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A conserved spider silk domain acts as a molecular switch that controls fibre assembly

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A huge variety of proteins are able to form fibrillar structures¹, especially at high protein concentrations. Hence, it is surprising that spider silk proteins can be stored in a soluble form at high concentrations and transformed into extremely stable fibres on demand^{2, 3}. Silk proteins are reminiscent of amphiphilic block copolymers containing stretches of polyalanine and glycine-rich polar elements forming a repetitive core flanked by highly conserved non-repetitive amino-terminal^{4, 5} and carboxy-terminal⁶ domains. The N-terminal domain comprises a secretion signal, but further functions remain unassigned. The C-terminal domain was implicated in the control of solubility and fibre formation⁷ initiated by changes in ionic composition^{8, 9} and mechanical stimuli known to align the repetitive sequence elements and promote β -sheet formation^{10, 11, 12, 13, 14}. However, despite recent structural data¹⁵, little is known about this remarkable behaviour in molecular detail. Here we present the solution structure of the C-terminal domain of a spider dragline silk protein and provide evidence that the structural state of this domain is essential for controlled switching between the storage and assembly forms of silk proteins. In addition, the C-terminal domain also has a role in the alignment of secondary structural features formed by the repetitive elements in the backbone of spider silk proteins, which is known to be important for the mechanical properties of the fibre.

Figure 1

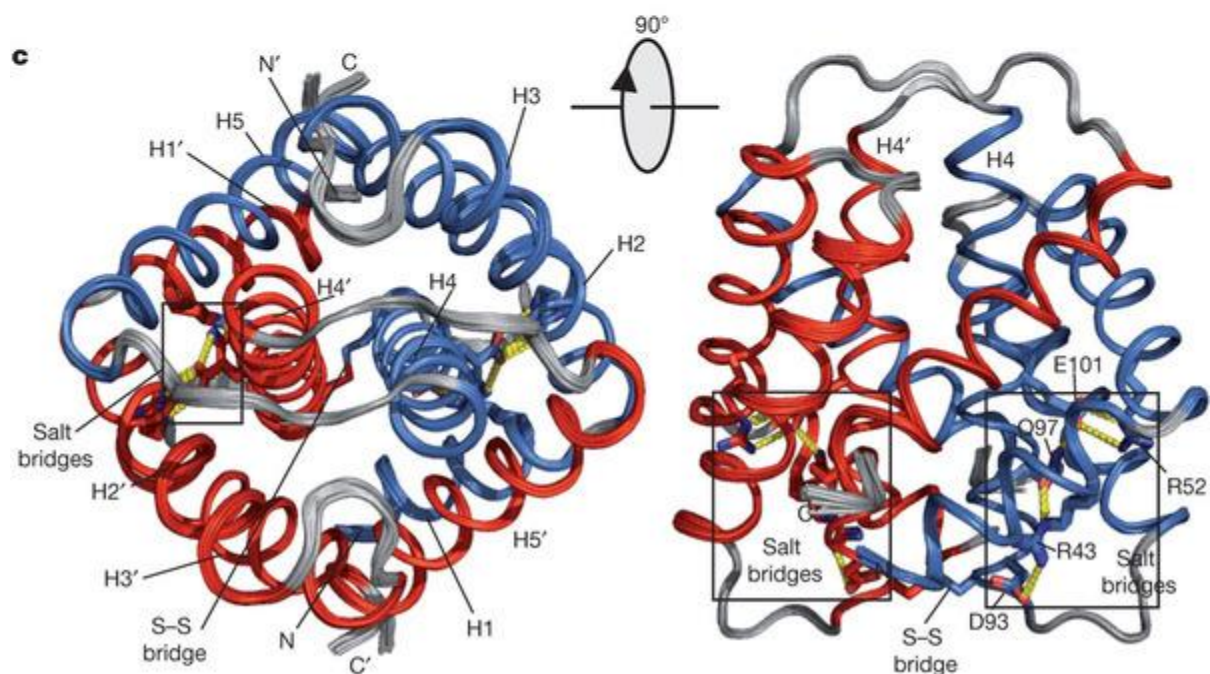
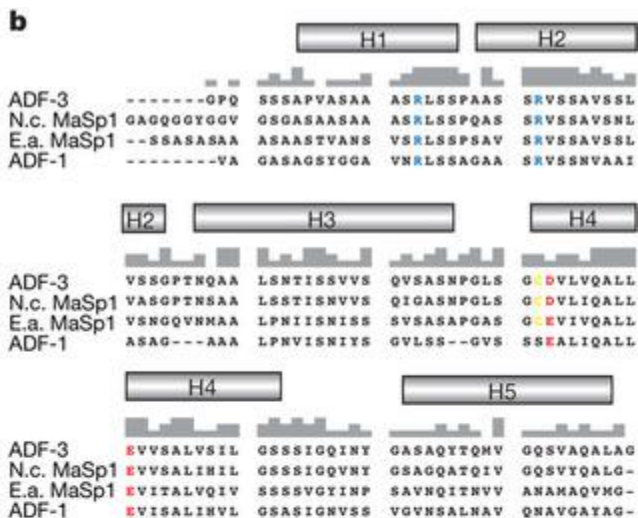
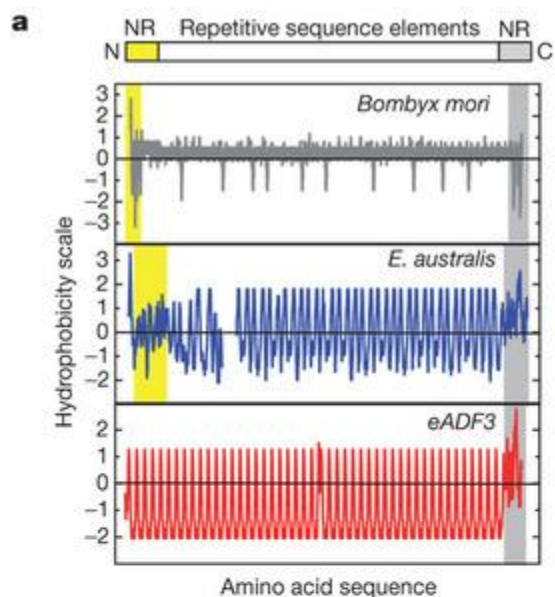


