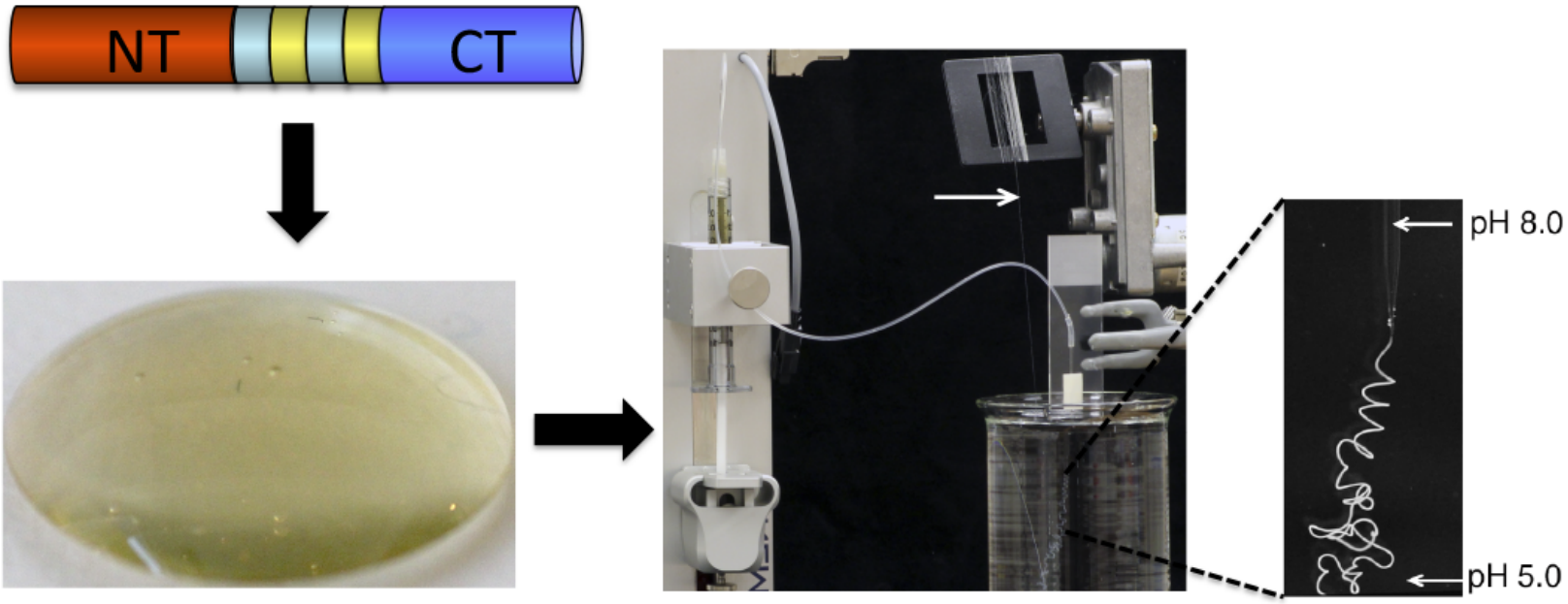
NT
CT
pH 8.0
pH 5.0

Biomimetic spinning of artificial spider silk from a chimeric minispidroin

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Abstract

Herein we present a chimeric recombinant spider silk protein (spidroin) whose aqueous solubility equals that of native spider silk dope and a spinning device that is based solely on aqueous buffers, shear forces and lowered pH. The process recapitulates the complex molecular mechanisms that dictate native spider silk spinning and is highly efficient; spidroin from one liter bacterial shake flask culture is enough to spin a kilometer of the hitherto toughest as-spun artificial spider silk fiber.

