Supplementary Information

for

3D self-assembly of cyclic peptides into multilayered nanosheets

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1. Materials

All reagents and solvents were purchased from Acros Organics, Aldrich, Fisher Scientific, Iris Biotech and Novabiochem. Dichloromethane was dried under reflux over calcium hydride. Glass slides for fluorescence microscopy were obtained from Ibidi (Cat# 80827). TEM grids (i. Cu carbon type-B, 300 mesh; ii. Holey carbon, 400 mesh) and PELCO® mica discs for AFM were purchased from Ted Pella.

2. Equipment

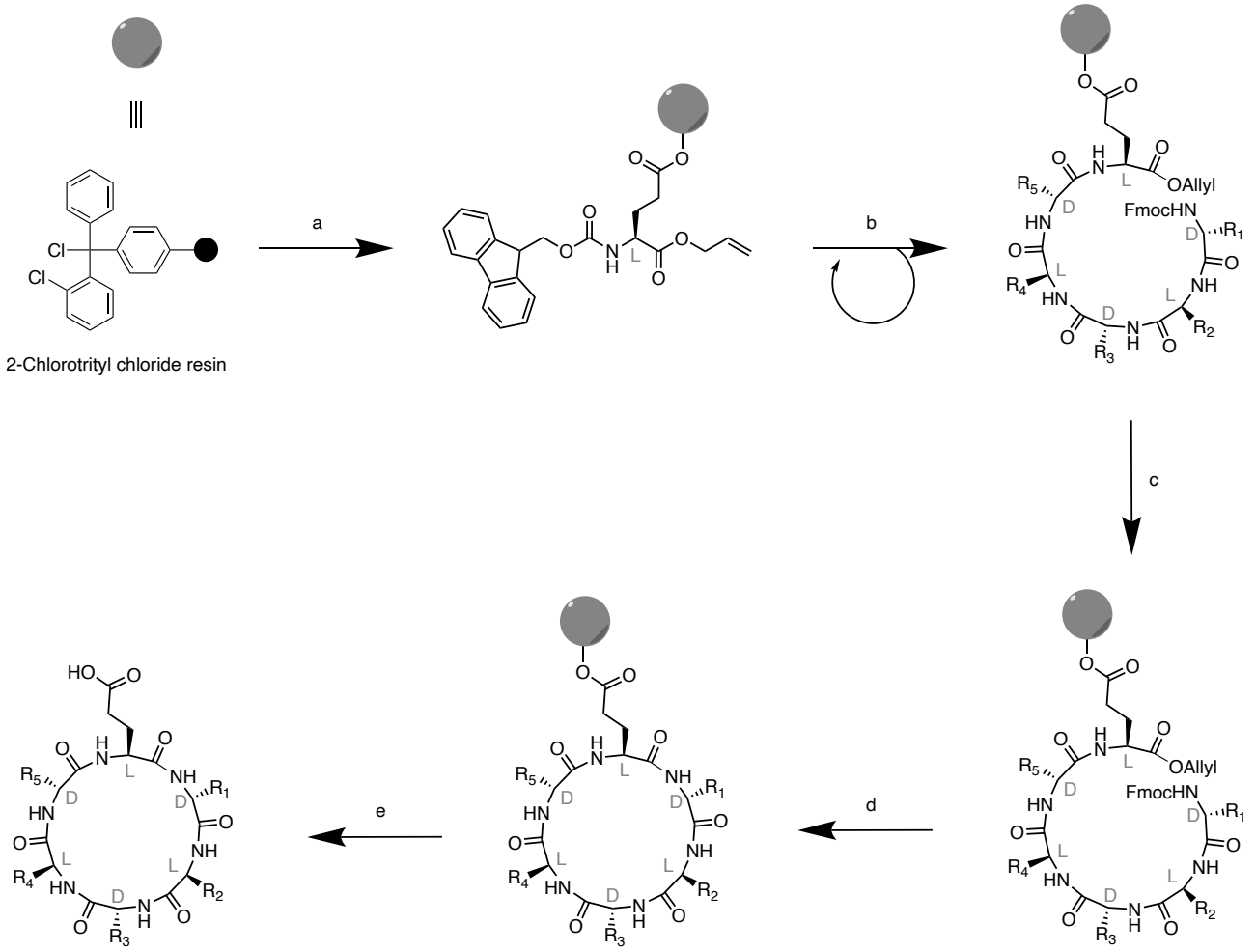
Peptide purification was carried out by preparative reverse-phase HPLC (Waters 1525 and 2489) using an Agilent Eclipse XDB-C18 column. 1H-NMR spectra were acquired on Varian 300 or 500 MHz spectrometers at 298K; chemical shifts (δ) are reported in ppm relative to that of DMSO (δ = 2.50 ppm). Analytical UHPLC of the peptide was performed on an Agilent 1260 Infinity II fitted with an Agilent SB-C18 column and connected to a 6120 quadrupole MS detector. HR-MS was acquired on a Bruker MicroTOF. Epifluorescence micrographs were taken with a Nikon Eclipse T*i* (60x immersion objective, FITC cube: Ex=475/35 nm; Em=530/43 nm). STEM images were acquired on a FESEM Ultra plus (Zeiss) operating at 20 kV from unstained samples. Non-contact mode AFM analysis was performed on a NX-10 instrument fitted with ACTA 10M cantilevers (Park systems). Cross-polarised microscopy was performed on an Olympus BX51 fitted with two rotary polarisers. Wide-angle X-ray scattering (WAXS) was measured on a Bruker D8 VENTURE PHOTON-III 14 κ-geometry diffractometer fitted with an Incoatec IμS 3.0 microfocus sealed tube (Cu Kα, λ = 1.54178 Å) and a multilayer mirror monochromator.

