Soft Matter

REVIEW

Silk nanoparticles: from inert supports to bioactive natural carriers for drug delivery

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Silk fibroin (SF) has a long history for the development of medical devices, scaffold and pharmaceutical dosage form while, only recently, silk sericin (SS) has gained attention as a natural polymer mainly in cosmetic field. Both materials have been studied and employed for drug delivery nanosystem production, showing biocompatibility, controllable biodegradability and non-immunogenicity, even if sometimes they require combination with other polymers to be processed. In this review, we focused on silk proteins as bioactive natural carriers, since they show not only optimal features as inert excipients, but also remarkable intrinsic biological activities. SF has an anti-inflammatory property, exploitable for the treatment of several diseases, while SS presents antioxidant, anti-tyrosine, anti-aging, anti-elastase and anti-bacterial features. We give a complete overview of both SF- and SS-based nanosystems, with particular attention on production techniques

Introduction

Nanotechnology is moving quickly to become a part of our everyday life, with a few examples including carbon nanotubes employed in lithium ion batteries (1), nanoclays in plastic glasses, and titanium oxide nanoparticles in sunscreens. Exactly 58 years ago (December 29th 1959), Richard Feynman, during the annual meeting of the American physical Society at Caltech (California, USA), introduced an unexplored nanotechnological concept with a speech entitled “There’s plenty of room at the bottom” which changed the way of thinking with scientific research and thus our lives (2).

Today, nanotechnology is at the cutting edge in a plethora of fields including physics, chemistry, pharmaceutics, biology, medicine, electronics and engineering. Nanomedicine simply means to apply a nanotechnological approach to conventional medicine in order to improve clinical results. Nanosystems are widely employed in research studies including lab-on-a-chip, as diagnostic tools (3), as nanodevices such as nano-needles (4), as therapeutic agents for personalized medicine purposes or in form of nano-chemotherapy and thus improving patient compliance, ameliorating both specificity and sensitivity of the therapy (5), and improving pharmaceutical formulation performances.

The major challenge of drug delivery systems (DDS) is to deliver the drug to the targeted location at the right time and at the right dosage (6). Today, nano-drug delivery systems (NDDS), namely nanoparticles or nanospheres, both synthetic and natural-based, have been developed to overcome the major limitations related to conventional DDS; for example, exploiting their enhanced permeation and retention effect (EPR), nanosystems are able to passively reach cancer tissues. NDDS are employed to ameliorate both free Active Principle Ingredient (API) technological features, increasing their cargo loading, controlling drug fate, avoiding off-target dissemination, thus obtaining a controlled release and limiting cytotoxic effects mainly due to free drug presence and correlated side effects (7) (8). NDDS are easily taken up by cells due to their small size, however this characteristic leads to their quick clearance from the bloodstream.

Generally, NDDS “smartness” is related to their tunable sensitivity in responding to different stimuli such as variations in temperature, pH, magnetic field, light, and salt concentrations (9). For example, the introduction of poloxamer or chitosan moieties leads to temperature responsiveness; conversely, the functionalization with polyacrylamides makes the polymer actively responsive to light stimuli (10). Unfortunately, these smart systems also suffer from several limitations including decreased mechanical strength, potential cytotoxicity (as occurs for polyacrylamides) drug instability, chance of drug leakage or retardation of drug release (11).

Scientists are always looking for innovative smart nano-platforms: they must be able to interact exclusively with targeted tissues or cells, in which they have to release API in a controlled and sustained manner to achieve a therapeutic effect. Biological carriers, such as mesenchymal stem cells (MSCs), have recently been proposed as smart DDS and NDDS due to their biocompatibility and stability in physiological conditions (12). MSCs show the spontaneous migration towards injured tissues or cell-secreted membranous vesicular systems (i.e. extracellular vesicles (EVs)) while avoiding tumorigenic risks related to replicant cell manipulation (13, 14). Some authors have proposed a combined approach between technological and biological nano carriers. Perteghella et al. proposed an

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innovative approach based on the combination of curcumin-loaded SF nanoparticles and MSC-derived extracellular vesicles, through which it is possible to harness beneficial properties from both SF nanosystems and EVs (15).

1. Silk structure

Silk is natural fiber produced in the glands of arthropods including silkworms, scorpions, bees, mites and spiders (Nephila clavipes), with similarities and differences in both structure and properties (16). In particular, Bombyx mori silkworm silk (Bombycidae family), also named mulberry silk, is the most renowned, characterized and employed silk; on the other hand, Saturniidae (Antheraea mylitta) produces non-mulberry silk. Otherwise, spider silk is not commonly employed due to the lack of commercially established supply chains, as occurs for sericulture, due to the smaller yield and wilder nature of spiders compared to silkworms (17).

