Chapter

Silk Fibroin Nanoparticles: A Biocompatible Multi-Functional Polymer for Drug Delivery

Faith H.N. Howard, Zijian Gao, Hawari Bin Mansor, Zidi Yang and Munitta Muthana

Abstract

The versatility of nanomedicines allows for various modifications of material type, size, charge and functionalization, offering a promising platform for biomedical applications including tumor targeting. One such material, silk fibroin (SF) has emerged, displaying an excellent combination of mechanical and biological properties characterized by its high tensile and breaking strength, elongation, stiffness and ductility. High stability allows SF to maintain its chemical structure even at high temperatures (around 250°C) and compared with other biological polymers like polylactide (PLA), poly(lactic-co-glycolic acid) (PLGA), and collagen, SF shows excellent biocompatibility and lower immunogenic response making it a very suitable material for drug delivery and tissue engineering. Here we describe the structure, synthesis and properties of SF nanoparticles. We evaluate its emergence as a multi-functional polymer for its utility as a nanocarrier to deliver cancer therapies directly to tumors together with considerations for its clinical use.

Keywords: silk fibroin, polymer, nanomedicine, nanocarrier, drug delivery

1. Introduction

As stated by the World Health Organization, cancer is the second leading cause of death globally, accounting for an estimated 9.6 million deaths, or 1 in 6 deaths, in 2018 [1]. GLOBOCAN also predicts an increase in cancer rates, with more than 20 million new cancer cases expected annually by 2025 [2]. Over time, as our understanding and knowledge of molecular and tumor biology has increased, the cancer treatment paradigms have notably changed, particularly during the past 20 years. Previously, cancer was listed and treated based on its origin or its unique histomorphologic characteristics. However, in 2002, Schiller et al. reported that third generation chemotherapy administered to non-small-cell lung cancer showed almost similar survival curves [3]. Although the results are only limited to lung cancer, this indicates that cancer treatment using general (non-specific) cytotoxic chemotherapies have reached a therapeutic plateau. Since then, this research area has evolved based on two focus

areas: tumor molecular profiling and molecular targets. Together, these efforts have further realized two recent revolutions in cancer research. Firstly, genotype-directed precision oncology which focuses on personalized therapies to treat specific genomic abnormalities regardless of the cancer type. Secondly, the targeting of particular components in the tumor microenvironment. As a result, the discovery of an abundance of anticancer drugs have showed a promising early step for cancer eradication. However, most of these therapeutics agents have undesirable characteristics, limiting their clinical usage and invalidating further drug research [4].

Recent studies have suggested that the use of nanoparticles as a drug carrier may be one of the best alternatives to improve the therapeutic effect of anticancer drugs. Nanotechnology, or in this case, nanomedicine, is the use of materials usually in nanometer scale in the fields of medicine and health [5]. Nanoparticle-based drug delivery systems have shown remarkable progress in overcoming the limitations of conventional drug delivery or drug therapy method. The unique characteristics that usually accompany these potential carriers include nanoscale size, high surface-to-volume ratio, auspicious physical and chemical properties and most importantly, endless possibilities for modifications that support cell targeting, gene delivery etc. [5] .

Nanoparticles fall into two different categories, namely soft/organic and hard/inorganic nanoparticles. Soft nanoparticles are based on organic material, typically prepared from polymers or molecules that can self-assemble (coacervation) into large particles. The materials can range from full synthetic polymer to natural materials such as silk proteins [6]. Hard nanoparticles, on the other hand, are inorganic and usually insoluble, e.g. silver, gold nanoparticles, and carbon nanotubes [7, 8]. The benefits of organic nanoparticles as drug nanocarriers have been reported numerously in recent years, citing desirable characteristics including, biodegradability, non-antigenic and superior biocompatibility [9]. Thus, the delivery system's advancement holds the promise of future precision medicine, which would greatly improve cancer survival rate by treating each cancer patient with the most effective drugs in the most efficient ways [10].