3. Abbreviations

ACN: acetonitrile; DCM: dichloromethane; DIPEA: *N*,*N*-diisopropylethylamine; DMF: *N*,*N*-dimethylformamide; DMSO: dimethyl sulfoxide; FITC: fluorescein isothiocyanate; Fmoc: 9-fluorenylmethyloxycarbonyl; HBTU: *N*,*N*,*N*',*N*'-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uranium hexafluorophosphate; PyAOP: (7-azabenzotriazol-1-yloxy)trispyrrolidinophosphonium hexafluorophosphate; TFA: trifluoroacetic acid, ThT: thioflavin-T, TIPS: triisopropylsilane, Trt: trityl.

4. General protocol for solid-phase peptide synthesis (SPPS)

Cyclic peptides were synthesised manually on solid phase as depicted in Scheme S1, following the protocols below:



Scheme S1. Synthetic protocol for the synthesis of ^{6}CP and its variant $^{6}CP_{2W}$. a) 2-chlorotrityl chloride resin functionalisation with the first amino acid coupled by its side chain: i. Fmoc-L-Glu-OAll, DIPEA, distilled DCM, 1 h. ii) Resin capping: DCM:MeOH:DIPEA (8.5:1.0:0.5) b) Peptide elongation: i. Piperidine in DMF (20% v/v), 15 min; ii. Fmoc-amino acid, HBTU, DIPEA, DMF, 30 min. This process was repeated 5 times to couple all the amino acids in the sequence. The last amino acid will maintain the Fmoc protecting group in the amine end. c) Allyl removal: $Pd(OAc)_2$, PPh_3, phenylsilane, 4-methylmorpholine, DCM, overnight (16-20 h). The final product was washed (30 min x2) with a solution of sodium diethyldithiocarbamate in DFM (0.5% w/v). d) Cyclization: i. Fmoc removal: piperidine in DMF (20% v/v), 30 min; ii. Peptide cyclization: PyAOP, DIPEA, DMF (2 h x2). d) Peptide cleavage: TFA:DCM:H_2O:TIS (90:5:2.5:2.5), 2 h.

4.1. Attachment of the first amino acid. For ^{6}CP [cyclo-(*D*-Leu-*L*-Leu-*D*-His-*L*-Glu-*D*-His-*L*-Glu)] and $^{6}CP_{2W}$ [cyclo-(*D*-Trp-*L*-Trp-*D*-His-*L*-Glu-*D*-His-*L*-Glu)], 2-chlorotrytil chloride resin (312.5 mg, 1.6 mmol chloride per g resin) was soaked for 30 min in freshly distilled DCM (5 mL). The resin was filtered and a solution of Fmoc-*L*-Glu(OH)-OAll (614.1 mg, 1.5 mmol) and DIPEA (425 µL, 3 mmol) in dry DMF (5 mL) was added over the resin and shaken for 1 h. Then the mixture was filtered and washed with DCM (3 x 3 mL). The free reactive groups of the resin were capped with a solution of DCM:MeOH:DIPEA (8.5:1.0:0.5, 5 mL) that was added and shaken for 30 min. Finally, the resin was filtered off, washed with DMC (3 x 3 mL), and dried under a high vacuum to determine the new loading by Fmoc quantification. For this, a small fraction of dried resin was reacted with a solution of piperidine in DMF (20% v/v, 5 mL) for 30 min. After this time, the new loading was calculated by measuring the absorbance of the adduct after Fmoc removal.[1]

