A microfluidic cell for studying the formation of regenerated silk by synchrotron radiation small- and wide-angle X-ray scattering

Anne Martel,¹ Manfred Burghammer,¹ Richard Davies,¹ Emanuela DiCola,¹ Pierre Panine,² Jean-Baptiste Salmon,³ and Christian Riekel¹

¹European Synchrotron Radiation Facility, B.P. 220, F-38043 Grenoble Cedex, France

²Xenocs SAS, 19, rue Francois Blumet, 38360 Sassenage, France

³LOF, Unité mixte CNRS-Rhodia, 178 av. Schweitzer, 33608 Pessac, France

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A tube-in-square-pipe microfluidic glass cell has been developed for studying the aggregation and fiber formation from regenerated silk solution by *in-situ* small-angle X-ray scattering using synchrotron radiation. Acidification-induced aggregation has been observed close to the mixing point of the fibroin and buffer solution. The fibrous, amorphous material is collected in a water bath. Micro-wide-angle X-ray scattering of the dried material confirms its β -sheet nature. © 2008 American Institute of Physics. [DOI: 10.1063/1.2943732]

I. INTRODUCTION

There is considerable interest in mimicking silk fibers produced by spiders and silk worms in view of their interesting mechanical properties. While the structure and function of spinning ducts in spiders¹ and silk worms^{2,3} have been studied extensively, microscopic models of silk protein (fibroin) aggregation and silk fiber formation have still to be experimentally verified.^{1,4,5} *In-situ* synchrotron radiation (SR) micro-small-angle X-ray and micro-wide-angle X-ray scattering (μ -SAXS and μ -WAXS) experiments during forced silking of *Nephila* spiders⁶ provide microstructural information on the nascent silk thread at the exit of the spigots. Information on the fiber-formation process taking place within the spinning duct however, cannot be obtained in this way and the influence of pH,^{7,8} ions,^{9–12} and stress¹³ on fibroin aggregation have only been addressed by *in vitro* studies. Several molecular transformations and structural steps (e.g., silk I) have been suggested as being involved in the formation of silk II type fibers.^{3,5,14–16} Our aim is to mimic silk protein aggregation in a microfluidic environment and to obtain microstructural information by SR-SAXS/WAXS techniques. Silk producing microfluidic devices made of silicon¹⁷ or polydimethylsiloxane¹⁸ (PDMS) are, however, not optimized for *in-situ* X-ray scattering studies requiring a high transparency and a low scattering background. A microfluidic cell optimized for X-ray scattering during silk fibroin aggregation made of glass will be described in the current article.

II. MATERIALS, METHODS, AND EXPERIMENTAL SETUP

A. Microfluidic cell

A tube-in-tube geometry^{19,20} has been chosen which allows the central fibroin solution to be confined by a surrounding buffer solution and permits the easy extraction of solid material (Fig. 1). The inner capillary and the outer capillary of the cell are made of borosilicate glass. The outer capillary has a square cross-section, which allows a constant X-ray path length when scanning laterally across the protein aggregation zone. By reducing the wall thickness of the outer capillary to about 50 μ m by etching with hot NaOH solution, the X-ray transmission at 13 keV (0.095 nm) is increased from about 58% to about 87%. The corresponding X-ray transmission of a 1.05-mm-thick water layer is about 80%. The inner round capillary fits tightly inside the square capillary so

FIG. 1. Schematic design of a tube-in-square-pipe microfluidic cell. The inset shows the cross section of the two tubes. Note that the inner capillary fits tightly into the outer square capillary so that it remains centered during operation.

that it stays well centered and the inner flow is surrounded by the outer flow. Another advantage of this geometry is that a symmetric flow profile is maintained around the fibroin stream, which does not interact with the walls of the tube. This is not the case of the laminar streams obtained using the hydrodynamic focusing geometries in planar microfluidic devices, in which velocity profiles vanishes at the walls.^{21,22} Such geometries are therefore more prone to protein aggregation at the walls and may require appropriate surface films for protection.¹⁸ A concentric mixing geometry is also closer to the geometry of the silk spinning duct¹ as compared to the geometry of planar microfluidic devices. The capillaries are connected to motorized *Harvard Apparatus* syringe pumps by fluorinated ethylene propylene (FEP) tubing (Fig. 1). The syringe pumps are controlled via a PC through an RS232 interface by custom control software. The fibrous material is spun from the exit of the capillary into a water bath. The setup used for SR experiments is shown schematically in Fig. 2.

