Supporting information for

pH-Triggered Self-assembly and Hydrogelation of Cyclic Peptide Nanotubes Confined in Water Microdroplets

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1. Experimental section

Materials and methods

Reagents were acquired from Fluka, Aldrich, Iris Biotech or TCI. Egg yolk lysophosphatidyl choline (EYPC) was acquired from Avanti Polard Lipids or Aldrich. Mineral heavy oil was acquired from Sigma. ^{1}H NMR spectra were acquired in a Varian 300 MHz spectrometer. Chemical shifts (δ) are reported in ppm relative to TMS (δ = 0). All spectra were normalized with respect the residual solvent signal. Infrared spectra were acquired in a PerkinElmer Spectrum Two ATR. Analytical HPLC was carried out in an Agilent 1260 Infinity II equipped with an Agilent SB-C18 column and connected to a 6120 Quadrupole LCMS. HR-MS was acquired in a Bruker Microtof.

Abbreviations

CP: Cyclic peptide, 2CTC: 2-Chlorotrityl chloride, DCM: dichloromethane; DIEA: *N*,*N*-Diisopropylethylamine, DMF: *N*,*N*-Dimethylformamide, Fmoc: 9-Fluorenylmethoxycarbonyl; Mtt: 4-Methyltrityl; *N*-HATU: *N*-[(Dimethylamino)-1*H*-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-*N*-methyl methanaminium hexafluorophosphate *N*-oxide; *N*-HBTU: *N*-[(1*H*-Benzotriazol-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HFIP: 1,1,1,3,3,3-Hexafluoropropan-2-ol; PyAOP: (7-Azabenzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate; TFA: Trifluoroacetic acid; TFE: 2,2,2-Trifluoroethanol; TIS: Triisopropylsilane; UV-vis: Ultraviolet-visible.

Synthesis of CP1 precursor

Cyclic peptides were prepared manually in solid phase. 2-Chlorotrytil chloride resin (2CTC, 500 mg, 1.6 mmol Cl^{-}/g resin) were soaked in freshly distilled DCM (4 mL) for 30 min. The solvent was filtered off, and a solution of *N*-α-(9-Fluorenylmethyloxycarbonyl)-L-lysine allyl ester hydrochloride (178 mg, 0.4 mmol) and DIEA (350 μL, 2 mmol) in freshly distilled DCM (4 mL) was added to the resin. After 2 h, the solvent was filtered off and the resin was washed with DCM (4 mL). A mixture of DCM-MeOH-DIEA (8.5:1:0.5, 4 mL) was added and the resin was shaken for 1 h, then washed with DCM (3 x 4 mL) and diethyl ether (4 mL). The

resin was dried under high vacuum and the loading was determined by quantification of the Fmoc group.[1] For this, a small portion of the resin (*ca* 10 mg) was treated with a solution of piperidine in DMF (1:4, 2 mL) for 30 min. An aliquot of this solution (20 µL) is diluted to 1 mL DMF and the absorbance was read between 290-301 nm. The concentration of the dibenzofulvene-piperidine adduct is obtained by using the extinction coefficients tabulated in the literature.

A portion of resin (0.1 mmol) was used for the synthesis of the peptide. The Fmoc group was removed by treatment with piperidine/DMF (1:4, 2-3 mL) for 30 min. The resin was washed with DMF (6 mL) and then treated with a solution of Fmoc-protected amino acid (0.4 mmol), *N*-HBTU (151 mg, 0.4 mmol) and DIEA (0.1 mL, 0.6 mmol) in DMF (3 mL). The resin was shaken for 45 min and then washed with DMF (3 mL). The procedure was repeated with each corresponding aminoacid (7 cycles). After the coupling of the last amino acid, the resin was washed with DCM (3 x 3 mL) and a solution of PPh_3 (39 mg, 0.15 mmol), *N*-methylmorpholine (110 µL, 1 mmol) and phenylsilane (120 µL, 1 mmol, 10 equiv) in DCM (2 mL) was added, and the mixture was bubbled with argon for 10 min. Then, a solution of $Pd(OAc)_2$ (6.7 mg, 0.03 mmol) in DCM (0.5 mL) was added dropwise and the resin was further bubbled with Ar for 2 min and the suspension was stirred overnight. The resin was washed with DCM (4 mL), DIEA in DMF (2% v/v, 4 x 4 mL), sodium diethyldithiocarbamate (0.5% w/v in DMF, 4 x 4 mL) and DMF (4 x 4 mL). The resin was stirred with piperidine/DMF (1:4, 2 mL) for 30 min. Finally, it was washed with DIEA in DMF (2% v/v, 4 x 2 mL) and DMF (4 x 4 mL).

Cyclization was carried out by adding a solution of PyAOP (208 mg, 0.4 mmol) and DIEA (0.1 mL, 0.6 mmol) in DMF (3 mL) and the suspension was stirred for 12 h. After washing with DMF (3 mL), the reaction was repeated twice in the same conditions. For the selective removal of the 4-methyltrityl group, the resin was washed with DCM (3 x 4 mL), then treated with a mixture of DCM/HFIP/TFE/TIS (6.5:2:1:0.5, 3 mL) and stirred for 1 h. The mixture was filtered off and washed with DCM (3 x 3 mL). The process was carried out again in the same conditions and washed with DCM (3 x 3 mL) and DMF (3 x 3 mL). Then, a solution of ((*tert*-butyloxycarbonylaminoxy)acetic acid (48 mg, 0.25 mmol) and *N*-HATU (94 mg, 0.25 mmol) in DMF (1 mL) was added to the resin followed by the dropwise addition of

DIEA (42 μL, 0.25 mmol) in DMF (1 mL). The resulting mixture was stirred mechanically for 45 min. Finally, the resin was washed with DMF (3 x 3 mL). These deprotection and coupling with (*tert*-butyloxycarbonylaminoxy)acetic acid steps were repeated once.