For $^{6}CP_{HQQE}$ [cyclo-(*D*-Leu-*L*-Leu-*D*-His-*L*-Gln-*D*-Gln-*L*-Glu)] and $^{6}CP_{QEQE}$ [cyclo-(*D*-Leu-*L*-Leu-*D*-Gln-*L*-Glu-*D*-Gln-*L*-Glu)]: Rink Amide resin (167 mg, 0.1 mmol) was swollen in DMF for 30 min and then treated with piperidine in DMF (20% v/v, 3 mL) to remove the Fmoc protecting group. The resin was washed with DMF (3 x 3 mL) and a solution of Fmoc-*L*-Glu(OH)-OAll (163.6 mg, 0.4 mmol), HBTU (132.5 mg, 0.35 mmol) and DIPEA (95 µL, 0.6 mmol) in DMF (3 mL) was added over the resin and shaken for 1h. Then, the mixture was filtered and washed with DMF (3 x 3 mL) and DCM (3 x 3 mL).

4.2. Linear peptide synthesis. The rest of the amino acids were coupled considering the new loading. Cycles of Fmoc removal, piperidine in DMF (20% v/v, 2 mL) for 15 min, and amino acid coupling, Fmoc-protected amino acid (0.4 mmol), HBTU (0.35 mmol) and DIPEA (0.6 mmol) in DMF (3 mL) for 30 min, with their respective washes (DMF 3 x 3 mL) were followed. After the coupling of the last amino acid , the resin was washed with DMF (3 x 3 mL), DCM (3 x 3 mL) and dried under vacuum.

4.3. OAll removal. The resin was reacted with a solution of PPh_3 (39.3 mg, 0.15 mmol), *N*-methylmorpholine (110 µL, 0.01 mmol), phenylsilane (123 µL, 1.0 mmol) and Pd(OAc)2 (6.7 mg, 0.03 mmol) in dry DCM (4 mL) overnight (16-20 h). The resin was then washed with DCM (3 x 3 mL) and DIPEA in DMF (2% v/v, 3 x 3 mL) and soaked in a solution of sodium diethyldithiocarbamate (0.5% w/v in DMF, 2 x 3 mL) for 30 min to remove all traces of Pd. Finally, the resin was stirred with piperidine/DMF (20% v/v, 3 mL) for 30 min for removal of the Fmoc group at the N-terminus.

4.4. Cyclisation. was carried out by shaking the resin with a solution of PyAOP (208.6 mg, 0.4 mmol) and DIPEA (95.1 mL, 0.6 mmol) in DMF (3 mL) for 2 h. After washing with DMF (3 x 3 mL) and DCM (3 x 3 mL), the cyclization was repeated two more times under the same conditions.

4.5. Peptide cleavage. The peptide was deprotected and cleaved from the resin by addition of a freshly prepared TFA cocktail (4 mL, TFA:DCM:H2O:TIPS, 0.9:0.05:0.025:0.025), this mixture was shaken for 2 h and then filtered. The resin was washed with TFA (0.5 mL) twice and concentrated under nitrogen. The resulting reaction mixture was precipitated by dropwise addition onto cold diethyl ether (40 mL) under stirring. The resulting suspension was centrifuged for 10 min at 3000 rpm and the pellet was dried with a stream of air and dissolved in MilliQ:ACN (80:20).

