

A water playground for re-assembly from fibrils to plates

Simone Adorinni,^[a] Marina Kurbasic,^[a] Ana M. Garcia,^[a,b] Slavko Kralj,^[c,d] Ottavia Bellotto,^[a] Erica Scarel,^[a] Paolo Pengo,^[a] Rita De Zorzi,^[a] Michele Melchionna,^[a] Attilio V. Vargiu*^[e] and Silvia Marchesan*^[a]

[a] Dr. S. Adorinni, Dr. M. Kurbasic, Dr. A. M. Garcia, Dr. O. Bellotto, Ms. Erica Scarel, Prof. P. Pengo, Prof. R. De Zorzi, Prof. M. Melchionna, Prof. S. Marchesan

Department of Chemical and Pharmaceutical Sciences

University of Trieste

Via Giorgieri 1, 34127 Trieste, Italy

E-mail: smarchesan@units.it

[b] Dr. A. M. Garcia

Facultad de Ciencias y Tecnologías Químicas,

Instituto Regional de Investigación Científica Aplicada (IRICA),

Universidad de Castilla-La Mancha,

Ciudad Real 13071, Spain

[c] Dr. S. Kralj

Materials Synthesis Department

Jožef Stefan Institute

Jamova 39, 1000 Ljubljana, Slovenia

[d] Dr. S. Kralj

Department of Pharmaceutical Technology, Faculty of Pharmacy,

University of Ljubljana

1000 Ljubljana, Slovenia

[e] Prof. A. V. Vargiu

Department of Physics

University of Cagliari

Cittadella Universitaria, S.P. 8 km. 0.700, 09042 Monserrato (CA), Italy

E-mail: vargiu@dsf.unica.it

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Abstract: Short-peptide amyloid assembly and disassembly play crucial roles in various research fields, which range from addressing pathologies that lack therapeutic solutions to the development of innovative soft (bio)materials. Hydrogels from short peptides typically show thermo-reversible gel-to-sol transition, whereby fibrils disassemble upon heating, and re-assemble upon cooling down to room temperature (rt). Despite ongoing intense research studies in this area, the majority focus on peptide-peptide interaction and neglect the structuring role of water in peptide supramolecular behavior. This study describes an unprotected tetrapeptide gelator that forms highly stable fibrils which, upon heating, re-organize into plates that persist upon cooling to rt. All-atom molecular dynamics (MD) simulations and experimental methods reveal water as a key player in the thermodynamics that accompany this irreversible morphological transition, and advance our understanding of supramolecular structures.

Introduction

Amyloid assembly and disassembly have attracted great interest in recent years, with Fmoc-derived gelators having gained wide popularity.[1] By contrast, mastering the supramolecular behavior of short peptides devoid of synthetic appendages, which are more relevant to natural processes, is still very challenging. On one hand, amyloid disassembly could be of high value in a therapeutic context, yet fibril re-assembly is unavoidable when it is the thermodynamic product. On the other hand, general rules for the design of building blocks of three or four unprotected amino acids (aa) that self-organize into macroscopic – and ideally functional - hydrogels, as opposed to insoluble aggregates, are not yet fully elucidated,[2] although integration of experiments with computational tools is enabling fast progress in the field.[3]

It is well-established that amyloids have an amphipathic nature. Hydrophobic components segregate in steric zippers that exclude water and stabilize the structure.[4] In particular, phenylalanine (Phe)-based zippers have been reported for minimalistic peptides.[5] Yet, hydrophilic components must favorably interact with water to yield a bulk hydrogel. Gel-to-sol transition can be conveniently induced by heating, and usually the gel reforms upon cooling to rt. Reported examples of unprotected gelling peptides as short as three[6] or four[7] residues indeed display such thermo-reversible behavior.

For the design of self-assembling short peptides, the combination of D- and L-residues in heterochiral sequences is an attractive approach. Syndiotactic stereochemistry in cyclic peptides is well-known to permit the formation of nanotubes, with potential applications spanning from membrane transporters to hydrogels in confined droplets.[8] We recently introduced D-aa in non-gelling, L-tripeptides as a convenient strategy to modulate self-assembly in linear sequences.[6a] Molecular dynamics (MD) simulations suggest that this approach may favor the segregation of hydrophobic and hydrophilic components on opposite faces of β -structures, thus yielding amphiphilic assemblies that favorably interact with water and gel.[6a] D-aa are advantageous for their known resistance to protease-mediated hydrolysis, and self-assembly of their derivatives has been used to develop new therapies.[9] Besides, D-aa can interfere with amyloid fibrillation and hold therapeutic potential in related pathologies.[10]. There is thus scope to study self-assembling peptides with D-aa.