The peptide was released from the resin by treatment with freshly prepared TFA cocktail (TFA-DCM-H_2O-triisopropylsilane, 0.9:0.05:0.025:0.025, 1 mL/40 mg resin) for 2 h and then filtered. The resin was washed with TFA (0.5 mL) and the combined fractions were evaporated to 1-2 mL by bubbling argon. The concentrated solution was added dropwise to cold diethyl ether (10 mL diethyl ether/ml TFA). The resulting precipitate was centrifuged for 10 min at 3000 rpm. The supernatant was discarded, then fresh diethyl ether was added and the suspension was sonicated and centrifuged. The resulting solid was dried under vacuum. The sample was dissolved in milliQ water and purified by semipreparative HPLC using a C18 column [gradient of H_2O-0.1% TFA-acetonitrile-0.1% TFA 95:5 (0 min) to 70:30 (20 min)] to give 16.5 mg of **1** (18 %); **^{1}H NMR** (300 MHz, D_2O) δ: 8.62 (s, 2 H), 7.29, 7.28 (2 s overlapped, 2 H), 4.65 (m, 2 H), 4.62 (s, 2 H), 4.43-4.25 (m, 6 H), 3.88-3.70 (m, 4 H), 3.40-3.05 (m, 6 H) 2.97 (t, 2 H, $J_{H,H}$ = 9 Hz), 1.9-1.3 (m, 8 H), 1.32-1.25 (2 d, 6 H, $J_{H,H}$ = 6 Hz); R_t = 7.06 min, RP-uHPLC (C18, H_2O-acetonitrile (dopped with 0.1% TFA) 100:0 (0 min) → 25:75 (15 min)); **FTIR** (neat): 3274 (b), 1669 (m), 1621 (m), 1537 (m), 1432 (m), 1182 (m), 1141 (w), 838 (w), 800 (w), 723 (w), 627 (w), 520 (w) cm^{-1}; **HRMS** calculated for $C_{38}H_{62}N_{15}O_{12}^+$: 920.4697, found: 920.4689.

Synthesis of CP1 (Scheme S1)

The purified peptide **1** (3.6 mg, 3.92 μmol) was dissolved in a DMSO (100 μL) solution containing of pyrenecarboxyaldehyde (11 mg/mL, 1.1 mg, 4.8 μmol, 1.2 equiv.). The solution was stirred at 60 ºC until no starting material was observed by HPLC-MS, typically in 1.5-2 h reaction time. Then, the solution was added to cold diethyl ether (2 mL) and vigorously shaken until a yellow precipitate was formed. The organic layer was decanted after centrifugation and the solid was sonicated for 10 min with fresh cold diethyl ether (2 mL), centrifuged and decanted. Sonication with diethyl ether and decantation was repeated twice. The solid was dried under vacuum to give 3.8 mg of **CP1** (86%); **^{1}H NMR** (300 MHz, DMSO-d_6) δ: 9.35 (s, 1 H), 8.77 (m, 3 H), 8.40-7.89 (m, 14 H), 7.67 (s, 2 H), 7.23 (2 s, 2 H),

5.05 (m, 1 H), 4.67 (s, 2 H), 4.58 (m, 1 H), 4.30 (m, 4 H), 3.60-2.67 (m, 17 H), 1.72-1.00 (m, 14 H); R_t = 9.24 min, RP-uHPLC (C18, H_2O-0.1% TFA-acetonitrile-0.1% TFA 95:5 (0 min) → 5:95 (15 min)); **FTIR** (neat): 3275 (b), 1670 (m), 1626 (m), 1542 (m), 1204 (w), 1129 (w), 844 (w), 799 (w), 722 (w), 625 (w) cm^{-1}; **HRMS** calculated for $C_{55}H_{70}N_{15}O_{12}^{+}$: 1132.5323, found: 1132.5327

Circular dichroism: Circular dichroism spectra were acquired in a Jasco J-1100 CD spectrometer. Data was collected at 20 ºC in a 2 mm light path quartz cuvette after subtraction of the solvent background signal. In pH titration experiments, a concentration of 0.4 mM **CP1** was fixed. pH was adjusted by adding small aliquots of NaOH (0.1 M) and the pH was measured using a drop pH-meter. Solutions were equilibrated for 10 min prior measurements. Experiments at a fixed pH and variable **CP1** concentration were carried out in HEPES (60 mM, pH 8.)

Measurement of viscoelastic properties of the gel: Gels of **CP1** (2% w/w) were measured in an Anton Paar MCR-302 rheometer in a parallel plate setup with a gap of 0.5 mm. In frequency sweep experiments, elastic (*G*') and viscoelastic (*G*") modulus were measured at angular frequencies of 0.1-100 rad/s at a constant shear strain of 5%. All measurements were carried out at a temperature of 20 ºC.

Fluorescence measurements: Fluorescence measurements were carried out in a Varian Cary Eclipse fluorescence spectrophotometer. Fluorescence spectra were acquired at 20 ºC with an averaging time of 0.5 s. Samples were prepared at different pH values and measured with a drop pH meter. Emission measurements were carried out by excitation wavelength of 340 nm. Thioflavin FRET experiments were carried out at a concentration of 20 μM of thioflavin.

Epifluorescence and LSCM: Epifluorescence measurements of droplets (see preparation below) were carried out in an Olympus BX51 at magnifications of 20x, 40x and 100x. Confocal measurements were carried out in a Leica SP5 exciting at 405 nm and operating at a magnification of 63x. Images were analyzed using ImageJ.[2,3] Confocal images of the gel were acquired from cyclic peptide samples (2% w/w) that were alkalinized with NaOH (0.1 M) until the gel was formed (typically pH around 8). All images were acquired at room temperature

Transmission and scanning transmission electron microscopy: Cu grids (300 mesh, coated with holley carbon grid, Electron _Microscopy Sciences) were used to deposit the samples at concentration of 0.02% w/w. Samples were stained with uranyl acetate (2%). STEM images were acquired in a Zeiss Ultra Plus scanning transmission electron microscope operating at an extra high tension of 20 kV. Transmission electron microscopy images were acquired in an JEOL JEM-1011 operating at 100kV.

Formation of gels: For gelification assays, a solution of the peptide in milliQ water (2% w/w, 150 μL) was prepared by sonication (pH 3-4). This solution is alkalinized with NaOH (0.1 M) (usually up to pH around 8) until no flow was observed after inversion of the vial.

AFM: Atomic force microscopy measurements were carried out in a Park Systems NX-10. ACTA tips were used (silicon tips, nominal values: spring constant = 40 N/m, frequency = 300 kHz, ROC less than 10 nm). Aqueous samples at pH 8 of **CP1** (15-20 μL, 1.8 mM) were deposited in freshly cleaved mica. After 15-20 min equilibration, the micas were thoroughly rinsed with milliQ water and dried with a steam of Ar, then measured immediately. Image analysis was carried out with Gwyddion.[4] Persistence length was roughly estimated from AFM images presenting isolated fibers with length longer than 500 nm. After loading the AFM height maps in Easyworm software[5], the persistence length was fitted using the mean square end-to-end model implemented in Easyworm.

