Cyclization and Self-Assembly of Cyclic peptides

Alejandro Méndez-Ardoy, Ignacio Insua, Juan R. Granja, Javier Montenegro

Centro Singular de Investigación en Química Biolóxica e Materiais Moleculares (CIQUS), Departamento de Química Orgánica, Universidade de Santiago de Compostela, Santiago de Compostela 15782, Spain.

Abstract

Cyclic peptides are a fascinating class of molecules that can be programmed to fold or self-assemble into diverse mono- and multidimensional structures with potential applications in biomedicine, nanoelectronics or catalysis. Here we describe on-resin procedures to carry out tail-to-tail peptide cyclization based on orthogonal protected linear structures. We also present essential characterization tools for obtaining dynamic and structural information, including the visualization cyclic peptide assembly into nanotubes (AFM, TEM) as well as the use of fluorescence microscopy.

Keywords: Cyclic peptides, solid phase synthesis, self-assembly, supramolecular chemistry, nanotubes

1. Introduction

From the vast diversity of peptides found in the natural world, cyclic peptides (CPs) have gained considerable attention. A range of structurally diverse CPs have been identified in

organisms ranging from bacteria to mammals, playing, among other functions, a significant role in host defense.[1, 2] A common feature observed in CPs is their chemical stability due to the lack of free amino and carboxy termini, which prevents, degradation by proteases.[3] From a structural point of view, naturally occurring and synthetic cyclic peptides can adopt different conformations ranging from β-strands and sheets, to helical turns or loop structures, in which the ring constraint plays an important role in inducing and stabilizing these secondary structures.[4] As a consequence, interactions between cyclic peptides and natural or synthetic receptors often benefit from an entropic gain due to the preorganization of the peptide skeleton.[5] This is crucial to facilitate the rational design of peptide analogues with enhanced activity.[6] In a broader sense, the preorganized spatial conformation of CPs opens the door towards the implementation of self-assembly pathways into nanomaterials with controlled properties.[7] As an example, cyclic peptides consisting of an even number of amino acids with alternating chirality have the ability to self-assemble into well-defined organic nanotubes.[8] The precise disposition of the amide functions pointing perpendicular to the ring plane favors the stacking of successive peptide units in a β-sheet-like conformation. Since their discovery, these self-assembling CPs have attracted attention as synthetic transmembrane ion channels,[9,10] antimicrobials,[11] drug delivery systems,[12] anti-atherosclerotic agents,[13] antivirals[14] or transfecting vectors.[15] Additionally, they have also been used to template the one dimensional distribution of a range of molecular structures.[16–19] Control over nanotube topology and assembly has been also addressed by the covalent incorporation of polymers,[20] which allowed the obtention of structured, hybrid nanotubular materials.[21,22] According to the application pursued, subsets of amino acid side chains have been commonly exploited to

impart specific activities. Finally, the tuning of the internal pore properties has been also addressed by the use of γ- or δ-amino acids.[23, 24] Table 1 shows some representative examples.

[Table 1 near here]

Efficient synthetic methodologies and suitable characterization methods of self-assembled structures have been well described in the literature. Current literature covers different approaches towards the macrocycle formation,[25–28] usage of coupling reagents[29, 30] and additives.[31, 32] On the other hand, self-assembly of CPs can be monitored by a collection of experimental procedures that provide both dynamic and structural information. On a molecular level, fluorescence spectroscopy can provide valuable information concerning self-assembly thermodynamics and kinetics by the addition of external[33] or anchored[34] fluorescent probes. Structural information about the nature of the nano/micro aggregates can be obtained by microscopy techniques. Transmission Electron Microscopy (TEM) provides a way to characterize diameter, shell thickness or bundling.[8, 20-23, 35] Atomic Force Microscopy (AFM) also provides an excellent way to measure nanotube diameters using height profiles.[23,36] Complementary characterization techniques such as Small Angle Neutron Scattering (SANS) or Stochastic Optical Reconstruction Microscopy (STORM) might be crucial for clarifying certain structural details such as assembly strength, multicomponent assembly[37-38] and will be fundamental to provide insights into assembly properties such as charge transport[39] and mechanical characterization.[40]

