

Optical trapping with high forces reveals unexpected behaviors of prion fibrils

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Amyloid fibrils are important in diverse cellular functions, feature in many human diseases and have potential applications in nanotechnology. Here we describe methods that combine optical trapping and fluorescent imaging to characterize the forces that govern the integrity of amyloid fibrils formed by a yeast prion protein. A crucial advance was to use the self-templating properties of amyloidogenic proteins to tether prion fibrils, enabling their manipulation in the optical trap. At normal pulling forces the fibrils were impervious to disruption. At much higher forces (up to 250 pN), discontinuities occurred in force-extension traces before fibril rupture. Experiments with selective amyloid-disrupting agents and mutations demonstrated that such discontinuities were caused by the unfolding of individual subdomains. Thus, our results reveal unusually strong noncovalent intermolecular contacts that maintain fibril integrity even when individual monomers partially unfold and extend fibril length.

Amyloid fibrils are self-associating protein aggregates with a common cross- β -sheet structure¹. Formation of amyloids under normal physiological conditions has long been associated with devastating human diseases, in which amyloids sometimes cause pathology² and sometimes protect against it^{3,4}. More recently, researchers have come to appreciate that some amyloidogenic proteins serve an astonishing variety of normal biological functions, including protein scaffolding, environmental adaptation, long-term memory and biofilm formation⁵. Moreover, the capacity to acquire an amyloid conformation is an inherent property of most proteins⁶, suggesting it is one of the most ancient folds in protein chemistry. These discoveries have produced an explosion of interest in the nature of amyloids, but the fibrous nature of these proteins generally prohibits standard structural analyses. Thus, the molecular forces that govern their structures have been difficult to elucidate.

Among the amyloidogenic proteins, fibrils formed by the Sup35 prion protein of *Saccharomyces cerevisiae* are of particular interest because they constitute a protein-based mechanism for the inheritance of biological traits. In its normal soluble state, Sup35 is a translation-termination factor. When it switches into the prion (amyloid-forming) state, less soluble protein is available to function in translation, causing the read-through of nonsense codons. Associated changes in gene expression create a wide variety of novel phenotypes, enhancing survival in diverse environments⁵. These phenotypes are heritable by a 'protein-only' mechanism: mother cells faithfully pass prion conformers on to their daughters, perpetuating the cycle of conformational conversion.

Intense study of Sup35 prions has already provided many insights. First and foremost, these studies have shown that the basis of prion replication is the ability of prion fibrils to template the conformational

conversion of protein in the nonprion conformation into the prion conformation⁷⁻⁹. The fibril-severing activity of Hsp104 then provides fragments for inheritance in daughter cells¹⁰⁻¹³. Further, like most amyloid proteins, Sup35 forms a variety of structurally related but distinct fibril types¹³. Biologically, these variants lead to phenotypically distinct transmissible prion states that are readily assayed *in vivo*. When different amyloid variants are taken up by prion-free (prion-minus) yeast cells after cell-wall removal (protein-only transformation), they create distinct stably propagating prion phenotypes, or 'strains'¹⁴. Amyloid variants that are more easily fragmented generate a greater number of seeds¹³ and leave a lower concentration of soluble Sup35 available for translation¹⁵. Thus, somewhat paradoxically, structurally weak fibrils create phenotypically strong prions.

Here we describe an optical-trapping technique we have developed to manipulate individual prion fibrils and directly characterize their fragmentation. Although optical trapping has provided a wealth of information on DNA-enzyme interactions¹⁶, motor protein movements¹⁷ and protein-folding landscapes¹⁸⁻²⁰, it has not been applied to amyloids because a suitable experimental geometry has not been available. Manipulation of amyloid fibrils has, to date, been achieved only by atomic-force microscopy. In these experiments, fibrils have been laid onto a supporting surface and forces have been applied perpendicular to the fibril axis²¹⁻²³. To directly assess the molecular forces that maintain fibril integrity, we sought to stretch fibrils parallel to the fibril axis. We also wished to keep them free in solution, to eliminate the complications of surface interactions. Taking advantage of the self-assembly properties of prion fibrils, we devised a method to attach one end of the fibrils to a glass surface and the other end

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to a polystyrene bead. This allowed us to optically trap and pull on the beads. Further, the advances in our instrumentation allowed us to apply much greater forces than those previously used in trapping experiments and permitted direct fluorescent visualization of fibrils during force application.

RESULTS

Fibrils with uniform biological properties are produced

Sup35 has three distinct regions. The C-terminal region contains the translation-termination domain but is completely extraneous to prion behavior *per se*^{8,9}. The ability of Sup35 to exist stably and heritably in one of two different states, the prion state or the nonprion state, derives from the divergent properties of the N-terminal region (N) and the middle region (M)²⁴. N is extremely rich in glutamine and asparagine residues and drives the protein into the prion amyloid. M is highly charged (with lysines and glutamates) and promotes the soluble nonprion state (Fig. 1a). To allow visualization of fibrils in optical-trapping experiments, we used a variant of NM, a protein only containing the N and M regions, with a single cysteine substitution at residue 184. The NM variant was labeled with an Alexa-555 fluorophore and mixed with an equal concentration of wild-type NM for polymerization. This substitution is in the M domain, lies outside the amyloid fibril core^{25–27} and has no effect on fibril formation^{25,28}. The biological purity of these fluorescently labeled fibrils was assayed by protein-only transformation (Fig. 1d–f).

Initially, the proteins were allowed to assemble into amyloid spontaneously. However, such fibrils produced a spectrum of prion strains with different amounts of nonsense suppression when they were assayed by protein-only transformation of prion-minus cells (Fig. 1b,c). To ensure that fibrils were both biologically homogenous and of clear relevance to the *in vivo* prion state, we seeded polymerization with lysates of cells carrying phenotypically strong or weak prion elements. For strong prions, polymerization was performed at 4 °C, a condition that favors assembly of amyloids conferring the strong prion phenotype¹⁴. Indeed, the resulting fibrils produced a uniform and strong nonsense-suppression phenotype when analyzed by protein-only transformation (Fig. 1d,f). For weak prion elements, polymerization reactions were seeded at 37 °C, which favors assembly of amyloids conferring a weak prion phenotype. When assayed by protein-only transformation of prion-minus cells, these fibrils produced only weak biological phenotypes (Fig. 1e,f). Thus, these procedures allowed the preparation of fluorescently labeled protein fibrils that carried true prion states but of single subtypes.