B. mori silk cocoons are constituted by two proteins, namely SF and SS, closely linked but different in terms of structure and properties. Generally, the percentage of SF is about 65-85%, while SS ranges from 15% to 35% (18) (19) (20). The fibroin fibrous core is composed of a heavy (about 350 kDa) and a light (about 25 kDa) chain, linked together by both a single disulfide bond and a 25 kDa glycoprotein, entirely covered by a sericin glue-like structure, holding them together. The light chain is constituted by no-repeated sequences; contrastingly, the heavy chain is enriched in glycine (Gly) (46%), alanine (Ala) (29%), serine (Ser) (12%) and tyrosine (Tyr) (5%) creating typical highly-repeated sequences of hexapeptides (Gly-Ala-Gly-Ala-Gly-Ser) or dipeptides (Gly-Ala/Ser/Tyr), directly related to the secondary structure (19) (21). Moreover, charged amino acids are present in fewer quantities, such as lysine, arginine, glutamate and aspartate (22) (23) (24).

SS has a globular structure and is enriched in aspartic acid (16%) and serine (37%), polar amino acids (25) able to limit the shear stress during the SF spinning formation process. In nature, the SS coating is important for the silkworm's survival by protecting the larva from atmospheric hazards (26). Unfortunately, the simultaneous presence of SF and SS dramatically decreases the biocompatibility of this biomaterial, thus limiting silk applications in its native form. For this reason, a degumming process is required to remove the SS coating from SF core, then the obtained degummed SF and SS solution are processed for the development of different DDS (19). Since SS is a water-soluble protein, it is easily removed by using boiling water or using alkaline and neutral proteases (27). The addition of detergents could alter the SS structure, molecular weight and functionalities (22). The degumming of cocoons in autoclave represents an effective strategy for obtaining an aqueous sericin solution, thus reusable for other purposes, but it is unable to remove all sericin, resulting in an impure SF. In fact, SS is constituted by three layers, characterized by different degrees of solubility due to their different amino acid composition (described in-depth below) (18).

It is crucial to underline that SF molecular weight (MW) varies in relation to the degumming process time and procedure. Generally, a MW of about 100 kDa is obtained after a degumming with Na2CO3 for 30 minutes (28). Moreover, the extraction time of SF strongly influences the nanoparticle creation, since it directly regulates the degradation of silk chains and amorphous regions (29). Regenerated SF is prepared by solubilizing it into chaotropic salts: the most common procedure is based on the use of highly concentrated lithium bromide solutions (LiBr), but other salts such as calcium chloride (CaCl2) or lithium thiocyanate (LiSCN) are employed (30) (31) (32). Recently, Zheng and colleagues (2016) demonstrated that Ajisawa's reagent, composed of CaCl2, ethanol and water mixture, is less expensive than LiBr, although it leads to SF aggregation during the subsequent dialysis steps, employed to remove solvent and salt (33).

Silk has been exploited for centuries due to its remarkable properties and today represents a suitable biomaterial for creating a spectrum of biomedical tools, starting from tissue engineering scaffolds to DDS (34) (35). In particular, nanoparticles could act as reservoir systems, achieving a sustained and controlled drug release with both high loadings and encapsulation efficiency (36) (37). For these reasons, several scientific research groups started working on silk-based nanosystems looking for new promising perspectives and therapeutic applications.

2. Silk fibroin: structure and properties

Since Galen's time, silk fibroin (SF) found its first application as a surgical suture material (38). Overtime, SF has been employed in textile, cosmetic and biomedical fields and more recently as an innovative biomaterial in electric, optical and food industries (19).

Native SF fibers and regenerated SF solutions have already been processed with different approaches to obtain several systems, including films (39), sponges (40), microparticles (41), non-woven mats (42), hydrogels (43), tubes (44) and nanoparticles (45). Notably, SF was employed for the development of rhodamine B-loaded microneedles by thermal drawing, thanks to its great thermo-stability. SF shows great stability even when processed at extreme temperatures (higher than 250°C) without affecting its stability and structure (46). Moreover, subsequent treatment with methanol influenced either SF microneedle mechanical strength and drug release (47).

In addition to thermostability, SF retains an excellent combination of mechanical and biological features, two features hard to find simultaneously in other natural or synthetic materials. Although silk evokes the idea of softness, it represents one of the most robust biomaterials in nature, retaining a toughness higher than Kevlar (para-aramid synthetic fiber employed for bulletproof vest production), a high tensile and breaking strength and elongation, stiffness and ductility (16). SF obtained from B. mori retains both the highest tensile strength and modulus when compared to other silkworm family silks (48). All of these unique features made SF a biomaterial officially recognized by Food and Drug Administration (FDA) for the development of a plethora of nanotechnological tools (49).