The majority of spider silk proteins (spidroins), including major ampullate spidroins (MaSps, that form the dragline silk) and minor ampullate spidroins (MiSps, that form the auxiliary web spiral), share a common architecture of a non-repetitive N-terminal domain (NT)[1] an extensive repetitive region, and a non-repetitive C-terminal domain (CT)[2]. Since spiders are territorial and produce small amounts of silk, any industrial application of spider silk requires production of recombinant spidroins and generation of artificial spider silk fibers. Recombinant spidroins can be produced in bacteria, yeast and insect cells, but the proteins studied to date often differ from their natural counterparts by lack of one or both of the terminal domains, and/or by having repeat regions engineered from consensus repeats[3-11]. Furthermore, the recombinant spidroins have been obtained in low yields and/or have low solubility in water[3,4,9,11-13]. Surprisingly, even when solvents such as hexafluoroisopropanol (HFIP) and formic acid are used, the solubility of the recombinant spidroins[3-5,9] is far from the solubility of spidroins in native spinning dope, which reach a concentration of 30-50% w/v[14]. These drawbacks probably owe in part to the inherent high tendency of spidroins to self-assemble, but suboptimal properties of the recombinant spidroins and spinning methods used likely also contribute. Current methods to generate artificial spider silk fibers use denaturing conditions, and include electrospinning[3], hand-drawing[7], and wet spinning into coagulation baths of aqueous alcohols[4-6,8,9,13]. Most as-spun fibers, i.e. fibers that have not been subject to further manipulations, have disappointing tensile properties, (c.f. below), which is why extensive post-spinning procedures in organic solvents have been employed. Biomimetic microfluidic devices have not yet generated fibers that can be tested for tensile strength[12].

Recent analyses of the conditions in the spider silk glands and spinning ducts revealed that pH is gradually lowered from 7.6 to <5.7 along the gland[15]. The pH gradient induces specific conformational changes in the terminal spidroin domains, which result in

fiber formation via a lock and trigger mechanism[15,16]. During storage in the silk gland, at neutral pH, the NT is monomeric and highly soluble, which contributes to the solubility of the entire spidroin[1,16,17]. When pH is lowered in the spinning duct, NT forms stable dimers, which lock the spidroins into large networks[16,18]. The decrease in pH has other effects on CT, which instead gets destabilized, unfolds, and turns into ß-sheet amyloid-like fibrils[15]. The structural conversion of CT is hypothesized to trigger the transition of the repetitive region into ß-sheet conformation[15,19], in analogy with the nucleation phenomenon seen in amyloid fibril formation. In addition to the pH gradient, dehydration of the spinning dope likely takes place along the duct of the silk gland[20] and simultaneously, shear forces generated along the narrowing duct affect the CT and the repetitive region[2,21]. It has been suggested that native spidroins are stored as micelles, probably with the terminal domains in the shell and the repetitive regions shielded in the core[22], which may mediate their extreme solubility. These micelles likely fuse into elongated structures upon shear[22].

The findings described above imply that at least two prerequisites need to be fulfilled in order to realize biomimetic spinning of artificial spider silk; firstly that the recombinant spidroins are highly soluble and pH responsive, and secondly, that the spinning system mimics the conditions of the spider silk gland. Reproduction of the extreme aqueous solubility of native spinning dopes has been difficult, and to date, the reported concentrations of artificial spinning dopes in aqueous solvents have been in the range of 0.4-2% (refs. 7,11). Even when organic solvents are used, protein concentrations remain in the range of 8-30% (refs. 3-5,13). It struck us that the aqueous solubility and pH responsiveness of NT and CT, respectively, differ between spidroins from different spider species and silk types. For example, in the case of MaSp1 from *Euprosthenops australis*, NT shows extremely high solubility and pH sensitivity, but CT has low solubility and is inert to pH changes[1]. However, for *Araneus ventricosus* MiSp, the CT is highly soluble and pH

sensitive but NT shows only moderate solubility[15]. From this, we hypothesized that a chimeric recombinant minispidroin consisting of a highly soluble NT and a likewise very soluble CT would be advantageous from solubility and production points of view. Therefore, we designed a minispidroin composed of NT from *E. australis* MaSp1 and CT from *A. ventricosus* MiSp bracketing a short repetitive region from *E. australis* (NT2RepCT).

The NT2RepCT was produced in shake flask *Escherichia coli* cultures and yielded around 125 mg protein/L after purification. Nearly all protein was soluble and bound efficiently to the Ni-NTA column (**Supplementary Results, Supplementary Fig. 1**). The eluate contained >95% pure NT2RepCT and the size of the protein on the SDS PAGE gel corresponded well to the expected molecular mass of 33 kDa (**Supplementary Fig. 1**). Size-exclusion chromatography performed at pH 8.0 indicated a mass of 100 kDa (**Supplementary Fig. 1**), in agreement with a dimer (due to the constitutive dimeric nature of CT) and a non-globular structure of the repetitive part. Remarkably, NT2RepCT could be concentrated to >500 mg/ml in aqueous buffer at pH 8 without precipitation. Protein at such high concentrations (referred to as the spinning dope) was transparent and viscous (**Supplementary Fig. 2**), similar in appearance to the native spinning dope in major ampullate glands. The spinning dope could be stored at 4°C for weeks and at -20°C for months with maintained ability to form fibers (c.f. below). The NT2RepCT assembled into micelles that fused and elongated upon shear (**Supplementary Fig. 3**), in analogy to what is suggested for native spidroins[22]. The chimeric minispidroin designed herein thus followed our predictions, since it was produced at very high yields in a bacterial host and showed high solubility (>50% w/v) in aqueous buffers.