2. Materials

1. Disposable and measuring material:

1. Cartridges for solid phase peptide synthesis (SPPS column): Polypropylene chromatography columns or glass columns of 10 mL are usually suitable. Cartridges should seal effectively in order to hold occasional small overpressures that develop, for example in the allyl ester deprotection reactions - see *note 1*.
2. Glass vials (1 and 5 mL).
3. 2 mL eppendorf tubes.
4. General laboratory material for measuring volumes: syringes (1, 2, 5 mL, glass or polypropylene), measuring cylinders (10, 100 mL) and micropipettes for organic solvents or glass low-volume syringes.
5. Needles (0.8 mm gauge).
6. Glass Pasteur pipettes and bulbs.
7. Glass flasks (10 mL).
8. Rubber septa.
9. 50 mL conical tubes.
10. Syringe HPLC filters (0.45 μm, nylon).
11. TEM grids (Cu grids, 300 mesh, carbon type-B coating).
12. AFM tips (*e.g.* silicon tips, nominal values: spring constant = 40 N/m, frequency = 300 kHz, ROC <10 nm).
13. Freshly peeled mica as AFM surface.

14. Glass slides for microscopy.

2. Reagents:

1. C-terminal deprotection cocktail A: Triphenylphosphine (39 mg, 0.15 mmol, 1.5 equiv), *N*-methylmorpholine (110 µL, 1 mmol, 10 equiv) and phenylsilane (120 µL, 1 mmol, 10 equiv) in dichloromethane (DCM, 2 mL).
2. C-terminal (or palladium) deprotection cocktail B: $Pd(OAc)_2$ (6.7 mg, 0.03 mmol, 0.3 equiv) in DCM (0.5 mL).
3. 0.5% w/v sodium diethyldithiocarbamate solution in *N*,*N*-dimethylformamide (DMF).
4. DIEA solution: 2% v/v *N*-ethyldiisopropylamine (DIEA) in DMF.
5. Piperidine solution: 20% v/v piperidine in DMF.
6. Cyclization cocktail: (7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP, 208 mg, 0.4 mmol) and DIEA (100 µL, 0.6 mmol) in DMF (3 mL).
7. Trifluoroacetic acid (TFA) cocktail: trifluoroacetic acid/DCM/water/triisopropylsilane (9:0.5:0.25:0.25).
8. TFA/EDT cocktail: trifluoroacetic acid/ethanedithiol/triisopropylsilane (2:0.3:0.2).
9. Thioflavin T or Nile red for fluorescence microscopy staining.
10. Uranly acetate or phosphotungstic acid for TEM visualization.

All reagents should be reagent grade (≥ 95 %) purity. C-terminal deprotection and cyclization cocktails are scaled for reactions over 0.1 mmol linear peptide.

3. Solvents:

1. DCM HPLC grade.
2. DMF peptide synthesis grade.
3. Diethyl ether laboratory reagent grade.
4. MilliQ water.
5. Acetonitrile (ACN) HPLC quality.

4. Equipment: Washing steps can be carried out by connecting the cartridges to a small pressure of nitrogen for mixing. Stirring of resin-containing columns can be carried out in different systems such as arm-based flask shakers and rotator mixers. Filtration can be supported by conventional chemical-resistant membrane pumps. Additionally, vortex, 1-2 mL microcentrifuge tubes and 15-50 mL centrifuge tubes are used in analytical or cleaving steps. Reaction monitorization can be carried out by analytical reverse-phase chromatographic equipment coupled to mass detectors, while purification is generally carried out by semipreparative or preparative HPLC setups. Characterization of final products will require access to NMR facilities (typically with a frequency range of 300 MHz or more), analytical HPLC set-ups and mass spectrometers (*e.g.* ESI-FIA-TOF). Additionally, specific equipment for microscopy; non-contact mode operated AFM, STEM or TEM equipment, epifluorescence or confocal fluorescence microscopes.