Figure 2

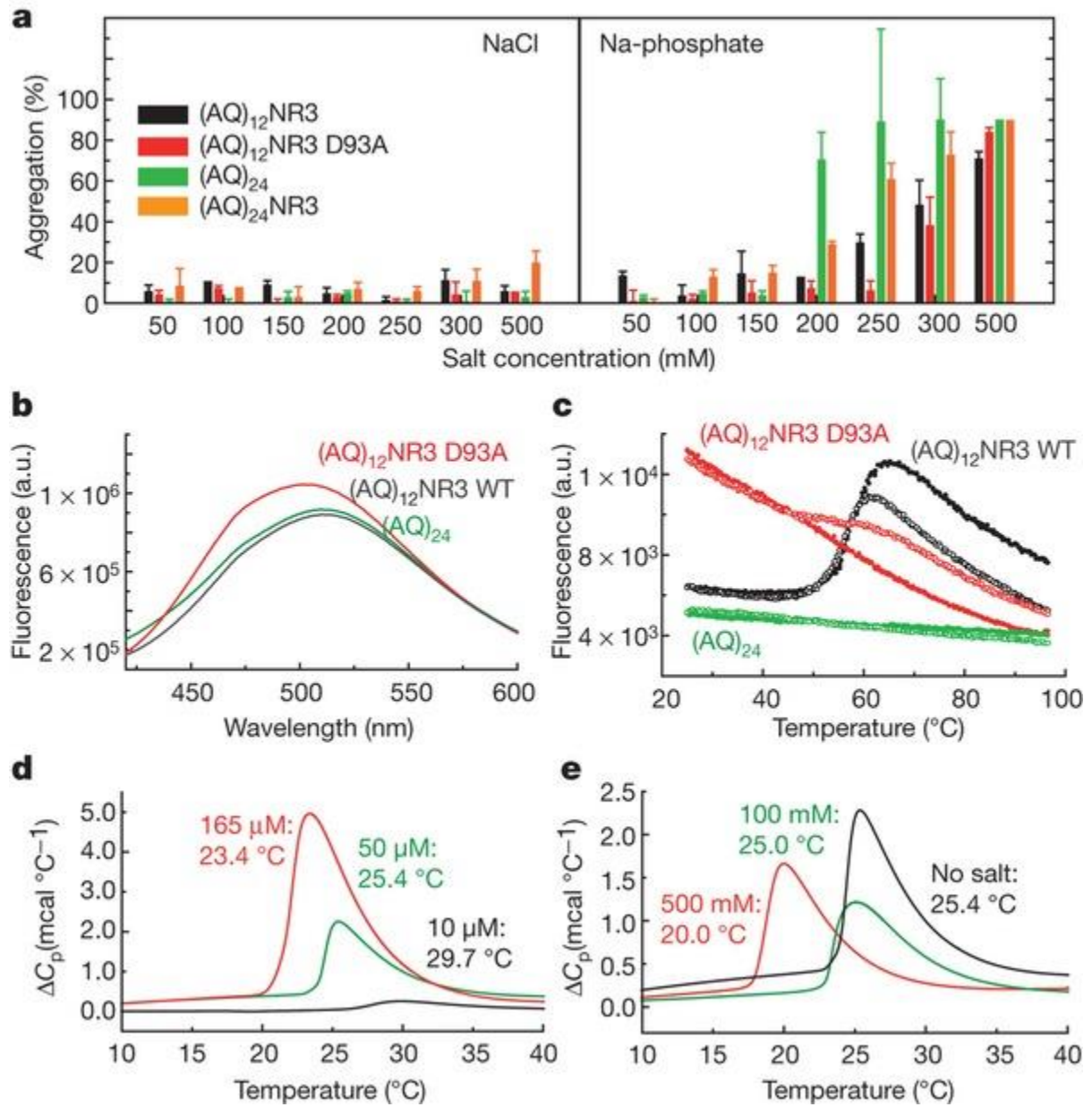


Figure 3

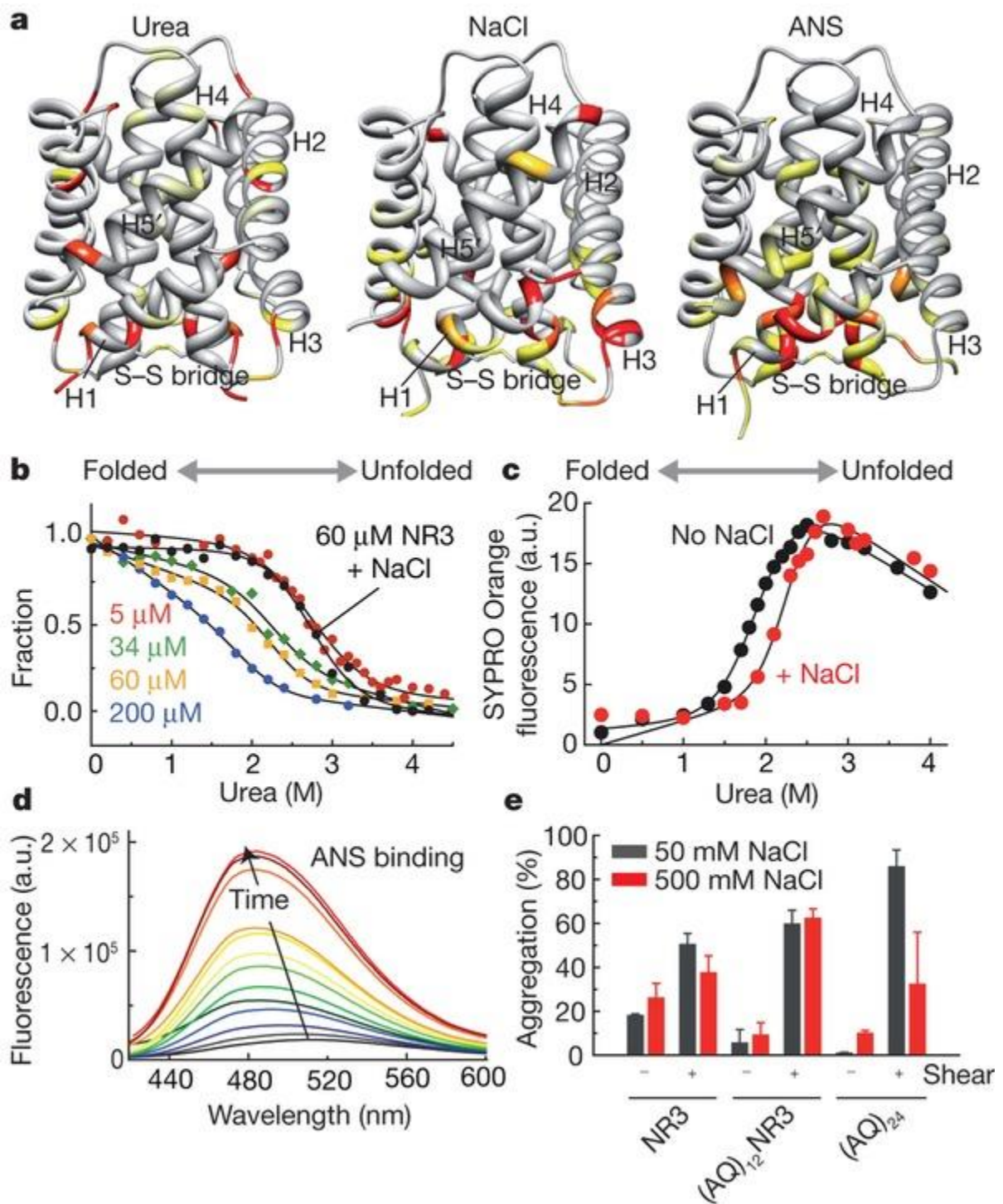