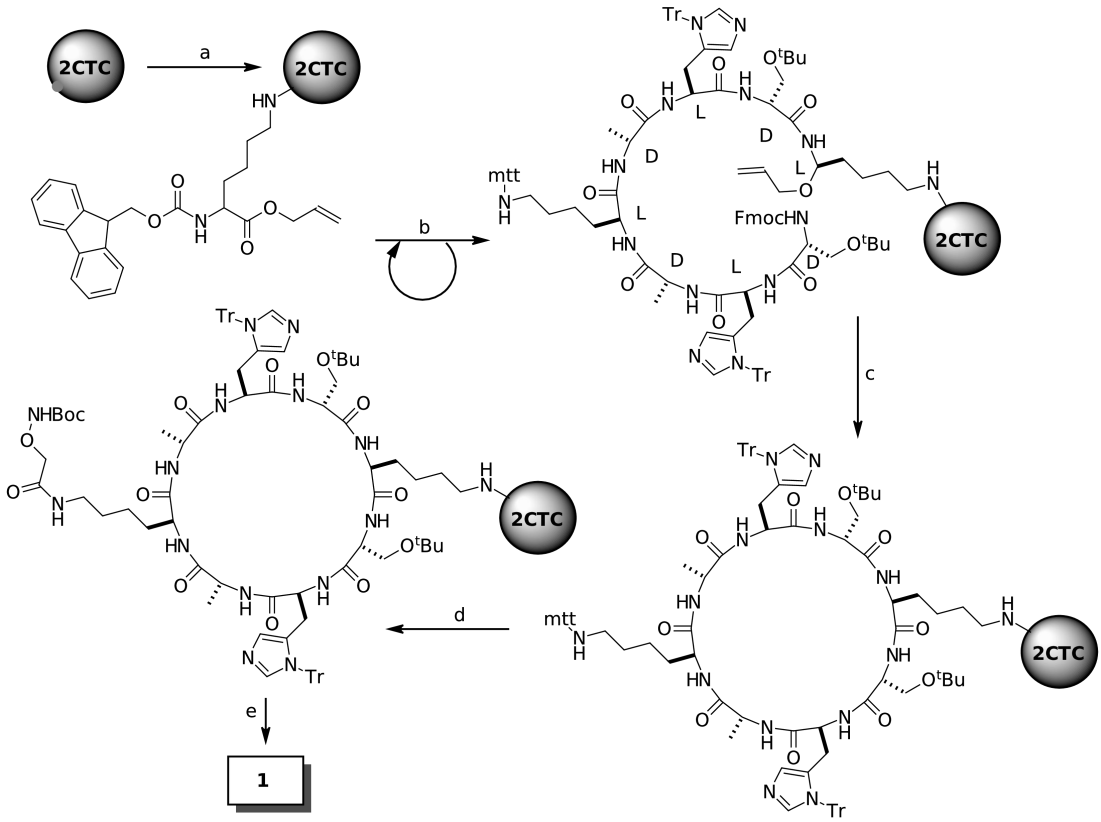
Preparation hydrophobic glass slides: Hydrophobic glass slides were prepared adapting a reported procedure.[6] Microscope glass slides were immersed in freshly prepared piranha solution for 40 min (H_2SO_4-H_2O_2 3:1; **WARNING:** piranha solutions react violently in contact with organic compounds and should be handled with care!). Slides were thoroughly rinsed with milliQ water, dried with a stream of air and further dried in the oven (65 ºC) for several hours. Slides were immersed in a solution of (1*H*,1*H*,2*H*,2*H*-perfluorooctyl)silane (3 mM) in chloroform (HPLC quality) for 20 min. The substrates were rinsed thoroughly with dichlorometane, dried with a stream of air and incubated in the oven for 20 min.

Preparation of water-in-oil droplets: Preparation of emulsions of aqueous droplets in oil were carried out by stirring. Typically, EYPC (10 mg) were dissolved in chloroform (HPLC quality, 1 mL) and a thin film was prepared by rotatory evaporation under reduced pressure.

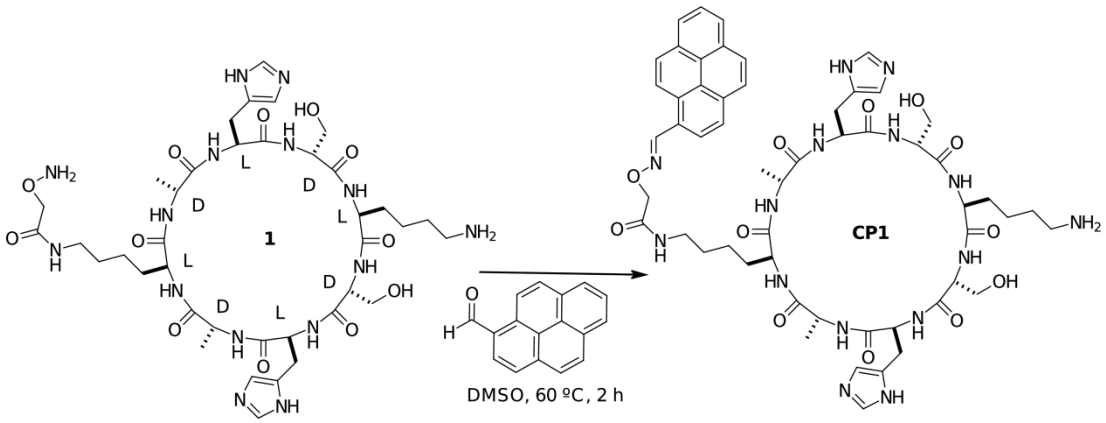
The film was further dried at high vacuum for several hours. Heavy mineral oil (5 mL) was added to give a phospholipid concentration of 2 mg/mL. The film was sonicated for 1 h and then equilibrated overnight at room temperature. Oil (200 μL) was mixed with an aqueous solution of **CP1** (1-2% w/w, 1 μL), and the suspension was stirred with a magnetic bar for 15-20 min at 700 rpm until a milky suspension was achieved. pH was changed by two different methods; *before droplets formation:* aqueous solutions of **CP1** were diluted with HEPES solution (20 mM, pH 8); *after droplets formation:* a mixture of Aldrich oil and propanamine (0.5-1% v/v) was added to the suspension. Images acquired immediately after droplet preparation. For this, a small chamber was prepared by mounting hydrophobic slides with imaging spacers (Secure-Seal Imaging Spacers, 0.12 mm depth, Grace Bio-Labs)