Previously used purification protocols for recombinant spidroins often involve precipitation of the expressed protein and/or solubilization procedures involving HFIP[4,6,8,9]

or guanidinium-thiocyanate[13]. Such spidroins are stable in aqueous buffers without precipitating for 3-5 days[13], in contrast to NT2RepCT that can be kept stable in aqueous solution for months. The presence of organic solvents or other denaturants during production likely prevents the formation of native-like three-dimensional structures of the spidroins, and we propose that the high aqueous solubility and stability of NT2RepCT over time is related to the presence of natively folded NT and CT domains with ability to assemble into micelles.

To enable biomimetic spinning, we designed a thin pulled glass capillary through which the NT2RepCT spinning dope was pumped into an acidic aqueous buffer collection bath (**Fig. 1a**) resulting in formation of a continuous as-spun solid fiber (**Fig. 1a-c, Supplementary Movie 1**). Fibers could easily be reeled onto rotating frames in air (**Fig. 1d, Supplementary Movie 2**). In this manner, we could produce around 1000 meters of fiber from NT2RepCT protein obtained from one liter of shake flask culture. The dope concentration interval at which fibers could be spun ranged from 100 to 500 mg/ml.

To explore the effect of pH on fiber spinning, we extruded the dope into aqueous baths with pH ranging from 2.0 to 7.5. Discontinuous fiber-like structures formed when the pH of the bath was between 6.0 and 6.5 (**Fig. 2a**). Continuous fibers formed when the pH was between 3.0 and 5.5, but if the pH of the collection bath was ≤2.5 no fibers were formed (**Fig. 2a**). Fibers formed in a bath with pH 5.0 were used for further experiments. As-spun fibers were homogenous as judged by scanning electron microscopy, with a diameter of approximately 10-20 μm, and fracture surfaces from fibers that were pulled until failure showed a compact and homogenous interior core (**Supplementary Fig. 4**).

The pH-dependent assembly of NT2RepCT was also studied using nano-electrospray mass spectrometry (nESI-MS). nESI-MS at pH 7.5 showed the presence of a major molecular species of 66560 Da, which corresponds to the native NT2RepCT dimer.

Lowering the pH to approximately 5.5 induced a significant shift in the quarternary structure. Oligomers could be observed up to one minute after addition of formic acid. Following loss of the higher oligomers, only low-intensity peaks corresponding to dimers could be detected, which further decreased to baseline over the course of a few minutes (**Fig. 2b**). NT on its own underwent rapid dimerization at low pH, while for CT in isolation, exposure to low pH caused gradual unfolding as indicated by an increasing amount of monomers and a shift towards higher charge states (**Supplementary Fig. 5**). In analogy to amyloid-like fibrils[23], addition of acetonitrile could not dissolve higher-order oligomers of NT2RepCT, while concentrated formic acid recovered the monomer signal (**Fig. 2b**).

Fourier transform infrared (FTIR) spectroscopy of NT2RepCT in solution showed amide I and II band maxima approximately at wavenumber 1545 and 1650 cm^{-1}, respectively, indicating a mainly α-helical structure, in line with a natively folded protein. In contrast, NT2RepCT fibers showed a higher contribution of β-sheet conformation in both the amide I and amide II peak (**Fig. 2c**), which is similar to what has been observed for native dragline silk upon spinning[24]. Spectral decomposition of the amide I band allowed us to quantify the fraction of β-sheet conformation, which was 25% for NT2RepCT in solution and 60% in the fibers. For *Nephila inaurata* dragline silk[25], the corresponding value is 47% (**Supplementary Table 1**).