Considering its secondary structure, SF exists in three different structural models named Silk I, Silk II and Silk III. The first is mainly composed of α-helix domains, mixed with random coil and β-turn structures, and represents a metastable and water-soluble conformation. Conversely, Silk II is characterized by an antiparallel β-

sheets/crystal molecular model, thus showing a higher stability and both water and solvent insolubility (50) (51). Finally, Silk III structure prevails at the water/air interface (52).

Native SF exists in Silk II conformation, while regenerated SF is generally in Silk I. The secondary structure can be tuned by different processes: the treatment with alcohols, including ethanol and methanol, the physical shear, the exposure to water annealing process, electromagnetic fields or autoclaving lead to SF conformational transition in its secondary structure, characterized by a dominant Silk II structure (19) (40) (53) (54). For example, the water annealing process is a physical aqueous-based approach able to promote the SF conformational transition to β-sheets, avoiding the use of organic solvents (green method); moreover, this method is efficient for food coatings (55).

The peculiar self-assembly behavior of SF, from a molecular level to a hierarchical structure, is commonly exploited for creating micro-nanospheres, micro-nanoparticles and micro-nanofilaments, even if the mechanism has been not completely understood (56): hydrophilic polymers cause the phase separation, while water miscible solvents (such as methanol, ethanol and acetone) induce SF precipitation. This process, leading to nano-fibrils and micro-nanoparticle production, is influenced by changing molecular mobility, charge, hydrophilic interactions and concentrations (57). For example, Bai and colleagues demonstrated that SF nano-fibrils have been developed by regulating the self-assembly mechanism during repeated drying-dissolving process steps (58).

The US Pharmacopeia (USP) lists SF as a non-biodegradable material, since it preserves more than 50% of its tensile strength after 60 days of implantation in vivo, yet this statement disputes what is reported in the literature by several authors (59) (60). This different behavior depends on the type of SF (native or regenerated status), type of system (e.g. porous or not) and implantation site. 3D scaffolds, based on regenerated SF, completely biodegrade within one year via host immune system activity (61). Since SF is a protein, it undergoes a proteolysis pathway lead by the host immune system reaction and also by the protease enzymes at the implantation site, which are responsible for the amino acid chain leakage. The implantation site, the animal model and the type of protease influence the SF biodegradation mechanism and rate. For example, protease XIV induces higher mass reduction of SF scaffolds than α-chymotrypsin (62). In addition, the inflammation raised by the SF scaffold itself promotes its degradation. Moreover, the SF conformation strictly influences the degradation rate: high β-sheet content increases scaffold permanence at the implantation site. Nevertheless, even if SF undergoes a biodegradation pathway, it does not show issues related to the release of acidic by-products as occurs when employing synthetic polymers, such as poly lactic acid (PLA), poly glycolic acid (PGA) and their co-polymer poly lactic-co-glycolic acid (PLGA) (63).

SF is universally recognized as a biocompatible material, its tolerability is comparable to that observed for synthetic materials (PLA, PGA and PLGA). Inflammatory responses and immune-events, such as hypersensitivity, are mainly linked to the presence of SS residues that still cover the SF after the degumming process (64).

Notably, in relation to all the described features, SF scaffolds can be sterilized in autoclave, a procedure not possible for other protein-based biomaterials including collagen without affecting their morphology and properties. Conversely, organic solvents such as ethylene oxide or γ-irradiations could produce conformational changes (65).

The presence of abundant carboxyl and amino groups in the side chains allows for suitable bio-functionalization, thus improving SF properties such as cellular adhesion and specific targeting (21). Surface modifications influence both cellular behavior and biological activity; for example, the presence of the arginine-glycine-aspartic acid (RGD) sequence facilitates cell adhesion and proliferation (66).

2.1 Silk fibroin nanoparticles

Nanoparticles are obtained from regenerated SF using a broad variety of methods, exploiting silk self-assembly behavior ruled by hydrophilic and hydrophobic chain interactions (50) (Figure 1).

Encapsulated Active	Silk Fibroin - PTX	Silk Fibroin - DOX	Silk Fibroin - Cisplatin	Silk Fibroin - Curcumin
Production Technique	Desolvation (ethanol)	Salting out (potassium phosphate 1,25 M)	Electrospraying	Microdot capillary
Dimensions	130 nm	130 nm	59-75 nm	100 nm
Therapeutic Effect	Superior anti-tumor activity than free PTX	Magnetic tumor targeting	Induced lung cell apoptosis	Efficacy against breast cancer
Reference	P. Wu et al., 2013	Tian, Jiang, Chen, Shao, & Yang, 2014	Qu et al., 2014	Gupta, Aseh, Rios, Aggarwal, & Mathur, 2009

Figure 1. Examples of silk fibroin nanoparticles loaded with different actives. Production techniques, nanosystem dimension and therapeutic effects are reported. PTX - Paclitaxel; DOX – Doxorubicin.