One such natural polymer which promises great potential as a drug delivery system is silk fibroin (SF). Silk has been recognized as a valuable natural material for the fabric industry for centuries, but during the last few decades, it has attracted immense attention as a promising biopolymer for biomedical and pharmaceutical applications [11]. Silk from the domesticated silkworm *Bombyx mori (B. mori)* is well characterized and has been approved as a safe biomaterial by the US Food and Drug Administration (FDA) [12]. This chapter focuses on the use of *B. mori* derived SF as a functional material for cancer drug delivery.

2. Silk fibroin

2.1 Structure

Most SF utilized for biomedical and commercial applications are derived from cocoons of *B. mori* domestic silk moths. Constructed from a continuous fiber strand comprised of two cores of fibroin protein held together by sericin protein [13, 14]. The primary structure of *B. mori* SF mainly consists of glycine (Gly) (43%), alanine (Ala) (30%) and serine (Ser) (12%) [15]. The secondary structure of SF exists in three different structural forms including silk I, silk II and silk III. Silk I consists of α-helix domains which is in a water-soluble state and easy to convert to silk II

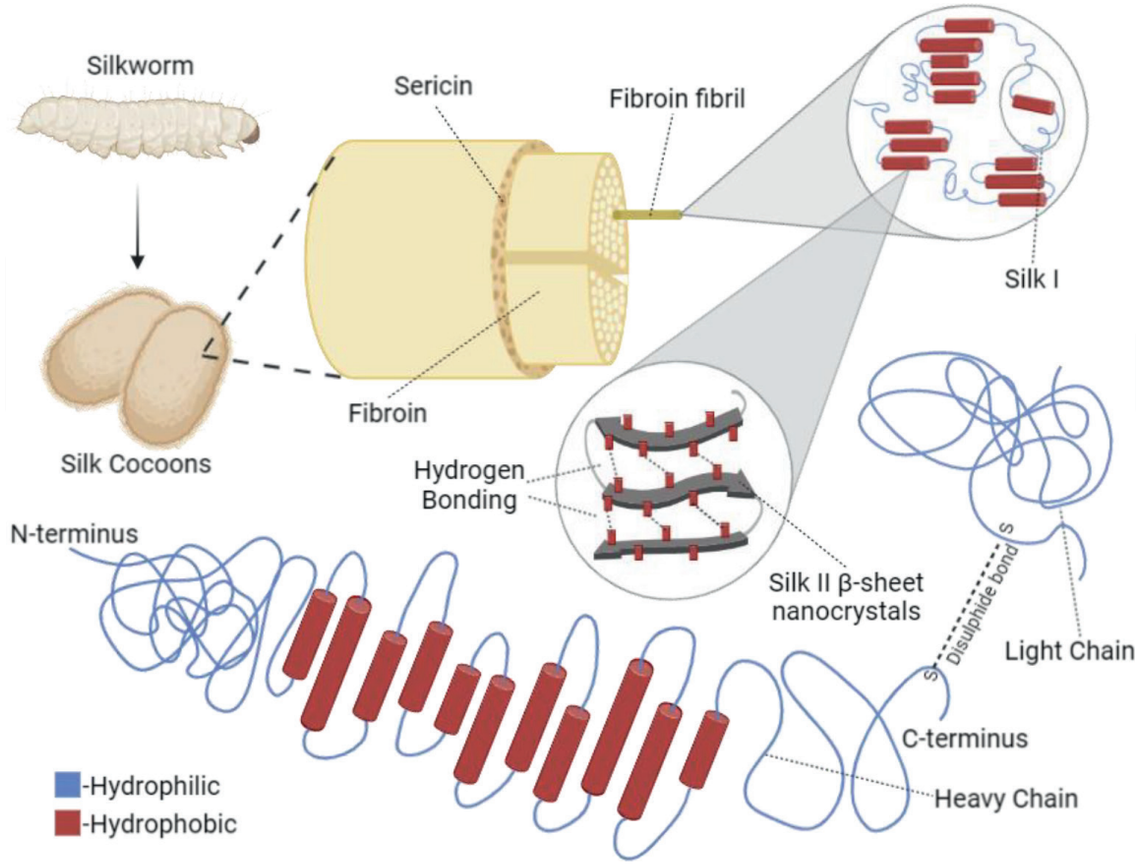

Figure 1.
Cross-sectional and structural composition of silk fibers. Created with BioRender.