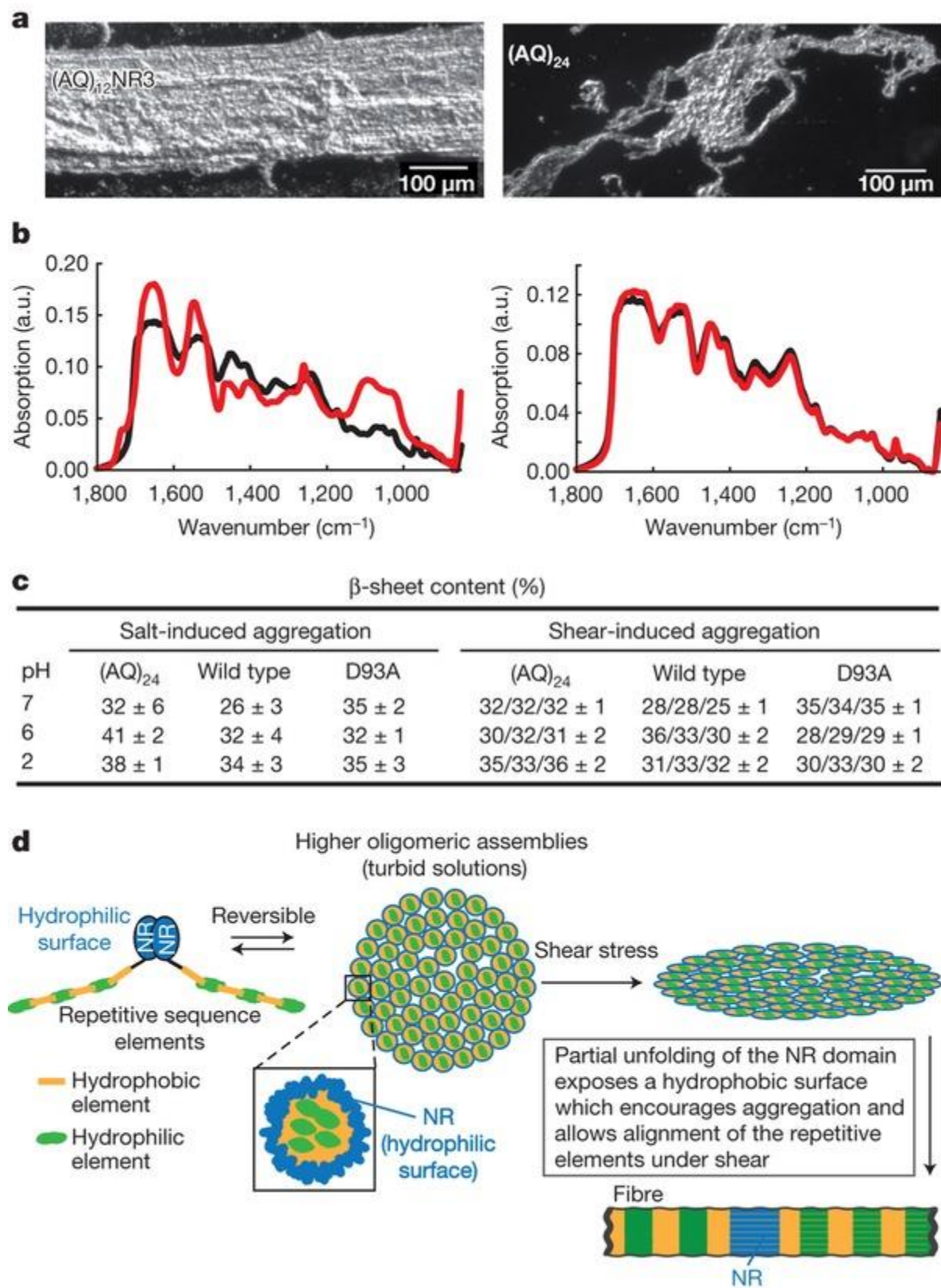
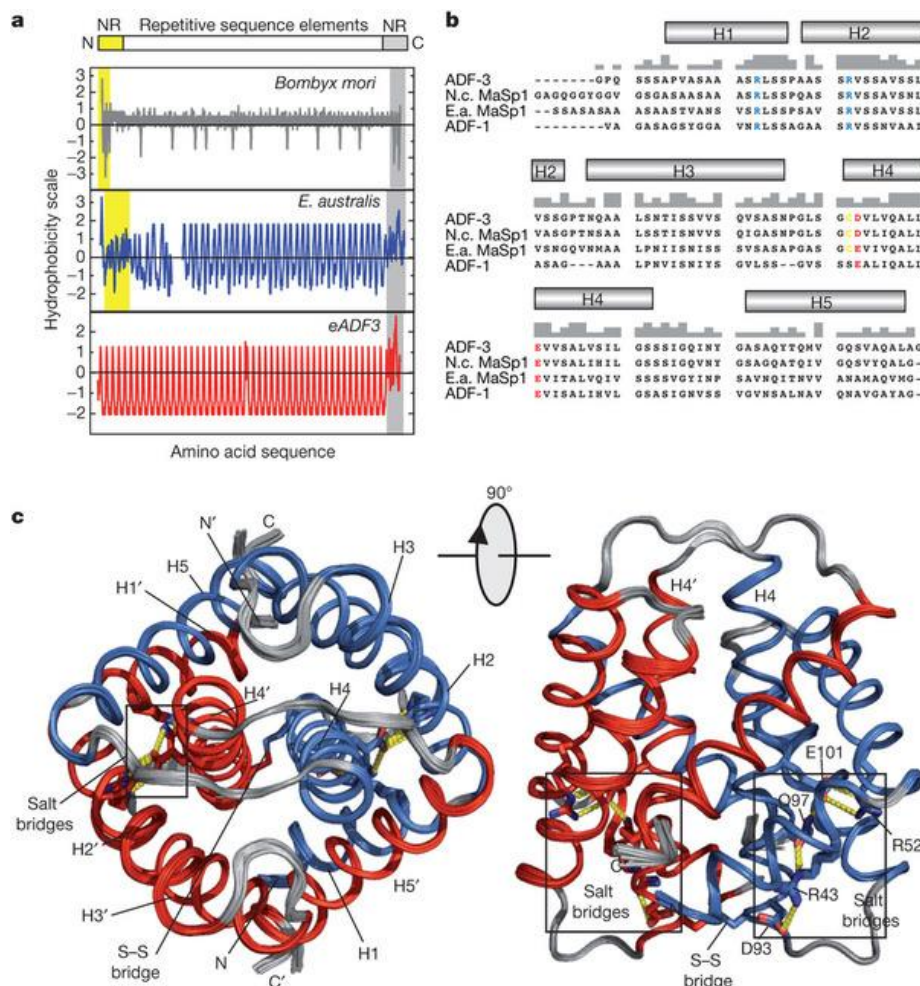