Notably, comparison studies of SF nanoparticles using mulberry and non-mulberry silks showed no significant differences (36). Since a protein is employed, it is possible to obtain SF nanosystems with a mean diameter ranging from 100 to 300 nm, depending on the employed technique (45).

A coarse technique employed for SF nanoparticle production is the milling procedure also known as mechanical comminution: a simple physical method based on chopping, grinding and crushing the SF degummed fibers into smaller systems (67). Unfortunately, this method required specific apparati (such as cutter mill, rotary milling and planetary ball milling) and long time period, and generally results in big nanoparticle aggregates with a wide size range (68). For these reasons it is not as commonly employed.

Desolvation represents the most common method for obtaining protein-based nanoparticles. Also known as simple coacervation, it is based on the reduction of the SF chain solubility in the presence of organic solvents such as acetone (29), ethanol (69), dimethyl sulfoxide (DMSO) (36) and methanol (70), leading to a phase separation (71). The organic solvent/dissolving agent leads to a sudden SF chain dehydration and packaging, leading to a change

from Silk I to Silk II conformation and providing the nanosystem formation. Thanks to the electrostatic repulsion with the negative charge, the nanoparticle aggregation does not occur (72) even if the SF concentration plays a crucial role in controlling the final nanosystem dimension. Therefore, finding the optimum SF/dissolving agent ratio is required. This easy process allows researchers to obtain nanosystems with controllable size; the inner core is composed of SF crystalline domains, while the core shell is characterized by the presence of hydrophilic tails (73).

The presence of organic solvents allows easy encapsulation of lipophilic active compounds by solubilizing them into the dissolving agents before carrying out the nanoparticle production. For example, curcumin- and 5-fluorouracil-loaded SF nanoparticles have been recently considered as innovative NDDS for breast cancer treatment (74). Notably, the nano-encapsulation of natural lipophilic drugs such as curcumin and resveratrol allows the drugs to overcome some of their typical hurdles including poor bioavailability and limited solubility, thus improving their therapeutic application potential (75).

The main drawback of dissolution is the presence of organic solvents, which must be removed by several centrifuge or strong dialysis cycles in order to avoid any cytotoxicity events.

In 2013, Wu and colleagues developed an innovative method to obtain nanoparticle production, exploiting the natural self-assembly of SF. Paclitaxel (PTX) ethanolic solution was added drop wise into the aqueous SF solution, limiting the amount of organic solvent and avoiding the use of surfactants. PTX-loaded nanosystems were round shape structures with a mean diameter of 130 nm and they were uptaken by gastric cancer cells, showing a higher antitumor efficacy than the systemic administration of free PTX (76). Seib and colleagues developed DOX-loaded SF nanoparticles by acetone nano-precipitation for the treatment of breast cancer and they showed a stimulus-responsiveness influenced by pH, highlighted in acidic conditions. Moreover, nanoparticles were taken up by breast cancer cells and DOX was released in a controlled manner once internalized in lysosomes (29).

Salting out represents another valid approach employed for SF nanoparticle production, even if it results in SF nanoparticles with higher dimensional range (500-1000 nm). This process starts with the preparation of a salting bath, based on potassium phosphate salts, typically K2HPO4–KH2PO4. The nanosystems resulted by the hydrophobic interaction between the SF protein chains and the decreased water molecules, which are replaced by protein-protein interactions (77) (45). The salt, pH, ionic strength and SF concentration influence the process yield, particle morphology, zeta potential, secondary structure, and nanoparticle stability. Alkaline pH is preferred over acidic to avoid non-dispersible aggregates. Otherwise, the secondary structure of nanoparticles obtained by salting out technology could influence the drug release (77). For example, Tian and colleagues developed doxorubicin (DOX)-loaded magnetic SF nanoparticles by a one step salting out approach, effective for targeted cancer therapy purposes. Briefly, magnetic nanosystems were added to the potassium phosphate bath before adding the SF solution, providing both the drug targeting, by the application of magnetic field, and the nanoparticle formation (78).