The assembly behavior of NT2RepCT thus followed the lock and trigger mechanism proposed for native spider silk formation[15] as determined by nESI-MS and FTIR experiments. To our knowledge, this is the first report of a successful recapitulation of the terminal domains' molecular mechanisms in an artificial spinning process. The protein design and method presented herein resulted in silk-like fibers that were qualitatively different from fibers generated in processes involving denaturing conditions. Denaturation of spidroins during production and/or spinning results in loss of the fine-tuned molecular

events that govern silk fiber formation, and instead generate fibrous aggregates of denatured proteins that should not be mistaken for biomimetic silk in terms of structure or function. To test whether coagulation can generate fibers from any protein, we spun bovine serum albumin (BSA) into a coagulation bath of methanol. Continuous BSA fibers were readily formed when the protein jet came in contact with methanol (**Supplementary Fig. 6**). On the contrary, no fibers were formed when BSA was extruded into a low pH aqueous collection bath.

The as-spun NT2RepCT fibers had a qualitatively similar stress-strain behavior as native spider silk in that they displayed an initial elastic phase until a yielding point after which plastic deformation occurred. The mechanical characteristics of the fibers were highly reproducible, although the toughness and ultimate tensile strength were lower than in native silk (**Supplementary Fig. 7, Supplementary Table 2**). One possible way to increase the toughness could be to spin NT2RepCT fibers with diameters closer to native dragline silk, as this apparently has an impact on the mechanical properties of silk fibers[26]. The toughness of NT2RepCT fibers (45 MJ/m^3) is, to the best of our knowledge, by far the highest hitherto reported for as-spun fibers. Our results support the concept that in order to obtain highly concentrated spinning dopes that are useful for producing fibers in a biomimetic setup, we need spidroins in which the terminal domains are highly soluble. Moreover, the spidroins need to be kept natively folded throughout the production, storage and initial spinning process, until lowered pH and shearing induce regulated conformational changes that result in conversion from soluble protein to β-sheet fibers. The method described herein may be further developed by, for example, the introduction of gradual changes of pH and ion composition.

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Author Contributions

MA, QJ, AA, XL, ML, and PP performed the experiments; AR, JJ, GP, QM, CVR, MT, HH, supplied equipment and expertise; AR and JJ conceived and designed the study; MA, AR and JJ wrote the manuscript. All authors edited the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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Figure 1. Biomimetic spinning of artificial spider silk. (a) Highly concentrated NT2RepCT spinning dope in a syringe is pumped through a pulled glass capillary with a tip size of 10-30 μm, with the tip submerged into a low pH aqueous collection bath. Fibers can be taken up from the collection bath (arrow) and rolled up onto frames. (b) Photo of a fiber as it is spun in the low pH aqueous collection bath. (c) Wet fiber nest in low pH buffer. (d) As-spun fibers on a frame. Fiber diameter in (b-c): approximately 40 μm (wet fibers, capillary tip diameter 30 μm). Fiber diameter in (d):,15 μm (dry fibers, capillary tip diameter 10 μm). Scale bar in (a) 3 cm (b) 3 mm (c-d) 5 mm.

Figure 2. pH-dependent assembly and spinning of NT2RepCT. (a) Spinning into aqueous buffers with pH values indicated above each panel, scale bar 2 mm. (b) nESI-MS of, from top to bottom, NT2RepCT at pH 7.5, incubated at pH 5.5 for 3 min, incubated at pH 5.5 for 30 min and then dissolved in formic acid, and

incubated at pH 5.5 for 30 min and then dissolved in acetonitrile. Ubiqutin was used as internal standard. Inset, fraction of NT2RepCT dimer over time at pH 7.5 (open symbols) and pH 5.5 (filled symbols). (c) FTIR spectra (254 scans for each spectrum, at least four spectra for each sample) of NT2RepCT at 10 mg/ml (blue dashed line), NT2RepCT fibers (red continuous line), and native *N. inaurata* dragline silk (black line and dots); reference ranges for the positions of the amide I and II peaks corresponding to different conformations are also displayed.

Online methods

Protein expression and purification

The construct NT2RepCT is composed of a 6xHis tag (MGHHHHHHM), an N-terminal domain based on the *E. australis* MaSp1 sequence (SHTTPWTNPGLAENFMNSFMQGLSSMPGFTASQLDDMSTIAQSMVQSIQSLAAQG RTSPNKLQALNMAFAS SMAEIAASEEGGGSLSTKTSSIASAMSNAFLQTTGVVNQP FINEITQLVSMFAQAGMNDVSA; EMBL Accession no. AM259067, ref. 27), a repetitive part consisting of two poly-alanine and glycine rich repeat regions from MaSp1 of *E. australis* (GNSGRGQGGYGQGSGGNAAAAAAAAAAAAAAGQGGQGGYGRQSQGAGSAA AAAAAAAAAA AAGSGQGGYGGQGQGGYGQSGNS; EMBL Accession no. AJ973155), and a C-terminal domain based on the *A. ventricosus* MiSp sequence (VTSGGYGYGTSAAAGAGVAAGSYAGAVNRLSSAEAASRVSSNIAAIASGGASALP SVISNIYSGVVA SGVSSNEALI QALLELLSALVHVLSSASIGNVSSVGVDSTLNVVQ DSVGQYVG; GenBank Accession no. JX513956).