5. Chromatographic purification of peptides

The sample was purified by semipreparative HPLC using an Agilent Eclipse XDB-C18 column [gradient of H2O+0.1% TFA:ACN+0.1% TFA→80:20 (0 min) to 25:75 (30 min)]. Peptide fractions were concentrated in vacuo to remove ACN and TFA and the remaining solution was freeze-dried.
A = H_2O + 0.1% TFA; B = ACN + 0.1% TFA; Gradient (A:B): 95:5 (0 min) → 5:95 (12 min)

6. Protocol for supramolecular self-assembly

Cyclic peptides were dissolved to a concentration of 50 μM in sodium phosphate buffer (20 mM, pH 6.5) in glass vials. Samples were sonicated for 5 min and then incubated at 80°C without shaking for 1.5 h. Next, peptide solutions were stored at room temperature for 1 h to promote an ordered assembly. To study the assembly behaviour at different pH, cyclic peptide solutions prepared likewise with pH adjusted with HCl or NaOH 1M before annealing. For fluorescence sample preparation, thioflavin-T (10 μM) was added to the cyclic peptide solution before annealing.

7. Epifluorescence microscopy

10 μL of cyclic peptide samples annealed in the presence of 10 μM thioflavin-T were deposited on glass slides and left to dry completely overnight in the dark at room temperature before imaging. Epifluorescence micrographs were taken at room temperature with a Nikon Eclipse Ti (60x immersion objective, Excitation=475/35 nm; Emission=530/43 nm). All images were analysed using ImageJ.

8. Scanning transmission electron microscopy (STEM)

Annealed nanosheet samples (5 μL; without thioflavin-T) were deposited on Cu Grids. Once dry, samples were rinsed twice with water (10 μL) for 1 min and stained with 5 μL of phosphotungstic acid (2% v/v in water) for 1 min, then the rest was removed. STEM images were acquired on a FESEM Ultra plus (Zeiss) operating at an extra high tension of 20 kV.

9. Atomic force microscopy (AFM)

Annealed nanosheet samples (5 μL, without thioflavin-T) were spotted on mica and were left to dry, then gently rinsed with water, left to dry again and imaged. AFM analysis was carried out on a NX-10 microscope (Park Systems) in non-contact mode and using ACTA cantilevers (silicon tips, nominal values: spring constant = 40 $N \cdot m^{-1}$, frequency = 300 kHz, ROC less than 10 nm). Image analysis was carried out using Gwyddion.

10. SAXS: Data acquisition and analysis

Solution SAXS experiments were performed on the BM29 automated BioSAXS beamline at the European Synchrotron Radiation Facility (ESRF, France). A sample-to-detector distance of 2.81 m and an X-ray wavelength of 0.9919 Å were employed, yielding a q-range from 0.058 to 5.2 nm^{-1}. Temperature was kept constant at 25 °C during measurement. Samples were continuously pumped through the 1 mm quartz capillary to minimize radiation damage during data acquisition using the automated sample changer. The signal from the buffer was subtracted and data were reduced using the standard protocols of the beamline [2] to obtain the scattered intensity in absolute units, *I(q)*, as a function of the reciprocal space, *q*.

Data analysis was performed using two convergent approaches. Initially, the pair-distance distribution function, *p(r)*, was determined using the indirect Fourier transform (IFT) method implemented in

BioXTAS RAW.[3] The p(r) is a probability function of the real space distances, *r*, within the scatterer, and thus relates to its shape and size. The second approach employed mathematical models that describe the shape and dimensions of the scatterer. Considering the observations from STEM and the potential coexistence of structures revealed by the IFT analysis, a model that combines the signal form 2D nanosheets and 1D cylinders was used. Data analysis was performed using the SasView 5.0 suite, where the Levenberg-Marquardt algorithm was used to minimize the differences between the mathematical model and the experimental data. It should be noted that the instrument configuration was selected to yield information on the smallest dimension of the structure (i.e., cross-section of the nanotubes and thickness of the nanosheets) to complement the microscopy results. Therefore, no information on the length of the nanotubes or overall size of the nanosheets was extracted from SAXS data analysis as these are beyond the resolution of the experiment.

11. WAXS: Data acquisition and analysis

WAXS profiles were recorded on a D8-ADVANCE (λCu = 1.5406 Å) and LYNXEYE XE-T detector from dry drop-cast samples of ^{6}CP nanosheets annealed at pH 6.5 deposited on a quartz surface. Difractograms were obtained in an angular range of $4<2\Theta<50$ with a 0.02° pass of 2 s.

12. Peptide characterisation

12.1. ^{6}CP [cyclo-(*D*-Leu-*L*-Leu-*D*-His-*L*-Glu-*D*-His-*L*-Glu)]

Yield: 36 mg (24%), white solid.