Electro-spraying provides the atomization of a starting SF solution via electrical field applications. A capillary nozzle passing through a high voltage field drops out silk solution. In this way, electrostatic forces break down the flow into small droplets. The obtained SF nanoparticles have to be treated with organic solvents to induce the conformational transition into the stable conformation (Silk II) (79). Moreover, the presence of two coaxial needles allows the creation of SF bubbles in which air is encapsulated within the protein solution (80). Kim et al. prepared SF microparticles via electro-spraying and demonstrated that both the concentration and the shear viscosity of SF solution played the most crucial role in affecting microsystem round-shaped geometry. Regular particles were obtained with short dissolution times (5 minutes in Ajisawa's reagent at 80°C) and high concentration (10% weight) of SF (81).

Characterized by a similar technology, the laminar jet break-up represents a non-harsh condition method, similar to spray-drying and electro-spraying techniques, although it avoids high temperature. The method consists of a vibrating nozzle that breaks the aqueous SF flow into small droplets. Due to high voltage application, droplets are then frozen in nitrogen. Wenk and colleagues observed that the SF nanoparticle diameter was directly influenced by the nozzle diameter and the treatment employed to obtain Silk II conformation. Moreover, authors encapsulated labile biological drugs, such as insulin-like growth factor, without affecting their properties due to the mild process (82).

An appealing technique is represented by supercritical fluid technology. CO2 is the most employed supercritical fluid mainly due to a-toxicity and low cost (83), for more details on this topic see review (84).

For the first time in 2009, Gupta et al. used the capillary microdot technique to create SF nanoparticles loaded with curcumin, characterized by a dimensional range lower than 100 nm and showing appealing anti-tumoral activity. The blending between SF and chitosan, proposed by the authors as a promoter of in vivo tissue regeneration, decreased both curcumin entrapment and release. The introduction of chitosan triggered the hydrophilic properties of nanosystems, resulting in a reduction of curcumin entrapment, which is a highly hydrophobic active, and consequently in a lower drug release (85).

Recently, an intriguing strategy for preparing SF nanoparticles directly during the dissolution step was suggested by Xiao and colleagues. Briefly, using specific concentration of a combination between formic acid and lithium bromide permitted to control the dissolution degree of SF degummed fibers. The obtained SF nanoparticles showed a spherical shape, no aggregation phenomena and a dimension of about 100-200 nm (86).

Generally, different approaches are used to load active molecules into SF nanoparticles: the encapsulation method, also named entrapment, is achieved by the solubilization of drug into bath in which nanoparticles are obtained. The absorption method, also known as the co-incubation technique, is based on loading of drug only onto nanosystem surface. Finally, the covalent binding is reached through the creation of drug-fibroin interactions of different natures, including chemical/physical reactions or crosslinking approaches (87).

SF nanoparticles appeared suitable NDDS for the encapsulation of labile drugs, such as growth factors. For example, vascular endothelial growth factor (VEGF) was loaded in SF nanoparticles by the co-incubation technique and a sustained drug release has been observed for over 3 weeks without any burst release (36). Similarly, bone morphogenetic protein-2 (BMP-2) was successfully encapsulated in SF nanoparticles, obtaining a controlled release of the encapsulated growth factor, making them effective for bone regeneration (37).

The absorption method is achieved by electrostatic bonds between the SF negatively charged nanoparticles and positively charged drugs by a simple incubation technique (36) (88), modifying the zeta potential of nanosystems (89). The surface decoration with positively charged moieties including chitosan or polyethylenimine avoids aggregation phenomena, thus improving cellular uptake (88).

The SF nanoparticle surface could be modified, thus improving drug loading, specific targeting and controlled release. It has many active sites, such as tyrosine residues and RGD sequences, which could be considered suitable anchorage sites for the bio-conjugation of surface molecules (90). PEGylation avoids nanoparticle aggregation phenomena by ameliorating their stability in suspension; this approach is commonly employed not only for SF, but also for all of the protein-based nanosystems (91). Wang et al. evaluated the stability of SF nanoparticles by coating them with glycol chitosan, N,N,N-trimethyl chitosan, polyethylenimine and PEGylated polyethylenimine by exploiting electrostatic interactions. Results showed that those coated with glycol chitosan and PEGylated polyethylenimine were easily re-suspended after lyophilization due to steric repulsion of the polymer chains (88).

Since SF is enriched in selective sites exploitable for PEG bonds, such as cysteine residues, this represents an interesting approach for overcoming the tendency of SF nanoparticles to aggregate after freeze-drying. Moreover, lyophilized nanoparticle aggregation problems could be overcame by adding cryo-protectants, such as glycine or mannitol, to the raw material (92).

Recently, RGD motifs have been added to SF nanoparticles to increase their targeting abilities to reach intestinal tissue; in particular, results showed that RGD functionalization reduces pro-inflammatory molecule release in bowel disease murine models (93).