2. Supplementary Figures

2.1. Synthesis of CP1 and precursors



Scheme S1. Preparation of precursor **1**: a) *N*-α-(9-Fluorenylmethyloxycarbonyl)-L-lysine allyl ester hydrochloride, DIEA, DCM, 2 h; b) i) piperidine/DMF (1:4), 30 min, ii) amino acid, *N*-HBTU, DIEA, DMF, 45 min, repeat 7 cycles with the corresponding aminoacid; c) i) $Pd(OAc)_2$, PPh_3, phenylsilane, 4-methylmorpholine, DCM, overnight, ii) piperidine/DMF (1:4), 30 min, iii) PyAOP, DIEA, DMF, overnight; d) i) DCM-HFIP-TFE-TIS (6.5:2:1:0.5), 1 h, twice ii) [(*tert*-butoxycarbonyl)aminoxy]acetic acid, *N*-HATU, DIEA, DMF, 45 min; e) TFA-DCM-H_2O-TIS (9:0.5:0.25:0.25).



Scheme S2. Preparation of **CP1**.

2.2. Assembly studies

Homodimer formation model:

For the equilibrium

$$A+A<\!\!-\!\!-\!\!>A\cdot A \qquad (1)$$

with association constant

$$K_a = \frac{[A\cdot A]}{[A][A]} \qquad (2)$$

Mass balance

$$[A]_{tot}=[A]+2[A\cdot A]=[A]+2K_a[A]^2 \qquad (3)$$

were $[A]_{tot}$ is the total concentration of monomer parted as free monomer and monomer in the complex. This be rearranged in the quadratic expression:

$$2K_a[A]^2+[A]-[A]_{tot}=0 \qquad (4)$$

Which can be analytically solved:

$$[A]= \frac{-1+\sqrt{1+8K_a[A]_{tot}}}{4K_a} \qquad (5)$$

At a given wavelength, the ellipticity is given by:

$$\Theta_\lambda=B_\lambda[A]\,l+C_\lambda[A\cdot A]\,l \qquad (6)$$

where B_λ and C_λ are the molar ellipticity constants for monomer and dimer respectively and l is the cell path length. Assuming $B_\lambda = 0$, the ellipticity response is given by:

$$\Theta_\lambda=C_\lambda\, l\,[A\cdot A]=C_\lambda\, l\, K_a[A]^2=C_\lambda\, l\, K_a\left(\frac{-1+\sqrt{1+8K_a[A]_{tot}}}{4K_a}\right)^2 \qquad (7)$$

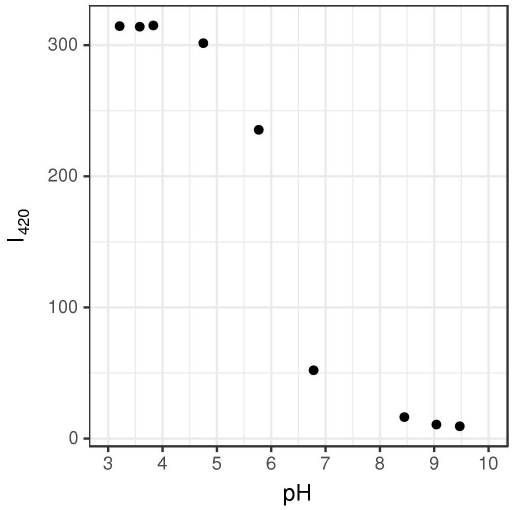


Figure S1: Evolution of fluorescence emission at 420 nm upon addition of NaOH (0.1 M) to a solution of **CP1** (0.2 mM) in milliQ water. λ_{ex} = 340 nm

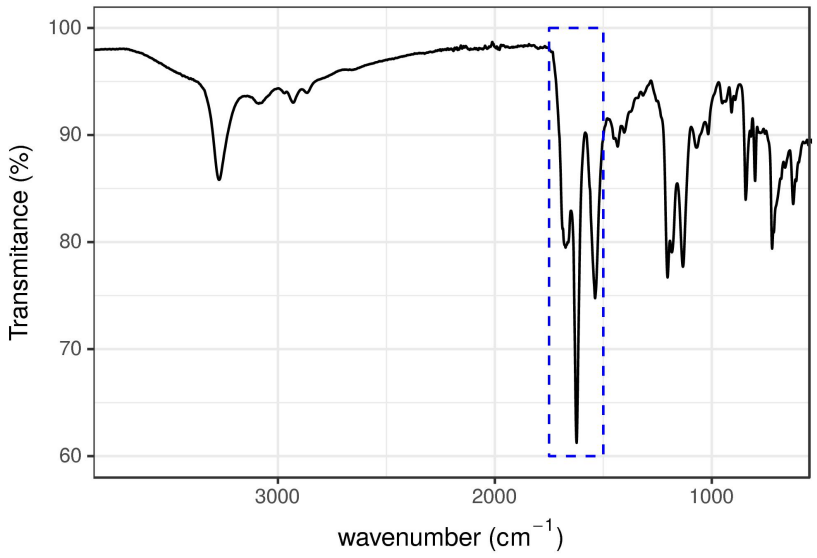


Figure S2: IR-ATR of a freeze-dried sample of **CP1** (2 % w/w) after alkalinization with NaOH (0.1 M). Base was added until a gel was obtained. The dashed blue box indicates the presence of intense peaks of the amides I and II at 1623 and 1538 cm^{-1} respectively.

