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An Engineered Spider Silk Protein Forms Microspheres**

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Natively unfolded or intrinsically unstructured proteins are typically characterized by a specific sequence containing distinct amino acids, a low overall hydrophobicity, and a lack of defined local structure.[1] In this article, we discuss a spider dragline silk protein that is stored at high concentration in the lumen of the major ampullate gland of the spider as partially natively unfolded protein.[2] The complex process of the a assembly of the protein into fibers starts in the spinning duct where chaotropic sodium and chloride ions are replaced by kosmotropic potassium and phosphate ions. The silk protein solution is further concentrated by solvent resorption and slightly acidified from pH 6.9 to pH 6.3.[3] To unravel the mechanistic details of the accompanying folding and assembly steps, we investigated the influence of solvent conditions on the intrinsically unfolded engineered eADF4(C16) (MW 47.7 kDa)[4,5] based on the known amino acid sequence of a dragline silk protein from the spider *Araneus diadematus*.

The addition of ions leads to the salting-out of proteins in general and induces the structure formation of unfolded in particular. The salting-out properties of salts are described by the Hofmeister series.[6,7] Since potassium phosphate plays an important role during the natural spinning process,[8,9] we investigated its influence on structure formation of eADF4-(C16) and analyzed microscopically the morphology of the resulting aggregates using atomic force and scanning electron microscopies (AFM and SEM). Low concentrations of potassium phosphate (< 300 mM) neutralize the coulombic repulsion between the negatively charged glutamates and lead to self-assembly of eADF4(C16) into nanofibrils with diameters between 2 and 10 nm. The thermodynamics of nanofibril formation of eADF4(C16) was influenced not only by ion strength but also by parameters like protein concentration, molecular weight, and pH (see the Supporting Information under http://www.angewandte.org). High salt concentrations (> 400 mM) induce formation of microspheres by altering the protein–water interactions (Figure 1).[1]

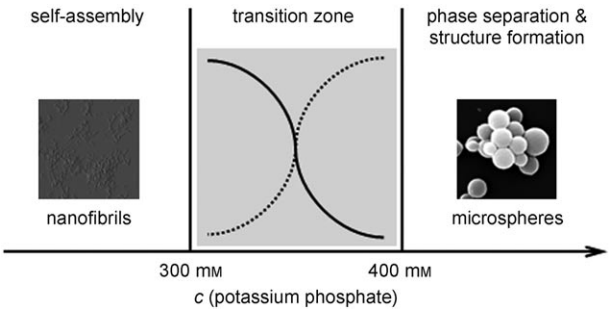

Figure 1. Nanofibrils and microspheres: At low potassium phosphate buffer concentrations nanofibrils self-assemble. At high salt concentrations microspheres are formed. In a transition zone the two species coexist.

In this study we focused on the assembly of microspheres. Since eADF4(C16) microspheres are as chemically stable as natural spider silk threads in protein denaturants such as 8 M urea and 6 M guanidinium chloride,[10] we used this extreme structural stability to compare the thermodynamics of salting-out and structure formation.

The salting-out of eADF4(C16) may be considered to be a liquid–liquid phase separation into a supernatant phase and a dense protein phase, as described previously for other proteins.[11] In the case of eADF4(C16), addition of 1 M potassium phosphate buffer led to an increase in light scattering which reaches a plateau after about 5 s (Figure 2), indicating the completion of liquid–liquid phase separation into an aqueous and a proteinaceous phase. We found that diluting the potassium phosphate buffer concentration below the minimum ionic strength required to maintain the separated phases led to suspension of the phases. In such suspensions only microspheres with a defined secondary structure were stable while unstructured aggregates disassembled upon dilution of potassium phosphate buffer to 166 mM which is below the required minimum ionic strength for salting-out. Analyzing the amount of resolubilized protein at various times, we determined that formation of microspheres was complete after 120 s (Figure 2).