Figure 4



Silk proteins are amphiphilic (Fig. 1a) and the C-terminal non-repetitive (NR) domain of such proteins produced by orb-weaving spiders is one of the most conserved regions (Fig. 1b). To determine the structure of the C-terminal NR domain (NR3) of *Araneus diadematus* fibroin 3 (ADF-3), we used high-resolution NMR with an optimized protein construct (Fig. 1c and Supplementary Tables 1 and 2). The structure is a new protein fold composed of a parallel-oriented dimeric five-helix bundle in which the longest helix (helix 4) is the main dimerization site (Fig. 1c, Supplementary Fig. 1). The single cysteine residue of each monomer involved in intermolecular disulphide formation is located at the N-terminal end of helix 4. The dimerization interface consists predominantly of hydrophobic residues. Packing of these residues is facilitated by a slight right-handed twist between helices 4 in both monomers. Helix 1 of one monomer and helix 5 of the second monomer interact in a clamp-like manner. Two salt bridges (R43–D93 and R52–E101) are located within each monomer, which fix helices 1 and 2 at one side of helix 4 (Fig. 1c). These salt bridges use the only charged residues of the entire known sequence of ADF-3 and are located in the most conserved parts of the NR domain (Fig. 1b).

Figure 1: Sequence analysis and structure of the non-repetitive (NR) domain of ADF-3.

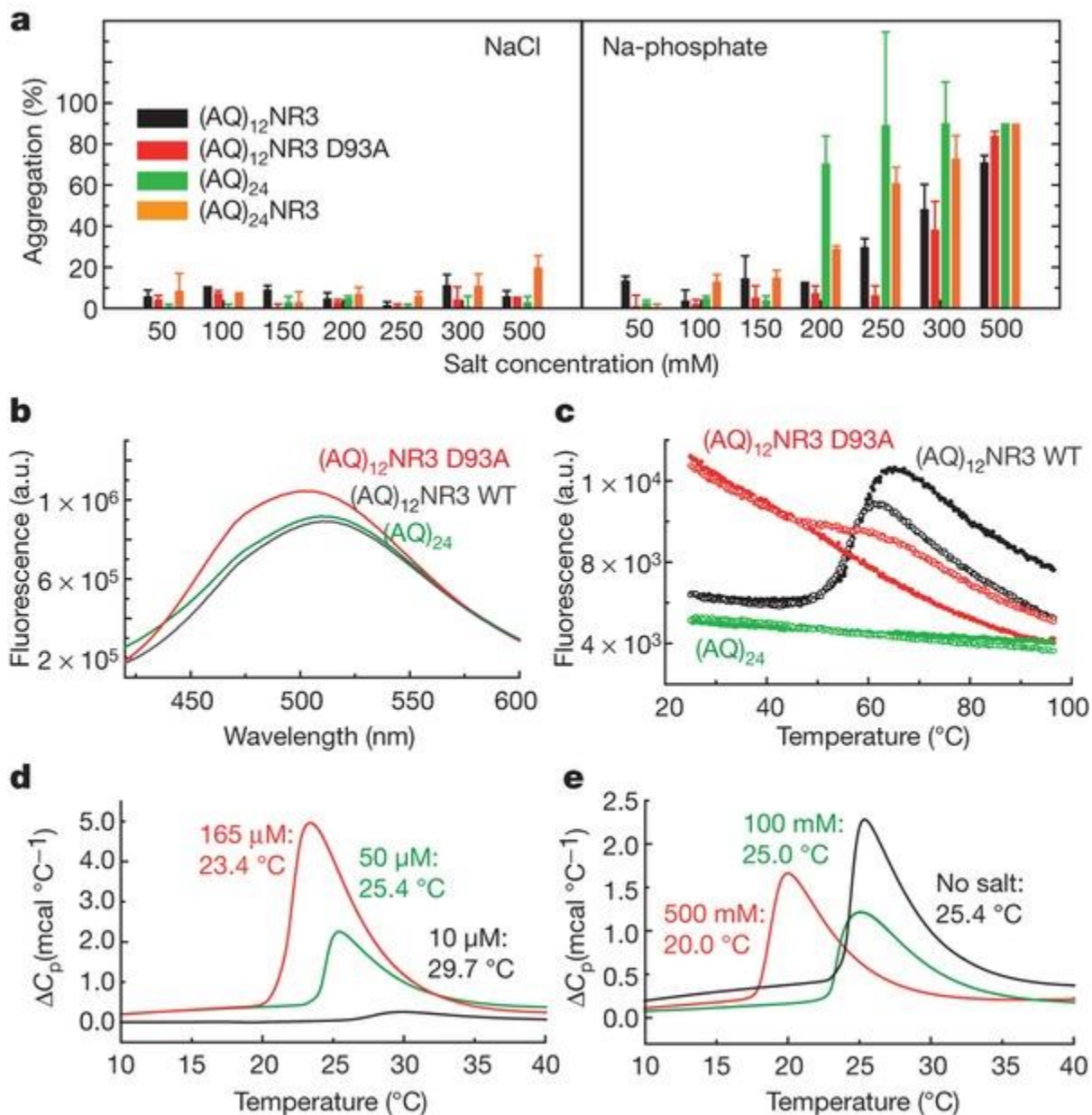


a, Hydrophobicity index of various silk proteins. b, Sequence alignment of the C-terminal NR domains of the major ampullate silk proteins (MaSp) of *Araneus diadematus*, *Nephila clavipes* and *Euprosthena australis*, and minor ampullate silk fibroin 1 (ADF-1) of *Araneus diadematus* (top to bottom). Grey bars indicate the degree of conservation between these sequences. The conserved

charged residues are coloured. c, Overlay of the 20 best-energy structures of the C-terminal NR domain of ADF-3 having a root mean square deviation (r.m.s.d.) of 0.18 Å.