Figure 1 The biological homogeneity of NM fibrils was confirmed by protein-only transformation. (a) Diagram of wild-type Sup35. The black segments in the N-terminal region of Sup35 (N) represent oligopeptide repeats. The highly charged nature of the middle region (M) is indicated. The C-terminal region (C) functions in translation termination. In b–e, biologically distinct prion states were induced when prion-minus (*[psi⁻]*) cells were transformed with fluorescently labeled fibrils formed under different conditions. (b) Colonies transformed with buffer alone. (c) Colonies transformed with spontaneously assembled NM fibrils. A mixture of prion phenotypes is highlighted in the blue box. (d) Colonies after protein-only transformation at 4 °C with NM fibrils seeded by lysates of yeast cells that carried a strong prion element. The uniform strong prion phenotypes (*[PSI⁺]*) are indicated by the light pink-colored colonies in the blue box. (e) Colonies after protein-only transformation at 37 °C with NM fibrils seeded by lysates from cells carrying a weak prion element. The uniform weak prion phenotypes are indicated by the dark salmon-colored colonies in the blue box. (f) Quantification of transformation efficiency and specificity of distinct prion fibrils. ‘Strong fibrils’ refers to the fibrils used in d; ‘weak fibrils’ refers to the fibrils used in e.

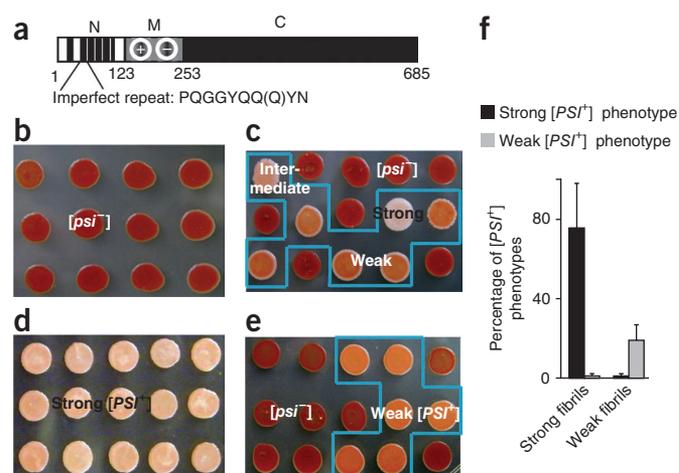
A strategy for optical manipulation was developed

To manipulate single fibrils with the optical trap, we attempted to attach one end of the fibrils to a glass surface and the other end to a polystyrene bead, providing ‘handles’ for optical pulling experiments stretching the fibrils from their ends. We tested several strategies reported in the literature for the attachment of other proteins for optical-trapping experiments but none achieved the desired geometries^{18,19,29}. As an alternative, we sought to take advantage of a quintessential property of yeast prions, their ability to serve as a template for the conformational conversion of soluble monomers. This recruitment of monomers occurs exclusively at fibril ends^{30,31}. We asked whether we could invert this process and use unstructured monomers bound to surfaces to recruit and capture preformed fibrils from solution.

We adsorbed NM monomers onto the glass surface of a flow chamber, with the expectation that the highly charged glass surface would interact with the highly charged M domain, leaving the amyloidogenic N domain free to interact with fibrils (Fig. 2a and Supplementary Methods). The remaining exposed glass surface was then blocked against nonspecific protein interactions with a solution of casein. Next, fluorescently labeled NM fibrils were introduced to the chamber for capture by the pre-affixed monomers. Finally, polystyrene beads (0.8 μm), which had themselves been prebound with NM monomers, were introduced for recruitment to the remaining free ends of the fibrils.

We visualized the fibrils with fluorescent imaging to optimize conditions and confirm the nature of their attachments (Supplementary Videos 1 and 2; see Online Methods for experimental details). Casein proved very effective in preventing nonspecific interactions between prion fibrils and the glass surface. The self-recognition properties of pre-attached monomers were necessary and sufficient to tether one end of the fibrils to the glass surface and capture the other end with the polystyrene bead.

To determine whether NM monomers captured the NM fibrils via bona fide prion interactions, we took advantage of the ‘prion species barrier’. This barrier inhibits prion transmission between Sup35 proteins that are derived from evolutionarily divergent protein sequences³². We asked whether the NM domain from the *Candida albicans* Sup35 protein could capture the NM fibrils of *S. cerevisiae*. The *Candida* protein has the same highly unusual amino acid composition as the *Saccharomyces* protein, rich in asparagine and glutamine residues (Fig. 2b), but the sequence is distinct and the two prions do not cross-seed each other³². When *Candida* monomers were



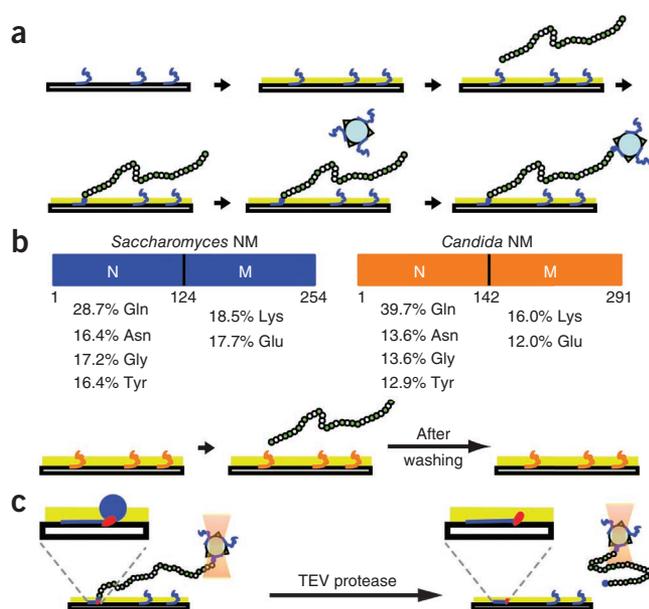


Figure 2 NM monomers effectively and specifically bind individual NM fibrils to a glass surface on one end and a polystyrene bead on the other. **(a)** Schematic diagram of the method for the attachment of NM fibrils. Monomers (blue) are adhered to the glass surface, and the remaining surface is coated with casein (light green). Green circles, fluorescently labeled NM monomers; white circles, wild-type NM monomers. Polystyrene beads (light blue circles) labeled with Alexa 488 (green triangle) and Alexa 555-NM (blue) are introduced and captured by the other free ends of the fibrils. **(b)** Top, the molecular architectures and amino acid compositions of *Saccharomyces* NM (blue) and *Candida* NM (orange). Bottom, schematic illustration of the experiment showing the specificity between prebound monomers and fibrils. From left to right, deposition of *Candida* NM on the glass surface; incubation of *Saccharomyces* NM fibrils in the flow channel; imaging after washing. **(c)** Schematic diagram of the detachment of NM fibrils from the glass surface with a treatment of TEV protease. The TEV recognition site is shown in red and highlighted in the enlarged segments. The M domain (blue line) binds with the glass surface, and the N domain (blue circle) interacts with the ends of NM fibrils. The bead was held in position by the optical trapping (light pink).

predeposited on the glass surface followed by casein blocking, fibrils of the *Saccharomyces* protein were very rarely tethered to the surface (Fig. 2b and Supplementary Video 3). Thus, the attachment between the surface-bound NM monomers and NM fibril ends shows the species-specific interactions characteristic of the bona fide prion fold.