structure once treated with organic solvents, electromagnetic fields, or physical spinning environments. Unlike silk I, silk II contains an antiparallel β-sheet/crystal molecular model which has hydrogen side chains from glycine and methyl side chains from the alanines resulting in higher stability and both water and solvent insolubility. Silk III prevails at the water/air interface (**Figure 1**) [16–18].

2.2 Properties

In the past centuries, silk has been used as a natural material for the fabric industry, but recently SF nanoparticles have been considered as a potential alternative carrier for anticancer drug delivery because of its physicochemical, mechanical, and biological properties [11]. Compared with other materials, SF is characterized by its high tensile and breaking strength, elongation, stiffness and ductility [19]. High stability allows maintenance of SF chemical structure even at high temperatures (around 250°C) [20]. In addition, compared with other biological polymers like polylactide (PLA), poly(lactic-co-glycolic acid) (PLGA), and collagen, silk fibroin shows excellent biocompatibility and lower immunogenic response [21–23] making it a very suitable material for drug delivery and tissue engineering. It is commonly used as a suture material, demonstrating comparable immunogenicity and biocompatibility to other natural and synthetic suture materials [24]. Moreover, it has consistently shown to be non-toxic and fully resorbable, this includes any degradation products [25]. Surprisingly, SF has been proved to have an intrinsic anti-inflammatory ability which could be used in the treatment of inflammatory bowel disease [26].

Degradability is another important property of silk fibroin. The degradation rate of SF is related to the molecular weight, the degree of crystallinity, morphological features and crosslinking [27]. This affords a tunability from seconds to years, another unique feature of SF. As a result of these properties, SF has been used in different nanosystems, including films, sponges, hydrogels, tubes [28]. Here, we focus on silk nanoparticles.

3. Silk nanoparticles

3.1 Synthesis of silk nanoparticles

Silk nanoparticles are an excellent candidate as a carrier for drugs, targeted therapies and contrast agents. They are synthesized from regenerated SF through a variety of methods based on their self-assembly behavior. Among all of the approaches, desolvation (**Figure 2**) is the most common method for SF nanoparticle synthesis, in which dissolving agents including ethanol, acetone, dimethyl sulfoxide (DMSO) and methanol could be used to dehydrate and package the silk chain, leading to the change from silk I to silk II structure and forming SF nanoparticles [29]. During the desolvation method, lipophilic active drugs (e.g. curcumin and 5-fluorouracil) can be easily dissolved in the organic solvents allowing nanoencapsulation of anticancer drugs [30]. The main challenge of the desolvation method is to find the ideal SF/dissolving agent ratio and ensure adequate mixing during SF nanoparticle formation which plays an important role in the control of the nanoparticle size.