Finally, we analyzed the morphology and structure of the microspheres in more detail. They have smooth surfaces and are solid with no apparent submicrostructure (Figure 3 a,b). The secondary structure of the microspheres was investigated using FTIR spectroscopy (Figure 3 c). A maximum at 1625 cm^{-1} indicated a significant amount of β-sheet structure (64 % as determined after deconvolution). Interestingly, the

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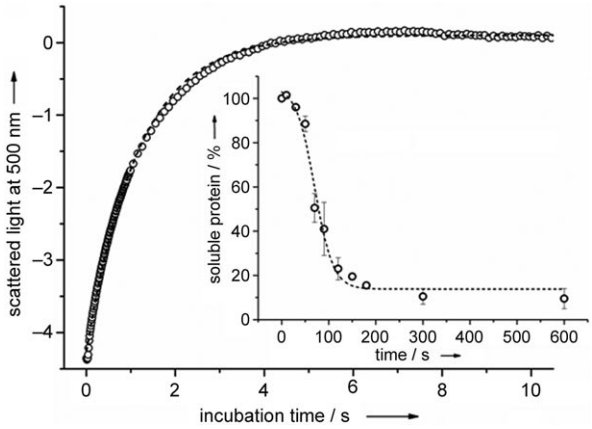

Figure 2. Time dependence and reversibility of the phase separation of eADF4(C16) solutions. Light-scattering was monitored after addition of 1 M potassium phosphate buffer (pH 8.0) and rapid mixing in a stopped-flow instrument. The scattering signal reaches a plateau after about 5 s (not reflecting instrumental limits), indicating that the separation in a proteinaceous and an aqueous phase is finished. To investigate the reversibility of phase separation, H_2O was added at various time points. The amount of soluble protein is plotted as a function of time of H_2O addition.

secondary structure of the microspheres is similar to that of nanofibrils formed by the same protein.[12] Based on these findings it can be concluded that the formation of spheres and the assembly of nanofibrils are structurally similar processes, which differ only in their kinetics.

Nanofibril assembly likely occurs through a mechanism of nucleation–aggregation like that described for amyloid fibrils (Figure 4). In the first step the dissolved unfolded protein forms a nucleus which interacts with additional protein, leading to the formation of protein fibrils.[12,13]

For spherical protein assembly several models have been discussed. Bohr et al. described two models for the formation of spherical structures of lysozyme and ribonuclease. In the bulk-growth model, spheres assemble at a nucleation point in a spherically symmetric way resulting in beads; whereas in the sheet-growth model, protein sheets assemble with a thermodynamically equilibrated curvature resulting in a hollow shell with further shells inside.[14]

Importantly, eADF4(C16) microspheres are solely formed above a critical potassium phosphate buffer concentration. Their formation follows a salting-out mechanism which can be considered a liquid–liquid phase separation. The "one-phase state" is the initial state displayed by a solution of monomeric and intrinsically unfolded protein molecules. Changing a constraint such as the ionic strength by addition of kosmotropic ions alters the free energy of the system and leads to phase separation into protein-rich and solvent-rich phases. This phase-separated state is energetically favored, and the protein concentration in the "protein phase" increases to a critical level. Results from stopped-flow experiments and Cryo-SEM studies indicating that the spheres are solid suggest that the bulk-growth model of spherical protein is the best explanation for the formation of silk microspheres. Upon reaching the critical concentration for nucleation, several structured nuclei are formed simulta-

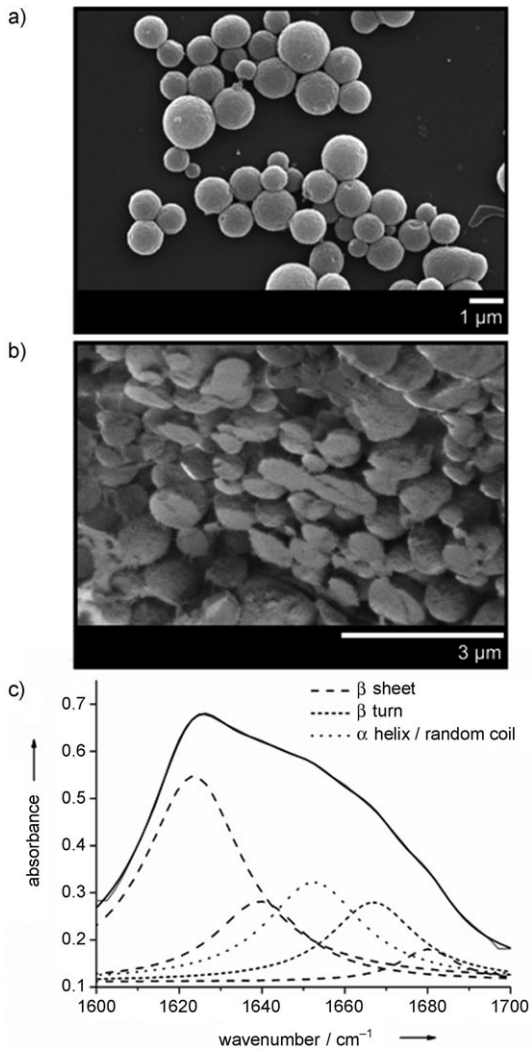

Figure 3. Morphology and structure of eADF4(C16) microspheres. SEM pictures of a) intact and b) freeze-fractured microspheres reveal a smooth surface and solid matrix. c) FTIR spectroscopy indicates that microspheres are rich in β-sheet secondary structure.