3. Methods

3.1. Cyclization of linear peptides by reaction between the carboxy- and amino-termini.

For the preparation of linear peptides used as precursors of cyclic products, the first amino acid of the sequence is protected on its carboxyl group by an allyl ester. The amino acids are anchored to the resin via the lateral side chain - see *note 2*. Several allyl-protected amino acids are commercially available (*e.g.* Fmoc-*L*-glutamic acid allyl ester, Fmoc-*L*-aspartic acid allyl ester, *N*-alpha-Fmoc-*L*-lysine allyl ester). The next coupling cycles can be carried out with Fmoc-protected amino acids, taking into account that residue chirality (*D* or *L*) must be alternated along the sequence. Due to the presence of the allyl ester protecting group at the C-terminus, the introduction of other palladium-sensitive protecting groups in the sequence should be avoided (*e.g.* other allyl esters, *N*-Alloc or 1,1-dimethylallyl, allyloxycarbonyl). An even number of amino acids is introduced, with ring sizes between 6 to 12 amino acids - see *note 3*. If possible, it is generally advised to design peptide growth leaving a non-bulky amino acid at the N-terminus to favor cyclization. A complete description of the experimental procedures for solid-phase peptide synthesis of Fmoc protected peptides can be found in the literature, which can be used in conjunction with the particularities discussed above.[41]

We will describe here the required steps used for sequential orthogonal deprotection of the Allyl and Fmoc groups, followed by cyclization through the reaction between the free carboxy and amino termini. The procedure is schematically depicted in Figure 1. The following protocol is indicated for the cyclization of 0.1 mmol of linear peptide anchored

on resin. Suggested **checkpoints** for evaluating the efficiency of the reaction are also indicated - see *notes 4 and 5*. Unless otherwise indicated, each resin washing step is carried out for 1 min.

[Figure 1 near here]

1. Synthesize linear peptide.
2. The resin (0.1 mmol in linear peptide) is transferred to a polypropylene or glass SPPS column (10 mL). The remnants of DMF are removed by washing with DCM (3 × 3 mL, 1 min). C-terminal (allyl group removal) deprotection cocktail is prepared in a glass vial (5 mL) and added to the SPPS column. The suspension is degassed by continuous flow of nitrogen for 5-10 min. - see *note 6*
3. Palladium cocktail B is prepared in a glass vial (1 mL) and added dropwise using a Pasteur pipette or a syringe- see *note 7* . The resin suspension is bubbled with an inert gas (N_2, Ar) for 2 min. At this point, the mixture color turns to pale yellow.
4. The resulting suspension is shaken for 6 h - see *notes 8 and 9.*
5. The solution mixture is filtered off, and the resin is washed with DCM (4 x 4 mL), DIEA solution (4 × 4 mL), sodium diethyldithiocarbamate solution (4 × 4 mL) and DMF (4 × 4 mL). **Checkpoint:** The success of the deprotection is confirmed by HPLC/MS. Using a gradient method of 95:5 water-ACN to 95:5 in 5 min (0.5 mL/min flow rate), the deprotected peptide has 0.3 min lower retention times, and a mass loss of 40 unified atomic mass units (taking into account an absolute charge of 1) in comparison with the starting material (Figure 2a) - see *notes 10 and 11*.

[Figure 2 near here]

6. The resin is then treated with a piperidine:DMF solution (2 mL, 1:4) for 10 min and successively washed with DIEA solution (4 × 2 mL) and DMF (4 x 4 mL). See *note 12*.
7. A solution of the cyclization cocktail is prepared in a glass vial (5 mL) and added to the SPPS vessel. The suspension is shaken for 3 h, then washed with DMF (4 × 3 mL). See *note 13*. **Checkpoint:** The success of the cyclization is evidenced by chromatographic peaks (gradient method of 95:5 water-ACN to 95:5 in 15 min and 0.35 mL/min flow rate) at higher retention times and a mass loss of 18 unified atomic mass units (assuming net charge of 1) compared to the starting material. If the reaction is not complete, this step can be repeated with the same conditions (Figure 2b).
8. The resin is washed with DCM (3 × 3 mL) and treated with freshly prepared TFA cocktail (3 mL) prepared in a 5 mL glass vial. The mixture is shaken for 2 h. See *notes 14 and 15* . After this time, the cocktail is filtered off and the resin is washed with fresh TFA (1 x 1 mL). The combined TFA layers are evaporated by pouring in a 10 mL glass flask and bubbling nitrogen, until a volume of about 1-1.5 mL volume is reached.
9. This solution is slowly added dropwise using a Pasteur pipette to an ice bath-cooled falcon tube containing diethyl ether (~ 10 mL/mL cocktail). The resulting suspension is centrifuged (10 min at 4000 rpm) and the organic layer is discarded.
10. The solid is washed with fresh diethyl ether by vortexing for 10 sec, centrifuged and decanted.