The anchoring monomers provide specific interactions

To determine whether the M domain of the individual monomers pre-attached to the surface was solely responsible for anchoring the fibrils to the glass surface, we engineered NM monomers to carry a tobacco etch virus (TEV) protease cleavage site (ENLYFQG) at the junction between N and M (residues 128–134). These monomers, prebound to the glass surface, were used to recruit fibrils that were entirely composed of NM proteins that did not contain a TEV site. We captured the fibril-tethered bead in the trap and then introduced TEV protease. This released the entire fibril from the glass surface (Fig. 2c and Supplementary Videos 4–6). We conclude that, except for the M domain of the single anchoring subunit, no interactions exist between the glass surface and the prion fibrils. Thus, individual prion monomers can function as a highly specific amyloid ‘glue’, with the M domain providing an anchor to the surface and the N domain capturing a preformed fibril at its end.

Prion fibrils are remarkably resistant to mechanical forces

We first pulled fibrils that created phenotypically strong prions in the optical-trapping experiments because, as discussed in the introduction, they are structurally weaker and more likely to be fragmented. We pulled fibrils with forces up to ~100 pN, which is sufficient to unfold many globular proteins at the low loading rates typically achieved in optical-trapping force spectroscopy ($1\text{--}100\text{ pN s}^{-1}$)^{18–20,33}. (Mechanical unfolding of proteins is strongly dependent on loading rate because of the nonequilibrium nature of the pulling experiments. Unfolding proteins at higher loading rates, such as those achieved with atomic-force microscopy ($\sim 10^4\text{--}10^6\text{ pN s}^{-1}$), generally requires greater forces, 100–500 pN.) We subjected fibril tethers to force-extension measurements by focusing the laser beam on the beads to trap them and then moving the flow chamber with attached NM fibrils on a piezoelectric stage with nanometer resolution (Supplementary Videos 7 and 8). Individual NM fibrils were first centered directly

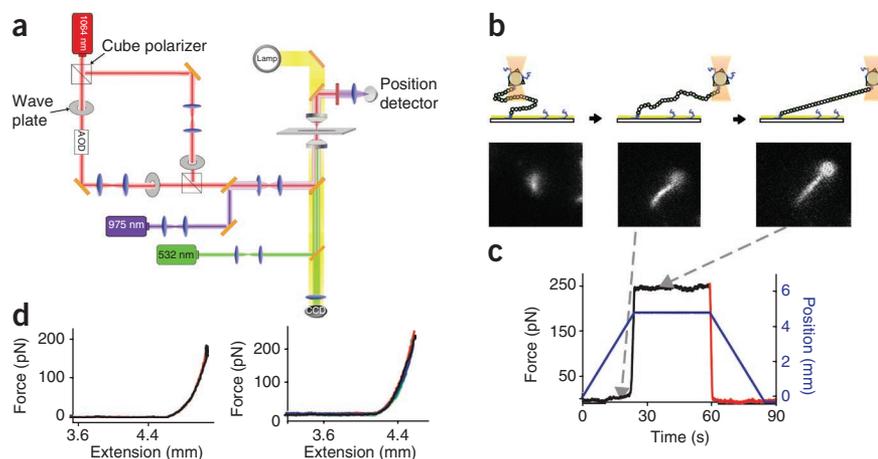
above the surface attachment point and then pulled from the center position to full extension (stretching phase). After being held at the extended position at high force for a prolonged period (holding phase, 40–80 s), the fibrils were finally relaxed (relaxation phase) back to the center position. The extension-relaxation had no effect on NM fibrils as the stretching curves and relaxation curves were overlapped (data not shown).

To achieve much higher forces, we used an optical-trapping instrument that was configured to include a second stationary trap (Fig. 3a). A lower-force primary branch was guided through acousto-optic deflectors (AODs) to enable precise calibration of the bead’s position³⁴. The second stationary branch, which had minimal optical components to reduce power loss, was then switched on to perform the pulling experiments. This allowed us to achieve forces of up to 250 pN using the same typical loading rate for optical-trap experiments (Fig. 3b,c and Supplementary Methods). Again, pulling had no effect on fibril integrity (Fig. 3c,d). Force-extension curves from the stretching and relaxation cycles were superimposable (Fig. 3d, left), even when individual fibrils were subjected to multiple stretching cycles (Fig. 3d, right), indicating that no rupture or permanent deformation had occurred. Thus, although NM fibrils are assembled via noncovalent monomer interactions, they are remarkably resistant to disruption by mechanical forces.

More extreme conditions produce complex traces

To increase the probability of detecting rupture events, we repeated fibril stretching in the presence of guanidine hydrochloride (GdHCl), a chaotropic reagent that decreases protein stability^{25,35}. NM fibrils with a strong prion phenotype unfold in the presence of GdHCl at $D_{1/2} \sim 1.5\text{ M}^{25}$. $D_{1/2}$ is the GdHCl concentration at which 50% of the protein is in the unfolded form. Under the greatest pulling forces we could achieve (250 pN), 0.4 and 0.8 M GdHCl had no effect. To our surprise, at 1.2 M GdHCl, instead of simple rupture most pulling trials yielded complex traces characterized by sudden force-dropping events. These occurred during both the stretching and holding phases. Several examples are shown in Figure 4a, with force-dropping events indicated by the black arrows and final rupture indicated by the red arrows. We were able to subject most fibrils to multiple cycles of stretching and relaxation, producing many associated force-dropping events before rupture (Fig. 4b shows two examples). In total, we recorded 50 stretching-relaxation cycles from 15 tethered NM fibrils and observed 116 force-dropping events before rupture.

