Interfacial rheological properties of recombinant spider-silk proteins

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Freestanding protein films are interesting for many applications ranging from microencapsulation to tissue engineering. Here, the authors use interfacial rheology to characterize the adsorption kinetics and the rheology of spider-silk films formed at an oil water interface. The high surface activity of the engineered spider-silk proteins results in a fast formation of highly stable films, which can be modified by the addition of phosphate ions to the solution. © *2009 American Vacuum Society.*
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I. INTRODUCTION

New release systems are required in many technical and industrial applications such as flavor encapsulation or drug delivery. Encapsulation of flavors and drugs in micron-sized capsules can be achieved by using synthetic polymers, inorganic materials, or colloidal particles. Recently, the spontaneous adsorption of recombinant spider-silk protein at an emulsion interface has been used to form solid and stable thin-shelled microcapsules, 5–30 μm in size with exceptional mechanical properties.[1,2] The silk protein was first dissolved in an aqueous phase. After emulsification in oil, the protein adsorbed at the oil/water interface and formed a stable film. This formation was followed by a conformational change in the proteins from random coil to β-sheet rich structure. The nature of the oil did not dramatically influence the adsorption process.[1]

To explore possible applications of the capsules (encapsulation and the controlled release of drugs or flavors), a detailed understanding of the formation process and the mechanical properties of the interfacial silk film is important. Because of the small size of the microcapsules, a detailed understanding of the formation process and final mechanical properties is difficult to obtain. Although the mechanical properties of such microcapsules can be tested with an atomic force microscopy, their spherical geometry makes a mechanical analysis complicated.[3,4] Especially the nonlinear properties are hard to access, as the geometry and the permeability of the membrane have to be taken into account. However, if the film is formed on a flat interface, the protein film would be accessible to standard rheological methods thus making it easier to investigate the interfacial adsorption.

Flat interfaces have been previously used to understand the adsorption of proteins at the air/water or oil/water interface.[5–11] Among these studies the adsorption of β-lactoglobulin and casein to flat interfaces has been use to better understand the behavior of these proteins in milk emulsions.

Here, we use a flat interface to better understand the behavior of silk protein during microcapsule formation by protein emulsification. In these studies the viscoelastic properties of interfacial spider-silk films are measured at an oil/liquid interface with a Du Noüy ring. The commonly found three step mechanism of film formation is observed: adsorption, aggregation and subsequent reorganization, and refolding of the proteins. Comparison with β-lactoglobulin demonstrates the outstanding efficiency of spider-silk proteins to form highly elastic films, already at very low concentrations.

II. METHODS

Dragline silk protein ADF-4 from the garden spider *Araneus diadematus* has been used as a template for the spider-silk construct C_{16} engineered for bacterial expression.[12–14] The repetitive part of ADF-4 is generally composed of a single conserved repeat unit displaying only slight variations. These variations were combined in one consensus module termed C (GSSAAAAAAAASGPGGYGPENQGPSGPGGYGPGGP), which was multimerized to obtain the repetitive protein C_{16}. The resulting C_{16} protein has a molecular mass of 48 kDa.[13]

The C_{16} silk gene was expressed in the *E. coli* strain BLR (DE3, Novagen). Cells were grown at 37 °C in lysogency broth (LB) medium to an OD600=0.5. Before induction with 1 mM isopropyl-β-D-thiogalactoside, cells were shifted to 25 °C. Cells were harvested after 3–4 h of induction. C_{16} protein was purified and protein identity and purity were