B. Materials

Fresh *Bombyx mori* cocoons were provided by Stazione Sperimentale per la Seta, Milano. The cocoons were softened in an ice/water mixture for 16 h and then degummed in 0.1% Na_2CO_3 at 80 °C for 5 min. The resulting fibrous material was washed in distilled water and dried in air. The dry material was then dissolved in saturated LiSCN at 60 °C for 3 min and centrifuged for 20 min at 4 °C and 5×10^4 g to remove the nondissolved residues. The fibroin solution was obtained by dialysis against H_2O and characterized by dynamic light scattering (DLS) and spectrophotometry. The fibroin concentrations used for the SR experiments were 5–9 mg/ml. In order to induce protein aggregation analog to the acidification conditions in the spinning duct,^{7,8} a H_3PO_4 20 mM, NaCl 100 mM pH 2 buffer was used.

C. Synchrotron radiation experiments

SR-SAXS experiments on fibroin solutions were performed at the ID02 beamline of the European Synchrotron Radiation Facility (ESRF).²³ This beamline is optimized for solution small-angle scattering experiments. A combination of three sample-to-detector distances (1500, 3000, and 5000 mm) provided a Q range $\approx 2 \times 10^{-2} \text{ nm}^{-1} < Q < 4 \text{ nm}^{-1}$, using a wavelength (λ) of 0.1 nm ($Q = 4\pi \sin \theta \lambda^{-1}$, θ being 1/2 of the scattering angle). The SAXS detector was a fiber-optically coupled CCD (FReLoN).²⁴ The incident and transmitted intensities were also simultaneously recorded. The fibroin solution was measured prior to aggregation using a temperature-controlled flow-through capillary (diameter ≈ 2 mm), allowing sample and solvent scattering to be measured in exactly the same conditions. The two-dimensional (2D) SAXS patterns were normalized to an

FIG. 2. Schematic setup of a microfluidic cell used for synchrotron radiation scattering experiments. The inset shows a zoom of the mixing zone and the positions (millimeters from the inner capillary exit) probed by the beam.

absolute scale using water standard and azimuthally averaged to obtain the differential scattering cross section per unit volume, $I(Q)$. 100 patterns were taken during mixing of water in the pH 2 buffer and averaged. The averaged pattern was subtracted from the fibroin data as a background.

The aggregation processes were studied at a fixed sample-to-detector distance of 3000 mm. The size of the beam was $150 \times 100 \mu\text{m}^2$. The fibroin solution (9 mg/ml) was injected into the inner tube and the pH 2 buffer into the outer tube (Fig. 2). SAXS patterns were collected during mixing of fibroin with the buffer at a distance of 0.5 mm and 7.5 mm from the inner capillary exit. Prior to mixing, a flow of buffer (0.66 ml/h corresponding to 0.36 mm/sec) and water (0.5 ml/h corresponding to 0.36 mm/sec) was maintained. The flow of water was stopped at a given time and replaced by a flow of fibroin solution (0.5 ml/h). The flow speed at the mixing point was 0.29 mm/sec. These flow rates have been chosen to minimize the risk of aggregation as fibroin and buffer arrive at the mixing point with the same velocity. Therefore, the protein flow is not constricted by the surrounding buffer flow, as depicted by Fig. 2. SAXS patterns were recorded at selected positions after the mixing point. Patterns of pure water and buffer solution collected prior to the onset of fibroin flow were taken as background. Note that the scattering function thus obtained does not correspond to an absolute intensity scale.

The formation of crystalline β -sheet material was detected in the air-dried fiber by μ -WAXS using an about $1 \times 1 \mu\text{m}^2$ beam with $\lambda = 0.096 \text{ nm}$ at the ESRF-ID13 beamline. Further details of the ID13 setup are reported elsewhere.²⁵

FIG. 3. (A) Absolute scattering function $I(Q)$ of fibroin solution in water (5 mg/ml). The inset shows the Guinier plot with an extrapolated R_g value of 10.8 nm. (B) Scattering function for aggregation at 0.5 and 7.5 mm distance from the inner capillary exit. The corresponding 2D SAXS patterns are also shown.