UHPLC-MS (C18-ESI, +eV): R_t = 5.4 min.; m/z = 759.45 ([M+H]$^+$), 380.30 ([M+2H]$^{2+}$).

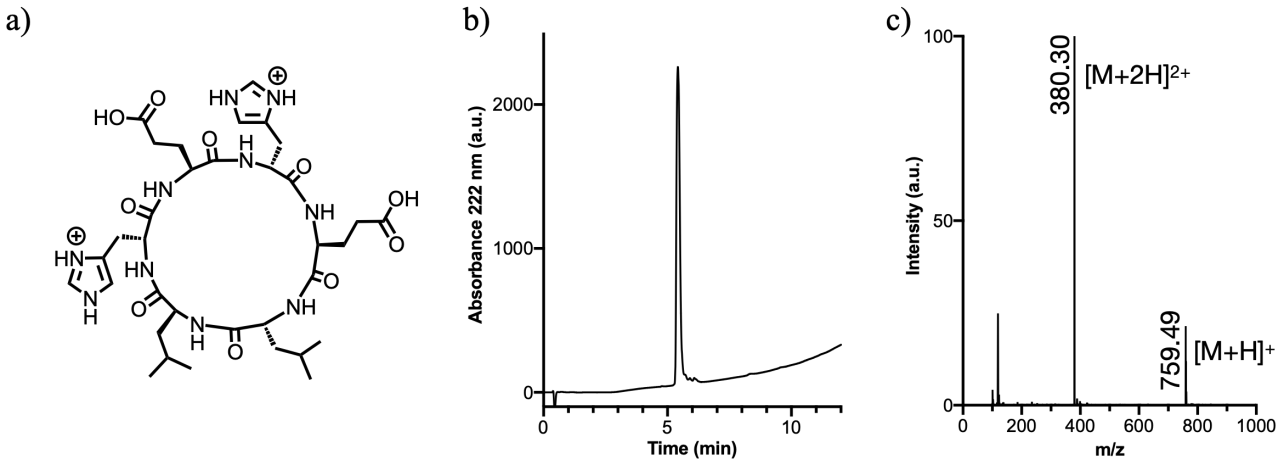


Figure S1. a) Structure of ^{6}CP; b) Chromatogram after purification; c) Mass spectrum.

^{1}H-NMR (300 MHz, CD_3OD): δ = 0.86-0.98 (m, 12H, Leu *i*-Bu x2), 1.53-1.65 (m, 6H, Leu-CH_2, Leu-CH), 1.94-2.42 (m, 8H, Glu-CH_2-), 3.05-3.37 (m, 4H, His-CH_2-), 4.23-4.77 (m, 6H, H_α), 7.35 (dd, *J*=13.25 Hz, 1.33, 2H, His-CH=), 8.80 (dd, *J*=5.75 Hz, 1.39, 2H, His-CH= x2) ppm.

