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Self-Coacervation of a Silk-Like Protein and Its Use As an Adhesive for Cellulosic Materials

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Supporting Information

**ABSTRACT:** Liquid–liquid phase separation of biomacromolecules plays a critical role in many of their functions, both as cellular components and in structural assembly. Phase separation is also a key mechanism in the assembly of engineered recombinant proteins for the general aim to build new materials with unique structures and properties. Here we describe the detailed characterization of spider silk (spidroin)-inspired protein coacervates and their use as adhesives for cellulosic materials. Applications involving cellulose are rapidly expanding due to its excellent structural and functional properties in combination with new ways to process and use it. Consequently, new types of biological materials are expected. We noted previously that a block-architecture in which a spidroin repetitive sequence is flanked in both N and C termini by folded domains leads to a self-coacervating phase behavior (Figures 1A and S1−S4). The general architecture was important for coacervation, and the same behavior was achieved even with other unrelated proteins used as terminal domains. The block design of the recombinant proteins was inspired by the structure of spidroins, but it does not necessarily follow that the mechanisms described here apply for condensates that function extracellularly also prevent the dissolution of the adhesive into the environment, which is especially important in marine adhesives. The high protein concentration and their increased interactions can also lead to a rapid trigger of adhesion and curing.

Here we describe the detailed characterization of spider silk (spidroin)-inspired protein coacervates and their use as adhesives for cellulosic materials. Applications involving cellulose are rapidly expanding due to its excellent structural and functional properties in combination with new ways to process and use it. Consequently, new types of biological materials are expected. We noted previously that a block-architecture in which a spidroin repetitive sequence is flanked in both N and C termini by folded domains leads to a self-coacervating phase behavior (Figures 1A and S1−S4). The general architecture was important for coacervation, and the same behavior was achieved even with other unrelated proteins used as terminal domains. The block design of the recombinant proteins was inspired by the structure of spidroins, but it does not necessarily follow that the mechanisms described here apply for condensates that function extracellularly also prevent the dissolution of the adhesive into the environment, which is especially important in marine adhesives. The high protein concentration and their increased interactions can also lead to a rapid trigger of adhesion and curing.

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native spidroins. The repetetive spidroin sequence used here was an engineered version containing 12 repeats of residues 325−368 of the load-bearing repetitive major ampullate spidroin 3 from *Aaraneus diadematus* (*eADF3*).14−16 The main feature in the primary structure of this protein is the repetition of short poly-A stretches flanked by P-, G-, and Q-rich stretches (Figure S5). This results in a sequence with alternating hydrophobic and hydrophilic properties17 and a high predicted disorder tendency.18 As terminal blocks, the structurally folded and thermally stable family 3 cellulose-binding module (CBM)19 from the *Clostridium thermocellum* cellulosome were used. These blocks show a high affinity toward cellulose.

Closer examination of the dense phase revealed that it consists of small droplets. To make a distinction to previously described phase separation that was achieved by adding potassium phosphate, we name the system under study here LLC (liquid-like coacervate (or condensate)).12 The LLCs form spontaneously when protein concentration is high and ionic strength is low. The LLC-containing phase remained stable at even 30% protein concentration. Interestingly, the ATR-FTIR spectra which is sensitive to secondary structure conformation showed similarity to dissected spider silk dope (Figure 1D). The LLC solution showed a high adhesiveness to surfaces and forming long filaments when pulled apart (Figure 1E).

To address the question of how LLC droplets formed and grew, we used spatial light interference microscopy (SLIM). With this interferometry-based method, dry mass can be measured in a microscope setup.21 We observed how independent drops emerged in a previously mixed sample (Figure 2A, Videos 1 and 2 and Figure S6). The curves showing an increase in dry mass were obtained by identifying spots were droplets appeared and recording integrated mass content of that location in video replays. After droplet initiation, a linear region of growth occurred until a slow leveling of the growth rate. Droplets grew larger by coalescence with droplet fusions adding to dry mass in an additive way as expected (Figure 2B, Video 3). The ratio of dry mass in a droplet to dry mass of a droplet-free surrounding of the same size was calculated for a sample of 15 droplets and gave a ratio of 140 with a standard deviation (SD) of 51.