SF is commonly employed as a coating material for scaffolds, exploited to ameliorate the biocompatibility of synthetic or metal materials and thus improving cell proliferation and adhesion to the support, as occurred for titanium bares (94) without altering the coated scaffold diameter and morphology (95). Similarly, SF-coated quantum dots (QD) allowed the combination of QD optical properties with SF biocompatibility, thus making them possibly employable in tumor labeling and intracellular tracking (96).

2.2 Silk fibroin composite nanoparticles

It is quite common in the nanotechnological field to combine polymers of different natures to exploit benefits from both of them. Although SF encloses a plethora of benefits, sometimes it is necessary to couple it in order to completely fulfill the desired target, even if the result could be worse. For example, SF-albumin blended nanoparticles were developed for methotrexate delivery. The presence of albumin improved SF mechanical properties and biodegradability, while drug nano-encapsulation avoided methotrexate cytotoxicity effects, including hepatotoxicity and pulmonary diseases (97).

Conversely, SF-chitosan non-covalently blended nanosystems were developed to deliver curcumin but the authors obtained worse results than when using the single polymer nanosystems. Curcumin entrapment reached 96% for SF nanoparticles and 64-73% for blended nanoparticles. This difference was also observed in terms of cellular uptake and efficacy against breast cancer cells (85). Contrastingly, SF-PEG blended nanosystems loaded with curcumin were produced for anti-aging purposes and provided a suitable support for tissue regeneration (39). Notably, the particle size of SF-PEG nanosystems was influenced by both PEG molecular weight and concentration. Moreover, when employing PEG with low molecular weight at a concentration between 40% and 60% w/v, hydrogels were formed (87). Similarly to PEG, poly vinyl alcohol (PVA), a FDA-approved polymer, was successfully employed for SF blended micro-nanosphere production because a natural phase separation occurred when the two polymers were combined to create films via a three step process (98). Briefly, blended SF-PVA solutions were employed to prepare dried films dissolved in water, and then centrifuged in order to obtain SF nanoparticles (97). Moreover, PVA is able to improve the nanoparticle superficial morphology in terms of smoothing and uniformity (99).

In this context, Numata and colleagues obtained a bimodular scaffold based on SF hydrogel, loaded with rhodamine B and containing SF nanoparticles loaded with fluorescein isothiocyanate (FITC). They observed a starting burst release for rhodamine B, whereas a sustained and controlled release for FITC was noted (100). This approach could be employed not only for dye models, but also to couple API with different biological properties.

2.3 Silk fibroin anti-inflammatory potential

Recent suggestions showed that SF possess an intrinsic anti-inflammatory therapeutic potential as already observed in a mice edema model (101) and in the treatment of inflammatory bowel disease (70), even if its mechanism has not been explained. SF anti-inflammatory activity could substitute traditional anti-inflammatory therapies based on NSAIDs, COX-2 and PGE-2 inhibitors, glucocorticoids, which can even worse the pathological condition. The biodegradation products of SF allowed to reduce the inflammatory conditions as reported in a mice edema model by suppressing the secretion of inflammatory markers including COX-2, IL-6, IL-1β and TNF-α. A synergic effect was observed when employing SF and the superoxide dismutase enzyme (101). Disaccording results were observed by Cui et al., where SF microparticles, obtained by grinding, up-regulated the production of IL-6, IL-1β and TNF-α, resulting in an inflammatory condition (67).

A synergic activity, accompanied with a boost of its anti-inflammatory activity, was observed in SF nanoparticles encapsulating resveratrol. The reduction of the expression of several mediators of inflammatory pathway, including chemokines, cytokines and adhesion molecules have been shown (70). The advantages to employ SF nanoparticles are that they can be easily targeted, thus adhering better to inflamed tissues compared than traditional drugs, increasing the therapeutic effect and limiting side effects and safety issues.

Its intrinsic anti-inflammatory activity, makes SF an effective active, as actually occurs for other natural-based compounds such as chitosan, and no more as an excipient employed for scaffold or drug delivery vehicle production.

3. Silk sericin: structure and properties

SS is a hydrophilic-based glycoprotein synthetized by the labial gland of B. mori, with a molecular weight of about 200 kDa. It consists of 18 types of amino acids including serine, glycine, glutamic acid, aspartic acid, threonine and tyrosine, with serine being the most prevalent (26) (102) (103). Notably, it becomes enriched in flavonoids and carotenoids, responsible for antioxidant and anti-tyrosinase activities (104). The typical globular shape of SS is due to the peculiar amino acid disposition and their properties confer it typical adhesive features. From an organic composition description, SS contains 46.5% carbon, 31% oxygen, 16.5% nitrogen, and 6% hydrogen (105).

