European Journal of Pharmaceutics and Biopharmaceutics 97 (2015) 392–399

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Review Article

From silk spinning in insects and spiders to advanced silk fibroin drug delivery systems

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ARTICLE INFO

Article history:
Received 22 December 2014
Revised 7 March 2015
Accepted in revised form 12 March 2015
Available online 20 March 2015

Keywords:
Silk fibroin
Drug delivery
Biomimicry
Crystallization
Spinning

ABSTRACT

The natural process of silk spinning covers a fascinating versatility of aggregate states, ranging from colloidal solutions through hydrogels to solid systems. The transition among these states is controlled by a carefully orchestrated process *in vivo*. Major players within the natural process include the control of spatial pH throughout passage of the silk dope, the composition and type of ions, and fluid flow mechanics within the duct, respectively. The function of these input parameters on the spinning process is reviewed before detailing their impact on the design and manufacture of silk based drug delivery systems (DDS). Examples are reported including the control of hydrogel formation during storage or significant parameters controlling precipitation in the presence of appropriate salts, respectively. The review details the use of silk fibroin (SF) to develop liquid, semiliquid or solid DDS with a focus on the control of SF crystallization, particle formation, and drug–SF interaction for tailored drug load.

1. Introduction

Silk either from spiders or from silkworms has been of high research interest during the last decades in a variety of scientific fields due to extraordinary properties, ranging from inkjet applications [1], bio-resorptive optics [2], implants [3], or drug delivery of proteins [4] and small molecules [5,6] in various application forms [7]. The silk fibers have a high toughness [8] comparable to high-performance polymers such as Kevlar [9] leading to the use of these fibers as suture material and for textiles since ancient times. Early studies linked the extraordinary mechanical properties of some silk threads to Nylon and reported linear mass density of the silk fiber from the ecribellate spider genus *Araneus* of 7.8 den (den = mass in grams per 9 km) and 8.7 den for Nylon [10]. A distinguishing feature of these silk threads from Nylon, however, is the tensile strength and ductility. One colorful description was that these threads could be extended up to a length of 80 km before these would break under their own weight [11]. The mechanical properties of processed silk differ from the native fibers [12–14] and are variable upon different treatment [15], but still make it an attractive material for musculoskeletal use and tissue engineering [16–22]. In contrast to manufacturing conditions of synthetic polymers, the natural process leading to silk is confined to physiologic conditions, i.e. all-aqueous processes at ambient temperatures, ambient pressure and within a moderate range of pH values. It is for these benign conditions that scaffolds, implants, or drug delivery systems (DDS) made from silk circumvent challenges arising from residual organic solvent or harsh conditions leading to stability challenges e.g. of co-formulated drugs [23–29]. Furthermore, the general cell- and biocompatibility has been demonstrated for sericin-free silk [30–33]. Another advantage is the stabilizing effect during storage and processing of co-formulated biologics in a silk matrix. This successful stabilization has particularly been shown for silk fibroin (SF) – the major component of whole silk for different proteins, as well as for small molecules [4,23,26,29,34–37] with excellent tolerability as demonstrated in pre-clinical studies [16,38,39]. The ability to tailor the biodegradability of silk [40] allows the balancing of mechanical stability and degradation rate, with SF being proteolytically degraded into uncritical degradation products [4,41–44].

2. Molecular structure and function of silk fibroin building blocks

The SF primary sequence, conformation and inter-molecular interaction allow for a perplexing self-assembly pattern which is key to understand the remarkable characteristics of this protein. This is based on a rather unusual amino acid composition, dominated by short side chained alanine, glycine, and serine, accounting for about 50–65% of SF [45–47]. Upon spinning, most of these are present in crystalline parts of SF, which was attributed to the

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http://dx.doi.org/10.1016/j.ejpb.2015.03.016
0939-6411/

tensile strength of the material, whereas intermediate amorphous regions account for the rubber like properties and ductility [48,49]. These properties are a function of absorbed water with a plasticizing effect [50]. The primary sequence is composed of hydrophobic blocks, combined with terminal and intermediate hydrophilic parts. The *in vivo* spinning process is tightly controlling the interaction of these hydrophobic blocks, thereby controlling the β-sheet formation and overall crystallinity. This control is at least in part by hydrophilic linkers positioned between the hydrophobic blocks. The charge of these linkers is a function of pH, ionic strength and composition of the surrounding phase. Furthermore, these linkers provide chain flexibility for protein folding and water solubility [51], allowing for micellar structures in which the larger and charged terminal blocks are discussed to point outside and interact with the surrounding phase [52]. The formation of micelles is favored by a folding of the SF molecules into loosely assembled structures allowing better interaction of the terminal hydrophilic blocks with the continuous phase and interaction of the hydrophobic blocks within the same molecules and/or with other SF molecules. At this stage, the molecules are still flexible and processes are reversible. With increasing interaction of these hydrophobic blocks the formation of β-sheets at least in part irreversibly locks the resulting structures. Increasing micelle concentration allows for increasing inter-micellar interaction, eventually leading to the formation of nanofibrils and liquid crystalline phases. These nanofibrils are further organized to fibers by stretching and progressing β-sheet formation. SFs from various species allow for this mechanism in spite of differences in their crystal forming regions, with e.g. *Bombyx mori* (*B. mori*) SF having glycine–alanine–glycine–alanine–glycine–serine repeats $(GAGAGS)_n$ and spider SF being composed of repeated poly(alanine) regions [53]. Each of these steps is governed by the composition of the surrounding phases, including pH, ionic strength of selected ions and protein composition comprising silk concentration. Aspects of these gained insights into the fascinating natural processes were recapitulated in laboratory experiments. The focus of this review is to delineate insights of the natural process in an effort to translate these for application in the design and manufacture of SF based DDS.