11. The solid is dried under a stream of nitrogen and then dissolved in water or water-ACN mixture according solubility (~ 2 mL) -see *note 16*- and filtered (0.45 µm syringe filter) prior to purification by HPLC. See for example reference [42].
12. Typically, analytical HPLC, mass spectroscopy and ^{1}H-NMR can provide sufficient information concerning the purity of the final product. Exact mass can be obtained from ESI-MS. High molecular weight cyclic peptides (typically > 2000 Da) can also be analyzed by MALDI-TOF.

3.2. Aqueous self-assembly of cyclic peptides

D/L-alternating cyclic peptides can self-assemble in solution through backbone H-bonding to generate supramolecular nanotubes with peptide side chains radially exposed on their surface (Figure 3). The chemical nature of these side chains can control (dis)assembly transitions and nanotube association. Therefore, different peptide sequences might require particular assembly conditions (*e.g.* concentration, heating, pH and ionic strength) based on their polarity and acidic/basic nature, as described below.

[Figure 3 near here]

3.2.1. Temperature annealing

Amphipathic cyclic peptides might need a heating-cooling cycle for their self-assembly.[35,43] While heating favors the contribution of the entropy to the assembly proccess, the generally enthalpic inter-backbone H-bonding contribution prevails upon cooling. In the formation of nanotubes and bundles from these type of peptides it seems possible that the heating step could improve hydrophobic packing while the cooling step brings additional structural control by the contribution of the hydrogen bonds formation.

In this plausible scenario, temperature annealing avoids the formation of disordered peptide aggregates - see *note 17*

1. Dissolve the cyclic peptide in 500 µL of sodium phosphate buffer (20 mM, pH 7.4) to a final concentration of 20-100 µM in a glass vial- see *note 18*.
2. Measure UV absorption for consistency across batches - see *note 19*.
3. Sonicate the sample for 5 min, then incubate at 80°C for 1.5 h on a heating block - see *note 20*.
4. Leave the sample at room temperature for 24 h to anneal - see *note 21*.
5. Samples can be purified by centrifugation (10,000 rcf, 10 min) and resuspended in fresh buffer or milliQ water by gentle pipetting or vial inversion.

3.2.2. Assembly triggered by pH

When electrostatic repulsions between like-charged peptides prevent their self-assembly, changes in pH can neutralize such repulsions and trigger nanotube formation. In these cases, the pH required for self-assembly will be close to the peptide's isoelectric point and hence it will vary with the number and combination of acidic and basic residues. This pH-dependence is more pronounced the more charged residues in the peptide, needing about 2 to 4 like-charged residues to control the self-assembly of an octapeptide with pH. This next protocol is taken from the base-triggered self-assembly of cyclic peptides containing basic residues (*i.e.* histidine; pK_a *ca*. 6), where neutralization of the repulsive protonated state of histidine's imidazole ring is achieved by addition of NaOH to a peptide solution in acidic medium.[44] Likewise, the assembly of cyclic peptides containing glutamic or aspartic residues (pK_a *ca*. 4) can be triggered by acidification of an alkaline solution.[8] In these cases, the neutralization of the carboxylates or imidazole moieties would turn these groups

from repulsive to attractive via H-bonding, and thus facilitate peptide ring stacking. The formation of these inter-side chain hydrogen bonds along the nanotube can be also mediated by water molecules.

