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Interfacial behavior of recombinant spider silk protein parts reveals cues on the silk assembly mechanism

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KEYWORDS

Recombinant spider silk, Self-assembly, Interfaces, Nanofibrils, Structural changes

17

18 ABSTRACT

19 The mechanism of silk assembly, and thus cues for the extraordinary properties of silk, can be
20 explored by studying the simplest protein parts needed for formation of silk-like materials. The
21 recombinant spider silk protein 4RepCT, consisting of four repeats of poly-alanine and glycine
22 rich segments (4Rep) and a C-terminal domain (CT), has previously been shown to assemble
23 into silk-like fibers at the liquid-air interface. Herein, we study the interfacial behavior of the two
24 parts of 4RepCT, revealing new details on how each protein part is crucial for silk assembly.
25 Interfacial rheology and quartz crystal microbalance with dissipation show that 4Rep interacts
26 readily at interfaces. However, organized nanofibrillar structures are formed only when 4Rep is
27 fused to CT. A strong interplay between the parts to direct the assembly is demonstrated. The
28 presence of either a liquid-air or a liquid-solid interface had a surprisingly similar influence on
29 the assembly.

30

31 INTRODUCTION

32 Silk is being explored for use as a biomaterial for a range of applications, including tissue
33 engineering, due to its exceptional mechanical properties in combination with good
34 biocompatibility and biodegradability.¹⁻³ Unveiling details in the mechanism of silk assembly is
35 needed to understand and reproduce the extraordinary properties of silk. Silk is made of large,
36 complex proteins that are produced in soluble form in glands of arthropods such as silkworms
37 and spiders. The proteins are assembled into silk upon pultrusion through a narrowing duct.⁴⁻⁶

Two of the most studied silk proteins are the fibroin from the domesticated silkworm *Bombyx mori*, and the major ampullate spidroin (MaSp) that forms dragline spider silk.^{7,8} A characteristic feature of these proteins is their highly repetitive amino acid sequences rich in glycine and alanine. The proteins are disordered and partly helical before transition into densely stacked β -sheet crystals upon silk assembly.^{8–11} A factor known to regulate silk assembly is the decrease in pH along the duct, caused by carbanhydrase production and proton pumps.^{12–14} It is also suggested that ion concentration gradients, dehydration of the protein dope, and hydrodynamic forces that increase along the narrowing duct facilitate the fiber formation.^{4,11,15–17} In this study, we focus on investigating the effect of interfaces on the assembly of silk, which has been less explored so far.

Silk fibroin extracted from *B. mori* cocoons has been shown to possess interfacial activity and to assemble at the liquid-air interface.^{18–20} The silk fibroin forms either helices or β -sheet structures at the interface depending on the surface pressure applied; an increased surface pressure induces β -sheet formation.¹⁸ In interfacial shear rheology, regenerated silk fibroin has been reported to form a viscoelastic gel layer at the liquid-air interface.²⁰ The silkworm fibroin protein has also been shown to assemble at the water-oil interface where the elasticity of the interfacial layer depends on the pH, ionic strength and the oil used.^{21–23} Solubilized spider silk from *Nephila clavipes* has likewise been shown to form a β -sheet rich, viscous layer at the liquid-air interface according to spectroscopic and interfacial rheology measurements.²⁴

Silk from spiders is challenging to obtain in sufficient quantities that meet regulatory demands. Therefore, recombinant technology has been used to produce miniaturized spider silk proteins in heterologous hosts, some with maintained ability to form silk-like materials.^{25,26} Recombinant partial spider silk proteins derived from MaSp1 and MaSp2 from *N. clavipes* were found to form

elastic films at the liquid-air interface.²⁷ The recombinant spider silk protein C₁₆ with 16 repeats of a consensus unit from the dragline silk protein ADF-4 has also been studied with interfacial shear rheology at the water-oil interface.²⁸ Herein we have investigated the interfacial behavior of the parts of an even smaller recombinant spider silk protein, 4RepCT, derived from MaSp1 of *Euprosthenops australis*, which spontaneously form silk-like fibers at the liquid-air interface.^{26,29} 4RepCT consists of four repetitions of alternated poly-alanine and glycine rich regions (4Rep) followed by a globular C-terminal domain (CT), which dimerizes via a disulfide bridge.²⁶ The N-terminal domain (NT) from the *E. australis* MaSp1 has previously been identified as a pH-dependent regulator, which increases the protein solubility at pH > 7 while stabilizing a dimer fold when the pH is decreased to below 6.3.³⁰ However, NT is not crucial for silk assembly to occur, and it has so far not been assigned to any properties of the formed silk fibers. On the other hand, both the C-terminal domain (CT) and a part of the alanine- and glycine rich repetitive region (4Rep) have been shown essential for spontaneous formation of silk-like materials to occur.²⁶ When the two parts of 4RepCT were produced separately, CT was shown able to assemble into fibers at the liquid-air interface, while 4Rep formed small aggregates.²⁶ The fibers of CT were fragile, and could be dissolved in 8 M urea, while fibers of 4RepCT were shown to be both chemically and thermally stable.³¹ The combined 4RepCT protein can also assemble into a stable foam by the introduction of air bubbles.³² Moreover, assembly of 4RepCT occur not only at the liquid-air interface but also on solid surfaces, which has been verified by monitoring the continuous, infinite build-up of a thin silk coating on surfaces via quartz crystal microbalance with dissipation (QCM-D).³³

We hypothesize that investigations of these partial silk proteins at interfaces, while limiting the influence of other parameters such as ion composition, pH, and shear forces induced by flow,

would give insights into the intrinsic properties of the protein parts and their role in silk assembly. Herein, we present studies on the silk protein parts 4Rep and CT separately, as a non-covalent mixture (4Rep+CT), and linked as a fusion protein (4RepCT) (Figure 1a). The proteins were studied at both the liquid-air and the liquid-solid interface (Figure 1b). Surface activity, formation of intermolecular interactions, viscoelastic properties, nanoscale morphology and changes in secondary structure were used to determine the role of each protein part in silk formation and to formulate a hypothesis on the mechanism of surface assembly.

Figure 1. a) Schematic representation of the proteins. 4Rep (left) and CT (right) were used separately, combined in a mixture, or as a fusion protein linked to each other via a peptide bond. The top illustration resembles the hypothesized fold in solution for 4Rep and a model of dimeric CT based on PDB-ID 2KHM. b) Two different interfaces were studied; the liquid-air interface (1) and the liquid-solid interface (2).