Figure 3 Stretching NM fibrils by optical trapping at high forces with simultaneous fluorescent imaging. (a) Schematic of the trapping instrument with dual trapping capabilities used for high-force experiments (see Online Methods for details). 1,024 nm, trapping laser; 975 nm, position-detection laser; 532 nm, fluorescence imaging. (b) Schematic representation and fluorescence snapshots of the experiments. From left to right, the bead was first centered on top of the attachment point, and then the stage was moved along the x axis until the fibril was fully extended. (c) Representative pulling and relaxation traces for NM fibrils in the presence of normal assembly buffer. The force and the position of the stage were plotted as a function of time. Black line, stretching and holding phase; red line, relaxation phase; blue line, position of the bead relative to the attachment point of the tether on the glass surface. (d) Left, force applied versus fibril extension length (force-extension curve) for the tethered fibril experiencing one stretching-relaxation cycle. Black line, stretching and holding phase; red line, relaxation phase. The x axis is expanded to illustrate the behavior in the fully extended position. Right, the force-extension curves for a single tethered fibril that experienced four sequential stretching-relaxation cycles, whose curves are superimposed upon each other. For clarity, the holding phase is not shown. Stretching and relaxation curves from the same cycle are shown in the same color, with subsequent curves in a different color.



When individual fibrils were subjected to multiple stretching-relaxation cycles, the stretching curves from subsequent cycles were not superimposable (Fig. 4b), in contrast to those produced in the absence of GdHCl (Fig. 3d, right). This was because discontinuities in force curves increased fibril length. These length extensions were generally retained in the relaxation phase, probably because the high concentration of GdHCl prevented the protein from returning to its previous state (see below and Supplementary Fig. 1).

Excluding artificial origins of the force-dropping events

As occurs with other proteins³⁶, our NM constructs might form branched or kinked amyloid fibrils, and such geometries could give rise to complex mechanical behavior. The use of simultaneous fluorescent imaging in our optical-trapping experiments allowed us to eliminate this concern. We were able to focus exclusively on uniform, unbranched fibrils with a single swivel point of attachment to the glass surface. It is possible, however, that fibrils adhere to the glass surface at multiple points that are too close to the surface to be resolved by fluorescence. If so, abrupt elongations and sudden force drops would then be due to these attachments being 'peeled' sequentially from the surface. The ability of TEV to release the entire fibril from the surface when the only molecule containing a TEV site was the anchoring monomer (see above) made this extremely unlikely, but it was possible that the addition of GdHCl allowed additional attachments to form. We found, however, that

1.2 M GdHCl prevented predeposited NM monomers from recruiting NM fibrils to the glass surface and very rarely produced additional points of attachment (Supplementary Fig. 2).

Another explanation for the force discontinuities is that they reflect sequential release of small segments of the M domain from the individual anchoring monomer. This seemed extremely unlikely because the total length extensions we obtained were often much longer than an individual M domain (see below). To investigate this possibility more directly, we visualized the position of rupture by fluorescence imaging. If the sequential force-dropping events were due to the release of anchoring attachments, rupture events would simply represent the final release of the last anchoring M-domain segment. To visualize the position of fibril rupture, we trapped the bead with fibrils in the extended position and manually aligned the fibril with the x axis of the piezo stage without the prolonged process of centering. Fluorescent images could then be stably recorded before and after rupture. (Without the prolonged process of centering, photobleaching that occurs due to simultaneous exposure to the trapping and visualizing laser beams was minimized, allowing direct visualization.) In the recorded movies (Supplementary Videos 9–14), fragments representing various fractions of the initial fibril could be seen still attached to the surface after rupture. Thus, rupture was indeed not due to detachment of the anchoring M domain from the glass surface (or, for that matter, of the streptavidin linkage to the bead) but to severing of the fibril itself.

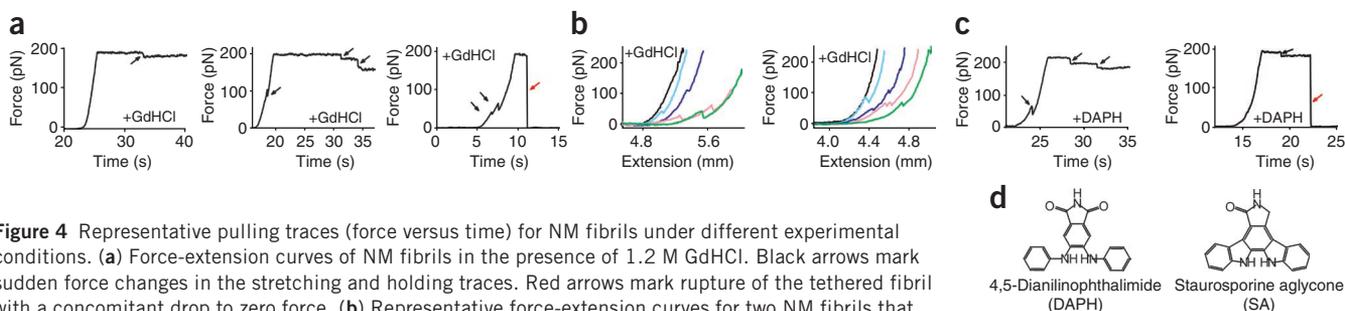


Figure 4 Representative pulling traces (force versus time) for NM fibrils under different experimental conditions. (a) Force-extension curves of NM fibrils in the presence of 1.2 M GdHCl. Black arrows mark sudden force changes in the stretching and holding traces. Red arrows mark rupture of the tethered fibril with a concomitant drop to zero force. (b) Representative force-extension curves for two NM fibrils that experienced multiple stretching-holding-relaxation cycles in the presence of 1.2 M GdHCl. Black, light blue, blue, light pink and green correspond to cycles 1–5, respectively, for each fibril. For clarity, only stretching phases are shown. (c) Force-extension curves in the presence of 50 μM DAPH. (d) The structures of DAPH and SA.

Complex traces reflect intrinsic properties of prion fibrils

Do discontinuities in the pulling traces derive from features integral to the structural and biological properties of the prion fibrils? We used several different types of experiment to investigate this question. First, we pulled fibrils in the presence of 4,5-dianilinophthalimide (DAPH), rather than GdHCl. DAPH is a highly specific disrupter of particular types of amyloid fibrils and, in particular, disrupts molecular contacts in the N domain of NM fibrils. It does not affect other types of protein folds³⁷. We recorded 51 stretching-relaxation cycles from 24 tethered NM fibrils in the presence of 50 μM DAPH and observed 146 force-dropping events before rupture. These force-dropping events were very similar in character to those observed in 1.2 M GdHCl (Fig. 4).

We then performed the experiments in the presence of staurosporine aglycone (SA). SA is a very close analog of DAPH, containing only two extra carbon-carbon bonds that impose planarity on the structure (Fig. 4d). In contrast to DAPH, however, SA does not dissociate NM fibrils and has no effect on the amyloid fold³⁷. In the presence of SA, we observed no discontinuities in force-extension curves. Thus, the force-dropping events that occurred in the presence of DAPH were specifically due to intrinsic structural properties of the amyloid fold.