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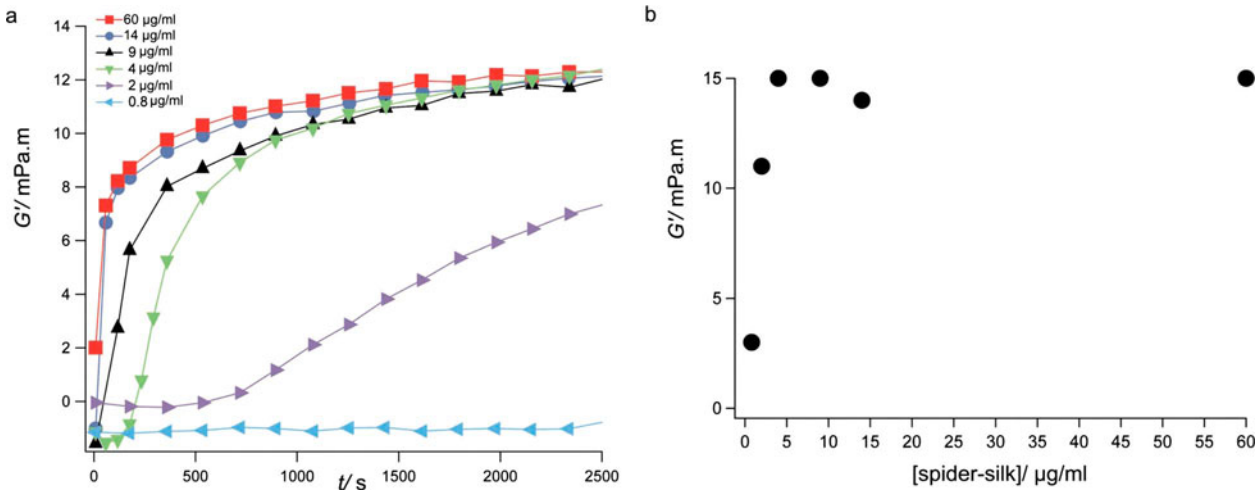

FIG. 1. (Color online) (a) Time dependence of the elastic modulus G' for different spider-silk concentrations. (b) Concentration dependence of the elastic modulus G' measured after 5000 s. Already above 4 μg/ml, a saturation is observable.

assessed, as described by Huemmerich *et al.*[13]

Interfacial rheology measurements at oil/water interfaces were performed with a commercial rheometer (AR-G2, TA Instruments, New Castle, USA) using a Du Noüy ring with a radius of 20 mm. The thickness of the platinum-iridium wire was 0.36 mm. The surface between the inner ring and the circular wall of the container was oscillatory sheared with constant maximum strain. Flow theory between concentric cylinders is applied and the two-dimensional (2D) storage and loss moduli were recorded. Raw data were obtained from the rheometer, and Lissajoux figures were plotted to ensure the linear response of the systems.[15]

10 mM of degassed trishydromethylaminomethane (TRIS) buffer was first added to the sample vessel at room temperature. Then, the sample ring was submerged to avoid oil contact and oil was added. After addition of oil, protein was added in the buffer to prevent adsorption at the air/water interface. Immediately before the measurements, each sample was thoroughly mixed by submerging the sample ring below the surface and shearing the sample quickly. The sample ring was then raised to the sample surface in less than 1.5 s and the surface measurements were performed at 1 Hz and at constant strain of 6% for spider silk and at 1 Hz and at a constant strain of 1.2% for β-lactoglobulin, purchased from Sigma. For oil/water interfacial experiments, 5 cSt polydimethylsiloxane (PDMS) oil, purchased from ABCR, was used. Competitive adsorption experiments were performed with dextran purchased from Sigma (464 kDa) at 20 mg/ml and lecithin purchased from Roth at 10 μM.

III. RESULTS AND DISCUSSION

We characterized the rheology of spider-silk films formed at the oil/water interface. Upon injection of protein solution into the aqueous phase, the formation of an elastic film is monitored. Increasing protein concentrations result in a faster formation of a stable interfacial film [Fig. 1(a)]. At very low spider-silk concentration (0.8 μg/ml), we observed a very slow increase in G', which takes up to 4000 s. The resulting film is hardly stable and a significant fluctuation of the elastic modulus (~6 mPa m) is observed at long times (not shown). At a higher concentration (4 μg/ml), a mechanically stable film is formed within 500 s. Thus only a few μg/ml are needed to form a elastically stable silk film at the oil water interface, which compares well with casein,[7] but which is two orders of magnitude lower than that observed for β-lactoglobulin, where significantly higher critical film forming concentrations are observed [~100 μg/m l for β-lactoglobulin (Fig. 4)].