Using mutagenesis experiments, our data show that the charged residues are essential for the structural integrity of the NR domain, as deletion of the salt bridges results in significantly reduced thermal stability of the entire domain ([Supplementary Fig. 2](#)). Of particular note is that although the NR domain is relatively hydrophobic in comparison to the repetitive backbone of the protein ([Fig. 1a](#)), in the folded state the hydrophobic residues are buried and the solvent-exposed surface displays hydrophilic amino acids such as glutamine and serine, assuring its hydration ([Supplementary Fig. 1b](#)). At low pH (pH 2, where the salt bridges are disrupted) NR3 shows markedly increased hydrophobicity and decreased secondary structure content as probed with 8-anilinonaphthalene-1-sulphonic acid (ANS) fluorescence, circular dichroism (CD) and NMR spectroscopy ([Supplementary Fig. 3a–d](#)). In addition, a destabilized NR3 salt bridge mutant (D93A) of an engineered ADF-3 analogue, (AQ)₁₂NR3 containing 12 repeats of the 'A' and 'Q' repetitive sequence elements¹⁶ (see Methods), also shows pronounced ANS binding ([Fig. 2b](#)) and molten globule-like thermal unfolding behaviour (a partially structured state that shows higher binding affinity to ANS than the native state), which can be stabilized by the addition of sodium chloride ([Fig. 2c](#)). This emphasizes the role of a correctly folded NR domain for silk protein storage and the inhibition of undesired aggregation.

Figure 2: Assembly and aggregation properties of our spider silk-like proteins.



a, Salt-induced protein aggregation. Error bars indicate standard deviation. b, Binding of hydrophobic ANS by proteins. a.u., arbitrary units; WT, wild type. c, Thermal transition experiments indicate molten globule-like behaviour of the D93A variant (red symbols), whereas the wild-type protein binds to the dye only during unfolding (black symbols). A structural transition of the D93A variant can be induced by sodium chloride (open symbols: in presence of 300 mM sodium chloride). Without the NR domain, no transition can be observed (green symbols). d, The concentration dependence of the LCST of (AQ)₂₄NR3. e, The sodium chloride dependence of the LCST of (AQ)₂₄NR3.

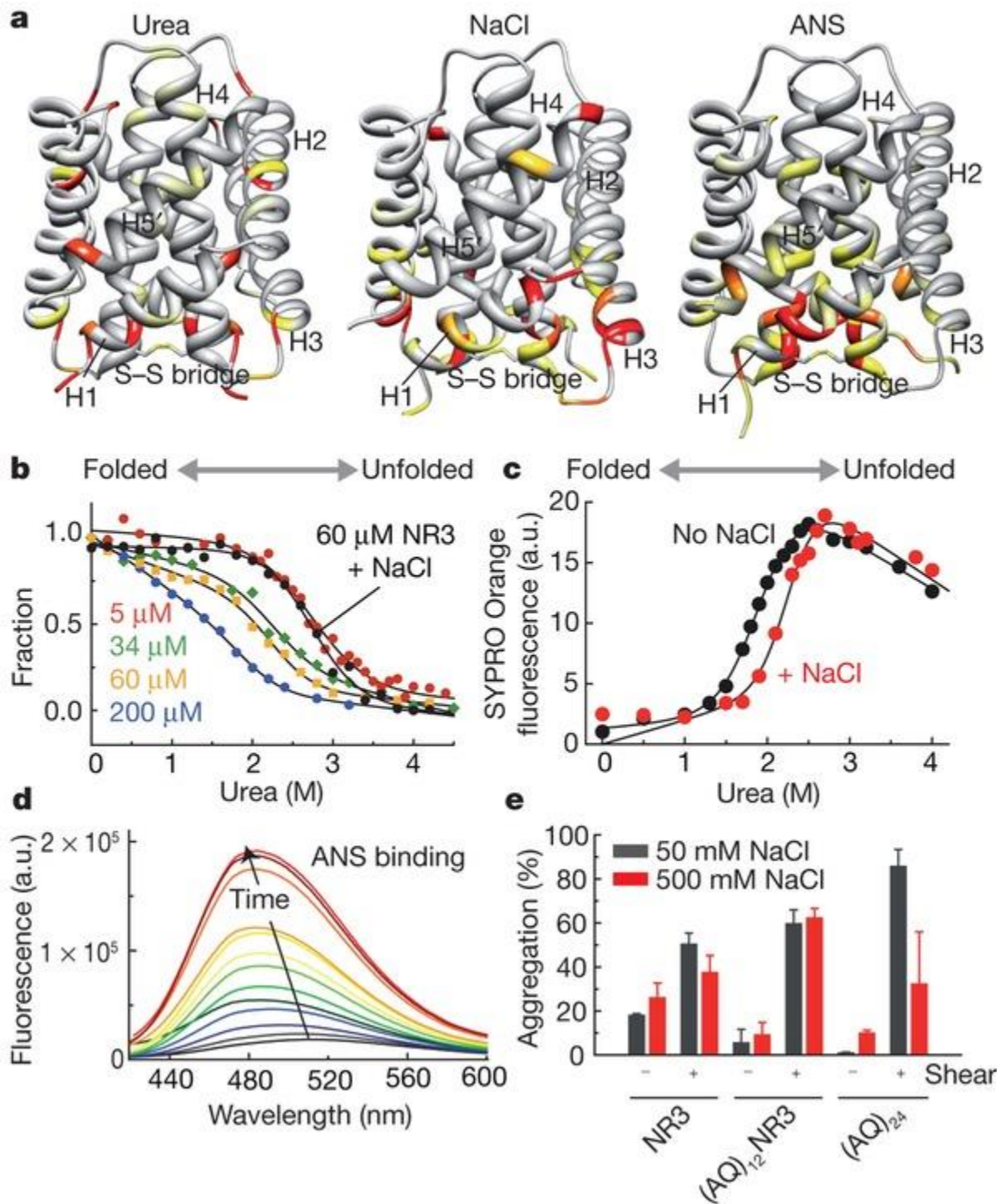
In vivo the transition from soluble protein to solid fibres involves a combination of chemical and mechanical stimuli (such as ion exchange, extraction of water, acidification and elongational flow^{3, 13, 17}). Hence, we were interested in determining the effect of such stimuli on spider silk-like proteins, and used dimeric (AQ)₁₂NR3 and monomeric (AQ)₂₄ (an analogue of approximately the same molecular mass as the dimer without the NR carboxy-terminal domain). In the absence of shear forces (as silk proteins are stored in the lumen *in vivo*) the extent of protein aggregation *in vitro* is determined by the concentration of salt, and even at extremely high concentrations of sodium chloride (up to

500 mM) the level of aggregation is low (less than 15%), whereas in the presence of equivalent concentrations of sodium phosphate the level of aggregation is markedly higher (up to 100%) owing to the more kosmotropic nature of the phosphate anion ([Fig. 2a](#)). Such a finding explains why the proteins are stored inside the lumen in the presence of the relatively chaotropic sodium chloride (approximately 100–150 mM)⁹, and emphasizes the requirement for ion exchange within the spinning duct.