3. Silk processing in insects and spiders

Insects and spiders have developed highly specialized glands in evolution. Some spiders produce up to seven different types of silks [54–56]. The focus of the following mechanistic description is on the gland for major ampullate silk, from which dragline and radial web fibers are built. This gland is divided into three main functional zones A to C (proximal to distal). In spiders' A-zone the core of the fiber is formed from spidroin 2 (spidroins are fibroins in spiders), which is secreted by tall cells from granules [57]. In these granules, Spidroin 2 is in a predominantly random coil conformation, already showing some β-sheet and -turn patterns [54]. This dope solution containing small spherical droplets is transported to the B-zone. In the B-zone, spidroin 1 is secreted and coating the spidroin 2. Morphologically, the duct is now narrowing, which in turn increases the shear forces on the colloidal solution and pressure difference. It is this increase in pressure favoring conformational change to a β-sheet structure along with controlled acidification (*vide infra*). The spidroin pre-formed fiber is now progressing into a segment within which water and sodium are further removed from the lumen and potassium, surfactants and lubricants are added [55]. Additionally, proton pumps are further decreasing the pH and the morphology of the duct is changing into small tube like, tapered structures. The liquid phase is exposed to low and uniform stress within this channel like part of the duct, allowing a careful orientation of the SF molecules along the gradient. Once the solution reaches the drawdown taper, the force required for moving the dope along the wall becomes larger than the force of drawing the thread and the dope detaches to form a narrow thread. A valve is located distally, through which a broken thread can be gripped, the spinning process can be restarted and the thread is repaired [58]. The spigot positioned at the distal outlet is further removing residual water and forcing the fiber outside. The morphology of the *B. mori* silk gland is different from spiders (*vide supra*) and divided into three anatomical units [59]. The thin and flexuous posterior part of the synthesizing unit is responsible for SF production and synthesis of the P25 accessory protein which are then transported to a wider middle part where they are stored as a concentrated hydrogel. In the following distal part the expression of sericin, which is coating the SF core, peaks. The fluids entering the subsequently tapered duct are exposed to a low and constant extensional flow. Furthermore, pumps are located in the proximal duct for the regulation of pH and ion type as well as ion concentration. By these means and in analogy to what was described above for spider silk processing, the interplay of precisely controlled fluid mechanics as well as appropriately balanced ion types, ion concentrations, and pH is responsible for the orientation of liquid crystalline phases. Continuing active removal of water and proceeding β-sheet formation lead to a thread at the exit of the spigot. These exciting insights as well as recent advancements in the understanding of the natural spinning processes prompted us to select three major input parameters, critically impacting the formation of silk threads *in vivo*. We translate the role of these parameters within the *in vivo* spinning process to the application for the manufacture of drug delivery systems. The factors are the (i) pH, (ii) ion composition and (iii) fluid flow of the dope in the natural spinning process (Fig. 1).

3.1. pH

A thorough understanding of the molecular structure of the SF molecule (*vide supra*) is key to unlock the biopolymer's responsiveness to pH gradients. As pointed out before, hydrophobic blocks are positioned between hydrophilic spacers and this "block polymer" like structure is flanked N- and C- terminally by a hydrophilic head and tail, respectively. The hydrophobic (and crystal forming) blocks do not carry charges and are not responsive to pH. However, the hydrophilic spacers are acidic as are the N-terminal head group and the C-terminally bound light chain of SF, leading to a negative net charge at neutral pH and as reflected by an isoelectric point (*pI*) of about 4 [4,60]. The role of the N-terminal domain is particularly well documented for the pH-dependent control of fiber formation, preventing premature formation of β-sheets at neutral pH and guiding fiber formation during acidification [61–63] and has been reviewed before [64,65]. The role of the C-terminus includes influence on both, storage and ordered assembly [66,67].

The pH in the gland of spiders can be actively adjusted by using different mechanisms [60]. In the A-zone the pH is approximately neutral, followed by gradual acidification in the B-zone by virtue of proton pumps as well as by the secretion of acidic polysaccharides, resulting in a pH of 6.3 in the duct [54,68]. Acidification lowers charges on the acidic SF, which lowers repulsive forces among molecules. Thereby, the hydrophobic blocks may reassemble leading to increased β-sheet content eventually leading to gelation. Structural studies using circular dichroism detailed conformational changes of SF as a function of acidification [60]. Another model emphasized the impact of potassium ion concentrations along with pH changes leading to an unfolding of the spider SF molecule through breakage of its water shell and facilitating molecular