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Deciphering the mechanism of protein interaction with silk fibroin for drug delivery systems

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ABSTRACT

Silk fibroin (SF) is an exceptional drug delivery carrier with respect to stabilizing, protecting, and delivering sensitive biologics. A synopsis of thermodynamic, static light scattering, hydrophobicity probing, and nanoparticle tracking analyses served as a basis to decipher the mechanism of interaction between SF and two model proteins, protamine and polylysine. The impact of salts aiding (chaotropic), not affecting (neutral), or opposing (cosmotropic) SF unfolding was a major determinant, ranging from complete abolishment to maximal interaction efficacy. Evidence is provided, that the underlying mechanism of the remarkable ability to tailor drug/SF interaction throughout such large ranges and by appropriate salt selection is the control of structural breakdown of SF micelles as present in pure SF *ad initium*. This study provides a mechanistically justified and hypothesis driven blueprint for future experimental designs addressing the controlled interaction of biologics and SF.

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1. Introduction

The drug delivery of biologicals is a high growth segment of the pharmaceutical industry, with key drivers of innovation being the stabilization of these sensitive molecules opening a potential for storage at room temperature and deployment of challenging administration routes [1]. Another frequent challenge in the production of current drug delivery systems is the need for processing in organic solvents, at extreme pH values, and mechanical stress all of which potentially challenging the protein's integrity [2,3]. Silk fibroin (SF), a biopolymer isolated from whole silk cocoons of the silkworm *Bombyx mori* (L., 1758), has demonstrated superb stabilization for and controlled release of biologicals [1], thereby providing exciting new avenues for drug delivery [4–10] while further opening the interface to biomaterials and/or tissue (re-)generation [11]. Protocols have been presented, allowing manufacturing of protein loaded SF drug delivery systems under mild condition [8] and by translating insights of the natural (all-water based) silk spinning processes into advanced biomaterial and drug delivery system production, respectively [12,13]. Successful development of delivery systems was reported for therapeutically relevant proteins and peptides, including nerve growth factor (NGF) [7], insulin-like growth factor I (IGF-I) [8], fibroblast growth factor 2 (FGF-2) [14], bone morphogenetic protein 2 (BMP2) [4,5,15], monoclonal antibodies (mAb) [11], enzymes [16], and others [1,6]. Most of these studies deployed the excellent SF material properties [17–19] and biocompatibility [20,21] for cell growth and differentiation [22,23] and combined these advantageous properties with bio-functionalities through co-processing the biopolymer and the therapeutic or by means of its covalent decoration before or after scaffolding into three dimensional carriers [1,5,24]. More recently, these solid SF drug delivery carriers were complemented by semisolid drug delivery systems and freeze-dried gels for mAb delivery, detailing the design space and mechanism of release by which SF may be used as a drug delivery biopolymer [11,25]. However, the precise mechanism of interaction of SF and biologicals in aqueous solution is still poorly understood and, therefore, scientists were largely forced to follow empirical approaches. Nevertheless, these studies linked better SF loading to positively rather than negatively charged drug molecules, hypothetically assuming Coulomb forces establishing between the negatively charged SF at neutral pH (pI ~ 4.5) [26] and positively charged, alkaline drugs [9,27]. Other hypotheses explained sustained delivery profiles with hydrophobic attraction, linking the rather hydrophobic patches of SF to the hydrophobicity of small molecule drugs, such as propranolol, or hydrophobic patches of antibodies [1,11].

This study aims at expanding this mostly empirically built knowledge base by elucidating the mechanism of SF interaction with biologicals, using the basic model proteins polylysine and

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protamine, respectively. These proteins were selected based on their net positive charge at neutral pH (facilitating electrostatic forces to the net negatively charged SF). Furthermore, these model proteins were chosen to include some structural variability, with polylysine representing a strongly and homogenously charged polyelectrolyte characterized by significant intramolecular electrostatic repulsion resulting in random coil conformation [28], while protamine provides more structural complexity, including hydrophobic sections/β-sheet [29]. In addition to the variation of the model drug, the effect of specific ions resulting in cosmotropic or chaotropic environment, respectively, on protein–SF interaction was studied.

2. Experimental details

2.1. Materials

Cocoons of *B. mori* (L., 1758) were obtained from Trudel AG (Zurich, Switzerland). Protamine sulfate salt from salmon grade x, amorphous powder and Poly-L-lysine hydrobromide (polylysine, 15–30 kDa by viscosity) was from Sigma–Aldrich (Munich, Germany). All other chemicals used were at least of analytical grade and from Sigma–Aldrich if not stated otherwise.