Γ-ray studies allow us to observe that SS structure is divided in three parts according to their different solubility and amino acid structure; each layer is produced and secreted by cells localized in different gland regions of the arthropods. Sericin A is the external layer and is easily removed by degumming silk cocoons by hot water; sericin B occupies the middle space and has lower polarity even if it is contains the same amino acids as sericin A; finally, the inner layer is represented by sericin C, strictly close to the SF filaments and showing poor water solubility. In order to completely remove all three sericin layers, alkali solutions must be used during the degumming process (106).

SS secondary structure retains a combination of β-sheets and random coil domains, thus giving it a double behavior, albeit the latter is often predominant. As occurs for SF, SS crystallinity could also be induced by the exposure to organic solvent (e.g. ethanol), increasing the mechanical or physical shear stresses or via the crosslinking with glutaraldehyde (107) (108). Its polar structure explains its solubility in water, even if it depends on the ratio of random coil to β-sheet structures. Interestingly, SS is soluble in hot water, while its β-sheet conformation prevails at lower temperatures resulting in a gel formation (109).

SS has been considered for centuries as a silk waste product in textile industries, extracted and removed during the degumming process, in order to ameliorate the quality of SF. Only recently, SS has been considered for biomedical, cosmetic and pharmaceutical purposes (Figure 2) (26). It is difficult to recycle SS after classic degumming processes due to the presence of chemicals, although Wu and collaborators recently developed an innovative degumming process able to recover SS by employing ultrafiltration (110).

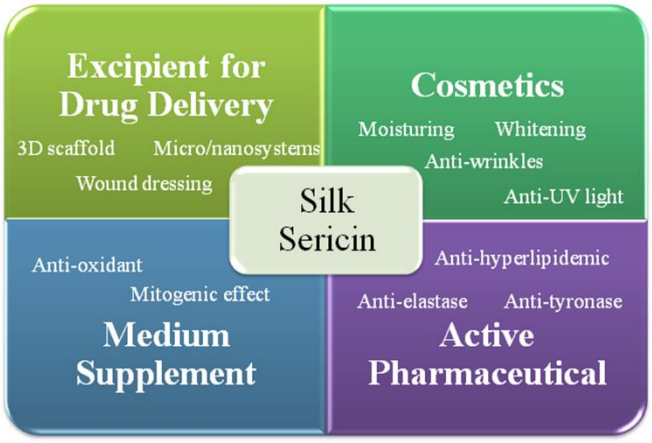

Figure 2. Possible application fields of silk sericin. Silk sericin is employed as an excipient for the development of drug delivery systems, in cosmetic field, as an effective active pharmaceutical ingredient or as a supplement for cell culture medium, as a substitute for fetal bovine serum.

SS shows several activities such as antioxidant (111), anti-aging (anti-wrinkle), anti-tyrosinase, anti-elastase (112), anti-bacterial (wound-healing), anti-coagulation (113), and cryo-protection. Moreover, SS shows a hypoglycemic effect by increasing insulin production and secretion as observed in rat models, and also by ameliorating diabetes-related complications (114) (115).

Otherwise, its addition to cell culture media improves cell proliferation, showing a mitogenic effect on several cell lineages and avoiding oxidative stress (116). In particular, Terada et al. reported positive results on four cellular lineages using low MW SS at a concentration between 0.01% and 0.1% weight/volume (117). SS is increasingly introduced as fetal bovine serum substitute as cellular supplement due to lower cost and no risk of zoonosis (118).

The potential of SS at a nanoscale level, for the design and development of nano-tools, has not been completely investigated. Based on our knowledge, results regarding the employment of SS "alone" for nanoparticle production, still lack, probably due to its instability influenced by extreme pH, water solubility and temperatures.

3.1 Silk sericin composite nanoparticles

SS is enriched in several polar side chains made of hydroxyl, carboxyl and amino groups providing a broad spectrum of functionalization, crosslinking and blending with other polymers to make it suitable for biomedical and therapeutic applications (Figure 3) (119). As already mentioned for SF, PEGylation technique was employed to improve SS nanoparticle stability in water, which is the principal goal during SS nanoparticle design. Notably, the introduction of PEG chains allowed the conformational change into SS structure, from random coil to β-sheet (120). Similarly, genipin, a non-toxic, natural cross-linker, was introduced to develop SS nanoparticles for the delivery of atorvastatin. Close to what was reported for SF nanoparticles, dissolution technique in ethanol was exploited to obtain SS nanosystems, followed by its bond with the cross-linker. Briefly,

nanosystems were characterized by a particle size of 166 nm with a high entrapment efficiency (91%) and a controlled drug release related to the cross-linking level; the higher the cross link with genipin, the slower drug release in 48 hours. Moreover, the anti-hyperlipidemic activity of SS potentiated the atorvastatin in vivo therapeutic potential maybe due to their synergic activity (121).