Second, we stretched an amyloid variant made from the same NM protein but embodying a weak prion element. As previously described¹³, such fibrils are more resistant both to chemical denaturation and to physical fragmentation by agitation. When the variant was stretched by optical pulling in the presence of DAPH, only two isolated force-dropping events were observed in 76 stretching-relaxation cycles with 16 fibrils. The two fibrils that experienced such an event were stretched several times thereafter with no further drops in force. These weak-phenotype NM fibrils also resisted unfolding in GdHCl at the highest technically feasible concentration (1.4 M; further increases in GdHCl caused the polystyrene beads to stick to the glass surface).

The repeat region defines the mechanical behaviors

Force-dropping events were induced by the specific amyloid-disrupting agent DAPH (but not by SA) and were profoundly altered when fibrils embodied a different prion subtype (with the same M domain). Together, these results establish that discontinuities in

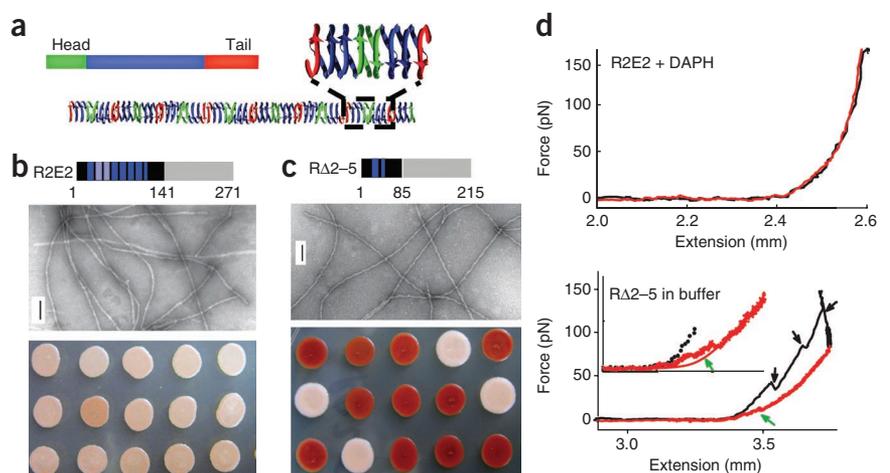
the force-extension curves reflect intrinsic structural and biological properties of the prion fibrils and are not due to extrinsic surface attachments. We therefore considered a rather unexpected explanation: individual subdomains of the fibrils have the capacity to sequentially unfold under mechanical force while noncovalent intermolecular contacts maintain fibril integrity.

If so, which segment of the protein might unfold to produce fibril-lengthening events? Although the atomic-level structure of NM fibrils is unknown, much evidence indicates that the M domain is not involved in the amyloid core^{25–27}. Indeed, we found that the M domain was readily cleaved from the fibrils and released as soluble fragments (Supplementary Fig. 3). The cleaved, N-domain-only fibrils maintained their fibril integrity, as confirmed by EM. Further, they had the same biochemical properties as wild-type NM fibrils.

As the N domain is responsible for fibril integrity, how might force-dropping unfolding events in this domain be associated with fibril lengthening? In a previously proposed β -helix model for NM fibrils^{25,38}, a continuous segment of the N domain forms the amyloid core, with the termini from neighboring monomers interacting in a head-to-head and tail-to-tail manner (Fig. 5a). The β -helical region between them, largely composed of the repeat region, is sequestered from intermolecular interactions³⁸. In such a structure, the repeat sequences might successively unfold, increasing the length of the fibril, while the integrity of the fibrils is maintained by head-to-head and tail-to-tail contacts.

To investigate how the repeat region influences the mechanical behavior of prion fibrils, we stretched fibrils assembled from NM protein carrying two additional repeats, R2E2 (ref. 39). We first determined that R2E2 fibrils seeded by cellular wild-type prion seeds uniformly produced strong or weak prion phenotypes (depending upon conditions, as described above) when assayed by protein-only transformation (Fig. 5b and Supplementary Fig. 4). This established that R2E2 proteins could faithfully receive strain-specific templating information from wild-type protein and transmit that information back to wild-type protein. In the presence of 50 μM DAPH, we stretched 11 R2E2 fibrils of the strong prion subtype, with five stretching-relaxation cycles for each. All fibrils remained intact, without unfolding or rupture, except for one fibril that ruptured without prior unfolding. The elimination of unfolding events by expansion of the repeat region, when

Figure 5 Expansion or deletion of the repeat region markedly changed mechanical behavior of prion fibrils. (a) A previously proposed β -helix model for NM fibrils. The M domain is not involved in the amyloid core and is not shown. Top left, color scheme for the N domain. Inset at top right, enlarged view of two monomers. (b,c) Top, schematic illustrations of R2E2 and R Δ 2–5. The blue segments in the N-terminal region represent oligopeptide repeats. Middle, R2E2 (b) and R Δ 2–5 (c) fibrils imaged by transmission EM. Scale bar, 100 nm. The R2E2 fibrils had an average diameter of 16 ± 2 nm, slightly wider than that of wild-type ‘strong’ NM fibrils, 12 ± 2 nm. The R Δ 2–5 fibrils had a diameter comparable to that of wild-type fibrils, but many showed a distinct wavy morphology. Bottom, protein-only transformation of R2E2 (b) and R Δ 2–5 (c) fibrils. (d) Representative force-extension curves for R2E2 (top) and R Δ 2–5 (bottom) fibrils. Black traces, stretching phase; red traces, relaxation phase; black arrows, force-dropping events in the stretching curves; green arrows, force plateaus in the relaxation curves at low forces. Inset in bottom graph shows expansion under the low-force regime. The stretching and relaxation curves were fit to modified WLC models. The deviation of the relaxation curves from the WLC fitting (red line in the inset) is indicated by the green arrow.