The concentration dependence of the elastic modulus (G' determined after 5 h) [Fig. 1(b)] clearly shows a saturation effect. Already with 4 μg/ml of bulk concentration of spider silk, the maximum plateau value of 15 mPa m is reached. For all stable films studied the loss modulus was negligible [Fig. 2(b)].

In general, the film formation process can be divided into three distinct regimes:[7] (i) induction regime, (ii) monolayer saturation by conformational changes in the proteins, and (iii) interfacial gelation.

In regime I, the observed induction times are attributed to the adsorption process of the protein to the oil/water interface, which is only observable at low concentrations of spider-silk protein (between 0.8 and 4 μg/ml). A transient increase in the loss modulus is observed directly after incubation, while no increase in the elastic modulus can be measured [Figs. 1(a) and 2(a)]. The presence of a small amount of unaggregated proteins at the interface increases only the viscosity without any change in the elasticity. Once a critical surface concentration of proteins is reached, the adsorption process moves to regime II, which is characterized by a rapid increase in the elastic modulus and a subsequent decrease in the loss modulus [Fig. 2(a)]. As soon as the proteins are predominantly in the aggregated form, the loss modulus decreases to reach a stable plateau at about 1 mPa m. In the latter regime, a logarithmic decay of surface tension as a function of time is observed for most proteins,[7] which is consistent with the fact that for the spider-silk films only a

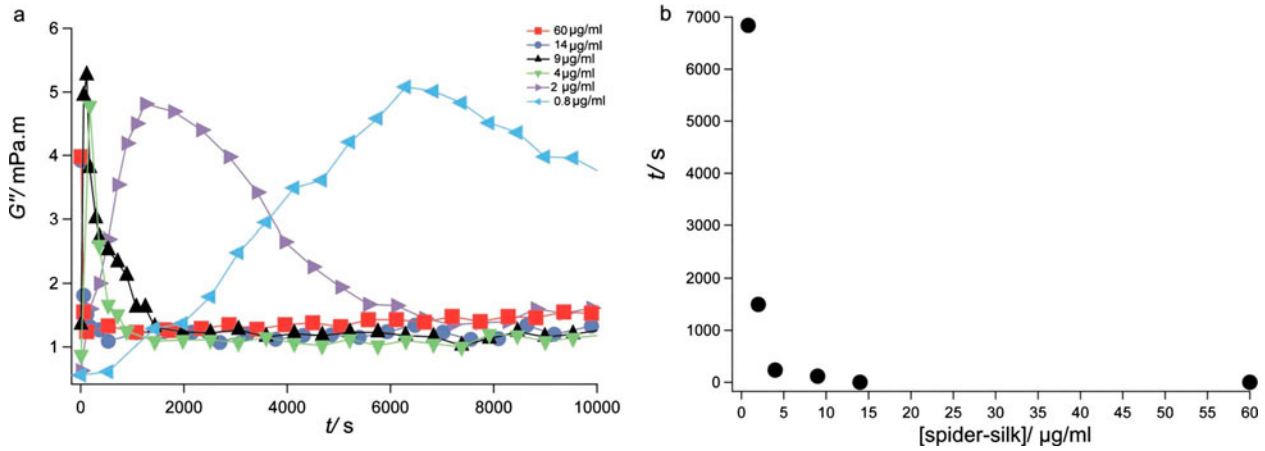

FIG. 2. (Color online) (a) Time dependence of loss modulus G'' for different spider-silk concentrations. Clearly, at low concentrations a transient increase in G'' is observable. (b) The time before a clear decrease in the viscous modulus G'' is observable as a function of the silk concentration. This indicates that concomitant with a higher adsorption rate a more rapid aggregation of the adsorbed proteins occurs.