1. Dissolve the cyclic peptide containing basic (*i.e.* histidine) residues in 500 µL of milliQ water to a final concentration between 10-20 $mg \cdot mL^{-1}$ in a glass vial - see *note 22*.
2. Adjust pH to 3 with aqueous HCl (0.1 M) and sonicate for 5 min to promote full dissolution.
3. Add aqueous NaOH (0.1 M) gradually up to pH 8, where assembly takes place - see *note 23*. Titration must be monitored with a pH-meter. Ideally, equilibration of the assembly should be monitored by suitable spectroscopic techniques (*e.g.* circular dichroism, fluorescence spectroscopy).
4. Alternatively, assembled samples can be directly prepared by dissolving the cyclic peptide (10-200 µM) in buffer solution (*e.g.* 30 mM HEPES) at pH 8.0 with sonication.

3.2.3. Assembly triggered by controlling the ionic strength

Similar to pH-triggered annealing (above), repulsions between like-charged peptides can be shielded by the addition of electrolytes to trigger self-assembly. The concentration of salt required is directly proportional to the number of charged residues in the peptide and their ionization state (*i.e.* pH). This next protocol describes the annealing of cationic cyclic peptides with different salts.[45]

1. Dissolve the cationic cyclic peptide (50 µM) in 500 µL of acidic buffer (50 mM MES, pH 5.6) in a plastic eppendorf tube with sonication.

2. Modify ionic strength to induce self-assembly by adding the corresponding salt solutions to a final concentration of: $CaCl_2$ (0.5 M), NaCl (1.5 M) or Na_2SO_4 (0.5 M) - see n*ote 24*.

3. Monitor peptide self-assembly by suitable techniques (*e.g.* fluorescence spectroscopy, circular dichroism, DLS) - see *note 25*. Alternative assembly characterization techniques are described in the following section.

3.3. Characterization of cyclic peptide assemblies

Microscopic characterization is highly empirical, and each peptide will show cleaner images with particular concentrations and sample preparations (*e.g.* washes, staining, *etc*.). Here, we will explain the preparation and analysis conditions of supramolecular cyclic peptide assemblies with microscopic and spectroscopic techniques.

3.3.1. (Scanning) Transmission Electron Microscopy

1. Annealed cyclic peptide samples (*vide supra*) are diluted with milliQ water to a final concentration of (1-10 μM) with very gentle mixing (*i.e.* vial inversions or smooth pipetting).

2. 4 μL of diluted sample are spotted on TEM grids and left to dry overnight at room temperature covered from dust.

3. If washing is required, 5 μL of milliQ water are deposited on the TEM grid and this liquid is immediately absorbed with filter paper. This process is repeated twice more.

4. If staining is required, 5 μL of the corresponding dye solution (*e.g.* 2% m/m uranyl acetate or 1 mg·mL^{-1} phosphotungstic acid), previously sonicated for 5 min and filtered

(0.45 μm), were deposited on the TEM grid. After 1 min of contact, the excess of staining solution is removed with filter paper as in the previous step.

5. After washing and/or staining, samples are left to dry overnight at room temperature and covered from dust before imaging.
6. STEM and TEM analysis are performed at 20 and 100 kV, respectively.

3.3.2. Atomic Force Microscopy

1. 5 μL of annealed cyclic peptide (*vide supra*) are spotted on freshly peeled mica surfaces and left to dry at room temperature covered from dust. Sample concentration usually varies from 0.1-100 μM; sample dilution can be done with milliQ water after annealing.
2. Once dry, the mica is washed with 10 μL of milliQ water deposited on the surface and immediately absorbed with filter paper sideways. This process is repeated twice more.
3. After washing, samples are left to dry overnight at room temperature and covered from dust before imaging.
4. Non-contact mode AFM analysis is performed with set points of *ca*. 7-12 nm and scanning rates between 0.2-0.5 Hz.

3.3.3. Fluorescence Microscopy

1. Annealing (*vide supra*) is performed in the presence of fluorescent probes (*e.g.* 10 μM thioflavin-T or Nile red) in the dark – see *note 26*.
2. 10 μL of annealed sample (10-200 μM) are deposited on microscopy glass slides and left to dry covered from dust in the dark.