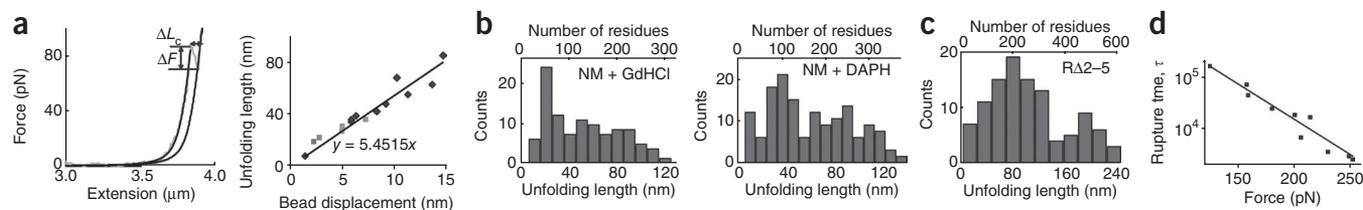


Figure 6 The distribution of the length of extension is broad. **(a)** Left, an example of WLC fits used to determine the length added to the fibril at each force-dropping event that occurred during the stretching phase. Right, a linear correlation was obtained between the length of extension determined from WLC fits and the instantaneous bead displacement that occurred upon unfolding. Black points, extension lengths from WLC fits to wild-type NM fibrils; gray points, extension lengths from WLC fits to RA2-5 fibrils. **(b)** Histogram of extension lengths for NM fibrils at each force-dropping event in the presence of 1.2 M GdHCl (left) or 50 μ M DAPH (right). **(c)** Histogram of extension lengths obtained with RA2-5 fibrils. A 12-nm and a 24-nm bin width were used for **b** and **c**, respectively (**Supplementary Methods**). **(d)** Rupture time versus force for the RA2-5 fibrils. The rupture events that occurred during the holding phase were fit to a Bell model as described in the Online Methods.

the intermolecular contacts that define prion strains remained intact, supports the idea that the repeat region is the origin of unfolding.

As expansion of the repeat region added mechanical stability, we asked whether deleting this region might destabilize fibrils enough to cause unfolding and rupture in the absence of denaturant. We tested an NM protein carrying a deletion of repeats 2 through 5, RA2-5. As confirmed by protein-only transformation, this protein faithfully received strain-specific templating information from the wild-type protein and transmitted it back to wild-type protein (**Fig. 5c** and **Supplementary Fig. 4**). Thus, for this protein, too, the strain-determining intermolecular contacts remained unaltered. Next, we stretched NM fibrils carrying the RA2-5 deletion in the optical trap. Under the same conditions used for the other fibril variants, unfolding and rupture occurred under physiological conditions, without the need for GdHCl or DAPH (**Fig. 5d**, black curves). We also observed plateaus in the relaxation curves at very low forces (**Fig. 5d**, red curves) that suggested refolding could occur in the absence of denaturant (**Supplementary Fig. 1**). In contrast to wild-type fibrils, which sustained many unfolding events before rupture (**Fig. 4b**), many RA2-5 fibrils ruptured after one unfolding event, and no fibrils were able to sustain more than three events without rupturing. Again, the marked changes in the mechanical behavior of the prion fibrils when the repeat region was contracted support the notion that this region is the origin of unfolding.

Characterization of length extensions and rupture events

To calculate the length of extension (additional contour length added to the fibril as a result of unfolding, ΔL_c), the force-extension curves with force-dropping events in the enthalpic stretching phase were fit with a modified worm-like chain (WLC) model to determine the contour lengths (L_c) before and after unfolding (**Fig. 6a**, left). A linear correlation was obtained between the length of extension of individual force-dropping events and the instantaneous bead displacement that occurred upon unfolding (determined by dividing the change in force by the trap stiffness; **Fig. 6a**, right). This provided a calibration factor to calculate the extension length of each force-dropping event occurring during the holding phase on the basis of the bead displacements obtained from the experiments.

In the presence of GdHCl or DAPH, the extension lengths of the unfolding events for NM fibrils from both the stretching and holding phases had a broad distribution (**Fig. 6b**). Such a distribution contrasts with the unfolding of protein polymers formed by multiple covalently linked globular domains strung together end to end with flexible linkers between them; these polymers often produce discrete and precise extension lengths by unfolding well-defined domains^{40–42}. The smallest population of extensions was approximately 15 to 20 nm, roughly

matching the length of the repeat region in the N domain (~ 9 residues per repeat $\times 0.34$ to 0.4 nm per residue $\times 5$ repeats = 15.5 to 18 nm). Individual extensions that are longer than the total length of the repeat region presumably derive from nearly simultaneous unfolding of multiple repeat regions. The extensions of RA2-5 fibrils were much longer than those of wild-type NM and often longer than the calculated length of the entire protein (**Fig. 6c**). The much longer extensions of RA2-5 fibrils must also represent events in multiple amyloid monomers along the length of the fibril occurring almost simultaneously.

Unfolding and rupture events with RA2-5 fibrils were not complicated by the presence of denaturants. This allowed us to use data obtained in the constant-force regime (holding phase) to assess the strength of the intermolecular interactions that maintain fibril integrity and are broken upon rupture. The lifetime of an interaction, τ , under a constant applied force, F , can be described by Bell's equation,

$\tau(F) = \tau_0 e^{-\beta F x^\ddagger}$, where τ_0 is the intrinsic lifetime under no load, $\beta^{-1} = k_B T$ (ref. 43) and x^\ddagger is the characteristic distance along the reaction coordinate required to achieve rupture (transition distance; **Supplementary Methods**). The lifetime prior to rupture showed a clear relationship to applied force ($\tau_0 = 99 \pm 28$ d and $x^\ddagger = 0.13 \pm 0.01$ nm; **Fig. 6d**). This indicates that the intermolecular contacts have extremely high mechanical stability.

DISCUSSION

Unexpected behaviors of prion fibrils under mechanical forces

We have described a method to tether amyloid fibrils to a glass surface on one end and a polystyrene bead on the other, using monomers of the same protein as a strong and highly specific molecular glue. This allowed us to apply optical trapping with simultaneous fluorescence imaging, a technique that, to our knowledge, has never been reported for any amyloid, to characterize the forces that govern the integrity of prion fibrils.

Under unusually high pulling forces, we discovered unexpected properties of prion fibrils. First, multiple force-dropping events were observed before fibril rupture. These force-dropping events (i) occurred in the presence of a highly selective amyloid-disrupting agent but not in the presence of the inactive analog, (ii) occurred with phenotypically strong prion fibrils but not phenotypically weak prions and (iii) were highly sensitive to mutations in the N domain, in proteins that carried the same strain-specific prion-determining monomer-monomer interface and identical M domains. These results collectively establish that the force-dropping events reflect properties intrinsic to the prion fold and are not a result of extrinsic aspects of surface attachments. Moreover, they establish that fibrils of a single prion type contain two regions with distinct