Higher supramolecular assemblies are required to achieve efficient protein storage⁸ and we have previously reported our proteins to be thermoresponsive, displaying fully reversible lower critical solution temperature (LCST, phase separation on increasing the temperature) behaviour owing to a self-assembly process¹⁶. This behaviour was displayed only for silk proteins incorporating the NR domain. Using differential scanning calorimetry (DSC) we show here that the LCST is decreased in the presence of higher concentrations of salt and/or protein ([Fig. 2d, e](#)), thereby stabilizing the proteins during storage.

We have also found that the NR domain has an impact on the fibre formation process. On titration of chemical denaturants (urea or guanidine hydrochloride) into solutions of ¹⁵N-labelled NR3, NMR chemical shift changes (indicating structural variations or binding) monitored with ¹⁵N HSQC (heteronuclear single quantum coherence) experiments could be observed mainly within helix 1 and around the salt bridges ([Fig. 3a](#), [Supplementary Fig. 4](#)), therefore this region seems to unfold first. Titrations with sodium chloride demonstrated chemical shift changes in the same region (where the two salt bridges are located, [Fig. 3a](#)), which also shows the highest hydrophobicity in the protein as monitored with chemical shift changes upon the addition of ANS ([Fig. 3a](#) and [Supplementary Fig. 4](#)). Interestingly, at higher protein concentrations the protein shows a greater tendency to unfold ([Fig. 3b](#)). Additionally, SYPRO Orange (an ANS-like fluorescent dye) binding experiments¹⁸ demonstrate that the partially folded state of NR3 is more hydrophobic than the natively folded or the unfolded state ([Fig. 3c](#)). It is also noteworthy that the presence of sodium chloride (present during protein storage) stabilizes the NR domain against thermal or chemical denaturation ([Fig. 3b, c](#) and [Supplementary Fig. 5a](#)) and can even compensate for the loss in stability during reduction of the inter-molecular disulphide bridge ([Supplementary Fig. 5b](#)).

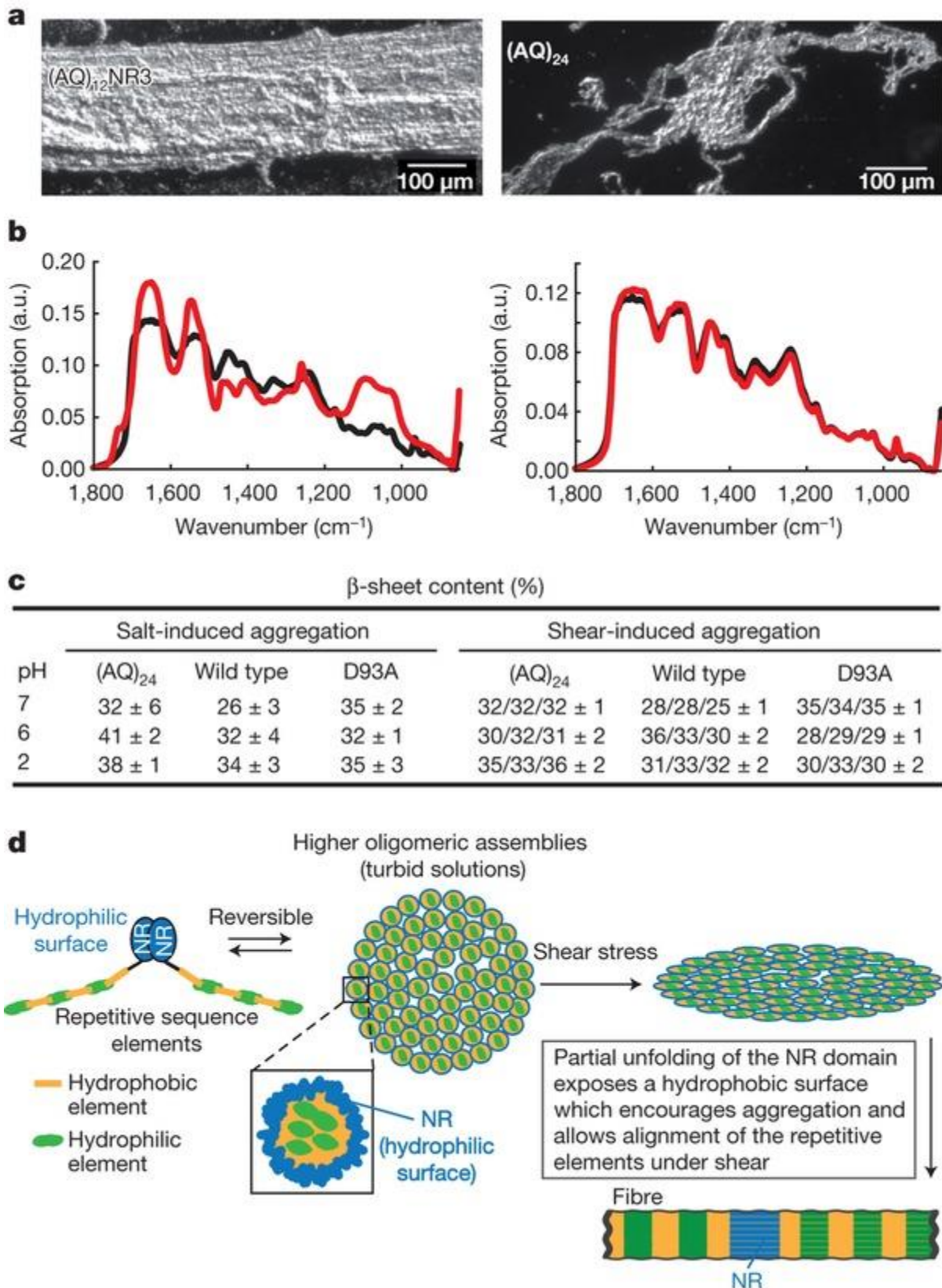
Figure 3: Stability and folding of spider dragline silk constructs.



a, NMR chemical shift perturbation experiments with urea, sodium chloride and ANS indicate that the structural elements around the salt bridges represent the most labile and hydrophobic region within the protein. b, Urea-induced unfolding of NR3 as determined by CD spectroscopy. c, Binding of hydrophobic SYPRO orange to NR3 during urea-induced unfolding. d, Shear-induced ANS binding of NR3. e, Aggregation of our spider silk-like proteins with and without shear stress. Shear stress leads to increased aggregation and is the most likely trigger for the assembly process in nature. Error bars represent standard deviation.