MATERIALS AND METHODS

Proteins

Spider silk proteins, recombinantly produced in *E. coli* and purified using affinity chromatography, were provided by Spiber Technologies AB. The proteins used were 4RepCT

(23 kDa), 4Rep (12 kDa), and CT (12 kDa). Proteins were stored frozen in 20 mM tris(hydroxymethyl)aminomethane (Tris) buffer, pH 8.0. After thawing, proteins were centrifuged or filtered using Acrodisc syringe filters with 0.2 µm Supor membranes to remove aggregates before use. Proteins were diluted and used in 20 mM Tris, pH 8.0. When a mixture of 4Rep and CT was used, equal amounts (g/L) were added to obtain the total concentration given in respective method section, i.e. 0.1 g/L of the protein solution with 4Rep+CT corresponds to 0.05 g/L 4Rep and 0.05 g/L CT.

Solid surfaces

Surface adsorbed protein samples for circular dichroism (CD) measurements were prepared on siliconized quartz slides. Bare quartz slides (fused and polished, 30 mm x 9.5 mm x 0.63 mm) were purchased from Chemglass Life Sciences. Slides were cleaned by heating in a solution consisting of a 5:1:1 mixture of Milli-Q water, 30% hydrogen peroxide and 25% ammonia for 10 min at 80 °C. After rinsing with Milli-Q, slides were put into a solution consisting of a 5:1:1 mixture of Milli-Q, 30% hydrogen peroxide and 37% hydrochloride acid for 10 min at 80 °C. Slides were rinsed with Milli-Q, dried using nitrogen gas, and placed standing in a glass desiccator so that both sides were exposed. 0.5 mL 99.5% dichlorodimethylsilane (Sigma Aldrich) was placed onto a watch glass inside the desiccator. The desiccator was purged with nitrogen gas and kept closed for one hour. The slides were dipped in 99% toluene, 99.5% ethanol, and Milli-Q, subsequently, for one minute in each solvent and then placed standing on a glass slide over a heating block at 120 °C for one hour. Slides were stored in 99.5% ethanol until use. After protein application and CD analysis, the quartz slides were cleaned by immersing them in 98% formic acid for 10 min followed by extensive rinsing with Milli-Q. The regained

baseline was confirmed after cleaning and they were reused for silanization and subsequent CD measurements.

Custom-made siliconized hydrophobic coverslips (Paul Marienfeld GmbH & Co. KG) were used for preparation of protein samples by liquid-solid adsorption for atomic force microscopy (AFM) imaging, whereas Langmuir-Blodgett (LB) films obtained from compressed proteins at the liquid-air interface were collected onto freshly cleaved mica (muscovite mica, Electron Microscopy Sciences).

QSX-301 Au sensors (Biolin Scientific) for quartz crystal microbalance with dissipation (QCM-D) were cleaned and rendered hydrophobic using 2 mM 1-undecanethiol as previously described.³³

Surface tension

The change in surface tension by interfacial adsorption was studied for 4Rep, CT, 4RepCT and a mixture of 4Rep and CT using a Sigma 701 force tensiometer (Biolin Scientific). A Wilhelmy plate was partly immersed in 20 mM Tris buffer and used to continuously monitor the change in surface tension after addition of protein (Figure 2a). A 8 mg/L solution of each protein was prepared in 20 mM Tris and allowed to reach ambient temperature (22 °C) for 15 min prior to experiments. The Wilhelmy plate was wetted in water before taring the balance. The protein solution was placed in a glass beaker with a diameter of 45 mm. The Wilhelmy plate was kept at a constant height, 3 mm into the buffer, during the measurements. The surface tension was monitored for 4 h at ambient temperature. The measurements were carried out in parallel with and next to interfacial shear rheology measurements. Each measurement was replicated at least twice.

Langmuir-Blodgett compression-expansion cycling

When applying proteins directly at the liquid-air interface by careful pipetting, a dense protein layer can be obtained. An increase in surface pressure in a Langmuir-Blodgett (LB) trough indicates that the proteins are surface active. By decreasing and increasing the surface area at a constant rate, the change in surface pressure reflects the distance between proteins (Figure 2b). Formation of molecular interactions is seen by a non-linear relationship between the changes in surface pressure during the compression phase as compared to the expansion phase.

Single isotherms and compression-expansion isotherms were measured using a KSV Minimicro trough (KSV Instruments), in which Langmuir-Blodgett (LB) films were also prepared. We use the term LB-film to describe molecular layers that have been transferred to a solid surface from the liquid-air interface by compressing the surface onto a hydrophilic substrate material, which is simultaneously pulled from the bulk liquid. LB-films therefore present the hydrophobic side of the surface layer that is directed towards the air during film assembly. The layers were prepared by careful injection of 50 – 60 μL protein solution, 1.0 g/L, onto the interface of 20 mM Tris buffer in the LB trough. After 10 min of stabilization, the monolayer was compressed by a constant rate of 3 mm min⁻¹ at a constant temperature of 22 °C. The isotherms (data not shown) were compressed as far as possible given the area of the trough. During the compression-expansion cycling the monolayers were compressed to a surface pressure of 35 mNm⁻¹ and then expanded back to 1 mNm⁻¹. Each measurement was replicated at least twice. The LB-films were collected onto mica substrates at a surface pressure of 20 mNm⁻¹ for all proteins except 4Rep, which was collected at 10 mNm⁻¹. The LB-films were dried for morphological studies using AFM.

Interfacial rheology

Intermolecular interactions between proteins at the liquid-air interface can be studied as a measure of viscosity using interfacial rheology. With a du Noüy ring placed at the liquid-air interface and oscillating at low amplitude, the torque needed to keep the amplitude at a fixed value will reflect the viscosity of the interface (Figure 2c). With increasing protein interactions at the interface, the elastic modulus (G' , storage modulus) will increase. Simultaneously, the liquid distortion is monitored and converted into viscous modulus (G'' , loss modulus). If silk proteins assemble at the interface, the ratio of the loss modulus to the storage modulus ($\tan \delta$) will be lower compared to that obtained from an interface with soluble proteins with few intermolecular interactions. The interfacial shear rheological properties of 4Rep, CT, 4RepCT and the mixture of 4Rep+CT were measured at the liquid-air interface at ambient temperature using a DHR-2 rheometer (TA Instruments, U.K.) equipped with a Pt-Ir du Noüy ring (13 mm diameter, Krüss, Germany, flamed prior to use) in an oscillatory mode. The protein samples were prepared at the same time as the samples for surface tension: a 8 mg/L solution of each protein was prepared in 20 mM Tris and allowed to reach ambient temperature (22 °C) for 15 min prior to experiments. The solution was placed in a glass beaker with a diameter of 60 mm and the du Noüy ring was placed onto the surface according to the manufacturer's instructions. The ring was set to oscillate at a constant frequency of 0.1 Hz with 0.1% amplitude. The interfacial storage modulus, G' , and loss modulus, G'' , were monitored for 4h at ambient temperature. The chosen frequency and strain were measured to be in the linear viscoelastic region. Each measurement was replicated at least twice.