small change in the surface elasticity is observable (~0.3%/h). Concomitant with the aggregation of the proteins at the interface is a conformational change in the secondary structure into a β-sheet rich conformation.[1,16] Even if the geometry of film formation between microcapsules and interfacial film is different, the presence of a hydrophobic/hydrophilic interface is enough to promote β-sheet rich conformation. At higher concentrations (~4 μg/ml) [Fig. 2(a)], the transient regime of the loss modulus is too short lived to be resolved and only the decay can be resolved. Consequently, increasing protein concentration results not only in a higher adsorption rate but also in a more rapid aggregation of the adsorbed proteins [Fig. 2(b)].[7,8]

In Fig. 3, the changes in the interfacial rheology, which result from the adsorption of spider silk at 0.02 mg/ml and β-lactoglobulin (0.1 mg/ml), are compared during 20 h.

Although the concentration for β-lactoglobulin is an order of magnitude higher, it has less effect on the interfacial rheology and exhibits an induction regime. For spider silk, an induction regime is only observable above at concentrations above 2 μg/ml. The elasticity of the formed interfacial film is a factor 3 lower than the elasticity of the spider-silk film and a significant loss modulus is observed resulting in a loss tangent six times smaller $G'/G''_{\text{lacto}}=1.98$. In contrast for silk, already after 10 000 s a highly elastic film is formed with a very low viscosity: $G'/G''_{\text{spider}}=12.4$.

Postcast application of phosphate ions induces a significant increase in the amount of β-sheet configuration in the secondary structure of the spider-silk protein films formed upon drying.[16–19] In order to test if the addition of phosphate also affects the mechanical properties of the interfacial films, potassium phosphate (0.3M) was added into the aqueous phase after 20 h of experiments. Upon addition, a sharp increase in the elastic modulus was immediately observed, which is consistent with an increase in the number of β-sheets and interconnections between the individual proteins. The film ruptures at 52 mPa m after applying a constant strain of 6%, indicating the resulting brittleness of the film. The linear response up to the rupture point was confirmed by recording the raw data of the rheometer.

In order to characterize the nonlinear behavior of spider-silk films, experiments are performed under a constant strain rate (10%/s). After formation of a stable film, and ensuring a well equilibrated system (~20 h), the shear stress was recorded as a function of a linear increase in strain from 1% to 100% (Fig. 4). The spider-silk interfacial film breaks at a value of 12% of strain. Although these films are completely different in protein composition and structure from natural spider-silk fibers, the maximum strains before breakage are comparable.[20] Also, the nonlinear behavior of β-lactoglobulin films is significantly inferior to spider-silk films. Breakage occurs already at 6% of strain and at a maximal shear stress of ~1 mPa

To test the relative surface affinity of the spider-silk protein, we conducted competitive adsorption measurements using both the highly surface active protein, lecithin, and the

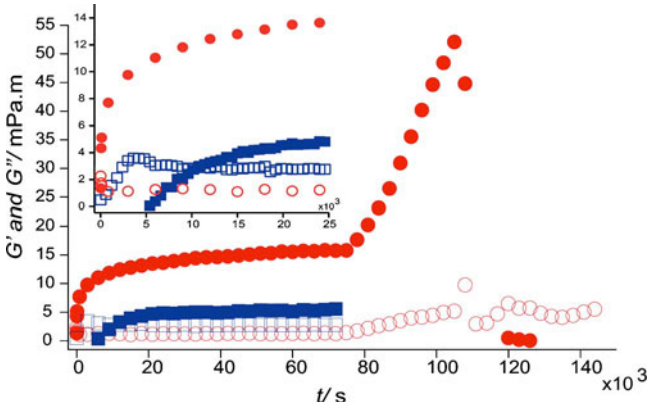

FIG. 3. (Color online) Time dependence of G' and G'' for spider silk (●) at 6% of strain and β-lactoglobulin (■) at 1.2% of strain. Full symbols represent G' and empty symbols G''. The spider-silk concentration is at 0.02 mg/ml. The β-lactoglobulin concentration is at 0.1 mg/ml. At 72 000 s, phosphate (300 mM) was added in the aqueous phase of the spider-silk film to induce β-sheet formation. The inset is a zoom of the adsorption process at short times.