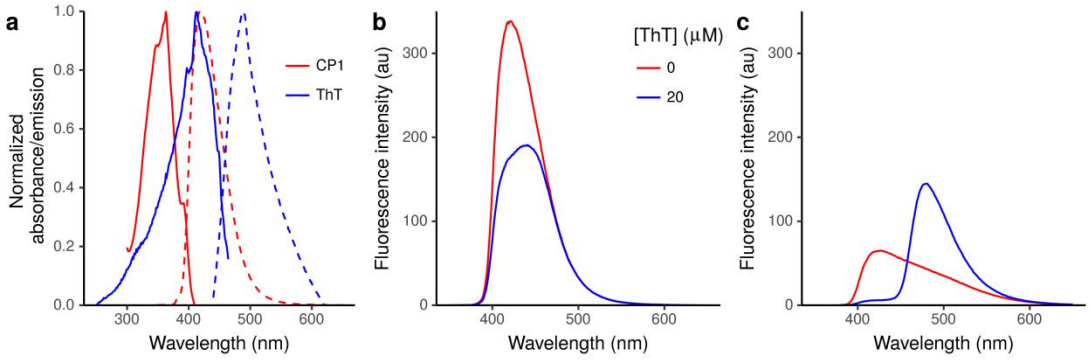


Figure S3: a) Normalized absorption (solid lines) and emission (dashed lines) spectra for **CP1** (0.2 mM) (red lines, λ_{ex} = 340 and λ_{em} = 420 respectively) in aqueous solutions (slightly acid) and ThT (10 μM) in water (blue lines, λ_{ex} = 420 and λ_{em} = 500, respectively); Fluorescence spectra of a solution of **CP1** (0.1 mM) in the presence (blue lines) or absence (red lines) of ThT b) at pH 3.8; c) at pH 8 in HEPES (20 mM).

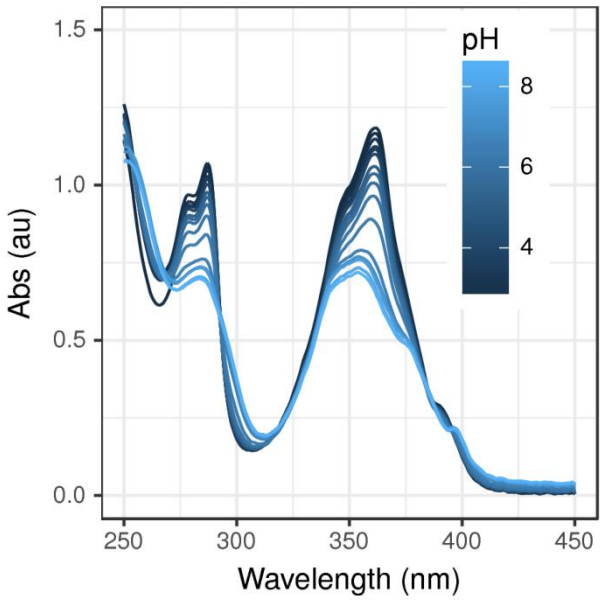


Figure S4: Absorbance changes after addition of small aliquots of NaOH (0.1 M) to a **CP1** (0.4 mM) in milliQ water.

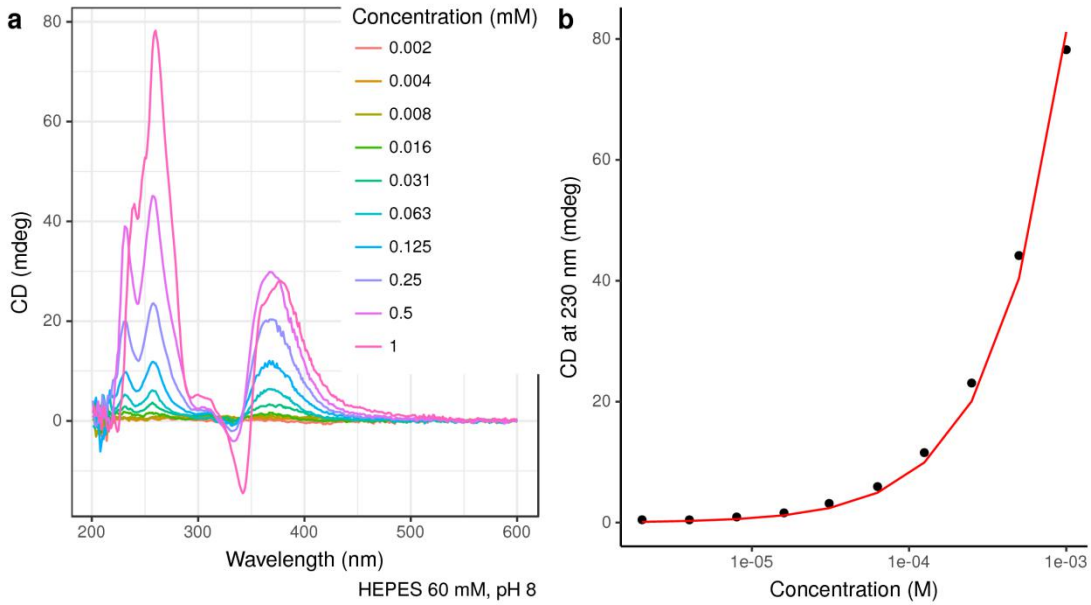


Figure S5: a) Circular dichroism of **CP1** at different concentrations in HEPES (60 mM) at pH 8; b) fitting to a homodimerization model. A K_a of 4 x 10^6 M^{-1} and a molar ellipticity constant of 820 deg cm^{-2} $dmol^{-1}$ was obtained.

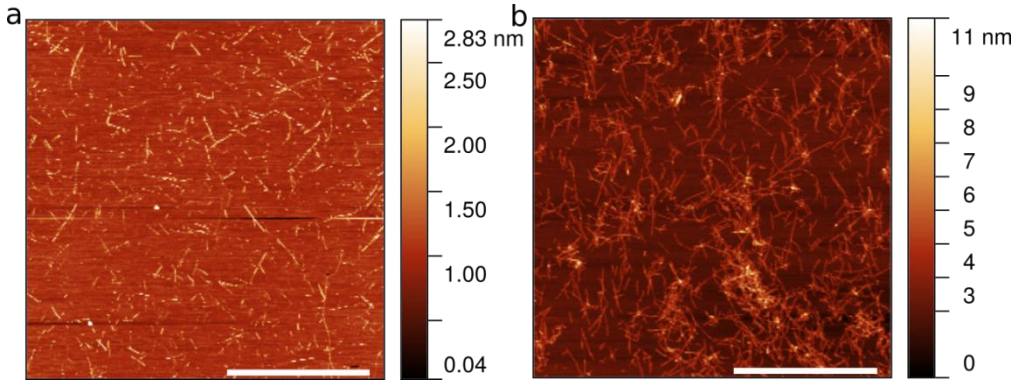


Figure S6: AFM images of **CP1** (0.2 % w/w) solutions at different pH deposited on mica surface: a) after dissolving in water (pH ~ 4) b) upon alkalinization (pH ~ 8). Scale bar is 4 μm.