Quartz Crystal Microbalance with Dissipation

The QCM-D technology utilizes the fact that an AT-cut quartz sensor oscillates at its resonance frequency and its overtones upon excitation. The oscillation frequencies depend on the

total sensor mass, and therefore sense changes upon adsorption of molecules onto the surface. With an increasing amount of adsorbed proteins, the frequency decreases. Concurrently, the dissipation of the oscillation increases as an effect of increased mass and viscoelasticity of the adsorbed layer (Figure 2d). When the surface is covered with proteins, the mass increase depends on protein-protein interactions instead of protein-surface interactions. To keep the concentration of the protein solution in the small sensor chamber constant, a low flow was used.

An E4 instrument (Q-sense AB) was used for QCM-D analyses. The temperature was set to 20 °C, and a flow rate of 20 μ L/min was used. When the flow rate is kept this low, the adsorption behavior is considered to be similar to that of a still solution. Previously reported results show that 4RepCT silk coatings obtained during QCM-D measurements under the same conditions as in our studies result in randomly oriented rather than aligned nanofibrils, confirmed by AFM.³³ A buffer baseline was established for at least 30 min. 0.1 g/L silk protein solutions were freshly prepared by dilution in 20 mM Tris before each measurement and kept on ice until use. Protein solution was flowed over the sensors for 120 min, followed by rinsing with buffer to regain the baseline. Measurements were performed in triplicates. Frequency and dissipation shifts were fitted to a one-layer model and a Voigt representation using the QTools software to calculate the corresponding mass adsorption from each experiment.

Figure 2. a) In the tensiometer, surface tension is measured continuously using a Wilhelmy plate partly dipped into the protein solution. The surface tension decreases as proteins reach the interface from the bulk. b) In our Langmuir-Blodgett set up, proteins are placed at the liquid-air interface and the surface pressure is measured as the proteins are packed together during compression-expansion cycles achieved by moveable barriers. c) A du Noüy ring is placed at the liquid-air interface of a protein solution in the rheometer. The small oscillation of the ring causes shear forces to the interfacial film that are sensitive to intermolecular interactions at the liquid-air interface. The development of storage and loss moduli of the proteins is monitored as a function of time. d) A QCM-D sensor is oscillating while covered by a protein solution. As proteins adsorb from the buffer onto the sensor surface, the oscillation frequencies decrease and the dissipations increase. The more proteins that has adsorbed, the greater the change.

Atomic Force Microscopy

The nanoscale morphology of proteins accumulated at liquid-air and liquid-solid interfaces was analyzed by AFM. The height images are obtained by scanning a selected area with a nm-range sharp tip mounted at the end of a flexible cantilever. The deflection of the cantilever is affected by the tip-surface interactions at each position, and the cantilever movement is monitored using laser reflection. Scanning line by line results in a 3D-image of the surface.

For imaging of the LB-films formed at the liquid-air interface, a NanoScope IIIa Multimode AFM instrument (E-scanner, Digital Instruments/Veeco) was used with an NSCI5/AIBS cantilever (μ MASCH). All images were recorded in tapping mode in air with a scan rate of 0.6–1.0 Hz, free amplitude about 0.65 V, and damping ratio of 0.7–0.85.

For imaging of proteins that had been adsorbed at the liquid-solid interface, hydrophobic coverslips were incubated in a 0.1 g/L protein solution for 60 min. Surfaces were washed three times with 20 mM Tris. Samples were imaged in a droplet of 20 mM Tris buffer in a Dimension FastScan instrument (Bruker), using ScanAsyst Fluid+ tips and PeakForce Tapping™. The scan rate was 0.5-1.0 Hz, and a set point of 0.01-0.02 V and amplitude of 20 nm were used.

All AFM images were flattened using the NanoScope Analysis 1.5 program to remove tilts in the image data. No further processing of the images was done.

Circular Dichroism

CD spectroscopy is based on circularly polarized light, which upon passing a protein sample results in different adsorption bands depending on the secondary structure of the proteins. Typically, α -helices results in a spectrum with minima at 208 nm and 222 nm, whereas β -sheet structures give rise to a sharp minimum at 218 nm. Disordered proteins instead show negative bands near 195 nm.³⁴ The method is commonly used for protein solutions. However, it is also possible to use it to assess the secondary structure of proteins adsorbed onto solid substrates as long as the substrate does not affect the polarized light. To increase the protein amounts and thus the signal of interfacial proteins, several quartz slides with adsorbed proteins were placed in parallel inside a cuvette (Figure 3). Similar set-ups have been reported previously.³⁵

CD spectroscopy was performed using a Chirascan CD Spectrometer (Applied Photophysics) and a 10 mm x 10 mm quartz cuvette. The temperature was kept at 20 °C, the wavelength scan

range was 200-260 nm (1 nm steps), the bandwidth was 1 nm, and the time-per-point was 1 s. Each sample type was prepared and analyzed at least twice, with triplicate measurements for each sample. After preparing 0.1 g/L protein solution, a measurement was done with protein solution in a cuvette. After this, Teflon holders with four parallel silanized quartz slides was placed in the cuvette with protein solution for preparation of samples with proteins adsorbed at the liquid-solid interface. After 60 min incubation, silk-coated slides were lifted from the cuvette, gently rinsed with 20 mM Tris, and placed in another cuvette with 20 mM Tris. The CD spectrum was in this way collected for eight parallel protein layers, since the light passes through four slides with protein on both sides of each slide. The baselines obtained with 20 mM Tris in cuvettes with or without slides were subtracted from respective spectra obtained from protein samples.

For CD measurements on proteins accumulated at the liquid-air interface, a protein solution of 0.1 g/L was left in a petri dish at ambient temperature for 60 min to allow the proteins to reach the interface. For 4RepCT the concentration was increased to 1 g/L and was left at ambient conditions for 12 h in order to increase the signal strength. A silanized quartz slide was lowered onto the interface to momentarily touch the surface to capture the accumulated proteins. Excess liquid was gently adsorbed by placing a light-duty tissue wiper at the side of the slide. A drop of 20 mM Tris was then slowly applied onto the surface by pipetting, and the liquid was gently removed again. This was done to remove any soluble bulk proteins captured in the drop that follows with the interfacial protein layer upon picking the sample. Samples were dried by evaporation. CD measurements were performed in air using two slides per sample type mounted in the Teflon holders. Measurements were performed using the same settings as described above.

Figure 3. Illustration of the set up for solid-state circular dichroism used in this study. Up to four quartz slides are mounted in parallel inside the cuvette using Teflon holders in the bottom and at the top.