III. RESULTS AND DISCUSSION

A. Characterization of fibroin shape

In order to establish the shape of the unperturbed fibroin molecule in pure water the radius of gyration (R_g) was determined in the Guinier approximation.²⁶ The absolute scattering function $I(Q)$ of a 5 mg/ml fibroin solution is shown in Fig. 3(A). From the Guinier plot (inset) a value of $R_g=10.8$ nm is determined. The same R_g value is obtained for a fibroin concentration of 9.34 mg/ml. DLS data on a 5 mg/ml fibroin solution show an apparent hydrodynamic radius of

FIG. 4. (A) Microscope image of artificial silk. (B) μ -WAXS pattern of artificial silk. The orientation of the equator is indicated by arrows. (C) Azimuthally averaged equatorial pattern fitted by three narrow Gaussians for the Bragg peaks, a broad Gaussian for the short-range order, and a zero-order polynomial. Miller's indices of the Bragg peaks are indicated. The data reduction was performed using the FIT2D software package.

15.7 nm. This value is in agreement with the R_g value as it includes the hydration shell. R_g has previously been determined for aqueous fibroin solutions extrapolated to infinite dilution as 8.15 nm by laboratory SAXS²⁷ and as 6.7 ± 0.3 nm from a 5 mg/ml solution by SR-SAXS.¹⁶ R_g values derived from the present study are considered to be more accurate in view of the extended Guinier region and more accurate background subtraction using a flow-through cell for measuring all solutions under the same conditions. At very low Q , the SAXS profile reaches a plateau (in the logarithmic scales representation, Fig. 3) which attests to the absence of aggregates.

B. Protein aggregation and β -sheet formation

2D SAXS patterns were obtained at selected positions after the inner tube. The scattering function measured at the positions shown in Fig. 2 suggests the presence of an aggregate structure [Fig. 3(B)], which is too large to allow the extrapolation of a radius of gyration as for the unperturbed fibroin molecule [Fig. 3(A)]. The increase in scattering intensity at low Q with a distance from the exit capillary suggests an increase in aggregate in the probed volume. We also observe a peak at the position of the fibroin molecule. For the beam position close to the exit capillary (0.5 mm) we assume that scattering function of unperturbed fibroin overlaps aggregate scattering. As the peak at the fibroin molecule position becomes, however, more prominent at the larger distance from the exit capillary, we assume the formation of an aggregate substructure with similar dimensions as the fibroin molecule. A more detailed analysis of the scattering function is in preparation. We note that no evidence for the formation of a crystalline β -sheet material from the WAXS range is obtained at this stage of protein aggregation. However, the aggregated fibrous material can transform, upon room temperature air drying, into a semicrystalline β -sheet-type material. Thus the μ -WAXS pattern of a typical dried fiber [Fig. 4(A)] shows *Bombyx mori* type silk II reflections²⁸ with a weak texture of the overlapping equatorial 020/210 reflections and an important short-range order halo [Fig. 4(B)]. The azimuthally integrated pattern can be fitted by three Gaussian functions simulating the strongest equatorial Bragg peaks and a broad Gaussian due to the short-range order contribution [Fig. 4(C)]. Intermediate hydrated structural phases were previously observed for aggregated fibroin obtained by shearing in a Couette cell.¹⁶ A similar analysis will be performed in the future on the fiber obtained in the present study.

The formation of a large aggregate structure prior to β -sheet formation resembles a proposed mechanistic model suggesting the formation of 100–200 nm fibroin micelles preceding the appearance of fibrillar structures due to shearing.⁵ An aggregation step is also observed upon shearing of an aqueous fibroin solution in a Couette cell.¹⁶ The flaky aggregate has β -bonding character according to Fourier transform infrared (FTIR) spectroscopy,¹⁶ which supports the assumption of shearing-induced hydrogen-bonding interactions and fibrillation in the micellar model.⁵ The conversion of the flaky aggregate to β -sheet material requires a further drying step¹⁶ as for the present aggregate material. Therefore, these results do not support a direct conversion of random coil silk protein into β -sheet material via a nucleation-dependent aggregation mechanism.⁴

IV. CONCLUSIONS

Fibroin aggregation has been observed *in-situ* by SAXS using a tube-in-tube microfluidic cell. The aggregate material suggests the presence of a large structure with a substructure retaining the size of the original fibroin molecule. The microfluidic setup and experimental techniques could be applied to other protein aggregation or conformation studies.

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