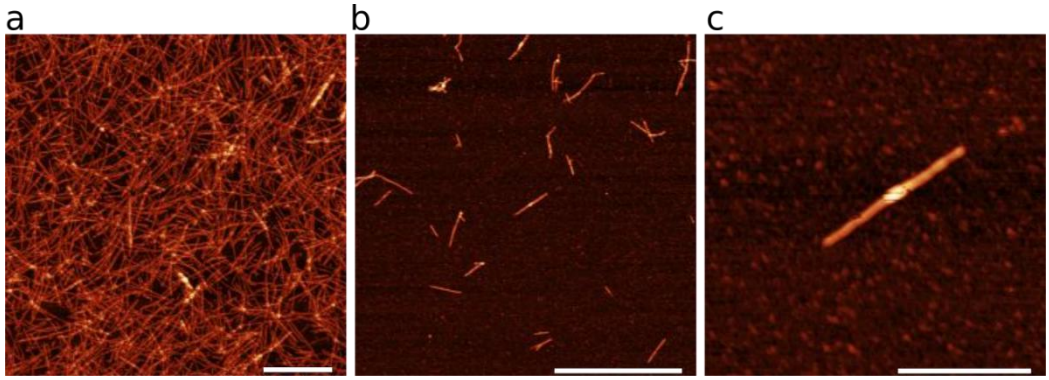


Figure S7: AFM images of **CP1** (0.2 % w/w) at pH *ca* 8 obtained by dilution of the gel: a) High density of nanotubes; b) low density; c) single nanotube. Persistence length was estimated from the later. Scale bars are 1 μm (a), 500 nm (b and c)

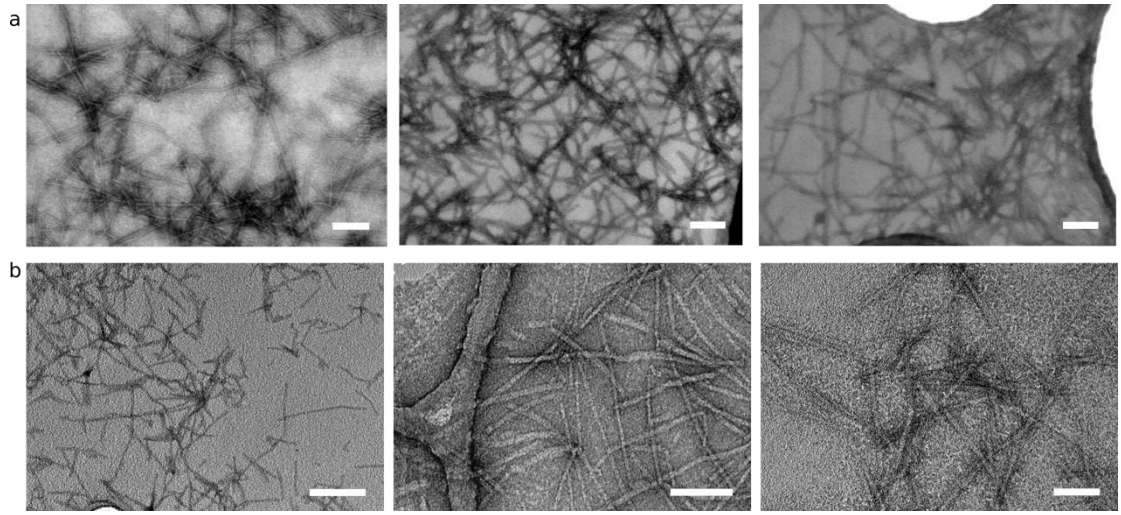


Figure S8: Micrographs of **CP1** (0.02 % w/w) after dilution of the gel (pH ca 8). Samples were stained with uranyl acetate. a) STEM images. Scale bar is 100 nm; b) TEM images. Scale bars are 200 (left), 50 (center) and 50 nm (right).

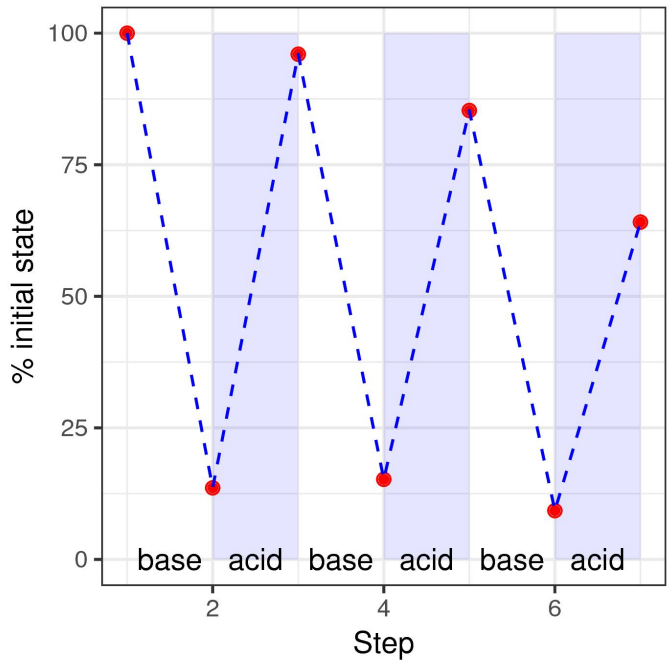


Figure S9: Reversibility of fluorescence quenching of **CP1** (0.2 mM) upon pH changes (acid, 3-4) or basic (*ca* 8).