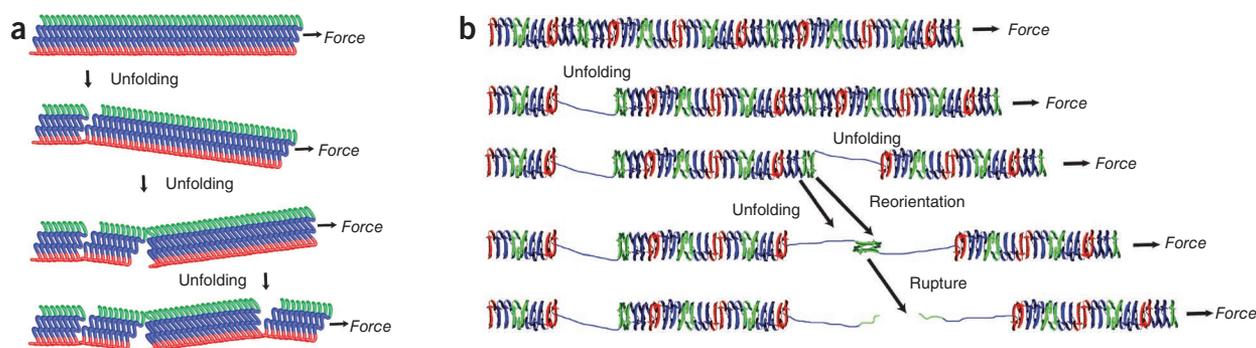


Figure 7 Cartoon representation of the mechanical unfolding of NM amyloid fibrils with different structures. (a) A possible mechanism with the β -pleated-sheet model. (b) A possible mechanism with the β -helix model. See Discussion for details.

mechanical stabilities. Unfolding, presumably of the repeat region, increases fibril length. Fibrils can sustain multiple unfolding events while maintaining fibril integrity, until mechanical failure of the stronger region causes rupture. These properties were unexpected because the prion fibrils show simple cooperative unfolding kinetics in chemical and thermal denaturation experiments^{14,25}.

Second, a broad distribution of unfolding lengths was observed for prion fibrils. This is in contrast to the unfolding of protein polymers, which have multiple identical well-folded globular domains linked with flexible peptide linkers. When individual domains of such polymers unfold, discrete extension lengths are evident^{44–46}. In contrast, in our prion polymers, a continuous amyloid core runs the entire length of the fibril, joining thousands of molecules, via both inter- and intramolecular interactions, into a rigid, highly cooperative structure^{7,25,47–49}. The mechanical pulling force is probably distributed to individual monomers in a very different way than it would be if flexible linkers intervened between folded domains, making simultaneous unfolding events more likely. Further, the amyloidogenic N domain of the prion protein has low sequence complexity, is extremely rich in glutamine and asparagine, and contains multiple tandem nine-mer repeats. We speculate that these sequence features may allow slightly different lengths of the repeat region to unfold in each event, cumulatively resulting in a broad distribution of fibril-lengthening events. This may also explain the different extension-length profiles observed with DAPH and GdHCl, as these compounds probably destabilize prion fibrils in somewhat different ways. Finally, although we made substantial efforts to generate uniform fibrils with biologically pure phenotypes, it is possible that the fibrils were not completely homogeneous but instead contained local structural fluctuations and imperfections.

Implications for the structure of prion fibrils

The molecular structure of NM fibrils has not been determined, but two general models have been proposed^{25,50–52}: a β -pleated-sheet model and a β -helix model (Figs. 5 and 7). Our observations cannot be reconciled with the β -pleated-sheet model. In that model, each monomer is stacked on top of the next along its entire length, one monomer per rung, and each amino acid residue on top of the same residue in the adjacent monomers (Fig. 7a)⁵². To increase fibril length in such an arrangement, both termini of the interfaces have to split open, alternating from side to side, with each split extending more than half of the fibril width but not so far as to cause rupture (Fig. 7a). This would require that the two termini of the interface have very similar mechanical integrities. But then the entire interface should dissociate and rupture the fibril immediately.

In contrast, the β -helix model does provide a framework for interpreting our data (Figs. 5 and 7). First, in this model the repeat region is largely sequestered from intermolecular contacts. Multiple repeat regions could unfold, with fibril length increasing at each event, while fibril integrity was maintained by intermolecular contacts. Indeed, mutations of the repeat region greatly altered the unfolding behavior. Fibrils with repeat expansions were mechanically stronger and fibrils with repeat deletions were mechanically weaker. This suggests that repeat expansions and deletions do not simply add or remove additional identical β -strands in the helix but slightly change the molecular geometry in some way. This is consistent with the fact that R2E2 and R Δ 2–5 fibrils showed different diameters and morphologies from wild-type fibrils (Fig. 5b,c).

Second, in the β -helix model, the termini from adjacent monomers contact each other head to head and tail to tail (Fig. 5a)²⁵. Simple geometry dictates that the β -strands at monomer interfaces are antiparallel (Fig. 5a, inset), whereas the β -helical strands within are parallel³⁸. Antiparallel and parallel β -strands might easily have somewhat different mechanical stabilities, constituting the mechanically stronger and mechanically weaker domains we observed. But, notably, if two neighboring repeat regions were to unfold, it would cause the intermolecular contact region between them to reorient relative to the pulling force (from perpendicular to parallel; Fig. 7b)⁵³. Breaking this interface would then necessitate the shearing of β -strands. The simultaneous shearing of all the hydrogen bonds requires much stronger mechanical forces than the simple stepwise unzipping of strands in a β -helix^{54,55}. Thus, even if the stabilities of inter- and intramolecular contacts are rather similar under the influence of chemical denaturants, under the influence of mechanical forces unfolding of the intramolecular domains would actually strengthen the intermolecular contact through reorientation to a geometry requiring simultaneous shearing of the entire interface. Supporting this concept, computational simulation of β helical proteins has predicted a reorientation of the β -strands after partial unfolding⁵³. Moreover, the distance along the reaction coordinate required to achieve rupture of our prion fibrils (x^\ddagger of 0.13 nm) was very similar to previously determined β -strand shearing transitions determined by optical trapping for globular proteins of known structure⁵⁵.

The same basic unfolding schema might be obtained with NM fibrils composed of more complex protein arrangements, which have been suggested for several other amyloidogenic proteins. For example, a domain-swapped trimeric β -helix model has been proposed for the mammalian prion protein⁵⁶, which presents additional intermolecular contacts and more intrinsic entanglements between the monomers. A cryo-EM study has suggested that β -(2)-microglobulin fibrils

consist of multiple β -sheet-rich globular subunits with multiple hierarchical inter- and intrasubunit contacts⁵⁷. In any case, whether NM fibrils have a structure that builds upon an underlying β -helix or not, our data establish that there must be distinct subdomains that can sustain multiple lengthening events while a mechanically stronger monomer-monomer interface maintains fibril integrity.