3. Images are taken on microscopes fitted with filter cubes suited for thioflavin-T (482/35, 510, 536/40 nm) and Nile red (543/22, 562, 593/40 nm). These values indicate excitation, dichroic and emission filter wavelength cut-offs, respectively.

3.3.4. Nuclear Magnetic Resonance

A variety of NMR experiments provide valuable conformational and thermodynamic information about the supramolecular structure of cyclic peptide ensembles. Most of these experiments are performed with one-faced *N*-substituted cyclic peptides (*i.e.* alternating backbone amide groups, see Figure 4), thus restraining the number of supramolecular oligomers to dimeric species, and allowing a clearer NMR interpretation that can be extrapolated to longer ensembles (*vide infra*). Also, such NMR studies can be performed in non-polar deuterated solvents to promote peptide self-assembly by limiting the dynamic exchange of H-bonding pairs with polar solvents. When D_2O is used as solvent, a frequent issue is deuterium exchange with a variety of the CP functional groups (amide, amine, hydroxy, carboxylic acids or thiols) - see *note 27*. A solution is to analyze the peptide dissolved in H_2O within a wider NMR tube including a D_2O reference. A variety of NMR water suppression techniques can then be applied to filter the water proton signals from the spectra.[46]

^{1}H-NMR: Allows the calculation of cyclic peptide association constant (K_a) and thermodynamic parameters (ΔG, ΔS and ΔH).[47]

1. Acquire the spectrum of a cyclic peptide at different concentrations (*e.g.* 1-40 mM). Peptide self-assembly (*i.e.* dimerization) shows a downfield shift of the backbone amide hydrogens from *ca*. 6.30 to 7.32 ppm (Figure 4).

2. Calculate K_a with Equation 1, where C represents peptide concentration and chemical shifts (δ) are denoted 'dim' for dimer (*i.e.* highest value), 'mon' for monomer (*i.e.* lowest value) and 'obs' for observed at a particular C.

$$\delta_{obs} = \delta_{dim} + \frac{(\delta_{mon} - \delta_{dim}) \cdot \left(-1 + \sqrt{1 + 8K_a \cdot C}\right)}{4K_a \cdot C} \qquad \text{(Eq. 1)}$$

3. Obtain K_a values at different temperatures to calculate the thermodynamic parameters ΔH and ΔS respectively from the slope and intercept of a Van't Hoff plot (Equation 2), where R represents the ideal gas constant (8.314 $J \cdot K^{-1} \cdot mol^{-1}$). ΔG is then calculated by ΔG=ΔH-TΔS.

$$-log(K_a) = \left(\frac{\Delta H}{2.3R}\right) \cdot \left(\frac{1}{T}\right) - \left(\frac{\Delta S}{2.3R}\right) \qquad \text{(Eq. 2)}$$

[Figure 4 near here]

Overhauser Effect Spectroscopy: Rotating frame (ROESY) and Nuclear (NOESY) Overhauser Effect ^{1}H-NMR experiments report on the spatial proximity of protons even if they are not bonded. These techniques are extremely useful to study the rotational isomerization of cyclic peptide stacks, the spatial conformation of pendant groups and the encapsulation of molecules in the lumen of cyclic peptide ensembles.[23,48,49] Additionally, Diffusion-Ordered (DOSY) experiments can provide valuable information regarding nanotube length and its relationship with CP concentration.[48]