Previous rheological studies emphasize the role of the NR domain for shear-induced aggregation of ADF-3, where solutions of proteins with the NR domain exhibit significant viscosity changes under shear, whereas solutions of proteins without the NR domain exhibit no viscosity changes at various shear rates^{14, 19}. ANS binding also increases under shear, indicating that the NR domain partially unfolds, thereby exposing hydrophobic surfaces ([Fig. 3d](#) and [Supplementary Fig. 6](#)), leading to oligomerization and ultimately β -sheet-rich fibre formation. In further *in vitro* protein aggregation assays under shear stress, we find that aggregation is vastly increased in comparison to corresponding assays in the absence of shear, and that the protein without the NR domain is much more prone to unspecific aggregation ([Figs 3e](#) and [4](#)). Exposure of solutions of proteins without the NR domain to shear stress resulted in the formation of ill-defined β -sheet-rich aggregates, typically with dimensions of micro- to millimetre scale ([Fig. 4a](#)). By contrast, proteins incorporating the NR domain formed relatively well-defined fibrous aggregates, which optical microscopy showed to be composed of ordered (aligned) bundles of long fibrillar aggregates, implying that the NR domain has a role in stabilizing the protein against undesirable aggregation. Polarized Fourier transform infrared spectra of the fibrous aggregates formed from our protein without the NR domain ((AQ)₂₄) show that the β -sheets are randomly aligned, whereas the β -sheets within fibres formed from our protein with the correctly folded NR domain (that is, wild type and not the D93A mutant) were predominantly aligned with the long axis of the fibres (in analogy to naturally spun fibres) owing to a controlled assembly process ([Fig. 4b, c](#)).

Figure 4: Fibre assembly mechanism of dragline silk proteins.



a, A well-defined fibre (dry state) of (AQ)₁₂NR3 and ill-defined fibrous aggregate of (AQ)₂₄ formed under shear. b, Infrared absorption spectra of (AQ)₁₂NR3 and (AQ)₂₄ fibres recorded at 0° and 90° relative to the long axis of the fibrous aggregates (black vs red line). c, Estimated β -sheet content of the aggregates. The values of β -sheet content for the aggregates formed under shear were recorded

at 0°, 45° and 90° relative to the long axis of the fibrous aggregates. d, The silk proteins are stored as higher oligomeric assemblies. Exposure of these assemblies to shear and salting out leads to partial unfolding of NR3 and controlled fibre assembly.

To examine the influence of the length of the repetitive backbone on the behaviour of the proteins, we performed aggregation assays with (AQ)₂₄NR3, finding that it was significantly more prone to aggregation than the equivalent (AQ)₁₂NR3 analogue (Fig. 2a), which leads us to speculate that the α -helical N-terminal NR domain⁴ (not included in our recombinant proteins) might also play an important function in protein storage and fibre assembly, especially in case of the natural system, where the proteins are larger.

Our data indicate that the C-terminal NR domain has a role in both spider silk protein storage and fibre assembly (Fig. 4d). During storage of spider silk proteins, the NR domain stabilizes a solution-competent state of the silk protein via the formation of higher supramolecular assemblies, and the C-terminal NR domain also acts as a trigger for the fast, controlled and efficient salting-out of the proteins in combination with the correct alignment of the β -sheet forming repetitive sequence elements during shear-induced assembly. The hydrophobic surface of the assembly state of the NR domain, in addition to the disulphide bridge, provides anchor points for the correct positioning of the repetitive sequence elements. This mechanism is reminiscent of other fibrillar proteins²⁰ like collagen²¹, where the highly conserved terminal pro-peptides form intermolecular disulphide bridges²² and regulate pro-collagen solubility and lateral assembly²³.

Methods:

Cloning, protein production and purification

The initial construct used for NMR resonance assignment (NR3-T7) was produced and purified as described previously²⁴. The gene of a shorter construct (NR3Δ19) used for NOESY experiments was inserted into a pET28a expression system (Merck Biosciences) encoding an N-terminal His₆-tag and expressed in *E. coli* BL21 (DE3) (Agilent Technologies) at 20 °C for 16–20 h. Purification was achieved by passage over a Ni-NTA column (GE healthcare) and a Superdex75 column. Between both columns the hexahistidine tag was removed by digestion with thrombin. Isotopically (¹³C, ¹⁵N) enriched protein samples were produced by growing bacteria in M9 medium supplemented with 1 g l⁻¹ ¹⁵NH₄Cl and 2 g l⁻¹ ¹³C glucose (Eurisotope). For NMR measurements, the proteins were concentrated up to 1 mM in 10 mM sodium phosphate pH 6.0. Addition of 1% (v/v) trifluoroethanol-d₃ (Eurisotope) was used for sample stabilization. The generation of a mixed ¹³C, ¹⁵N-¹²C, ¹⁴N dimeric sample was achieved by mixing equal amounts of labelled and unlabelled His₆-tagged protein samples and a subsequent addition of guanidine hydrochloride to a final concentration of 4 M and 50 mM DTT to achieve unfolding and reduction of the inter-molecular disulphide bridge. For refolding, the mixture was dialyzed against 50 mM Tris/HCl pH 8.0 resulting in an almost quantitative refolding yield. By this procedure, the monomers are allowed to exchange resulting in a 1:2:1 population of the homo-unlabelled, the hetero-labelled and the homo-¹³C, ¹⁵N-labelled species. Genes encoding proteins containing the repetitive elements (AQ)₁₂ and (AQ)₂₄ (A, hydrophobic polyalanine-rich motif: GPYGPASAAAAAGGYGPGSGQQ; Q, hydrophilic glutamine- and glycine-rich motif: GPGQQGPGQQGPGQQGPGQQ) were cloned into a pET29 expression vector (Merck Biosciences) encoding an N-terminal T7-tag and were expressed in *E. coli* strain BLR (DE3) (Agilent Technologies) at 25 °C for 3 h. Purification was carried out as described previously²⁴. Site-directed mutagenesis experiments with the gene of NR3Δ19 were done using the QuikChange mutagenesis kit (Agilent Technologies).

Circular dichroism (CD) spectroscopy

CD spectra and stability measurements were carried out on a Jasco J-715 spectropolarimeter (Jasco). For spectra and thermal transitions, a protein concentration of 10 μM was used, and thermal unfolding was monitored by the CD signal at 222 nm in a 1 mm path length cuvette using a heating rate of 60 K h⁻¹ in 10 mM sodium phosphate pH 6.0. For chemical unfolding experiments, samples of 5, 34, 110, 200 and 230 μM concentration were incubated with increasing amounts of urea overnight at 4 °C, and the CD signal at 222 nm was recorded with a path length of 0.1 or 1 cm, respectively, at 20 °C. A bandwidth of 5 nm and a response of 2 s were used. Fitting of the thermally and urea-induced unfolding profiles was done as reported previously²⁸ assuming a two-state folding mechanism.