RESULTS

Individual parts of the silk protein show distinct characteristics at the liquid-air interface

By monitoring surface tension (γ) at the liquid-air interface of a protein solution, the surface activity of the proteins and the formation of an absorbed protein film can be investigated. When proteins from the bulk reach the liquid-air interface, water molecules at the interface are rearranged, resulting in a decrease in surface tension. The larger the decrease in surface tension the more surface active the protein is. CT accumulated faster than 4Rep at the liquid-air interface (Figure 4). Both the mixture of the two proteins (4Rep+CT) and the fusion protein (4RepCT) resulted in intermediate responses compared to CT and 4Rep alone. All samples containing the 4Rep part showed a characteristic rearrangement phase, indicated by an intermediate plateau, which was most prominent for the non-covalent mixture of 4Rep and CT.

Figure 4. Surface tension of CT (dashed-dotted), 4Rep (dashed), 4RepCT (solid) and 4Rep+CT (dotted) solutions measured by a tensiometer.

To further explore the properties of the protein layer formed at the liquid-air interface, a LB-trough was used to evaluate the influence of compression and expansion of the protein layer. Proteins were applied to the liquid-air interface by carefully placing drops of protein solution onto the buffer bath. The surface pressure ($\pi = \gamma_0 - \gamma$) was measured continuously during three compression-expansion cycles and a final compression.

During compressions, the surface pressure increased slightly when the proteins were close enough to exhibit long-range interactions but still had space to move around, indicative of liquid-like behavior of the proteins within a monolayer. At a state where less space was available, further compression resulted in a more drastic increase in surface pressure, indicative of a solid-like behavior when the proteins have less freedom to move around. These two phases were most prominent for 4Rep (Figure 5a). In the first cycle, a large compression was needed before the surface pressure increased rapidly. When expanding after the first compression, a much lower surface area was reached, and in the following cycles the molecular area hardly changed. A

different cycling pattern was observed for all samples containing CT (CT, 4RepCT and the 4Rep+CT mixture). The expansion curves did not follow the same route as their respective compression curves, thus giving a slight shift of the molecular area for each cycle (Figure 5b-d). These samples showed a liquid-phase behavior once the pressure had been released. Protein interactions formed during compression of these samples were mainly reversed during expansion and built up again in the subsequent rounds. Samples of CT showed a slight decrease of the slope in the middle of the compression curve, i.e. a slight pressure release, and then it increased again before reaching the target pressure.

The area inside each compression-expansion curve was calculated for each protein sample. This visualizes the prominent irreversible reduction in surface area of the 4Rep sample compared to the other samples, including those that also contained 4Rep (Figure 5e).

Figure 5. Surface pressure isotherms from compression-expansion cycles in a Langmuir-Blodgett trough for 4Rep (a), CT (b), 4RepCT (c) and 4Rep+CT (d). e) Calculated area between in and out strokes of the compression-expansion curve for each cycle, for 4RepCT (circle), CT (diamond), 4Rep (square), and 4Rep+CT (pentagon).

Protein-protein interactions at the liquid-air and liquid-solid interface reveal insights about silk assembly

Interfacial surface rheology can be used to measure the storage modulus (elastic, G') and loss modulus (viscous, G'') of the proteins adsorbed at the liquid-air interface from a bulk solution. An increase in storage modulus is interpreted as an increasing amount of intermolecular interactions. According to the very low storage modulus obtained from the CT sample, almost no interactions seemed to occur between CT molecules at the liquid-air interface (Figure 6a). A small rise in loss modulus was seen in the beginning of the measurement (Figure S1a), indicating that proteins were present at the surface from an early time point, in accordance to the previously described surface tension measurements, although the viscosity of the interface remains low. Interestingly, both 4Rep and 4RepCT showed persistent increases in storage modulus and loss modulus. Although 4Rep needed a longer time than 4RepCT before the moduli started to increase, 4Rep showed the highest rate of change once the process had started. This indicates that a certain amount of 4Rep has to accumulate at the interface before interactions become efficient. To further analyze the interfacial rheology results, $\tan \delta$ values (G''/G') were plotted (Figure S1b). This revealed that CT reached values above unity, indicating a viscous-dominant (liquid-like) behavior. In contrast, the values obtained for all samples containing 4Rep (4Rep, 4Rep+CT and 4RepCT) were below unity, indicating elastic-dominant (solid-like) behaviors, which for silk proteins is due to formation of a film at the interface.

By plotting the surface pressure, π (from surface tension measurement), against the storage modulus, G' (from interfacial rheology) at corresponding time points (Figure 6b), it is clear that interactions starts to form readily at the interface when reaching a certain surface pressure,

resulting in an increase in G' . For 4Rep, this threshold occurred at the lowest values (~ 10 mN/m) where a rapid increase of G' was seen. This increase was accompanied with a slight decrease in surface pressure. For CT, G' remained stable although a steady increase in surface pressure was occurring. For 4RepCT there was an initial gradual increase in G' after ~ 11 mN/m followed by another rapid increase from ~ 20 mN/m. At the latter breakpoint the surface pressure started to decrease, as for 4Rep alone. For the 4Rep+CT mixture, the same first gradual increase in G' was seen, but in contrast to the fusion protein, a second phase with steeply increasing G' values was absent for the mixture.

Viscoelastic properties of proteins adsorbed to a solid surface can be revealed by monitoring the oscillation frequencies of a QCM-D sensor covered with a protein solution. Thus, this method was used to relate the observations from the liquid-air interface to protein behaviors at a liquid-solid interface. The decrease in oscillation frequency and increase in dissipation of the oscillation corresponds to increasing mass and viscoelasticity at the surface, including contributions from entrapped water. A heterogeneous surface will result in spreading of overtones, and by fitting the raw data to a model (see the Experimental section), a more realistic estimation of the adsorbed mass onto the measured surface is obtained. After modelling of the QCM-D data (Figure 6c), strikingly similar patterns of adsorption were seen for the liquid-solid surface as for the liquid-air interface (Figure 6a,c). For comparison, the third frequency overtone for each sample type is also shown (Figure 6d, primary measurement output). As observed at the liquid-air interface, 4Rep showed a similar lag phase at the liquid-solid interface, after which the adsorption increased drastically. 4Rep adsorbed much faster than 4RepCT also to solid surfaces. Both 4Rep and 4RepCT then continued to adsorb, indicating a silk-like assembly via protein-protein interactions. CT initially adsorbed onto the solid surface, but without a subsequent

formation of protein-protein interactions. Although the fusion protein 4RepCT has a high propensity to assemble, the solution containing 4Rep and CT in a mixture lacks this propensity. Instead, surface adsorption of the 4Rep+CT mixture, as measured by QCM-D, was soon saturated similarly to the sample with only CT. The initial adsorption of the 4Rep+CT mixture occurred at a rate similar to that of 4Rep, but was within a few minutes outrivaled by a slower process, resembling that of the adsorption of CT alone (Figure 6d, inset). Interestingly, the relation between frequency decrease and dissipation increase is constant for CT but consist of two phases for both 4RepCT and the 4Rep+CT mixture. The first phase is more rigid than the second phase, as interpreted from the lower D/f ratio for the first phase (Figure S2).