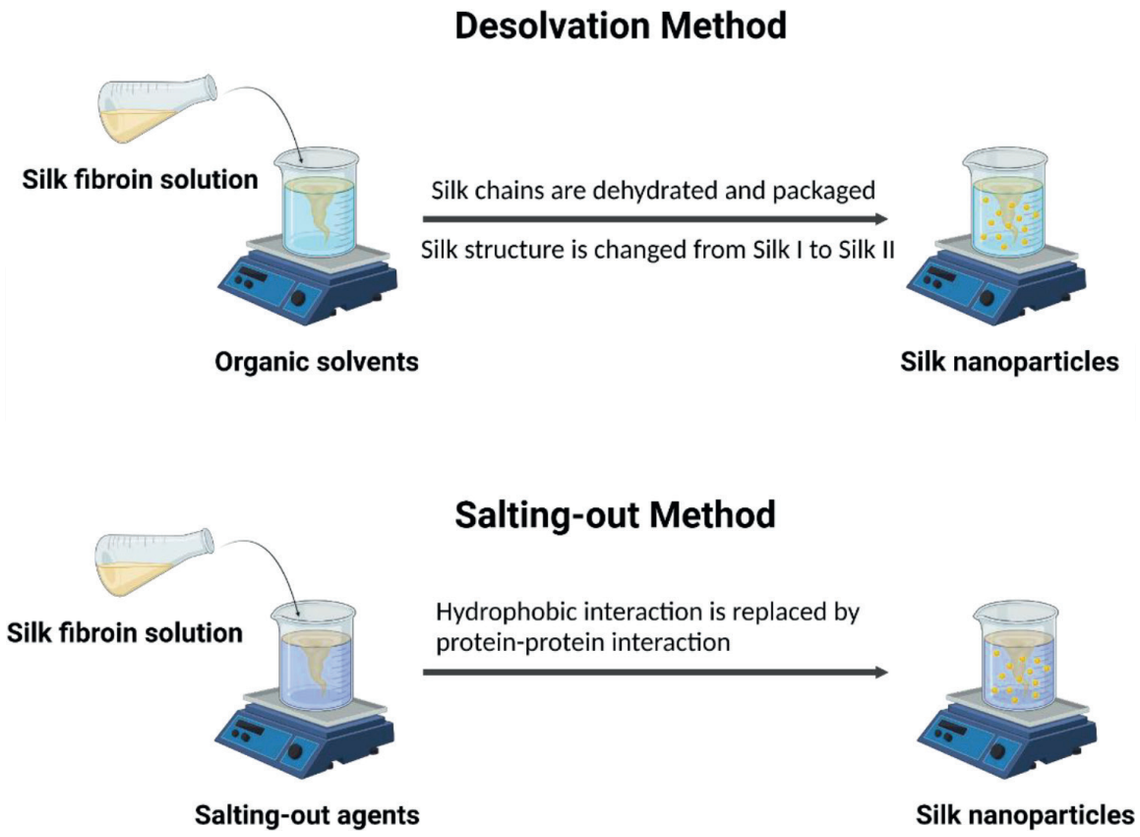

Figure 2.
Representation of the desolvation and salting-out method for the production of silk fibroin nanoparticles. Created with BioRender.

Salting-out (**Figure 2**) is another widely used method in the preparation of protein-based nanoparticles by removing the water barriers between protein molecules and increasing the interactions between proteins, leading to aggregation and precipitation from the solution [31]. The salt, pH, and ionic strength are major factors influencing the yield, particle morphology, zeta potential, and nanoparticle stability [30]. The salting-out method has advantages in avoiding the usage of toxic solvents, therefore maintaining the activity of the protein. However, the synthesized particle size is relatively large (500–2000 nm), and the high amounts of salts are difficult to remove [29].

The electrospraying method uses electrical forces to obtain liquid atomization where the liquid flowing out of a capillary nozzle is dispersed into small droplets by an electric field [32, 33]. Similar to the desolvation method, the main limitation of electrospraying is the organic solvents used during the synthesis that may damage the bioactivity of their cargo (e.g. enzymes, genes, and cell vitality) [33]. Other silk nanoparticle synthesis methods which are not used widely used include supercritical fluid technologies [34], mechanical comminution [35], capillary-microdot technique [36], and microemulsion [37].