NMR spectroscopy and structure calculation

Nuclear magnetic resonance (NMR) spectroscopic measurements were conducted at 298 K on Bruker DMX600, DMX750 and Avance900 spectrometers (Bruker Biospin). Backbone resonance assignment was achieved by a set of standard triple resonance experiments²⁵. Side-chain assignment was carried out using a combination of CCH-COSY (correlation spectroscopy via the nuclei C,C,H²⁹) and HCH-TOCSY

(total correlation spectroscopy³⁰) on double-labelled samples. Stereospecific assignment of prochiral H^β methylene and valine methyl groups and the resulting rotamer assignment were made using ³J_{NHβ} couplings observed in the HNHB experiment and patterns of NOESY connectivities. Distance data were derived from a set of three-dimensional NOESY experiments including ¹⁵N-HSQC-NOESY, NNH-NOESY on a ¹⁵N-labelled sample and ¹³C-HSQC-NOESY and heteronuclear edited 3D-CCH NOESY and 3D-CNH NOESY experiments²⁶. For obtaining inter-subunit NOE distance restraints, we used a ¹⁴N,¹²C-filtered/¹³C edited two-dimensional-NOESY experiment on a mixed ¹³C, ¹⁵N-¹²C, ¹⁴N labelled sample. NOESY cross-peaks in the three-dimensional spectra were converted into distance ranges after rescaling according to the corresponding HSQC intensities. Cross-peaks were divided into the following four classes: strong, medium, weak and very weak, which resulted in restraints on upper distance of 2.7, 3.2, 4.0 and 5.0 Å, respectively. Lower distance restraints were also included for very weak and absent sequential H^N-H^N cross-peaks using a minimum distance of 3.2 Å and for medium intensity or weaker sequential and intraresidual H^N-H^α cross-peaks using a minimum distance of 2.7 Å. Allowance for the use of pseudo atoms (using *r*⁻⁶ averaging) were added for methyl groups (0.8 Å for one methyl and 1.5 Å for two methyls) and nonstereospecifically-assigned methylene groups (0.7 Å). Dihedral angle restraints were derived for backbone φ and ψ angles based on C^α, C^β, C' and H^α chemical shifts using the program TALOS³¹. Restraints were applied for predictions consistent with the input chemical shifts, ³J_{HNHα} coupling constants measured from an HNHA experiment and the observed NOE pattern within sequential residues. Accepted predictions were applied using the tolerance calculated by the program ± 5°. The ³J_{HNHα} couplings were also applied as direct coupling constant restraints on the backbone φ angles. Hydrogen bond restraints were applied for 73 residues in secondary structural elements with low hydrogen exchange rates, as measured with MEXICO experiments^{32, 33} and where donor and acceptor were consistently identified in preliminary calculations. The restraints were applied with the inclusion of pseudo covalent bonds as described previously³⁴. Structures were calculated with Xplor-NIH²⁷ using standard protocols. Experimental restraints were applied to only one monomer, with non-crystallographic symmetry restraints over the backbone of ordered residues (S34-L138) used to assure the symmetry of the dimer. Sets of 50 structures were calculated and a final set of 20 structures chosen on the basis of lowest restraints violations. An average structure was calculated and regularized to give a structure representative of the ensemble. The quality of the regularized average structure was assessed with the program PROCHECK-NMR³⁵. Titration experiments were performed with 200–500 μM NR3 in 10 mM sodium phosphate pH 6.0 at 293 K using standard ¹⁵N-HSQC experiments. Chemical shift deviations were calculated as the mean of ¹H

and ¹⁵N chemical shift differences using following equation:

$$\Delta\delta_{av} = \left(\sqrt{\Delta\delta_H^2 + (\Delta\delta_N/5)^2} \right) / 2$$

Fluorescence spectroscopy

Fluorescence spectra of 10 μM ANS and SYPRO Orange in 10 mM sodium phosphate pH 6.0 were recorded with a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon) using excitation at 350 nm (5 nm bandwidth) and by collecting emission spectra from 420 to 600 nm with a bandwidth of 5 nm. With SYPRO Orange, excitation was set to 485 nm and emission was recorded from 520 to 700 nm. Thermofluor experiments³⁶ were performed with a real-time PCR machine (Agilent Technologies) by detection of ANS fluorescence (10 μM ANS was used). Heating rate was 30 K h⁻¹, protein concentration was 50 μM and sample volume was 50 μl in a 96-well plate. Five individual measurements were done for each protein sample and solvent condition.

Differential scanning calorimetry (DSC)

DSC experiments were performed using a VP-DSC Micro-Calorimeter (MicroCal). Standard measurements were performed at a protein concentration of 50 μM in 10 mM Tris/HCl, pH 8.0, with a heating rate of 20 K h^{-1} . Evaluation of the LCST behaviour was done using the ORIGIN software (MicroCal).

Rotation-induced aggregation

Before the experiment the proteins were dissolved in guanidine thiocyanate solution (6 M) and then dialyzed against 10 mM Tris/HCl, pH 8.0. Then the proteins were diluted into buffer containing 10 mM Tris/HCl, pH 8 with 50 mM NaCl. Final protein concentrations were adjusted to 40 μM of NR3, 14 μM of AQ₁₂NR3 and 8 μM of AQ₂₄. The samples were incubated for 12 h at 25 °C rotating with 25 r.p.m. After rotation, the clearly visible aggregates were transferred onto glass slides for optical microscopy (DMI3000B, Leica). The samples were then centrifuged at 125,000g to remove non-visible aggregates, and the amount of remaining soluble protein in the supernatant was determined using an ultraviolet spectrometer (NanoDrop ND1000, Thermo Fischer).

Fourier transform infrared (FTIR) spectroscopic studies

FTIR spectra of fibres were recorded on a liquid-nitrogen-cooled Bruker Tensor 27 FTIR spectrometer, fitted with a liquid-nitrogen-cooled Bruker Hyperion microscope with IR polarizers supplied by Optometrics Corporation. Spectroscopy was performed on fibres formed during the previously described aggregation assay that were washed with water to remove salts, air dried and placed on highly polished optical grade calcium fluoride disks (Crystal GmbH). Spectra of all aggregates were recorded in absorbance mode at 21 °C, with a 4 cm^{-1} resolution and 600 scans (corrected for background and atmosphere using OPUS software), and polarized FTIR spectra of aggregates of micrometre scale (and larger) were recorded at three different orientations (0°, 45° and 90° relative to the long axis of the fibrous aggregates) to determine if any of the secondary structural elements were aligned with the fibres.

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Author Information The resonance assignment obtained was deposited at the BMRB data bank under accession code 16249 and the atomic coordinates of the best 20 structures plus a regularized average structure have been deposited at the Protein Data Bank under accession code 2khm. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to H.K. (horst.kessler@ch.tum.de) or T.S. (thomas.scheibel@uni-bayreuth.de).