicellular aggregates (an indicator of tumor progression) when cultured in 3D culture conditions. The resistance of HepR21 cells cultured in silk scaffolds to the hyaluronan synthase inhibitor (4-MU) was approximately 2.5 times higher than that of cells cultured in standard 2D conditions. Moreover, 4-MU treatment of HepR21 cells led to decreased levels of HA and growth promoting factors, such as pAKT and PKC, and to increased expression of the p53 tumor suppressor. Because the level of hyaluronan (HA) (a major component of ECM) is known to be elevated in most cancerous microenvironments and because HA performs a regulatory role in the progression of tumors and metastasis, 4-MU may be used as an anticancer agent in the treatment of malignancies characterized by high level of

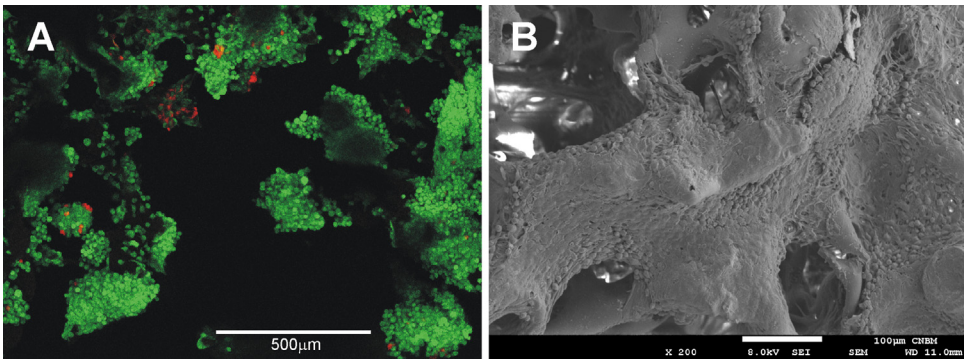

Fig. 3 – 3D breast cancer model build on silkworm silk scaffold. Breast cancer cells and fibroblast were grown in co-culture. (A) Confocal laser scanning microscopy (CLSM) and (B) scanning electron microscopy (SEM) images of cells after 14 days of co-culture. Cells were modified with reporter fluorescent proteins: red – fibroblast; green – breast cancer cells.

HA production. The applied 3D cancer model was successfully used not only to reconstitute the *in vivo* environment but also to indicate and explain the mechanism of 4-MU.[63]

Silk scaffolds were used as homing sites to study cancer metastasis *in vivo*.[64] *B. mori* silk scaffolds were prepared by salt leeching, were coupled with bone morphogenetic protein (BMP-2) and were seeded with bone marrow stromal cells (BMSCs). Following 1 day, 4 weeks and 7 weeks of culture in osteogenic media, the constructs were subcutaneously implanted into NOD/SCID mice. After 1 month, SUM1315 breast cancer cells were injected into mice mammary fat pads. The metastatic growth of breast cancer cells was measured in the silk scaffolds and was found to be present only in the 1-day scaffolds. The detailed analysis indicated that the highest metastatic incidence only occurred in the scaffolds coupled with BMP and in the scaffolds only seeded with BMSCs compared with scaffolds both coupled with BMP and seeded with BMSC. The scaffold development and implantation affected the metastatic spread. An undeveloped bone environment was more attractive for breast cancer cell metastasis. Because the nature of human bone tissue makes it highly difficult to analyze metastasis, this silk-based 3D bone model offers a good alternative for breast cancer invasion studies.

Silk scaffold-based tumor models can also be enriched with stromal cells to better recapitulate the tumor environment. Such co-culture studies are extremely important to understand the interaction between cancer cells and their surroundings. The tumor stroma consists of many cell types, including fibroblasts, activated fibroblasts, immune cells, inflammatory cells, and endothelial cells.[65] To treat cancer, complex knowledge regarding the tumor environment is needed. We constructed a co-culture breast cancer model consisting of both cancer cells and fibroblasts using a silk scaffold system (data not published). The preliminary results showed very good attachment of both fibroblasts and cancer cells on the silk scaffolds. Cells cultured in 3D proliferated slower than those cultured in 2D conditions. Moreover, both cell lines proliferated well on the scaffolds in monoculture, whereas in co-culture experiments, cancer cells overgrew the fibroblasts (Fig. 3). The cytotoxic effect of doxorubicin on the cells cultured in a 2D environment and on 3D silk scaffolds was compared, showing 10–20 times higher resistance in the 3D cultured cells (manuscript in preparation).

Silk scaffolds were used to generate a cell based-drug delivery system, as shown by Reagan et al.[66] The ability of mesenchymal stem cells (MSCs) to locate the tumor site was used as a method of targeted therapy. MSCs were modified to express a proapoptotic peptide, TRAIL (FLT-MSCs full length TRAIL-expressing MSCs). Three delivery routes of FLT-MSCs were compared: co-injections with tumor cells, tail vain injections after tumor formation and implantation on scaffolds before tumor formation. The MDA-MB-231 cancer cell growth in the presence of FLT-MSC stromal cells was inhibited.[66] FLT-MSCs, when injected intravenously or intratumorally were rapidly cleared from the organism; however, when the cells were seeded on the silk scaffolds and the scaffolds implanted subcutaneously in mice, a long, sustained delivery was achieved.[66] The cell/scaffold-based (implant-based) delivery system of therapeutic agents is a new method of anticancer therapy.

5. Conclusions

Biologically derived silk is a biodegradable and biocompatible material. Due to its extraordinary mechanical and physical properties, as well as its capacity to be processed into a range of structural forms, silk is a promising biomaterial for biomedicine research and therapy for the field of oncology, among others. So far, chemotherapeutic delivery and structural support for *in vitro* 3D cancer models are being intensively explored by researchers. Various implantable and injectable silk-based drug delivery systems enable the delivery of chemotherapeutics without causing side effects. The regional sustained drug delivery tools may improve post-resectional cancer treatment and reduce side effects, whereas intravenously delivered vehicles may improve the delivery, retention and biological efficacy of conventional anticancer drugs. Although spider silk is not as easily accessible as silk fibroin from *B. mori*, the use of the biotechnological strategies not only solves this accessibility problem but also enables modification of the properties of silk and the addition of new

features to silk biomaterials. Genetic engineering provides the possibility of controlling of the sequence and length of the bioengineered silk protein chain, which can influence the silk–drug interactions. Thus, the silk carriers can be tailored to incorporate any desired anticancer drug by either electrostatic or hydrophobic interactions. The release rate and kinetics can be modified by controlling the beta-sheet content in the silk structure. Moreover, the addition of a functional group can be used to control the drug loading/release profile and the specificity of the cell interactions (targeted therapy). Silk with dual functions can be obtained by mixing different hybrid silks. It may be useful to decorate silk matrices with complex proteins to promote attachment and growth of cells in a 3D *in vitro* cancer model. Although genetic engineering broadens the potential applications of silk, it may carry some limitations. A new bioengineered silk or hybrid silk (functionalized silk) must retain the properties of native silk, *i.e.*, the ability to self-assemble. Moreover, by adding new domains, immunogenicity or toxicity of the silk may be induced, which is of relevance for *in vivo* applications. Therefore, a detailed assessment of the properties of modified silk must be conducted.

Silk-based matrices such as spheres, capsules, scaffolds, films, and hydrogels can be successfully applied for cancer research and therapy. However, their application is not limited to the oncology field. The diversity in morphology, processing conditions, and functionalization expands the applications of silk materials to many areas of biomedicine.

Conflict of interest

None declared.

Financial disclosure

None declared.

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