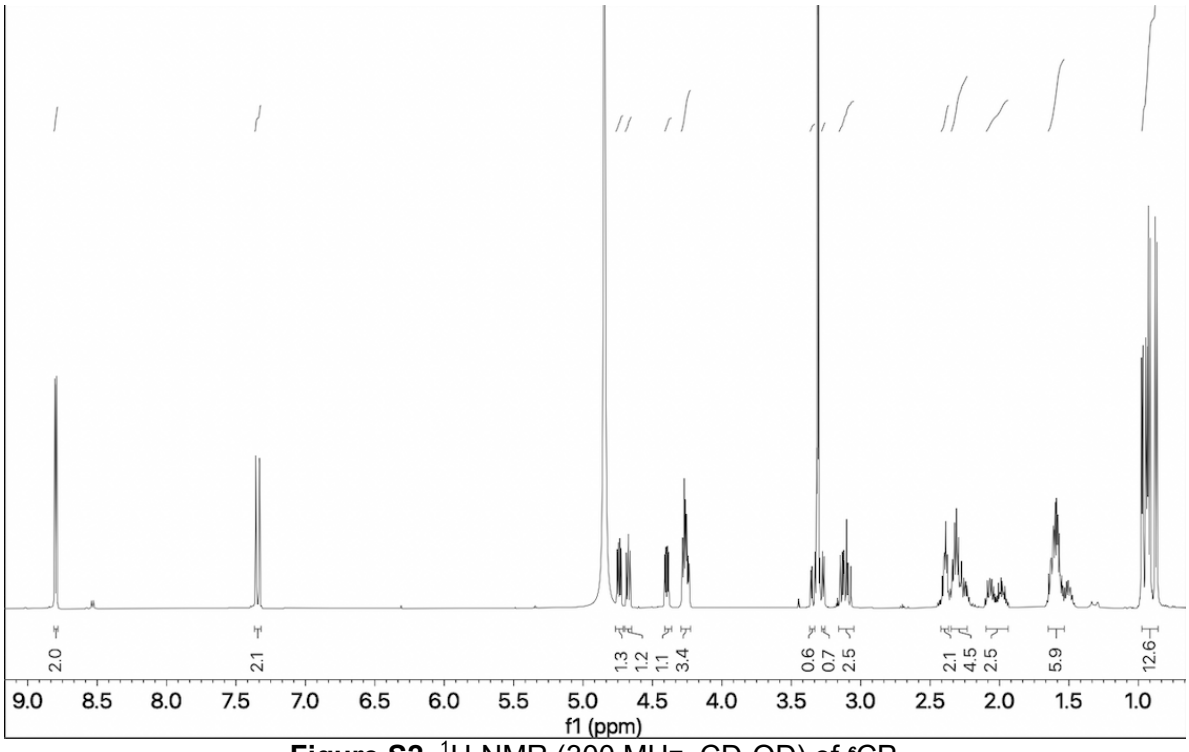


Figure S2. ^{1}H-NMR (300 MHz, CD_3OD) of ^{6}CP.

12.2. $^{6}CP_{2W}$ [cyclo-(*D*-Trp-*L*-Trp-*D*-His-*L*-Glu-*D*-His-*L*-Glu)]

Yield: 28 mg (16%), white solid.

UHPLC-MS (C18-ESI, +eV): R_t = 8.7 min.; m/z = 905.45 ($[M+H]^+$), 453.25 ($[M+2H]^{2+}$).

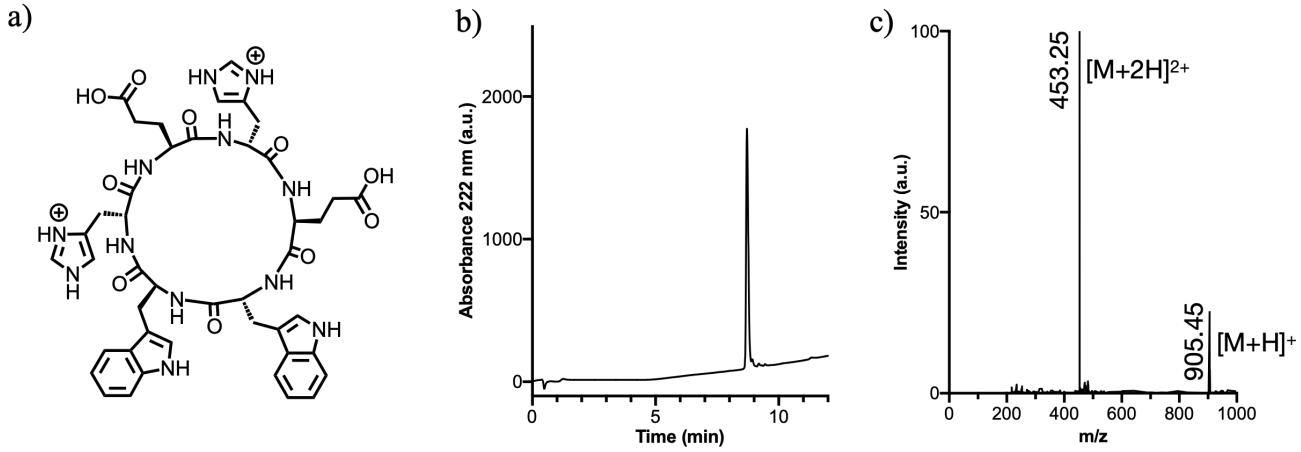


Figure S3. a) Structure of $^{6}CP_{2W}$; b) Chromatogram after purification; c) Mass spectrum.

12.3. $^{6}CP_{QEQE}$ [cyclo-(*D*-Leu-*L*-Leu-*D*-Gln-*L*-Glu-*D*-Gln-*L*-Glu)]

Yield: 32 mg (22%), white solid.