In a solution containing LLCs, the diffusivities of molecules in both the surroundings and within the LLCs were measured using fluorescence recovery after photobleaching (FRAP; Video 4). Proteins in the surroundings showed diffusion constants ranging from $2.3 \times 10^{-7}$ cm$^2$ s$^{-1}$ to $7 \times 10^{-6}$ cm$^2$ s$^{-1}$ (Figure 2C). The proteins within the coacervates moved much slower with diffusion constants from $1 \times 10^{-9}$ cm$^2$ s$^{-1}$ to $10 \times 10^{-9}$ cm$^2$ s$^{-1}$. Probing droplets of different size showed significant variation in the diffusion rates (Figure 2C). Analyzing the internal structure of the condensed LLC droplets using cryo-fracturing and high-resolution SEM imaging (Figure 2D) showed that the proteins are internally assembled in an interconnected bicontinuous network.

Shear rate dependent viscosity measurements showed that a solution containing LLCs showed shear thinning with increasing shear rates. At the onset of shear, the LLC containing solution had a viscosity of 490 Pa·s (Figure 3A). However, above the critical shear rate of 0.002 s$^{-1}$, the solution showed shear thinning. At a shear rate of 0.87 s$^{-1}$ the viscosity had dropped to 60 Pa·s. In an oscillatory shear stress test, LLC containing solutions showed a shear-induced gelation with gradual viscoelastic stiffening with $G' \gg G''$, that is, a dominant elastic response (Figure 3B).
However, as the bulk viscosity properties must be affected by the properties of the LLC droplets contained inside, an analysis of the properties of individual LLC droplets within the dilute phase was made. Measurements were made by aspiration with a micropipette submerged in the solution (Figure 3C, Video 4, Figures S7 and S8). In addition to viscosity, we could measure additional characteristics (Figure 4A), such as modulus and surface tension, by independent analysis in situ. The individual LLC droplets demonstrated viscoelastic properties. This was evident from the creep behavior in the aspiration and retraction curves (Figure S9), with an initial elastic regime which was followed by a linear viscous regime. Droplets had low viscosities ranging from 1 to 74 Pa·s, and Young’s moduli ranging from 1.3 to 43 Pa. The surface tension was also very low, with values ranging from 7 to 76 μN/m. As with measurements of diffusion rates, there was a considerable range of values spanning an approximately 10-fold range for all parameters. However, all measured droplets had an overall very low interfacial energy and a high deformability. As expected from the combination of low surface energy and low viscosity, the LLC droplets were easily deformed during flow (Figure 4A and Video 6). Freezing a sample very quickly after deformation using liquid ethane and cryo-fracturing showed by SEM imaging how the internal bicontinuous structure deformed and elongated together with the droplet (Figure 4A) with compression and stretching of the bicontinuous structure, leading to a partial alignment of the network components.

The behavior of the droplets provides an understanding of the bulk rheology in general terms. The elongation of droplets during deformation is expected to lead to a shear thinning, as elongated drops slide more easily past each other. The observed shear-induced gelation with a high ratio of elastic modulus to loss modulus is likewise expected since elongated droplets are induced by shear. The elastic recovery is expected as elongated droplets regain their spherical shapes. Overall, the bulk properties are reminiscent of many previously described occurrences of coacervates, such as marine adhesives and in the formation processes of squid beaks. In the squid beak formation, the combination of properties allows efficient impregnation and cross-linking of a chitin network. Interestingly, both systems have a very similar viscosity of around 300–500 Pa·s at the onset of shear. Furthermore, recDGHP-1 also exhibited shear-thinning behavior, however, at a greater shear rate 0.3 s⁻¹.

Following the above considerations, we studied how droplets would interact with stiff networks of cellulose fibrils. In Figure 4B, it can be seen how droplets of LLC easily stick to cellulose fibers and infiltrate networks of these. The LLC droplets easily penetrated networks and filled space in-between fibers. Individual droplets showed stickiness to cellulose fibers.