2.2. Preparation of SF solution

SF solutions were prepared from silk cocoons as previously described with modification [30]. Cocoons were boiled in a stirred aqueous solution of 0.02 M Na_2CO_3 twice for 1 h each, and then washed with water 10 times. The dried fibers were dissolved in 9 M aqueous LiBr solution at 55 °C until completely dissolved, yielding a concentration of 20% (w/v) SF which was filtered through a 5 μm-syringe filter (Versapor, Pall Life Sciences, Washington, NY) and dialyzed against ultrapure water for 2 days, changing the water 5 times and using SpectraPor dialysis membranes (MWCO 6–8000 Da, Spectrum Labs, Rancho Dominguez, CA). The SF solution had a final concentration of 1.5–2% (w/v) and was stored at 2–8 °C. Prior to use, the SF solution was filtered through a 5 μm-syringe filter (Versapor, Pall Life Sciences, Washington, NY).

2.3. Isothermal titration calorimetry (ITC)

The ITC titrations were performed using a MicroCal iTC200 (GE Healthcare, Buckinghamshire, GB) to measure the heat of reaction when a protamine solution (1.622 mM) was titrated into an SF solution (32 μM) or a polylysine solution (98 μM) was titrated into an SF solution (19 μM) at 25 °C and stirred at 400 rpm. Both, SF and titrant (protamine or polylysine) were dissolved in the same medium, composed of a 25 mM histidine buffer, pH 7.6 supplemented with sodium chloride, sodium thiocyanate, or sodium sulfate yielding an ionic strength of 192 mM, respectively. Each injection consisted of 2 μl of titrant injected during 4 s each (first titration at 0.2 μl of titrant injected during 0.4 s), with a spacing of 150 s between each of a total of 20 injections. The cell volume was 200 μl. The first injection was always omitted to minimize the impact of equilibration artifacts, following the manufacturer's recommendations. All curves were corrected by a blank titration, performed by titrating the respective model protein (protamine or polylysine) in buffer/salt solution without SF.

2.4. Static light scattering (SLS)

Light scattering was performed using an LS 50B luminescence spectrometer (Perkin Elmer, Waltham, MA) at a fixed angle of 90°. Excitation- and emission wavelength and -slits were set to 638 nm and 2.5 nm, respectively. Scattered light was observed for 60 s with an integration time of 0.1 s and a data interval of 1.2 s. The samples were produced as described before but volumes were increased by a factor of 10 to allow reliable measurement in a 3.5 ml quartz cuvette (cuvette type 101-QS, Hellma, Müllheim, Germany).

2.5. Zeta potential measurements

The surface charge was determined using a Delsa Nano HC (Beckmann Coulter, Brea, CA) using a 658 nm laser and a scattering angle of 15°. The samples were produced as described before but were diluted 1:12.5 for protamine and 1:7.5 for polylysine with ultrapure water immediately before each measurement.

2.6. Nanoparticle tracking analysis (NTA)

The colloid kinetics of SF and protamine alone, as well as of SF/protamine mixtures at different mixing ratios, were determined with a NanoSight NS 500 instrument (NanoSight, Wiltshire, UK) using a 40 mW laser at 638 nm and the NTA Software Version 2.3. All samples were prepared as described before, and were diluted 1:10 using the respective buffer/salt solution immediately before each experiment. Movies were cut using Movie Maker (Microsoft, Redmont, WA).

2.7. Fluorescence spectroscopy

Fluorescence intensities were recorded using an LS 50B luminescence spectrometer (Perkin Elmer, Waltham, MA) setting excitation at 490 nm and emission at 570 nm. Samples were prepared as follows: 2036 μl of an SF solution at 3.2 μM in the respective buffer/salt solution were mixed with 4 μl of a Sypro Orange (SO) solution, diluted 1:10 in DMSO from a stock solution (total dilution of working solution 50,000×). The protamine solution was added in steps of 20 μl each, measuring the fluorescence spectrum at each titration point (*vide supra*). Relative fluorescence was calculated by setting the fluorescence of the SF solution without protamine as reference (corresponding to a relative fluorescence of 1).

2.8. Fluorescent labeling of protamine

8 ml of a 1.0 mg/ml solution of Fluorescein isothiocyanate was added drop-wise to 100 ml of a stirred 2.0 mg/ml solution of protamine in 0.1 M sodium carbonate buffer, pH 9.0. The mixture was stirred for 8 h at 4 °C protected from light. Afterward the solution was dialyzed against ultrapure water for 36 h, using a SpectraPor dialysis membrane (MWCO 6-8000 Da, Spectrum Labs, Rancho Dominguez, CA) and the solution was freeze-dried for 24 h at 5 °C and 0.16 mbar (Alpha 1-4, Christ, Osterrode, Germany).

2.9. Microscopic particle analysis

A 32 μM SF solution was mixed with a 1.622 mM solution of fluorescently labeled protamine at a molar ratio of 10.4. An Axio Observer.Z1 microscope equipped with a Plan-Apochromat 40×/0,95 objective and an AxioCam MRm3 camera (Carl Zeiss Microscopy, Jena, Germany) was used. Excitation wavelength was set to 450–490 nm and emission wavelength to 500–550 nm.

3. Results

The protamine/SF interaction was studied by titrating SF into a solution of protamine in low ionic strength buffer of 0.31 mM, respectively. Endothermic signals with progressively declining intensity were recorded up to a ratio of 1.5 at which the reaction

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