Figure 6. a) Storage moduli from surface rheology measurements of the proteins in solution. b) Storage modulus versus surface pressure. c) Mass adsorption of the proteins to a solid surface as calculated from all frequency and dissipation overtones from quartz crystal microbalance data. d) Frequency changes of the third overtone, with the inset showing the first 10 min in more detail. The legends are the same in all four figures, with 4RepCT (solid), CT (dashed-dotted), 4Rep (dashed) and 4Rep+CT (dotted).

Morphology of proteins assembled at the interfaces

Further insights into the assembly process are given by nanomorphological studies of proteins adsorbed at the two types of interfaces using AFM. Proteins at the liquid-air interface were collected onto a solid substrate via the LB-method at a certain surface pressure after a single compression in a LB-trough (Figure 7a-d). Samples of proteins at the liquid-solid interface were obtained by direct adsorption onto a hydrophobic cover slip (Figure 7e-h).

It was clear that much 4Rep protein had assembled at both the liquid-air and liquid-solid interface (Figure 7a,e). The thick layer formed at the liquid-air interface was difficult to image at a high resolution (Figure S3), and therefore a $2 \times 2 \mu\text{m}$ scan is shown in Figure 7a. From the liquid-solid interface, it was clear that 4Rep alone forms a very thick, heterogeneous layer, with height distances varying up to several hundreds of nanometers. A region of intermediate thickness is shown in Figure 7e. At a few positions, the adsorbed layer was thin enough to reveal tiny fibril-like structures (Figure S3). In contrast, samples of CT collected from the liquid-air interface showed few and very small structures with a very low maximum height of ~ 1 nm as a dried film (Figure 7b). Similarly as for the liquid-air interface, hardly any morphological features could be distinguished when CT was allowed to adsorb onto a solid surface (Figure 7f). In

samples of the non-covalent 4Rep+CT mixture compressed at the liquid-air interface, some fibril structures similar to 4Rep could be seen as well as a very thin layer with similar features as the CT sample (Figure 7c). Again, a $2 \times 2 \mu\text{m}$ scan is shown, as the sample was difficult to image with higher resolution due to high adhesiveness. Some short fibrils were found also on the solid surface incubated with the 4Rep+CT mixture although less dense than on other samples containing 4Rep (Figure 7g). Notably, only samples of the 4RepCT fusion protein showed a uniform nanofibrillar pattern (Figure 7d,h), implying that CT is necessary for a regulated fibril assembly to occur on both the liquid-air and liquid-solid interfaces. All the different protein parts show very similar morphological characteristics at the two types of interfaces.

Figure 7. Atomic force microscopy images of compressed proteins films formed at the liquid-air interface (top row, a-d) and proteins adsorbed onto a hydrophobic solid surface (bottom row, e-h). The scan size is $2 \times 2 \mu\text{m}$ for image a and c, and $1 \times 1 \mu\text{m}$ for all other images. The height bar has been scaled so that it shows the maximum height for each image.

Secondary structure of silk proteins assembled at the interfaces

By analyzing the secondary structure of silk proteins collected at the interfaces compared to bulk solutions, occurrence of assembly into silk can be confirmed. Rearrangement of proteins into silk involves a change in protein secondary structure from mainly helical/random to a substantial fraction of β -sheet structures.⁸⁻¹¹ In this study, CD spectroscopy was used to analyze the secondary structure of the different silk protein parts. In solution, all proteins showed the characteristic spectrum of a helix dominated secondary structure with two minima, at 208 and 222 nm (Figure 8a). The secondary structure of silk proteins that had adsorbed onto solid surfaces (siliconized quartz slides) could be measured by placing several slides in parallel inside a cuvette (Figure 3). Proteins accumulated at the liquid-air interface could also be collected on such slides and analyzed with CD using the same approach.

The CD signal from slides with CT was very low, both when adsorbed directly onto the solid surface and when collected from the liquid-air interface (Figure S4), indicating a low amount of protein adsorbed. Similar results were obtained from the mixture of 4Rep and CT, which is also in line with QCM-D, rheology and AFM analyses.

For 4Rep alone, which adsorbs readily on surfaces according to QCM-D and AFM, a spectrum with a single minimum at 218 nm indicating β -sheets was confirmed for samples collected at both interfaces (Figure 8b,c). Samples of the assembled 4RepCT fusion protein gave rise to spectra with a broader minima centered at 215 nm for the liquid-solid interface (Figure 8d), thus indicating contributions from β -sheets but also helical structures. A narrower peak at 218 nm was obtained for 4RepCT collected from the liquid-air interface (Figure 8e).

Figure 8. Circular dichroism measurements of a) 4RepCT (black), CT (dashed-dotted), 4Rep (dashed) and 4Rep+CT (dotted) proteins in soluble form, and b) 4Rep at the liquid-solid interface, c) 4Rep at the liquid-air interface, d) 4RepCT at the liquid-solid interface and e) 4RepCT at the liquid-air interface.

DISCUSSION

Investigations of the mechanism of silk assembly needs careful consideration of each step in the process, for each part of the silk protein. Assembly and alignment of the silk proteins in the duct of spiders is regulated by a range of different factors e.g. pH and ionic composition, which have been studied elsewhere.^{4,11,16,17} At the most elementary stage, the presence of an interface will affect the structure of proteins, and for metastable proteins such as silk proteins, this might lead to structural rearrangements and silk-assembly. Interfaces also act to concentrate proteins locally and can thus affect the assembly process. Therefore, we have studied silk proteins at both

the liquid-air and liquid-solid interface to elucidate the process with which they interact, rearrange, and form stable silk.

Surface tension measurements at the liquid-air interface reveal that all samples containing the 4Rep part show similar behaviors upon accumulating at the interface (Figure 4). After a first phase of relatively fast surface tension decrease, the following slower phase indicates that the 4Rep part interacts in a different way when a certain density of protein is reached. This potentially leads to a rearrangement of the protein fold. QCM-D measurements show that the first minutes of adsorption are similar for both the 4Rep+CT mixture and pure 4Rep. However, shortly thereafter the adsorption rate for the 4Rep+CT mixture is reversed for a few minutes, indicating loss of proteins from the surface, after which the adsorption continues with the same slow rate as for pure CT. It thus seems as CT, in addition to hindering further adsorption of 4Rep onto the surface, also outcompetes some of the adsorbed 4Rep molecules. Possibly, this can occur for 4Rep proteins that have not refolded into β -sheet crystals. This is in line with the AFM images of adsorbed 4Rep+CT, where only a few short nanofibrils are found (Figure 7c and g).