3.2 Functionalization

Many drug delivery systems lack a targeting mechanism, resulting in poor accumulation at tumor targets. To achieve efficacious concentrations at the tumor, saturating drug doses are often administered which can lead to non-specific or toxic effects. Nanoparticles have been designed to overcome this problem by delivering the drug to specific tissue instead of a more generalized treatment. Currently, four different targeting mechanism have been explored; passive targeting, targeted recognition, triggered release and guided delivery (**Figure 3**). Historically, the enhanced permeability and retention (EPR) effect (a unique phenomenon of solid tumors) has been utilized whereby nanoparticles can extravasate through the leaky blood vessels of the tumor tissue without the need for surface modification [5, 38, 39]. Some surface modification that affects circulation time may also indirectly affect passive targeting such as PEGylation of the nanoparticles. This is due to EPR effect increases proportional to the circulation time [40]. However, some tissues may also contain fenestrated blood vessels resulting in a similar effect on nanoparticles, causing them to accumulate there [5]. Additionally, the tumor microenvironment varies depending on the tumor type and passive targeting may not be as efficient in that particular condition.

Targeted recognition involves the use of targeting molecules as an attachment to the drug-loaded nanoparticles. Targeting molecules such as ligands have high specificity to receptors and other cancer-specific target molecules available on the surface of cancerous tissue such as glycans [4]. Conjugation of nanoparticles with ligands such as transferrin, folic acid, enzymes, antibodies and other macromolecules has demonstrated enhanced uptake of nanoparticles by cancer cells [5, 39]. However, they still rely on passive accumulation at the tumor site.

Traditional cancer treatment methods suffer from a lack of specific regional and temporal activation leading to off-target effects. The concept of smart nanosystems uses the intrinsic and environmental differences between normal cells and cancer cells to trigger activation or release of drugs at a tumor site [41]. Intrinsic activation strategies include using the differences in pH, enzyme expression level, the concentration of membrane proteins and soluble molecules between healthy cells and cancer cells to trigger drug release at a specific location in the