The construct was cloned into a pT7 plasmid and transformed into BL21 (DE3) *E. coli*. Luria broth media with kanamycin (70 mg/l) was inoculated with a glycerol stock of *E.*

coli containing pT7-NT2RepCT and grown over night at 37°C with shaking (200 rpm). The overnight culture was used for a 1/100 inoculation of 500 ml LB media with kanamycin, which was then cultured at 30°C with shaking (200 rpm) until OD600 reached 0.8, after which the temperature was lowered to 20°C and protein expression was induced by adding isopropylthiogalactoside to a final concentration of 0.3 mM. The cells were cultured over night at 20°C with shaking (200 rpm) and were then harvested by centrifugation for 20 minutes at 5000 rpm, 4°C. The pellets were resuspended in 20 mM Tris pH 8 and frozen at -20°C, or lysed immediately after resuspension.

Lysis was performed in a cell disrupter (T-S Series Machine, Constant Systems Limited) at 30 kPsi, after which the lysate was centrifuged at 27 000 g, at 4 °C for 30 minutes. Supernatants were loaded on a Ni-NTA column and the protein was eluted with 300 mM imidazole. The eluted protein was dialyzed against 20 mM Tris pH 8, at 4°C over night, using a Spectra/Por dialysis membrane with a 6-8 kDa molecular weight cutoff. SDS-polyacrylamide (12%) gel electrophoresis and Coomassie Brilliant Blue staining was used to determine the purity of the protein. Broad Range Protein Ladder (ThermoFisher Scientific) was used as a size standard.

The protein was concentrated using centrifugal filter units (Vivaspin 20, GE healthcare) with a 10 kDa molecular weight cutoff at 4000 g in rounds of 20 minutes. To determine the protein concentration, 1 µl protein was diluted 400 times in 20 mM Tris and the absorbance at 280 nm was recorded. The highly concentrated NT2RepCT forms a transparent and viscous spinning dope.

Size exclusion chromatography

A Superdex 200 HR column (Amersham Biosciences) was used to run 200 µl of purifed protein sample in TBS running buffer (20mM Tris, 150mM NaCl and 1mM EDTA, pH 8.0).

The flow rate used was 0.5 ml/min. Molecular mass standards Ribonclease A (13.7 kDa), Carbonic anhydrase (29 kDa), Ovalbumin (43 kDa), Conalbumin (75 kDa), Aldolase (158 kDa) and Ferritin (440 kDa) were used for calibration.

Study of micellar structures using cryo-EM

NT2RepCT (5 mg/ml) was diluted to 0.001 mg/ml in 20 mM Tris buffer, pH 8.0. Alternatively, NT2RepCT (5 mg/ml) was dialysed against 20 mM Tris buffer, pH 7.5, after which the protein was pumped through a glass capillary with a tip diameter of 20 µm to induce shear, and diluted to 0.001 mg/ml in 20 mM Tris buffer pH 7.5. The cryo-samples were prepared in a controlled environment, at 16 °C and 100 % humidity, using automated Vitrobot (FEI). Data was acquired with a JEOL JEM-2100f microscope operated at 200 kV and a nominal magnification of 80000. Images were collected with TVIPS TemCam-F415 4k x 4k CCD-camera (Tietz Video and Image Processing Systems GmbH). The size of the micelles was estimated using the image processing program ImageJ.

Fourier Transform Infrared (FTIR) spectroscopy

FTIR analysis was carried out on liquid and solid samples using a Thermo Scientific Nicolet iS5 with iD5 ATR at room temperature, 254 scans were collected for each spectrum with a resolution of 4 cm-1. NT2RepCT protein (10 mg/mL in 20 mM TrisHCl pH 8), as-spun NT2RepCT fibers immersed in water, and *N. inaurata* dry fibers were studied. The background spectra of 20 mM TrisHCl or of water were acquired under the same conditions and subtracted from the scans of protein samples in solution, using Thermo Scientific OMNIC software. At least four spectra were obtained for each type of sample and averaged for the curves shown. The absorbance was normalized by a linear transformation to fit between 0 and 1 in the wavenumber range of interest, 1480-1720 cm^{-1}. To obtain a robust