2.3. Assembly in water-in-oil droplets

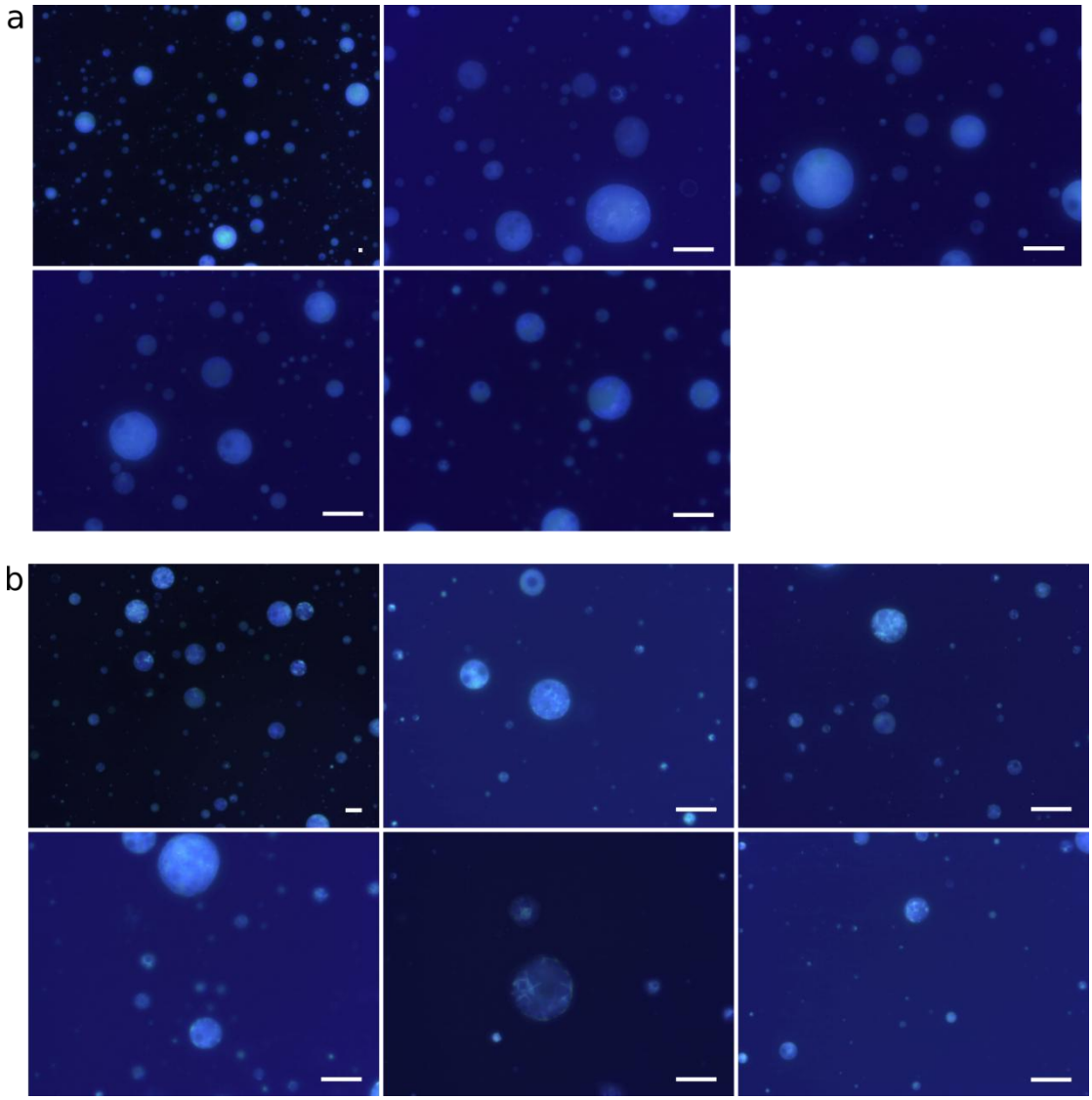


Figure S10: Formation of fibers in water-in-oil emulsions of **CP1** (2% w/w); a) dissolved in water and b) diluted in HEPES (30 mM) at pH 8. Scale bar is 10 µm.

2.4. Characterization of cyclic peptides

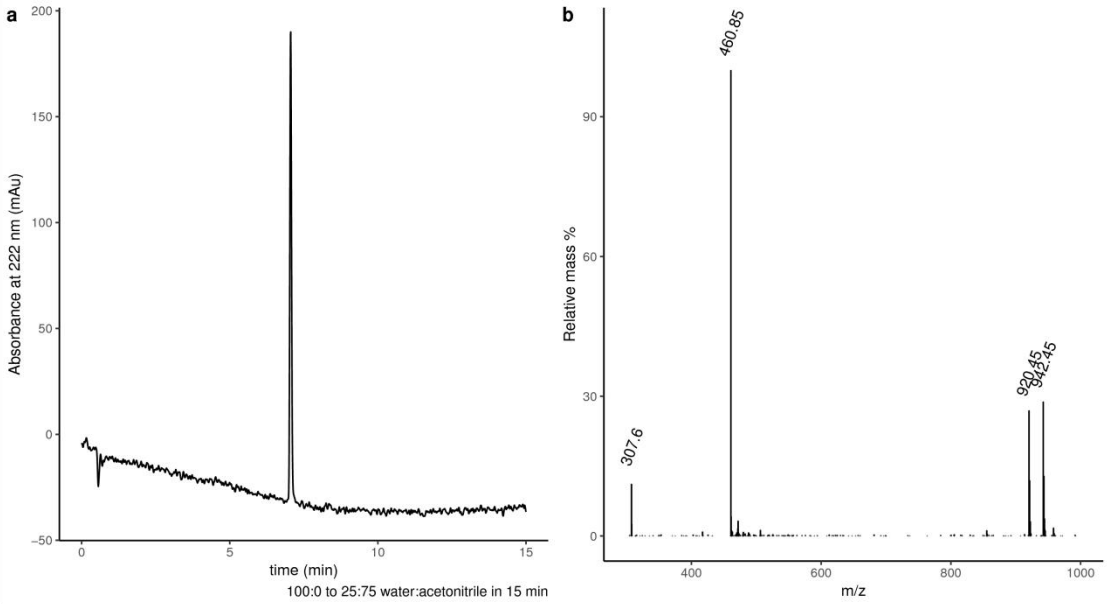


Figure S11: a) Chromatogram of **1** after purification; b) MS spectra from the main peak.