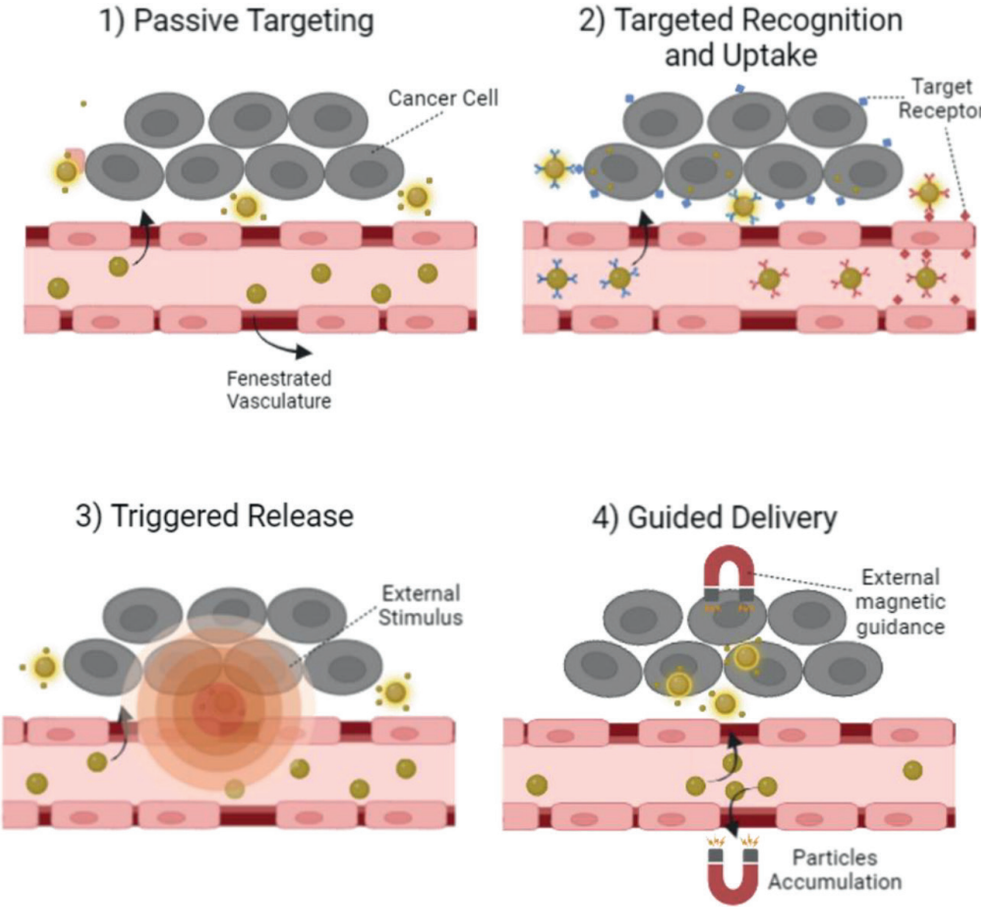

Figure 3.
Schematic of different drug targeting approaches for nanoparticles. Created with BioRender.

body [5, 41–43]. Extrinsic activation includes using ultrasound, magnetic field, light and photodynamic therapy to provide an activatable system with less toxic, safe, and minimal adverse effects [44–46]. At the moment, the development of nanoparticles activation strategy is not only satisfied with a single treatment but also presume a multimodal trigger system. Therefore, the synthesis and analysis of multifunctional nanomaterials have been extensively researched *in vitro* and *in vivo* for cancer [47, 48].

The above strategies, however, lack a navigational force to the desired target as well as the ability to penetrate tumors beyond diffusion limits. Magnetic tumor targeting, using magnetic carriers and an external magnetic field is demonstrating promise for enhanced tumor accumulation of chemotherapy [49] and virotherapy [50] following their systemic administration. In the presence of a magnetic field, peptide-functionalized magnetic silk nanoparticles demonstrated increased cellular uptake of an anticancer agent (ASC-J9) by HCT116 colorectal cancer spheroids [51]. Additionally, in an orthotopic model of breast cancer, magnetic targeting enriched Doxorubicin-loaded magnetic SF nanoparticles at the tumor site with a concomitant suppression of uptake by the liver, resulting in a significant decrease in tumor volume and survival [52]. The provision of an external driving force expands therapeutic use to a wide variety of tumors, independent of specific receptor expression.

3.3 Nanocarrier for cancer

3.3.1 Chemotherapy delivery

Due to excellent stability in the change of temperature [53], humidity [54], and pH [55], silk-based nanocarriers have been widely studied for the delivery of numerous chemotherapeutic substances such as doxorubicin, cisplatin, paclitaxel, 5′-fluorouracil, and floxuridine for cancer treatment (**Table 1**). In addition, silk nanoparticles can also be used in the delivery of natural plant-derived therapeutics including curcumin [65], celastrol and triptolide [70], which are limited by their poor water solubility. To reduce systemic toxicity and adverse side effects of chemotherapeutics, targeted delivery can be achieved by conjugation of various targeting ligands to the silk material (as described above), recognizing overexpression of particular epitopes on the surface of target cells [71]. For example, Lei Huang et al. [63] designed a folate (FA) conjugated silk nanoparticle double loaded with doxorubicin (FA-SFPs-DOX-DOX), which provided a pH-dependent targeted drug release, lasting for over 30 hours. This