A continuous build-up of proteins, as observed for samples of 4Rep at both interfaces, did not occur with samples of pure CT (Figure 6). On the other hand, CT reaches both the liquid-air and liquid-solid interface faster than 4Rep (Figure 4 and Figure S1a). As a consequence, in a solution containing both protein parts, CT hinders 4Rep from reaching the interfaces. This leads to a stagnation of assembly, which was seen in both rheology and QCM-D measurements. The blocking effect and the low storage modulus of CT indicate a low tendency of CT to form intermolecular interactions.

Rheology and QCM-D measurements show that 4Rep proteins readily form intermolecular interactions. However, it has previously been shown that 4Rep alone was unable to form

macroscopic fibers.²⁶ Together, this indicates that these proteins are not organized enough at the liquid-air interface to form fibers on their own. This is consistent with AFM images of 4Rep adsorbed onto solid surfaces, where a thick and adhesive layer of irregular fibrils was formed. In LB compression-expansion cycles, the protein-protein interactions that were formed during the first compression of 4Rep at the liquid-air interface are interpreted as irreversible, due to the lack of hysteresis during the following cycles (Figure 5a). This indicates that 4Rep forms a protein sheet in which the created intermolecular interactions remained stable instead of detaching from each other and spreading out as a liquid-like phase upon expansion. Since a large compression was needed to give a rise in surface pressure for 4Rep, a high density of proteins seems to be needed for the interactions to form. Once this rise was achieved, the proteins transformed into a stabile solid-like phase.

Results from the LB compression-expansion cycles indicate that CT and 4Rep interact readily with each other at the liquid-air interface, as both the 4RepCT and 4Rep+CT mixture showed a different compression-expansion profile compared to CT or 4Rep alone (Figure 5). This can also be seen in the relation between surface tension and storage modulus G' from the interfacial rheology measurements (Figure 6b). For samples where CT and 4Rep are present together, a gradual build-up of G' is seen, followed by a drastic increase of G' with a slight decrease in surface tension. The sample of 4Rep alone lacks the first phase. Thus, it is likely that the interactions occurring first are formed by 4Rep and CT together, whereas the rapid increase in G' is triggered by interactions between 4Rep parts.

Two recombinant silk proteins containing both a repetitive region and a C-terminal domain from the spider *N. clavipes* showed a similar continuous build-up at the liquid-air interface as studied by ellipsometry, surface rheology and surface tension measurements.²⁷ Comparable to

results from CD analyses herein, they could confirm increasing amounts of β -sheets (using PM-IRRAS). The β -sheets were assumed to be formed mainly from the hydrophobic poly-alanine blocks, which also correlate with the results obtained in this study.

It is noteworthy that a different behavior was reported for the engineered spider silk protein C₁₆ in the study published by Vézy *et al.*²⁸ At corresponding mass concentrations, the elastic modulus of C₁₆ at the water-oil interface (polydimethylsiloxane oil) had stagnated after approximately 70 min. C₁₆ (48 kDa) is constructed from 16 repetitions of a consensus sequence of the repetitive part from the spider *Araneus diadematus*, whereas the sequence of 4Rep (12 kDa) is derived directly from the native sequence from the spider *E. australis*. Moreover, the C₁₆ protein had been subjected to a denaturing agent, while 4Rep was purified under non-denaturing conditions. This, together with the difference in the interface (oil vs. air), may explain the differences in the observed behaviors.

The QCM-D and interfacial rheology measurements correlate remarkably well with each other (Figure 6a,c). After a lag phase, 4Rep shows the highest rate of formation of protein-protein interactions, while CT do not seem prone to form interactions at all. The stagnating signals from the 4Rep and CT mixture clearly show that it is important to have 4Rep and CT covalently linked for a regulated assembly to occur. The coherence of these two different techniques reveals that the protein behavior at an interface is the same for both the liquid-air and hydrophobic liquid-solid interface. Thus, protein-protein interactions induced at interfaces leads to silk assembly regardless of the fluidity of the hydrophobic/hydrophilic interface.

To compare the characteristics of the different silk protein parts assembled at the two interfaces, both the nanomorphology (AFM) and secondary structure (CD) of the protein samples were analyzed. As expected from QCM-D and rheology measurements, samples of CT from both

530 interfaces showed very low signals in CD measurements due to low amounts of adsorbed
531 proteins. When obtaining spectra of CT collected from the liquid-solid interface, low signals
532 could be distinguished that indicate presence of some proteins with a helical fold (Figure S4).
533 Similar results were obtained for the 4Rep+CT mixture, likely due to limited adsorption at the
534 interfaces. CD spectra of assembled 4Rep reveal that this protein part is effectively changing its
535 secondary structure to mainly β -sheets upon adsorption at both interfaces (Figure 8b,c).
536 However, the resulting protein layer is highly inhomogeneous (Figure 7a,e) and only some
537 shorter fibril-like structures can be observed in areas between the large amounts of quickly
538 adsorbing aggregated 4Rep proteins (Figure S3). AFM imaging shows that only the 4RepCT
539 fusion protein is able to assemble into nanofibrillar networks, which happens on both the liquid-
540 air and liquid-solid interface (Figure 7d,h). Secondary structure analyses of 4RepCT confirmed
541 that the intermolecular interactions revealed in the interfacial rheology and QCM-D
542 measurements are associated with secondary structure changes to β -sheet rich structures. These
543 results thus prove that the mere presence of an interface is sufficient for silk assembly to occur,
544 without influences from other environmental changes. However, CD spectra of assembled
545 4RepCT have a different signature than that of 4Rep (Figure 8). The minimum at around 215 nm
546 shows that 4RepCT formed β -sheets at the liquid-solid interface, although the width of the peak
547 indicates that also other secondary structures exists. Such contributions are even more evident for
548 samples from the liquid-air interface, at which also markedly little protein could be captured for
549 analyses, resulting in low signals. Contribution from helical content probably arises mostly from
550 the presence of CT. However, it is worth noting that CT has previously been shown to form
551 irreversible β -sheets after heating above its melting temperature.²⁶

The observation that 4RepCT but not CT alone assembles via protein-protein interactions at interfaces may be related to the previously reported difference in melting point for CT (72 °C) and 4RepCT (47 °C).²⁶ Thus, CT is more stable than 4RepCT, and thereby less likely to unfold and rearrange into tightly packed β -sheets. In the experiments conducted in this study, it is clear that only the presence of an interface is not sufficient to trigger a change of secondary structure and assembly of CT. It has previously been shown that CT is capable of assembling into silk-like fibers without 4Rep.²⁶ However, these fibers were obtained when the CT protein solution was subjected to gentle wagging, indicating that hydrodynamic motions can trigger a rearrangement of the CT structure. In the same study it was shown that 4Rep alone formed aggregates and a few smaller fibers when subjected to the same conditions.²⁶ This is in line with the herein observed propensity of 4Rep to form intermolecular interactions very fast, yielding unordered structures as visualized by AFM.