UHPLC-MS (C18-ESI, +eV): R_t = 7.9 min.; m/z = 741.37 ($[M+H]^+$), 371.05 ($[M+2H]^{2+}$).

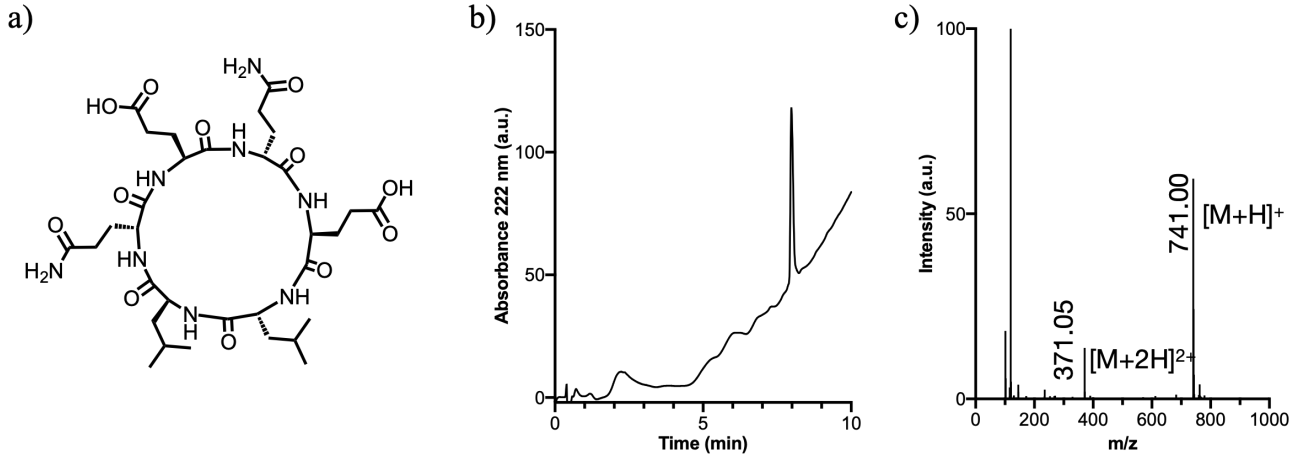


Figure S4. a) Structure of $^{6}CP_{QEQE}$; b) Chromatogram after purification; c) Mass spectrum.

12.4. $^{6}CP_{HQQE}$ [cyclo-(*D*-Leu-*L*-Leu-*D*-His-*L*-Gln-*D*-Gln-*L*-Glu)]

Yield: 38 mg (25%), white solid.

UHPLC-MS (C18-ESI, +eV): R_t = 7.33 min.; m/z = 749.40 ($[M+H]^+$), 375.30 ($[M+2H]^{2+}$).

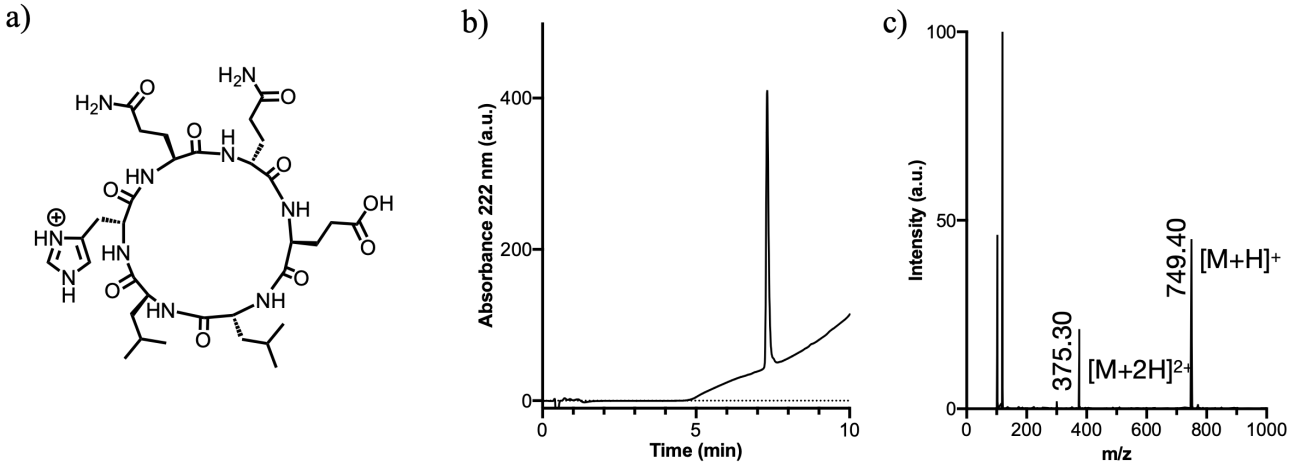


Figure S5. a) Structure of $^{6}CP_{HQQE}$; b) Chromatogram after purification; c) Mass spectrum.