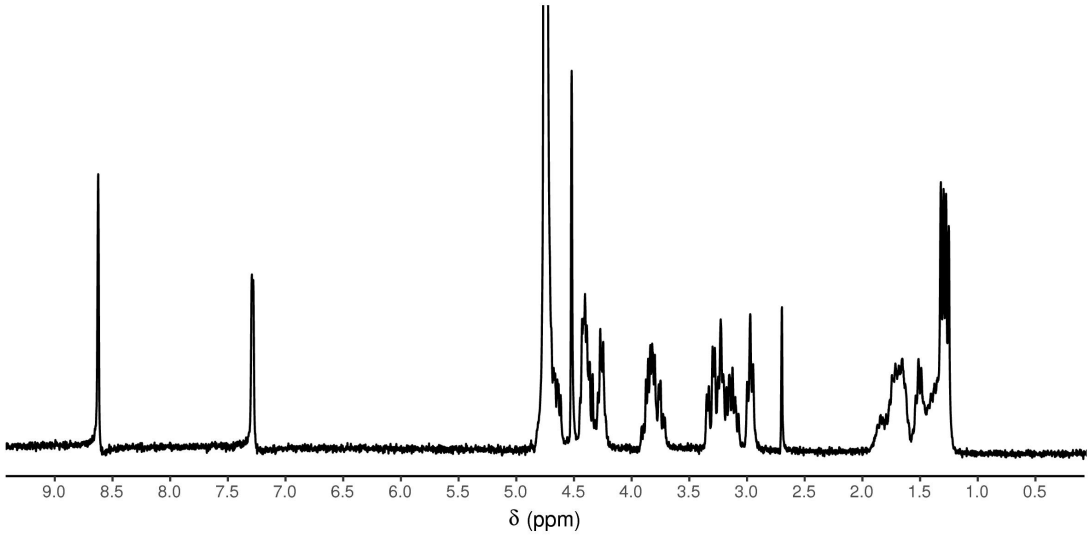


Figure S12: ^{1}H NMR (300 MHz) of **1** in D_2O.

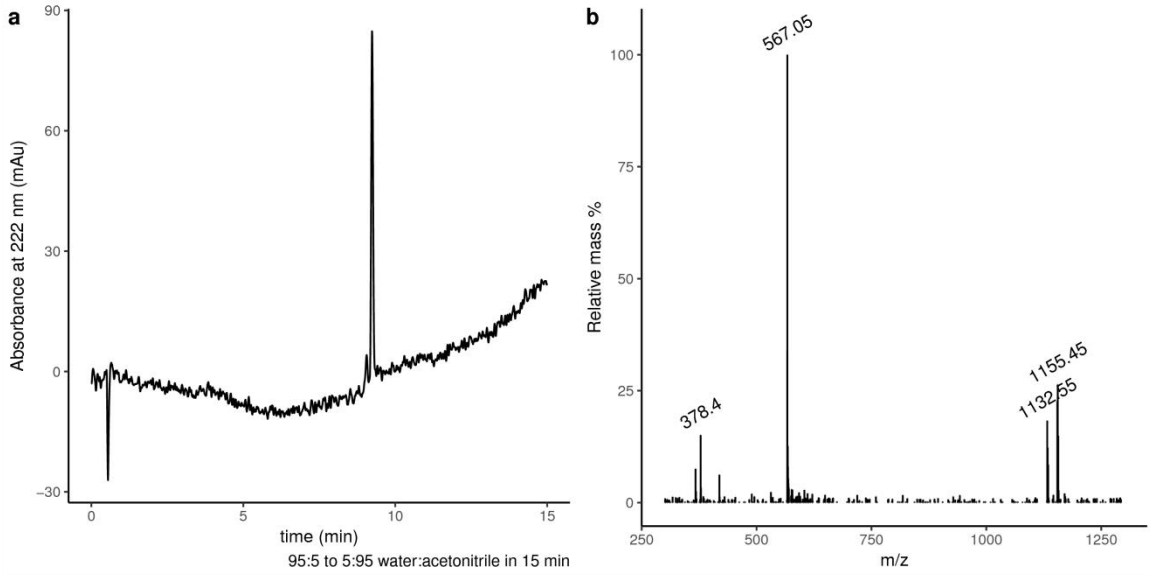


Figure S13: a) Chromatogram of **CP1** after purification; b) MS spectra from the main peak.

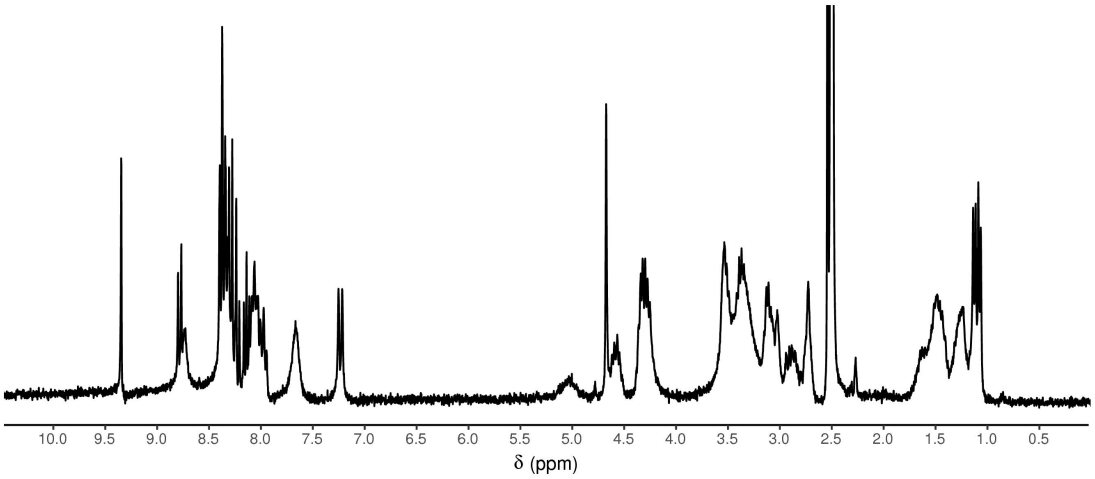


Figure S14 ^{1}H NMR (300 MHz) of **CP1** in DMSO-d_6.

3. References

[1] J. Meienhofer, M. Waki, E. P. Heimre, T. J. Lambros, R. C. Makofske and C.-D. Chang, *Int. J. Pept. Protein Res.*, 2009, **13**, 35–42; A. Romieu, T. Bruckdorfer, G. Clavé, V. Grandclaude, C. Massif and P.-Y. Renard, *Org. Biomol. Chem.*, 2011, **9**, 5337–5342.

[2] C. A. Schneider, W. S. Rasband and K. W. Eliceiri, *Nat. Methods*, 2012, **9**, 671–675.

[3] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid and others, *Nat. Methods*, 2012, **9**, 676–682.

[4] D. Nečas, P. Klapetek, *Cent. Eur. J. Phys.* 2012*,* **10***,* 181–188.

[5] G. Lamour, J. B. Kirkegaard, H. Li, T. P. Knowles and J. Gsponer, *Source Code Biol. Med.*, 2014, **9**, 16.

[6] J. M. Gorham, A. K. Stover and D. H. Fairbrother, *J. Phys. Chem. C*, 2007, **111**, 18663–18671.