Therapeutic agent	**Silk source**	**Functionalization**	**Cancer Type**	***In vitro* model**	***In vivo* model**	**Reference**
Doxorubicin	Silk Protein	RGD peptide	Breast	4 T1	4 T1 (F)	[56]
	Silk sericin	—	Breast	4 T1	—	[57]
	B. mori	—	Breast	4 T1	4 T1 (F)	[58]
	B. mori	—	Breast, colon	4 T1, MCF7, Caco2-BBE	4 T1 (OT)	[59]
	Spider silk	Herceptin	Breast	D2F2, D2F2E2	D2F2, D2F2E2 (OT)	[60]
	RSF	Hyaluronic acid	Lung	A459	A459 (X)	[61]
	B. mori	—	Breast	4 T1	4 T1 (F)	[62]
	B. mori	Folate	—	KB, C2C12	—	[63]
Cisplatin	*B. mori*	—	Lung	A549	—	[64]
Curcumin	*B. mori*	—	Breast	MDA-MB-231	—	[65]
Curcumin/ 5-FU	*B. mori*	Hyaluronic acid	Breast	4 T1	4 T1 (F)	[66]
	B. mori	—	Breast	4 T1	4 T1 (OT)	[30]
5-FU	RSF	—	Colorectal	HT-29	—	[67]
Paclitaxel	*B. mori*	—	Liver	H22	H22 (F)	[68]
	B. mori	—	Gastric	BGC-823, SGC-7901	BGC-823 (X, F)	[69]

RSF = regenerated silk fibroin, 5-FU = 5-fluorouracil, F = flank, OT = orthotopic, and X = xenograft. All in vivo models performed in mice.

Table 1.
Silk nanoparticles as nanocarriers for chemotherapy.

study demonstrated the importance of the target ligand, with FA-SFPs-DOX-DOX inducing greater cytotoxicity against HeLa cells compared with SFPs-DOX-DOX.

3.3.2 Peptide and protein delivery

Silk-based nanocarriers can also bind with peptides and proteins improving their *in vivo* stability. Lactoferrin is one such protein showing anti-cancer properties, whereby apo-bovine lactoferrin loaded silk nanoparticles induces significantly higher internalization and cytotoxicity towards the MDA-MB-231 and MCF-7 breast cancer cell lines [72]. Peptide-based cancer vaccines are another important therapeutic agent in cancer treatment. However, peptide vaccines suffer from short *in vivo* stability caused by proteolytic degradation and rapid clearance from the bloodstream [73]. A silk nanoparticle delivery system is an effective way to improve the bioavailability and stability of peptide tumor vaccines [74]. Using engineered spider silk nanoparticles a peptide-based vaccination resulted in successful activation of cytotoxic T-cells, without unspecific immune responses [75]. How these antigens are delivered can also influence the developing vaccination response. It is thought that controlled, persistent antigenic signals elicit stronger responses than transient bolus vaccine exposure [76–78], such as that seen with microneedle skin patches. Microneedle vaccines exploit the skin's accessibility, both in terms of ease of administration as well as access to densely populated areas of antigen presenting cells. Silk microneedles therefore represent an attractive prospect due to their tunable release kinetics of encapsulated cargo as well as their overall biodegradability. This system demonstrated a > 10-fold increase in ovalbumin (OVA)-specific T cell and humoral responses in C57/Bl6 mice when compared with parenteral immunization [79], warranting further investigation.

3.3.3 Gene delivery

Viral vectors are traditional carriers for gene delivery, however, their drawbacks in inducing high systemic toxicity and immune responses limit their application in cancer treatment [22]. Thus, non-viral vectors have emerged to address challenges surrounding improving transfection efficiency, target specificity and cytotoxicity [80]. Among various materials, silk-based nanocarriers have been reported to provide biodegradability, biocompatibility, high transfection efficiency, and DNase resistance in gene delivery [9]. Through genetic engineering, the transfection efficiency of silk nanoparticles could be further improved. Numata et al. [81] combined silk protein-based nanocarriers with poly(L-lysine) (PLL) for gene delivery, resulting in improved transfection efficiency of pDNA in human embryonic kidney (HEK) cells. Additionally, to further enhance target specificity of the silk-based gene delivery system, they included tumor homing peptides (THP) [80]; F3 peptide (specifically targeted towards nucleolin expressing tumor and endothelial cells) and Lyp1 peptide (shows target specificity towards the p32 receptor overexpressed in tumor cells) [82, 83]. The use of cationic polymers with silk-based nanocarriers is another popular strategy due to their high cellular uptake efficiency, good water solubility, excellent transferability and easy synthesis [84]. Polyethyleneimine (PEI) is one of the commonly used cationic polymers which easily assembles with gene therapies and demonstrates improved cellular uptake due to their positive charge [85]. Song et al. [65] designed magnetic-silk/PEI core-shell nanoparticles for targeted delivery of c-myc antisense oligodeoxynucleotides (ODNs), which had high uptake efficiencies and significantly inhibit the growth of MDA-MB-231 cells.