13. Additional SAXS data analysis of ^{6}CP

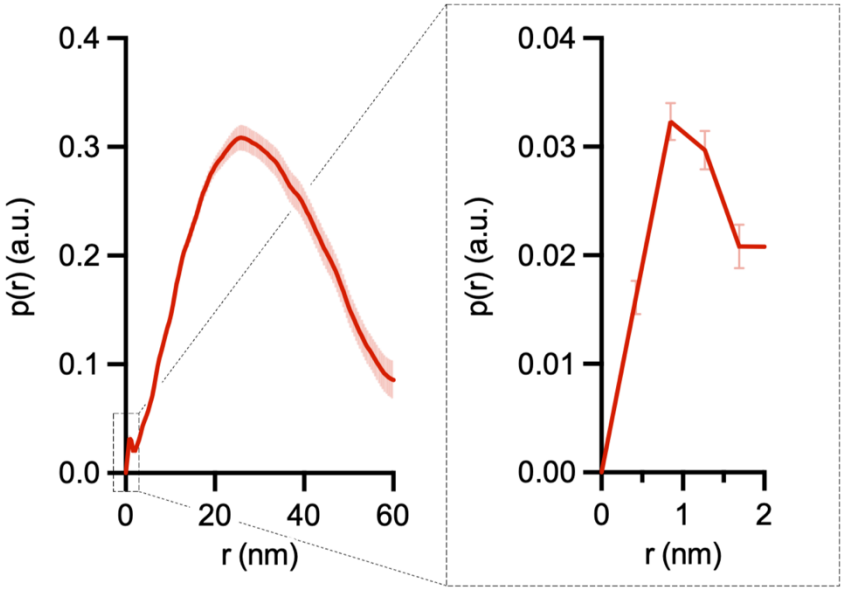


Figure S6. Pair-distance distribution function of the scatterers.
Left: overall signal. Right: signal at low values of the real-space dimension.

The results show the coexistence of small structures with a characteristic dimension of ca. 1 nm and large assemblies with a characteristic dimension around 20 nm. Also, the much higher values of the p(r) associated to the large structures confirms a higher volume fraction of those compared to the smaller assemblies. The values used for the modelling are shown in Table S1:

Table S1. Parameters used in the IFT method for the p(r) calculation.

D_{max} (nm)	Regularization constant	Scaling coefficient
61.0	8.0	1.0

14. Supporting references

(1) Kay, C.; Lorthioir, O. E.; Parr, N. J.; Congreve, M.; McKeown, S. C.; Scicinski, J. J.; Ley, S. V. Solid-phase Reaction Monitoring—Chemical Derivatization and Off-bead Analysis. *Biotechnology and Bioengineering* **2000**, *71* (2), 110–118. https://doi.org/10.1002/1097-0290(2000)71:2<110::aid-bit1002>3.0.co;2-2.

(2) Tully, M. D.; Kieffer, J.; Brennich, M. E.; Aberdam, R. C.; Florial, J. B.; Hutin, S.; Oscarsson, M.; Beteva, A.; Popov, A.; Moussaoui, D.; Theveneau, P.; Papp, G.; Gigmes, J.; Cipriani, F.; McCarthy, A.; Zubieta, C.; Mueller-Dieckmann, C.; Leonard, G.; Pernot, P. BioSAXS at European Synchrotron Radiation Facility – Extremely Brilliant Source: BM29 with an Upgraded Source, Detector, Robot, Sample Environment, Data Collection and Analysis Software. *J. Synchrotron Radiat.* **2023**, *30* (1), 258–266. https://doi.org/10.1107/s1600577522011286.

(3) Hopkins, J. B. BioXTAS RAW 2: New Developments for a Free Open-source Program for Small-angle Scattering Data Reduction and Analysis. *J. Appl. Crystallogr.* **2024**, *57* (1), 194–208. https://doi.org/10.1107/s1600576723011019.