We next studied how the silk inspired protein functioned as an adhesive, as it showed properties expected to be beneficial,
such as low surface energy, high protein concentration, high infiltration capability, and intermolecular interactions (Figure 1F). The CBM-eADF3-CBM interactions are evident even in conditions where the sample has been dried, as shown by breaking a sample of dried on a surface12 (Figure S10). The protein forms long fibers that span the cracks that formed during breakage, demonstrating a possible cohesive mechanism similar to that known as necking in polymer science.22 Figure 5A displays the bond strength of coacervated CBM-eADF3-CBM at 30% w/v that was used to adhere different cellulosic materials such as nanofibrillated cellulose of both bacterial and plant origin, cotton, office paper, and wood. After application, samples were allowed to solidify and cure at ambient conditions after water evaporation (Figure S11). Bond strengths varied between 15 and 75 Ncm⁻², depending on the type of cellulosic substrate. This suggested an influence of inherent nano- and microstructured features on the bond strength, also possibly reflecting differences in pore size and surface properties of the different cellulosics. Of all samples, bacterial cellulose had the highest bond strength. Decreasing the mass of the adhesive solution showed a substantial decrease in the bonding strength (Figure S12A). Controls made with different variations of blocks or the unrelated bovine serum albumin (BSA; Figures 5B and S12B) showed lower strength but also a much lower work of fracture, corresponding to the area under the fracture curve. SEM of the fractured surface of the specimens after tensile measurement test revealed that almost in all the cases debonding occurred due to structural failure of the bulk material, not the adhesive

Figure 3. Properties of bulk and individual LLC droplets. (A) Viscosity of 30% w/v protein solution containing LLC droplets as the function of shear rate, showing shear-thinning (red squares show viscosity and blue circles force). (B) Shear-induced gelation during oscillatory viscosity measurement (G': storage modulus (black squares) and G'': loss modulus (red circles)). (C) Phase contrast images of an individual LLC droplet during micropipette aspiration experiment. Plots show an aspiration curve and the calculated interfacial energy, Young’s modulus, and viscosity as a function of droplet size.

Figure 4. Fluid behavior of LLC and infiltration of cellulose fiber networks. (A) SLIM image of LLC deformation under shear. High-resolution cryo-fractured SEM image showing the deformation of the internal bicontinuous structure under shear. The blue arrow indicates direction of shear flow. (B) Infiltration and adhesion of LLC droplets (indicated with yellow arrows) on the surface and at the junctions of cotton fibers.
(Figures 5C and S13), suggesting that the adhesive formed a very strong interfacial bond (Figure 5D and Videos 6 and 7) to the cellulosic surfaces. However, debonding of the wood and cotton textile happened cohesively, and left adhesive on both surfaces illustrating that cracks propagated through the adhesive.

In summary, protein condensates should be seen as an essential aspect of polymer behavior for proteins and represent a key step for understanding when expanding the use of proteins for new uses such as adhesives. By using highly accurate methods of in situ protein concentration measurements, diffusivity measurements, and rheology of individual droplets, we found that the droplets showed a range of properties indicating that they are thermodynamically metastable, with varying properties between individual drops. Overall, the protein showed unique phase behavior and molecular structuring into highly deformable and low-interfacial energy assemblies. We showed how the preassemblies of the highly concentrated droplet in combination with low interfacial energy and cohesiveness could be efficiently employed as an intermediate state for strong adhesive assembly between cellulosic surfaces.

**ASSOCIATED CONTENT**

* Supporting Information
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Details for all the materials and experimental methods (PDF).

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Video 1: Formation and growth of multiple LLC droplets in water (AVI).
Video 2: Formation and growth of single LLC droplet in water (AVI).
Video 3: Coalescence of three individual LLC droplets, all varying in size and protein content, results in the expansion and increase of the protein content after each fusion (AVI).
Video 4: Fluorescent recovery after photobleaching part of the LLC droplet that was used to calculate diffusion coefficient of CBM-eADF3-CBM molecules (AVI).
Video 5: Micropipette aspiration of a LLC droplet (AVI).
Video 6: Illustrating deformation of LLC droplet into fluid threads under shear forces (AVI).
Video 7: Illustrating adhesive behavior of the LLC solution by gluing two pieces of normal paper to each other (AVI).
Notes
The authors declare no competing financial interest.

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