3.3.4 Diagnostics and theranostics

In addition to therapeutic delivery, silk nanoparticles are also promising as non-invasive imaging components and provide an opportunity to augment existing imaging modalities for diagnostic purposes. These modalities are often limited by inadequate contrast between healthy and diseased tissue, contributing to failure to detect signs of illness, particularly early signs. Silk nanoparticles can be used as a vehicle for loading magnetic resonance contrast agents, overcoming agglomeration limitations of magnesium oxide nanospheres [86]. Alternatively the use of fluorescent dyes and carbon dots for modification of the silk fibroin itself has applications for live cell imaging or visualizing degradation of silk-fibroin implants [87]. The production of fluorescent silk nanoparticles can be created using simple dyes, chemical modification of the fibroin, conjugation or entrapment of fluorescent proteins and even doping the silkworm larvae's diet with fluorescent dyes such as rhodamine and fluorescein [88]. Additionally, carbon quantum dots (CQDs) generated from SF are strongly fluorescent, resist photobleaching, can be further functionalized [89–91] and in comparison to other colloidal materials, avoid the need for toxic heavy metals. However, this process does require controlled and pressurized heating of the fibroin for carbonization into CQDs.

By combining these imaging modalities with their role as a drug carrier, silk nanoparticles are becoming an important theranostic device. Theranostics is an approach that combines cancer treatment and diagnosis, in which efficient imaging guidance of therapy is necessary for detecting the drug loading, targeted delivery, and release[123]. For example, Levodopa (a PTT agent) and manganese dioxide particles (a contrast agent) were formulated with silk sericin from *B. mori* cocoons to create a one-step method for MRI-guided photothermal therapy [92]. The composition of spheres made of spider silk and iron oxide nanoparticles have also demonstrated drug loading and release capacity with potential to be used in both hyperthermia and magnetic resonance imaging (MRI) applications combined with drug delivery against tumor cells [93].

4. Conclusion

Nanoparticles used for drug delivery require desired physicochemical properties including size [94], shape [95], structure [96], rigidity [97], and surface modification [98]. Translation and application of nanoparticles, including silk, to the clinic must first overcome a number of challenges including their heterogeneity, reproducibility and upscale production. Silk derived from different sources will possess different amino acid sequences and morphology, whilst LPS contamination of recombinant silk is a major obstacle for progression to clinic, requiring careful characterization of its toxicity and immunogenicity. Additionally, traditional nanoparticle preparation methods involving breaking down of large particles, nanoprecipitation, or self-assembly of monomers, suffer from wide size distribution and large batch-to-batch variability [99]. To obtain more stable and controllable nanoparticles, microfluidics has emerged for manipulating tiny fluids (1×10^{-9} L–1×10^{-18} L) in micro-channels with dimensions of tens of micrometers [100]. Several flow patterns including laminar flow, turbulent flow and droplet flow could be achieved under microfluidic control with potential to enhance fluid mixing, reduce reagent consumption and batch-to-batch variations [101, 102]. Interestingly, the introduction

of superparamagnetic magnetic nanoparticles (used to provide magnetic targeting capabilities) during the SF formation process provided artificial regulation of this process as well as drug entrapment, preventing agglomeration of SF and resulting in uniform, spherical nanoparticles [52]. Ultimately, SF nanoparticles provide many attractive properties for multi-functional drug delivery strategies but future use relies on reliable, reproducible manufacture to ensure appropriate comparisons can be made for their translation.

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