Based on the novel data of silk protein properties presented in this study we suggest the following mechanism by which 4RepCT assembles at interfaces, without the influence of other external factors (Figure 9). First, CT aids 4Rep in concentrating it to the interface by being more surface-active. Secondly, 4Rep starts to form intermolecular interactions, concurrently unfolding and rearranging into β -sheets. This process is constrained by the presence of CT, regulating the rate and character of the assembly. The intrinsic properties of the two important silk protein parts (4Rep and CT) at an interface are thus complementary. While 4Rep easily transforms from helical/random structure to β -sheets, the process is delayed due to the need for a sufficient density of 4Rep at the interface before extensive intermolecular interactions begin to occur. CT is efficient in reaching the interface and is thus responsible for accelerating the initial arrival of 4RepCT at the interface, while assuring that the 4Rep molecules refolds slow enough to allow

organization. This leads to arrangement into nanofibrils, from which further silk assembly can occur, leading to formation of macroscopic silk structures. If CT and 4Rep are present in the solution without covalent linkages, the two proteins will interact with each other but not form an organized fiber network. In this non-covalent mixture, the CT will not bring the 4Rep to the interface but possibly replace it.

Figure 9. Schematics showing adsorption of silk proteins at the two interfaces. CT (left) efficiently reaches the interfaces but does not form intermolecular interactions or cause structural rearrangements alone. 4Rep (middle) rapidly undergoes a structural change to β -sheets at both interfaces and interacts laterally leading to unordered morphologies. 4RepCT (right) reaches the interfaces and rearranges into nanofibrillar structures.

CONCLUSIONS

By studying the behavior of the recombinant partial spider silk protein 4RepCT and parts thereof both at the liquid-air interface and the liquid-solid interface in combination with secondary structure analysis and imaging with AFM, detailed insights into the important characteristic of silk assembly could be gained. This work establishes that CT serves as a float to reach the interface without forming intermolecular interactions. When covalently linked to 4Rep in the fusion protein 4RepCT, the two protein parts cooperate so that CT facilitates surface

adsorption, whereas 4Rep form intermolecular interactions and rearranges into β -sheets. The influence of CT is further visualized by AFM. 4Rep alone adsorbs into unordered morphologies, whereas distinct nanofibrils are formed when the assembly is slightly decelerated by fusion to CT. Altogether, both silk protein parts, 4Rep and CT, can be considered crucial for a proper silk assembly to occur, and thus for the unique properties of silk-like materials.

ASSOCIATED CONTENT

Supporting Information. Loss modulus and $\tan \delta$ from interfacial rheology, D/f from QCM-D, AFM images of 4Rep and CD spectra of CT and 4Rep+CT.

The Supporting Information is available free of charge on the ACS Publications website at <http://pubs.acs.org/>.

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Author Contributions

L.N. and M.H. designed and performed experiments related to the liquid-solid interface and wrote the manuscript together with M.K. All authors planned and performed experiments related to the liquid-air interface and contributed to the manuscript writing. M.K., L.N., S.A. and A.P.

analyzed the data and prepared figures. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest: M.H. have shares in Spiber Technologies AB, a company that aims to commercialize recombinant spider silk.

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ABBREVIATIONS

AFM, Atomic Force Microscopy; CD, Circular Dichroism; LB, Langmuir-Blodgett; QCM-D, Quartz Crystal Microbalance with Dissipation; Tris, Tris(hydroxymethyl)aminomethane.

REFERENCES

- (1) Aigner, T. B.; DeSimone, E.; Scheibel, T. Biomedical Applications of Recombinant Silk-Based Materials. *Adv. Mater.* **2018**, DOI: 10.1002/adma.201704636.

- (2) Omenetto, F. G.; Kaplan, D. L. New Opportunities for an Ancient Material. *Science* **2010**, 329 (5991), 528–531.
- (3) Rising, A.; Widhe, M.; Johansson, J.; Hedhammar, M. Spider Silk Proteins: Recent Advances in Recombinant Production, Structure–function Relationships and Biomedical Applications. *Cell. Mol. Life Sci.* **2011**, 68 (2), 169–184.
- (4) Andersson, M.; Johansson, J.; Rising, A. Silk Spinning in Silkworms and Spiders. *Int. J. Mol. Sci.* **2016**, 17 (8), 1290.
- (5) Gupta K, A.; Mita, K.; Arunkumar, K. P.; Nagaraju, J. Molecular Architecture of Silk Fibroin of Indian Golden Silkmoth, *Antheraea Assama*. *Sci. Rep.* **2015**, 5, 12706.
- (6) Sparkes, J.; Holland, C. Analysis of the Pressure Requirements for Silk Spinning Reveals a Pultrusion Dominated Process. *Nat. Commun.* **2017**, 8 (1).
- (7) Altman, G. H.; Diaz, F.; Jakuba, C.; Calabro, T.; Horan, R. L.; Chen, J.; Lu, H.; Richmond, J.; Kaplan, D. L. Silk-Based Biomaterials. *Biomaterials* **2003**, 24 (3), 401–416.
- (8) Rising, A. Controlled Assembly: A Prerequisite for the Use of Recombinant Spider Silk in Regenerative Medicine? *Acta Biomater.* **2014**, 10 (4), 1627–1631.
- (9) Giesa, T.; Perry, C. C.; Buehler, M. J. Secondary Structure Transition and Critical Stress for a Model of Spider Silk Assembly. *Biomacromolecules* **2016**, 17 (2), 427–436.
- (10) Malay, A. D.; Sato, R.; Yazawa, K.; Watanabe, H.; Ifuku, N.; Masunaga, H.; Hikima, T.; Guan, J.; Mandal, B. B.; Damrongsakkul, S.; et al. Relationships between Physical Properties and Sequence in Silkworm Silks. *Sci. Rep.* **2016**, 6, 27573.
- (11) Knight, D. P.; Knight, M. M.; Vollrath, F. Beta Transition and Stress-Induced Phase Separation in the Spinning of Spider Dragline Silk. *Int. J. Biol. Macromol.* **2000**, 27 (3), 205–210.
- (12) Andersson, M.; Chen, G.; Otikovs, M.; Landreh, M.; Nordling, K.; Kronqvist, N.; Westermarck, P.; Jörnvall, H.; Knight, S.; Ridderstråle, Y.; et al. Carbonic Anhydrase Generates CO₂ and H⁺ That Drive Spider Silk Formation Via Opposite Effects on the Terminal Domains. *PLoS Biol.* **2014**, DOI: 10.1371/journal.pbio.1001921.
- (13) Domigan, L. J.; Andersson, M.; Alberti, K. A.; Chesler, M.; Xu, Q.; Johansson, J.; Rising, A.; Kaplan, D. L. Carbonic Anhydrase Generates a pH Gradient in Bombyx Mori Silk Glands. *Insect Biochem. Mol. Biol.* **2015**, 65, 100–106.
- (14) Dicko, C.; Vollrath, F.; Kenney, J. M. Spider Silk Protein Refolding Is Controlled by Changing pH. *Biomacromolecules* **2004**, 5 (3), 704–710.
- (15) Spiess, K.; Lammel, A.; Scheibel, T. Recombinant Spider Silk Proteins for Applications in Biomaterials. *Macromol. Biosci.* **2010**, 10 (9), 998–1007.
- (16) Knight, D.; Vollrath, F. Changes in Element Composition along the Spinning Duct in a Nephila Spider. *Naturwissenschaften* **2001**, 88 (4), 179–182.
- (17) Tillinghast, E. K.; Chase, S. F.; Townley, M. A. Water Extraction by the Major Ampullate Duct during Silk Formation in the Spider, *Argiope Aurantia* Lucas. *J. Insect Physiol.* **1984**, 30 (7), 591–596.
- (18) Valluzzi, R.; Gido, S. P.; Muller, W.; Kaplan, D. L. Orientation of Silk III at the Air–Water Interface. *Int. J. Biol. Macromol.* **1999**, 24 (2-3), 237–242.
- (19) Valluzzi, R.; Gido, S. P. The Crystal Structure of Bombyx Mori Silk Fibroin at the Air–water Interface. *Biopolym. Orig. Res. Biomol.* **1997**, 42 (6), 705–717.