Applications of the new methodology

The methods we have developed should provide a broadly applicable tool for investigating many questions in the amyloid field. The mechanically resistant prion fibrils we described are fragmented in the yeast cytoplasm by the protein-remodeling factor Hsp104 (refs. 12,58). The fibril-severing activity of Hsp104, providing fragments for inheritance in daughter cells, is key to the extraordinary ability of prions to serve as protein-based elements of inheritance¹³. Our methods offer an opportunity to investigate the mechanism by which Hsp104 deploys 12 ATP sites to convert chemical activity into mechanical rupture of prion fibrils.

Moreover, our method for tethering amyloid fibrils should also be applicable to investigating forces that govern the integrity of other amyloids, as most such proteins template polymerization by recruiting soluble monomers at their ends, acting as strong and specific molecular glue. Several functional amyloids whose functions rely on their mechanical properties could be interesting candidates for investigation. For example, the amyloids in biofilms provide a mechanism for extremely strong organismal adhesion. If these fibrils undergo a β -strand reorientation like that we have suggested, it would explain their essential biological properties. Our method allows for testing of this possibility. The properties of the fibrils studied here, with their highly efficient and specific tethering capacity and their ability to sustain multiple length-extending unfolding events before rupture, should also be useful in the design of protein-based nanostructures.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/nsmb/>.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

J.D. initiated the project and carried out sample preparation and yeast biology; J.D. and C.E.C. designed and carried out the optical-trapping experiments and data analysis; M.C.B., M.J.L. and S.L. supervised the projects and interpreted the results; J.D. and S.L. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Protein purification, labeling and fibril assembly. Wild-type NM and NM mutants were cloned, expressed, purified and labeled as described¹ (see **Supplementary Methods** for details).

Validation of fibril attachment and optimizing conditions. We validated the success of the fibril attachment method by fluorescence visualization. Fibrils were visualized either with a commercially available inverted microscope (Axiovert, Carl Zeiss microscope, field of observation $60\ \mu\text{m} \times 60\ \mu\text{m}$) or with a customized Nikon TE2000 microscope on the optical-trapping instrument. With the customized microscope, images were recorded on a back-thinned EMCCD camera (ANDOR, field of observation $15\ \mu\text{m} \times 15\ \mu\text{m}$).

When casein was not used to block nonspecific adsorption, fibrils littered the glass surface, whereas when casein was used without predepositing NM monomers, fibrils were rarely detected. Thus, the casein successfully prevented nonspecific interactions between prion fibrils and the glass surface. When NM monomers were prebound followed by casein blocking, many fibrils were tethered in the field of observation ($\sim 60\ \mu\text{m} \times 60\ \mu\text{m}$; **Supplementary Video 1**). This confirmed that in the presence of casein the self-recognition properties of prebound monomers were required to capture the fibrils. In the final step—incubation of NM-coated beads with affixed fibrils—beads attached efficiently only if they had been predecorated with NM monomers and if the incubation was long enough to allow for specific capture.

Because of variations in experimental conditions, it proved necessary to test different concentrations of surface monomers and incubation times for each experiment, surveying the flow chambers to identify the best samples for pulling experiments. We typically worked with samples in which five to ten fibrils were tethered in the optical-trapping visual field (which is smaller than the viewing field in a standard inverted microscope, $\sim 15\ \mu\text{m} \times 15\ \mu\text{m}$; **Supplementary Video 2**). With such a density, most fibrils were tethered to the glass slide at only one end and carried a single bead at the other end for optical manipulation. When fibril density was higher than this, many fibrils were captured on the glass surface at both ends, although the main body of the fibril remained mobile in solution (data not shown).

Cleavage of NM fibrils from the glass surface by TEV protease. A NM variant was engineered with a TEV protease recognition sequence (ENLYFQG) substituting for amino acid residues 128 to 135 of wild-type NM. This region is located at the beginning of the M domain and is not involved in the formation of the amyloid core. These TEV-cleavable monomers were introduced into the flow channel and allowed to attach to the glass surface exactly as was done with wild-type protein. They were then used to tether wild-type NM fibrils as described in Results. The polystyrene bead was coated with wild-type NM and attached to the other end of the fibril. The bead that attached to the end of a fibril was trapped and held in a fully extended position before the protease was introduced to the chamber by flow. After transient fluorescence imaging of the intact fibrils, $20\ \mu\text{l}$ of

$20\ \mu\text{l}^{-1}$ TEV protease was introduced into the chamber by flow. An additional fluorescent image was then recorded after a 10-min incubation of TEV protease. To better resolve the fibrils that were released from the surface but still attached to the beads, a constant flow of $1 \times$ CRBB buffer (5 mM potassium phosphate, 150 mM NaCl, pH 7.2) was introduced through the flow channel to extend the fibrils along the direction of flow.

Stretching NM fibrils at high forces with a double-trap instrument. We designed the instrument for dual trapping capabilities by splitting the trapping laser (1,064 nm, 10 W; IPG Photonics) into two branches of approximately equal power using a cube polarizer (ThorLabs). One branch (the primary branch) was guided through AODs (IntraAction), enabling precise steering of the beam; however, the AODs also impart $\sim 50\%$ power loss. The second branch, which experienced minimal power losses along its path, was used to apply the maximum possible force. Half-wave plates (CVI), which can rotate the polarization of incoming light, were placed in each path to ensure maximum power throughput at the second cube polarizer. The maximum force capacity of our trapping instrument was determined by using the force-extension behavior of the fibrils as an internal control (see **Supplementary Methods** for details).

Before running an experiment, we aligned the secondary branch with the primary branch and the position detection laser (975 nm, Avonex) in the image plane by adjusting the position of the first lens in the telescope pair of the second branch using a three-axis micrometer stage. The bead was initially trapped with the primary branch and centered over the surface attachment point of the tethered fibril. The bead was subjected to position calibration as described⁵⁹. Briefly, the bead was raster scanned with the AODs through a grid of positions to map detector signal in two dimensions³⁴. The bead was then held in the center of the detector zone while position was registered to determine trap stiffness via the equipartition method⁵⁹. Then the bead was exchanged between the primary branch and the secondary branch for a stiffness calibration. After performing the stiffness calibration, we executed the pulling experiments as described in Results by using the secondary branch.

Fitting the force-extension curves with a modified worm-like chain model. Under no externally applied forces, NM fibrils showed end-to-end distances comparable to their fully extended length (contour length, L_c), indicating that persistence length (L_p) is of the same order as L_c . Therefore, the Palmer-Boyce WLC model, which is appropriate when $L_p \sim L_c$, was used to model the force-extension data (**Supplementary Methods**)⁶⁰. To fit the force-extension curves with discontinuities in the stretching phases, force-extension data before unfolding were fit first, and then L_c was adjusted to fit the data after unfolding. ΔL_c was determined by the L_c before and after the discontinuities occurred.

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