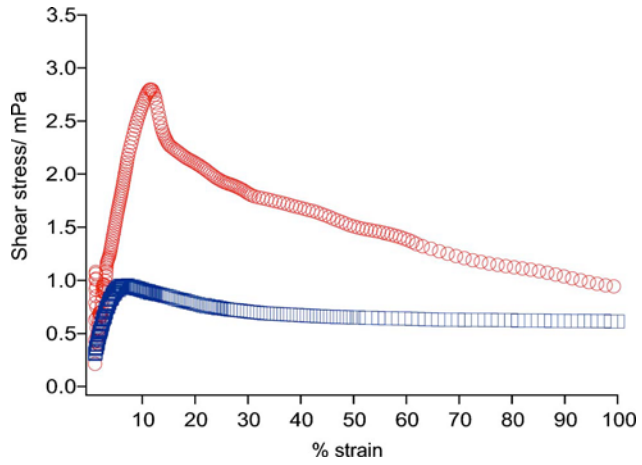

FIG. 4. (Color online) Nonlinear behavior of spider-silk (○) and β-lactoglobulin (□) films. The shear stress was recorded as a function of the applied strain at a constant strain rate (10%/s).

low surface active molecule dextran as competitor. Dextran or lecithin alone did not have any effect on the surface elasticity. To prepare the measurements, dextran was first dissolved in water and lecithin was dissolved in PDMS oil. When dextran and the silk protein were added together, an adsorption behavior typical of normal spider silk was observed with a sharp increase in the elastic modulus and a sharp decrease in the loss modulus (Fig. 5). This fact suggests that spider silk adsorbs favorably over dextran. Figure 5 shows that lecithin has an effect on the interfacial rheology, indicating that either the lecithin coadsorbes with the spider-silk protein or inhibits the adsorption of the silk protein. Lecithin is known to have a high surface activity and has been shown to displace proteins like casein from an emulsion droplet surface.[21,22] These measurements qualitatively show that spider-silk's affinity for the oil/water interface is between that of dextran's and lecithin's.

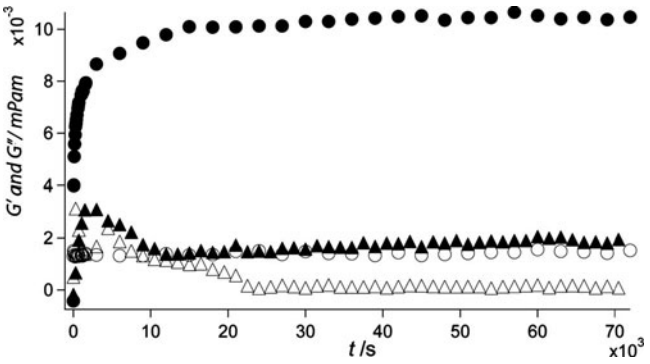

FIG. 5. Time dependence of G' and G'' during the competitive adsorption of spider silk and either dextran or lecithin. Black symbols represent G' and empty symbols represent G''. ●: spider silk at 0.02 mg/ml and dextran at 20 mg/ml. ▲: spider silk at 0.02 mg/ml and lecithin at 10 μM.

IV. CONCLUSIONS

In summary, we characterized the interfacial activity of spider-silk proteins by monitoring the 2D elastic and loss moduli of interfacial films formed at an oil/water interface. Spider-silk proteins form mechanically stable elastic films, which are useful in various applications, by adsorbing the proteins at an oil/water interface. This is consistent with elasticity measurements made on spider-silk microcapsules.[1] Measuring the viscoelastic surface properties of spider-silk films is ideally suited for determining the three different regimes of adsorption and aggregation at an interface and for observing the reorganization regime at low concentration. The elastic properties of the interfacial films can be easily tuned by adding phosphate and thus by inducing a higher content of β-sheet configurations.

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