- (20) Yang, Y.; Dicko, C.; Bain, C. D.; Gong, Z.; Jacobs, R. M. J.; Shao, Z.; Terry, A. E.; Vollrath, F. Behavior of Silk Protein at the Air–water Interface. *Soft Matter* **2012**, *8* (37), 9705.
- (21) Tang, X.; Qiao, X.; Sun, K. Effects of pH on the Interfacial Viscoelasticity and Stability of the Silk Fibroin at the Oil/water Interface. *Colloids Surf. Physicochem. Eng. Asp.* **2015**, *486*, 86–95.
- (22) Tang, X.; Qiao, X.; Miller, R.; Sun, K. Effect of Ionic Strength on the Interfacial Viscoelasticity and Stability of Silk Fibroin at the Oil/water Interface. *J. Sci. Food Agric.* **2016**, *96* (15), 4918–4928.
- (23) Qiao, X.; Miller, R.; Sun, K. Interfacial Adsorption, Viscoelasticity and Recovery of Silk Fibroin Layers at Different Oil/water Interface. *Colloids Surf. Physicochem. Eng. Asp.* **2017**, *519*, 179–186.
- (24) Renault, A.; Rioux-Dubé, J.-F.; Lefèvre, T.; Beaufiles, S.; Vié, V.; Paquet-Mercier, F.; Pézolet, M. Structure and Mechanical Properties of Spider Silk Films at the Air–Water Interface. *Langmuir* **2013**, *29* (25), 7931–7938.
- (25) Vendrely, C.; Scheibel, T. Biotechnological Production of Spider-Silk Proteins Enables New Applications. *Macromol. Biosci.* **2007**, *7* (4), 401–409.
- (26) Hedhammar, M.; Rising, A.; Grip, S.; Martinez, A. S.; Nordling, K.; Casals, C.; Stark, M.; Johansson, J. Structural Properties of Recombinant Nonrepetitive and Repetitive Parts of Major Ampullate Spidroin 1 from *Euprosthops Australis*: Implications for Fiber Formation †. *ACS Biochem.* **2008**, *47* (11), 3407–3417.
- (27) Renault, A.; Rioux-Dubé, J.-F.; Lefèvre, T.; Pezennec, S.; Beaufiles, S.; Vié, V.; Tremblay, M.; Pézolet*, M. Surface Properties and Conformation of Nephila Clavipes Spider Recombinant Silk Proteins at the Air–Water Interface. *Langmuir* **2009**, *25* (14), 8170–8180.
- (28) Vézy, C.; Hermanson, K. D.; Scheibel, T.; Bausch, A. R. Interfacial Rheological Properties of Recombinant Spider-Silk Proteins. *Biointerphases* **2009**, *4* (3), 43–46.
- (29) Stark, M.; Grip, S.; Rising, A.; Hedhammar, M.; Engström, W.; Hjälrm, G.; Johansson, J. Macroscopic Fibers Self-Assembled from Recombinant Miniature Spider Silk Proteins. *Biomacromolecules* **2007**, *8* (5), 1695–1701.
- (30) Askarieh, G.; Hedhammar, M.; Nordling, K.; Saenz, A.; Casals, C.; Rising, A.; Johansson, J.; Knight, S. D. Self-Assembly of Spider Silk Proteins Is Controlled by a pH-Sensitive Relay. *Nature* **2010**, *465* (7295), 236–238.
- (31) Hedhammar, M.; Bramfeldt, H.; Baris, T.; Widhe, M.; Askarieh, G.; Nordling, K.; Aulock, S. von; Johansson, J. Sterilized Recombinant Spider Silk Fibers of Low Pyrogenicity. *Biomacromolecules* **2010**, *11* (4), 953–959.
- (32) Widhe, M.; Johansson, U.; Hillerdahl, C.-O.; Hedhammar, M. Recombinant Spider Silk with Cell Binding Motifs for Specific Adherence of Cells. *Biomaterials* **2013**, *34* (33), 8223–8234.
- (33) Nilebäck, L.; Hedin, J.; Widhe, M.; Floderus, L. S.; Krona, A.; Bysell, H.; Hedhammar, M. Self-Assembly of Recombinant Silk as a Strategy for Chemical-Free Formation of Bioactive Coatings: A Real-Time Study. *Biomacromolecules* **2017**, *18* (3), 846–854.
- (34) Greenfield, N. J. Using Circular Dichroism Spectra to Estimate Protein Secondary Structure. *Nat. Protoc.* **2007**, *1* (6), 2876–2890.

727 (35) North, S. H.; Taitt, C. R. Application of Circular Dichroism for Structural Analysis of
728 Surface-Immobilized Cecropin A Interacting with Lipoteichoic Acid. *Langmuir*
729 **2015**, 31 (39), 10791–10798.
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