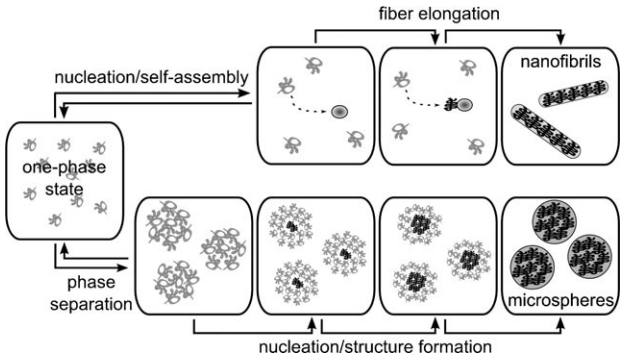

Figure 4. Assembly processes of nanofibrils and microspheres. The first step leading to nanofibrillar assembly is the formation of a nucleus. Protein attaches to this nucleus, its secondary structure converts, and the fiber starts to grow. The first step of microsphere formation is a salt-induced phase separation. A nucleus is formed within the protein-rich phase, which induces a structural transition in the associated protein followed by growth of the microspheres.

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neously in the protein-rich phase. The nuclei start to grow in a spherical manner, interacting with additional monomers and converting their structure. Spherical growth stops when the protein concentration in the protein-rich phase is below the equilibrium of solubility. Hence, the sphere diameter does not increase further. This model is supported by the fact that sphere size depends on protein concentration and mixing conditions.[15]

Which of the described silk protein assembly pathways might be relevant for the natural silk-spinning process and thread formation remains unknown. However, nanofibrils and nanofibrillar structures have been reported in the B-zone of the glands of spiders,[16] as well as in insect and spider cocoon silks.[17] We have shown that the thermodynamics of nanofibrillar assembly of eADF4(C16) is sensitive to factors like pH and salt concentration as well as protein concentration, and it is possible that nanofibril assembly is an early stage of the assembly of larger structures like threads. However, the faster kinetics suggests that phase separation, as observed during microsphere formation, might also be important for silk thread assembly.

Experimental Section

Sample preparation: eADF4(C16) was produced and purified as described previously.[8] To obtain aqueous solutions, lyophilized protein was dissolved in 6 M GdmSCN and dialyzed against subsequent buffers. The samples were centrifuged at 125000 g to remove remaining aggregates.

Stopped-flow measurements: The process of microsphere assembly was monitored by light-scattering after rapid mixing in a stopped-flow instrument (Bio Sequential SX.18MMV, Applied Photophysics, UK). eADF4(C16) protein solution (1 mg mL^{-1}) and potassium phosphate buffer (1 M, pH 8.0) were loaded into the syringes. After rapid mixing of equal volumes of both solutions, the light-scattering signal at 500 nm was recorded as a function of time. Importantly, the plateaus seen in the experiments do not reflect limitations of the photodiode but are real end points of the reaction.

SEM: Samples for SEM analysis were washed three times with doubly distilled water to remove salts and subsequently applied to Thermanox plastic cover slips and allowed to air-dry. After being sputtered with gold, the samples were imaged in an SEM (JSM 5900 LV, JEOL, Japan).

Cryo-SEM: Microspheres were applied to metal holders and frozen in liquid nitrogen. Frozen samples were transferred into a cryo-stage preparation chamber (Gatan ALTO 2500, Gatan Inc., UK) and fractured with a cold metal blade, sublimated at the temperature difference of 50 °C (sample: −90°, stage: −140°), sputtered with gold–palladium, and studied in the frozen state in a cryo-SEM (Hitachi S-4800, Hitachi, Japan) at an accelerating voltage of 3 kV and a temperature of −120 °C.

FTIR spectroscopy: Polarized absorbance spectra of fibrils were recorded at room temperature using a Bruker IFS 66 s spectrometer (Bruker, Germany) between 700 and 6000 cm^{-1} with a resolution of 4 cm^{-1}. The samples were prepared on CaF disks and air-dried.

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