and reasonable fit for spectral decomposition of the amide I band of the individual sample spectra, we assigned fixed positions for the centres of Gaussian peaks for β-sheets (1624 cm^{-1}), random coil and α-helix (two peaks at 1644 and 1663 cm^{-1}) and β-turn (1682 cm^{-1}) (ref. 28); centred in the wavenumber ranges for different secondary structures[29,30] (see **Fig. 2c**). Before curve fitting, a linear baseline was subtracted in the amide I and II regions (1450-1750 cm^{-1}). The software Fityk 0.9.8 was used for the fittings.

Fiber spinning

Round glass capillaries (G1, Narishige) with an outer diameter of 1.0 mm and inner diameter of 0.6 mm were pulled (Micro Electrode Puller, Stoelting co. 51217) to a tip diameter of 10-30 μm. A 1 ml syringe with Luer Lok tip (BD) was filled with NT2RepCT of a high concentration (100-500 mg/ml) and connected to a 27G steel needle (Braun) with an outer diameter of 0.40 mm. The needle was connected to the pulled glass capillary via polyethylene tubing. A neMESYS low pressure (290N) syringe pump (Cetoni) was used to eject the NT2RepCT at a flow rate of 1-20 μl/min into a low pH collection bath. Fibers could be spun in aqueous solutions with low ionic strength (20 mM sodium acetate pH 5.0). For tensile tests, FTIR, and scanning electron microscopy, fibers were spun into collection baths consisting of 500 mM sodium acetate buffer and 200 mM NaCl (pH 5) and rolled up onto frames. For testing the influence of pH on spinning, different buffer systems and molarities of those buffer systems were used; sodium phosphate (100 mM) for pH >5.5, sodium acetate (100 mM) for pH 5.5-4, and citric acid (200 mM) for pH<4.

Spinning bovine serum albumin

Freeze-dried BSA (Sigma-Aldrich) was dissolved to a concentration of 200-350 mg/ml in 20 mM Tris pH 8.0, after which spinning was attempted as described for NT2RepCT (above). Alternatively, BSA (200-350 mg/ml) was spun into a collection bath with 99% methanol.

Mass spectrometry

For MS analysis, purified NT, CT or NT2RepCT (1 mg/ml) was reconstituted into 100 mM ammonium acetate, pH 7.5 using biospin buffer exchange columns (Bio-Rad Laboratories). Proteins were either subjected to nESI-MS analysis at pH 7.5, or after the addition of formic acid to a final concentration of 0.02% in a microcentrifuge tube, resulting in a pH of 5.5. As a reference for time-dependent assembly of spidroin, bovine ubiquitin (Sigma) was added to NT2RepCT samples at a final concentration of 0.2 mg/ml prior to the addition of formic acid[31]. Samples were then immediately loaded into in-house produced gold-coated borosilicate capillaries and spectra were acquired continuously at 1 scan/sec for 10 minutes. For fibril dissolution, either concentrated formic acid or acetonitrile were added to a final concentration of 50% after 30 min incubation of NT2RepCT at pH 5.5. Spectra were acquired on a Synapt G1 T-wave mass ion mobility spectrometer (Waters) operated in ToF mode and equipped with a 32k quadrupole for high-mass analysis. The settings were: capillary voltage, 1.4 kV; sample cone 20 V; source temperature, 20 °C; trap collision energy, 100 V; transfer collision energy, 10 V; trap DC bias 8 V. Backing pressure was maintained around 7 mbar. Data were analyzed using the MassLynx 4.1 software package (Waters). For each time-point, 60 scans were combined and spectra intensities normalized to the ubiquitin signal. Relative intensities were extracted using mMass[32] and plotted using GraphPad 5.0.

Scanning electron microscopy of fibers

Dry fibers were put on scanning electron microscopy stubs, coated with gold/palladium for two minutes, and observed and photographed on a Zeiss Supra 35VP scanning electron microscope.