Notes

1. Reuse of cartridges: Polypropylene columns can be generally washed and reused a number of times. However, extended reaction times (*e.g.* overnight reactions) might result in development of color on the polypropylene cartridges. Additionally, filters might block eventually.
2. Conventional resins (polystyrene, generally 1% DVB crosslinking, 100-200 mesh particle size) are suitable for typical cyclization reactions, and their election is mostly dependent on the peptide sequence. Lysine side chains are generally anchored to 2-chlorotrityl chloride resin (2CTC, loading 1.0-1.6 mmol/g); while glutamic/aspartic side chains can be anchored to 2CTC or Wang (loading 0.5-1.3 mmol/g) resins when it is require to preserve the carboxylic side chain functional group, or Rink Amide AM (loading 0.4-0.75 mmol/g,) when the amide lateral side chain is pursued.
3. The ring sizes described in the literature are 6 to 12 when using α-amino acids, and up to 16 with γ and δ amino acids, with the ability to self-assemble into capsules. For a review, see reference [50].
4. Assessing the efficiency of the reaction: A portion of resin (10-20 mg) can be treated with freshly prepared acidic cleaving cocktail (TFE or TFE/EDT cocktail, 100 μL) in a 2 mL eppendorf for 2 h, then the peptide is precipitated by addition of diethyl ether (1-2 mL). After centrifugation, the solution is discarded and the residue is briefly dried with a stream of air, then dissolved in water or water-ACN, filtered through a syringe filter (0.45 μm) and analyzed by reverse phase HPLC.

5. Before checking the efficiency of on-resin reactions, it generally convenient to remove the DMF from the solid support. This will help in the precipitation of the cleaved peptide. For this, washing with DCM (3 mL, 3 x 1 min) is enough.
6. Because the gas flow enhances evaporation of the solvent, flow rates should be not excessive.
7. It is recommended to degass C-terminal (or palladium) deprotection cocktail B for 5 min. This can be done by bubbling an inert gas (nitrogen, argon) through a thin needle. The vial can be closed with a rubber septum and a second needle to allow pressure release. Beware that DCM will evaporate.
8. With time, the reaction mixture becomes darker, occasionally even black.
9. **CAUTION**: At this point, a substantial pressure can develop in closed columns; it is recommended that pressure is released slowly by opening the column tip.
10. Typically, the allyl ester deprotection mixture presents a clear yellow color, which slowly turns into dark brown solutions. However, uncareful manipulation might lead to the formation of a dark brown to black solution right after preparation, which might lead to less efficient deprotections. This usually happens when solutions are not degassed. When a dark solution is developed this way, it is very recommendable to first check the success of the reaction by analytical HPLC. If the conversion turns incomplete, the whole step can be repeated again.
11. It is desirable to optimize reaction times. While 6 h is enough for the deprotection of the C-terminal group allyl ester, we have observed completion at shorter times (1-2 h). In a similar way, some cyclizations can undergo almost completion in 1 h. In this case, small portions of resin can be retrieved at different reaction times and analyzed

by HPLC. On the other hand, extended reaction times with PyAOP generally generate colored subproducts

12. This step corresponds to the deprotection of the Fmoc group.
13. This step corresponds to the cyclization step.
14. This step corresponds to the resin release step
15. When thiol side chains (*e.g.* Cys) are present, the preferred cocktail is the TFA/EDT mixture to prevent thiol oxidation. (**CAUTION**, ethanedithiol is highly malodorous and toxic).
16. Typically, a mixture of water-acetonitrile is used to dissolve the pellet; however, in our experience, the addition of ACN should be minimized for samples of high polarity since it could worsen the separation.
17. The chosen annealing temperature and heating-cooling rate can influence the size and structure of the final supramolecular ensemble.
18. The use of glass or plastic containers can have a strong impact on self-assembly, as peptides might stick to some of these surfaces. Consider testing different ones.
19. For batch consistency, concentration can be cross-checked by UV at 222 nm. If tryptophan or tyrosine are present, quantitative results can be obtained with their extinction coefficients at 280 nm: 5,690 and 1,280 $M^{-1} \cdot cm^{-1}$, respectively.[51]
20. This heating step dissociates any kinetically trapped aggregates formed upon peptide dissolution.[35]
21. Annealing times may vary from hours to days, and these are also affected by temperature. Consider sampling at different times and cooling temperatures.