Figure 3. Examples of silk sericin nanoparticles combined with

Encapsulated Active	Silk Sericin - Atorvastatin	Silk Sericin - Fenofibrate	Silk Sericin - FITC & PTX	Silk Sericin - DOX
Composite Material	Genipin	Poly(ethyl cyanoacrylate)	Pluronic F-127 and F-87	Folate
Dimensions	166 nm	175 nm	100-110 nm	≈ 50 nm
Therapeutic Effect	Increase of anti-hyperlipidemic activity	Increase of bioavailability/efficacy of fenofibrate	Bimodular device for both hydrophilic and hydrophobic drugs	Tumor targeting and pH responsiveness (acidic conditions)
Reference	Kanoujia, Singh, Singh, & Saraf, 2016	Parisi et al., 2015	Mandal & Kundu, 2009	Huang et al., 2016

different molecules, including non-toxic crosslinkers such as genipin, Pluronic, folate-based targeted moieties and poly(ethyl cyanoacrylate).

SS-poly(ethyl cyanoacrylate) nanospheres were developed for delivery of fenofibrate, a lipophilic agent for lipid regulation. The presence of SS conferred muco-adhesive properties to poly(ethyl cyanoacrylate), creating stable NDDS able to stay for 6 hours in the gastric environment. These nanosystems were produced by interfacial polymerization, simply by adding dropwise cyanoacrylate to SS acidic solution, and then fenofibrate was loaded exploiting the incubation method (reported by the authors as soak method). The fenofibrate loading into SS nanospheres improved its oral bioavailability, increasing its in vivo activity and avoiding hepatic lipid accumulation (122).

SS nanoparticles blended with Pluronic F-127 and F-87 were developed as bimodular NDDS for both hydrophilic and hydrophobic drugs (FITC and PTX, respectively). The obtained nanosystems showed high stability in aqueous solutions and were rapidly internalized by cells due to their small dimensions of 100-110 nm. Moreover, results showed the efficacy of nanosystems to deliver and release the encapsulated drug, inducing cancer cell apoptosis (123).

Unfortunately, drawbacks of SS blended nanoparticles are not lacking: the presence of synthetic and no biodegradable polymers/cross-linkers in the formulation, necessarily added to develop and stabilize the SS, the rapid drug leakage, and the absence of targeting capabilities for tumor purposes are some of blending approach limitations (124). In relation to tumor targeting decoration, Huang and colleagues developed folate-SS-DOX nanoparticles: these nanosystems showed the ability to reach tumoral tissues by conjugating them with folate moieties. The presence of hydrozone bonds, by which DOX is linked to SS structure, showed a pH responsiveness resulting in a controlled drug release only selective for acidic conditions, which are typical of tumor tissues. Moreover, the interaction between highly hydrophilic SS and lipophilic drugs, such as DOX, resulted in nanoparticle formation due to the self-assembly behavior of SS (124).

Conclusions

There is still much to learn about the nanotechnological world despite the great progress that has been made within the past few years. The encapsulation of drugs into nanocarriers ameliorates their bioavailability and solubility profile, avoiding their biodegradation and instability. Silk proteins appeared as suitable and innovative natural-inspired excipients, exploitable for NDDS development in multidisciplinary fields, as well as bioactive compounds able to improve and support some API effects.

There are several in vitro or pre-clinical studies regarding silk nanosystems, showing their efficacy in the treatment of a broad spectrum of diseases, starting from cancer to inflammatory diseases. Unfortunately, these are not enough. Albeit silk-based polymers are capturing the interest of the scientific community, we are still far from their clinical application; this statement is enforced by the fact that until today there is no clinical trial based on silk-based polymers (searching for SF/SS nanoparticles, last access June 1st, 2017) Yet, different types of nanocarriers are commercially available, mainly liposomes and polymeric micelles, leading to good clinical results (125). The question arises spontaneously: “Why this diffidence in performing experiments on human beings?” “Why are clinical applications/trials based on silk nanosystems still missing?” One answer could be the fact that there are still several doubts concerning nanoparticle toxicity, regardless of their components, as occurred for Ferugulose ® (NC100150) and Resovist ® (126). Another possible response is that in vitro results related to silk nanoparticle employment do not completely match the prefixed target. Moreover, the tuning of a scalable and cheap production method could open the industrial production to silk nanosystems, thus opening their clinical applications.

Nevertheless, we believe that silk nanoparticles will find their clinical applications, opening innovative, undiscovered and exciting perspectives for the treatment of fatal diseases to future generations.

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