Tensile tests of fibers

Dry fiber samples were mounted onto plastic frames with 20 mm gauge length using tape and glue (Loctite® Super Glue Professional). Fibers were visualized under a light microscope (Leica DMI3000 B) using 40x object lens. Three photomicrographs were taken along the length of the fiber, and the diameter was measured from the photomicrographs using Image J (http://imagej.nih.gov/ij/) to get an average diameter of the individual fiber piece. The sides of the plastic frame were cut off and the specimens were mounted in an MTS Nanobionix (MTS Systems Corporation) tensile testing machine (resolution ± 1μN in force and 1 μm in cross-head displacement). The length of fiber where it was taut but not subjected to load was determined. Tensile test was performed at a pulling rate of 1 mm/min under nominal environmental conditions 24°C and 30% relative humidity. Eight different NT2RepCT fibers were tested to check for reproducibility. All the tensile properties were calculated using the software Testwoks (MTS Systems Corporation), which also allows controlling the tensile test. Mean values and standard deviations were calculated from the raw data.

Data availability

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

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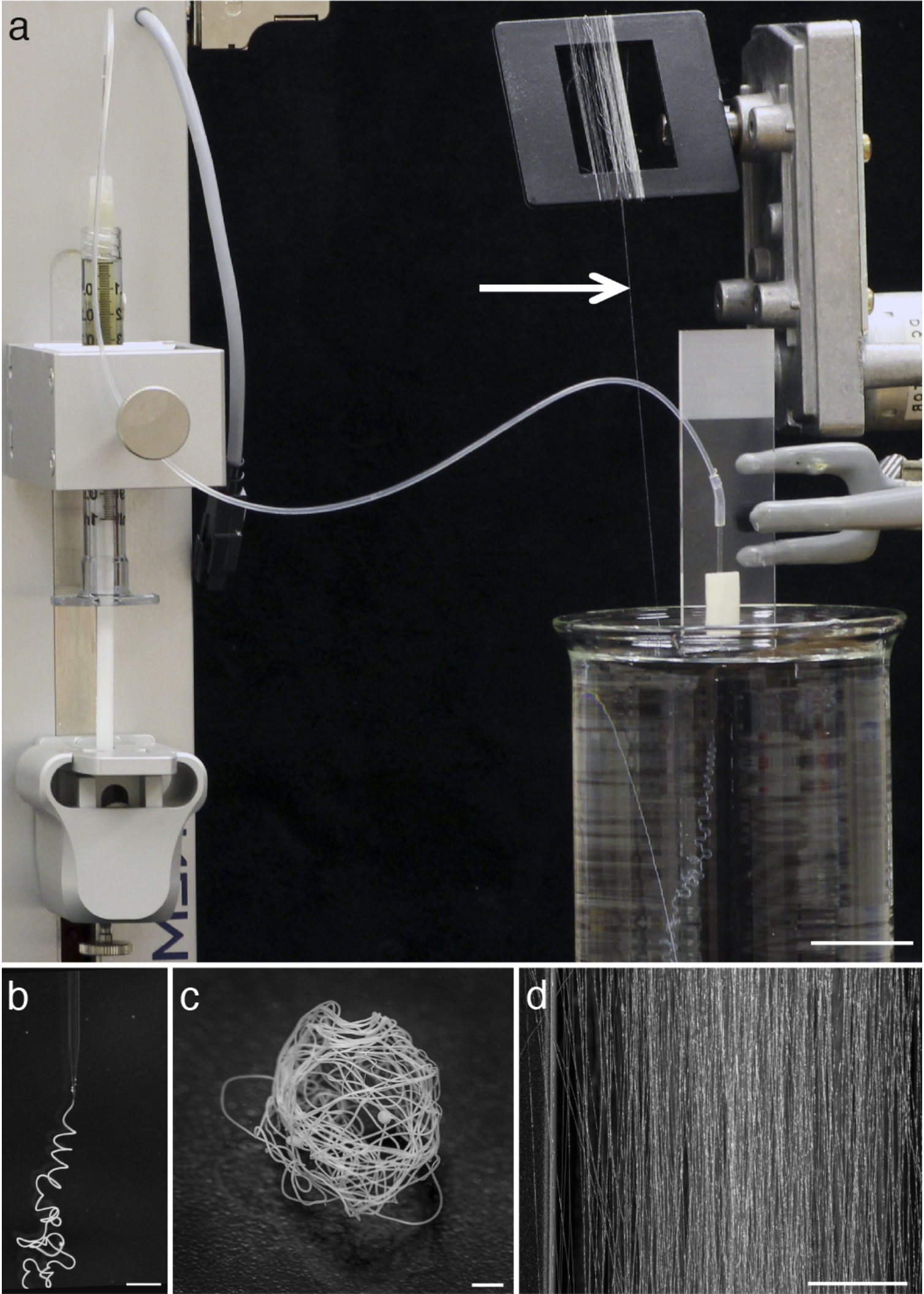
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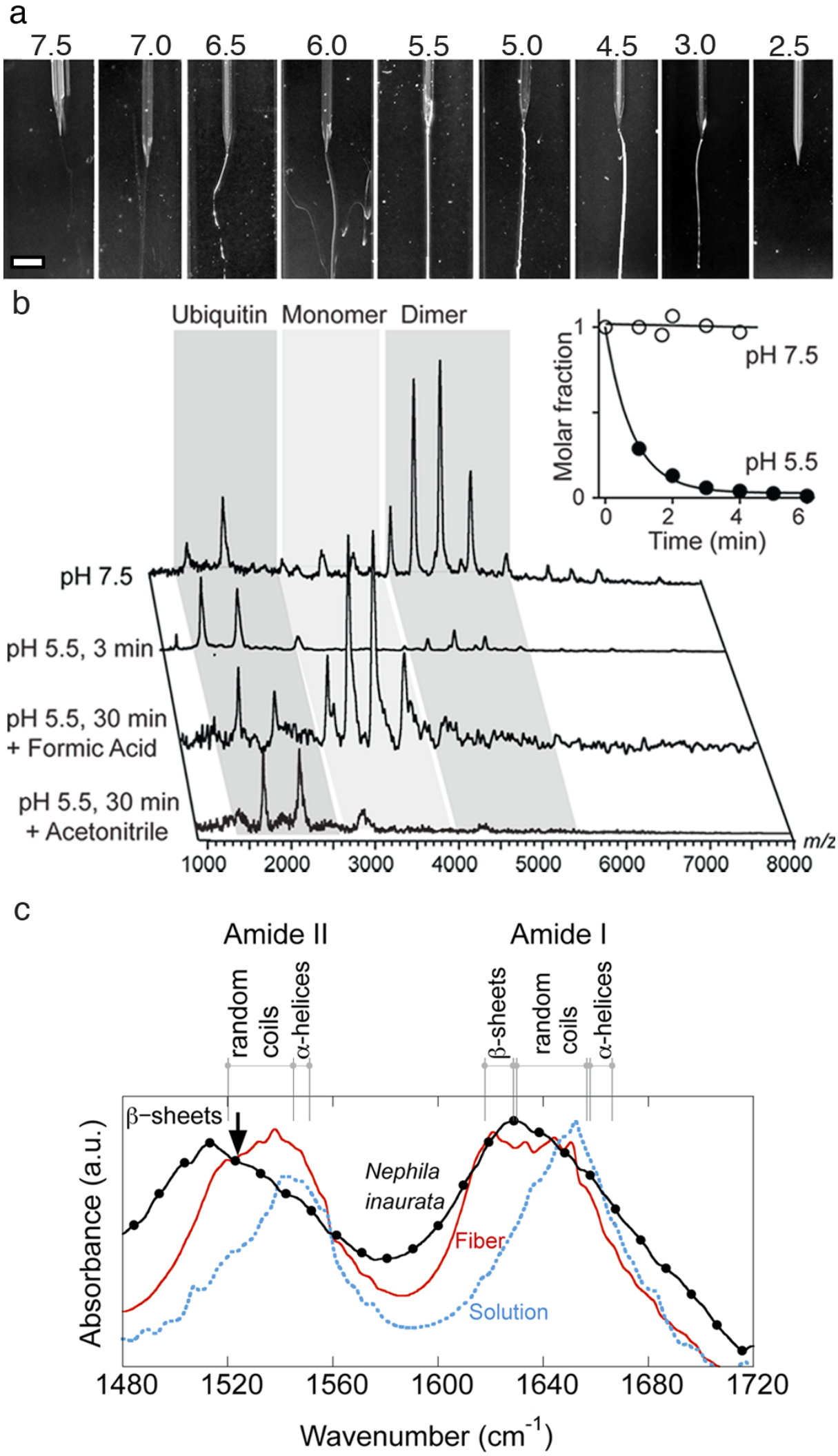
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pH 7.5
pH 5.5, 3 min
pH 5.5, 30 min
+ Formic Acid
pH 5.5, 30 min
+ Acetonitrile
m/z
1000
2000
3000
4000
5000
6000
7000
8000
c
Amide II
Amide I
random coils
α-helices
β-sheets
random coils
α-helices
β−sheets
Absorbance (a.u.)
Nephila inaurata
Fiber
Solution
1480
1520
1560
1600
1640
1680
1720
Wavenumber (cm-1)