22. This high peptide concentration (10-20 mg·mL^{-1}) was used to assemble hydrogels from dense cyclic peptide nanotubular networks. The same protocol also works for samples at concentrations used to assemble dispersed peptide nanotubes (10-200 μM).
23. Having His' imidazolyl side chain a pK_a of *ca*. 6, self-assembly begins around this pH (~50% neutralization), reaching full assembly at pH 8 (~99% neutralization).
24. Higher ion multivalency usually leads to more efficient charge shielding and hence self-assembly. The salt concentrations given correspond to equivalent ionic strengths (I), which can be calculated with Equation 3, where c and z indicate the concentration and charge of the ion, respectively.

$$I = \frac{1}{2}\sum_{i=1}^{n} c_i \cdot z_i^2 \qquad \text{(Eq. 3)}$$

25. The self-assembly of cyclic peptides bearing fluorescent pyrene pendants leads to the quenching and shift of pyrene's emission band to its excimer at 470 nm. [44,45]
26. Concentrated dye stocks (5 mM) can be prepared in water (thioflavin-T) or water-miscible organic solvents (Nile red) such as acetone or tetrahydrofuran. Samples are stained with 1 μL of dye stock (5 mM) in 500 μL of cyclic peptide solution (10-200 μM).
27. Addition of small amounts (*ca*. 1% v/v) of polar deuterated solvents (*e.g.* CD_3OH) can help disrupt peptide aggregates that may interfere with the measurements.

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Abbreviatures

2CTC: 2-Chlorotrityl chloride, *ACN*: Acetonitrile, *DCM*: Dichloromethane, AFM: Atomic Force Microscopy, *DIEA*: *N*-Ethyldiisopropylamine, DLS: Dynamic Light Scattering, *DMF*: *N*, *N*-Dimethylformamide, DVB: Divinylbenzene, *EDT*: 1,2-ethanedithiol, *FRET*: Förster resonance energy transfer , *PyAOP*: (3-Hydroxy-3H-1,2,3-triazolo[4,5-b]pyridinato-O)tri-1-pyrrolidinyl-phosphorus hexafluorophosphate, *N-TBTU*: Tetrafluoroborate Benzotriazole Tetramethyl Uronium, *N*-HATU: Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium, *TFA*: Trifluoroacetic acid, THF: Tetrahydrofuran, *TIS*: Triisopropylsilane, *HEPES*: 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid, *His*: Histidine, *MES*: 2-(*N*-morpholino)ethanesulfonic acid, *NCM*: Non-contact mode, *Rcf*: Relative centrifugal force, ROC: Radius of curvature, *SANS*: Small-Angle Neutron Scattering, *SPPS*: Solid-Phase Peptide Synthesis, *(S)TEM:* (Scanning) Transmission Electron Microscopy

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Figures and Tables

Figure 1. Schematic representation of the on-resin cyclization process: i) $Pd(OAc)_2$, PPh_3, phenylsilane, NMM; ii) piperidine/DMF; iii) PyAOP/DIEA, DMF; iv) TFA/DCM/H_2O/TIS (0.9 : 0.1 : 0.5 : 0.5).

Figure 2. a) HPLC chromatograms showing the deprotection of the allyl ester of cyclic peptide precursor (‘linear’ denotes the starting material, ‘- allyl’ denotes the deprotected peptide); b) HPLC chromatogram showing the crude mixture after cyclization conditions (PyAOP/DIEA).

Figure 3. Sequential self-assembly of D/L-alternating cyclic peptides into one-dimensional (1D) nanotubes via H-bonded stacks of ß-sheets (dashed lines) and two-dimensional (2D) nanosheets with hydrophobic interiors made of leucine (Leu)

interdigitation and tryptophan (Trp) stacks. Reprinted with permission from Insua et al. [35] Copyright 2020 American Chemical Society.

Figure 4. ^{1}H-NMR ($CDCl_3$, 300 MHz, 293K) and chemical structure of one-faced *N*-substituted cyclic peptide **CP1** at different concentrations (see labels). Reprinted with permission from Fuertes et al [48]. Copyright 2017 Royal Society of Chemistry.

Amino acid side chains	Role	Applications	Examples
Arginine, Lysine	Electrostatic interaction with membranes, DNA encapsulation	Antivirals, gene delivery	[14, 15]
Leucine, Tryptophan	Integration with membranes, cell membrane permeability	Antibacterial, membrane channels	[9, 11]

Table 1. Use of amino acid side chains in diverse applications envisaged for cyclic peptide nanotubes.