We report here the first unprotected D,L-tetrapeptide (Figure 1) that assembles into a highly stable hydrogel at physiological pH and undergoes a thermally-induced irreversible re-organization from fibrils to plates. We support experimental data with all-atom MD simulations that focus not only on peptide-peptide but also on peptide-water interactions. Interestingly, the supramolecular re-organization is not dictated by a key conformational change of the peptide, but rather by an irreversible change in its hydration, revealing water as a key player in the process.

Results and Discussion

The peptide was designed with two aliphatic D-aa at the N-terminus and the L-Phe-Phe self-assembling motif[11] at the C-terminus, synthesized in solid phase, purified by HPLC and characterized by NMR and ESI-MS (Fig. 1a and ESI Section S2). In particular, the design featured the elongation of the reported gelator DLeu-Phe-Phe[5b] with D-Nle, which displays a linear sidechain favoring intermolecular packing.[6a] While design rules for self-assembling D,L-tripeptides are emerging,[3b, 6a] to date

there is limited understanding of the supramolecular behavior of unprotected D,L-tetrapeptides, and filling the gap answers an interesting and still open research question.[12] It should be noted that even subtle chemical modifications of these systems can dramatically affect their supramolecular behavior. Indeed, N-acetylation of DLeu-Phe-Phe completely hindered self-assembly, and analogous results were obtained for the L-homochiral analog Nle-Leu-Phe-Phe (see ESI Section S9). These data confirmed the importance of both termini in establishing intermolecular salt bridges, as evident from the single-crystal XRD structure of a similar D,L-tripeptide, as well as heterochirality in hydrophobic short peptides to enable hydrogelation of amphipathic superstructures.[6a]

MD simulations of DNle-DLeu-Phe-Phe in explicit water revealed that the most representative conformation of the zwitterion is a turn with all hydrophobic chains on the same side of the backbone (Fig. 1b). Such isotactic spatial arrangement was recently reported to be key for the self-assembly of hydrophobic D,L-tripeptides into stable hydrogels.[6a] In both cases, the backbone is kinked. Peptide molecules stack thanks to hydrogen bonding between amides, with a pattern similar to that characterizing β -sheets and the aromatic rings running up the stack in a helical arrangement (Fig. 1c). Dihedral angles of the peptide backbone were calculated for the 2nd (D-Leu) and the 3rd residue (L-Phe) and are shown in the Ramachandran plot (Fig. 1d).

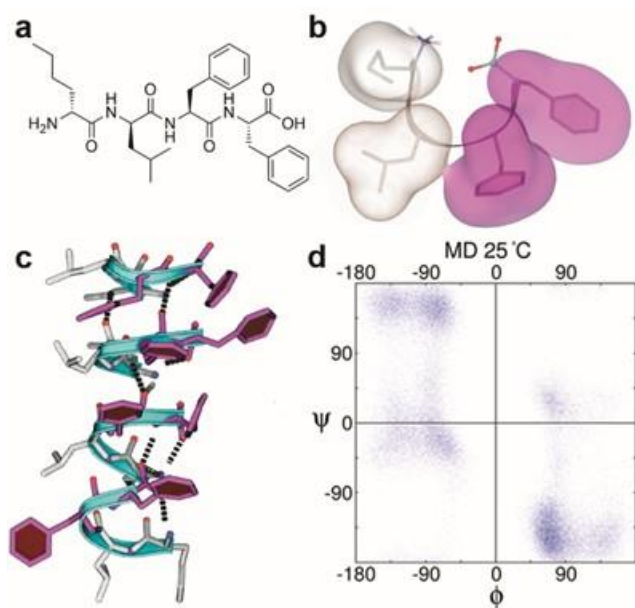


Figure 1. a) tetrapeptide structure. b) zwitterion conformation resulting from MD simulations (aliphatic and aromatic side chains in grey and purple, respectively, with transparent molecular surfaces). c) tetrapeptide stack resulting from MD simulations. H-bonds between amides (dashed lines) display a pattern similar to β -sheets. d) Ramachandran plot reporting the values of Φ and Ψ for the 2nd (D-Leu) and 3rd residues (L-Phe) of the tetrapeptide (the only ones for which both angles can be calculated),

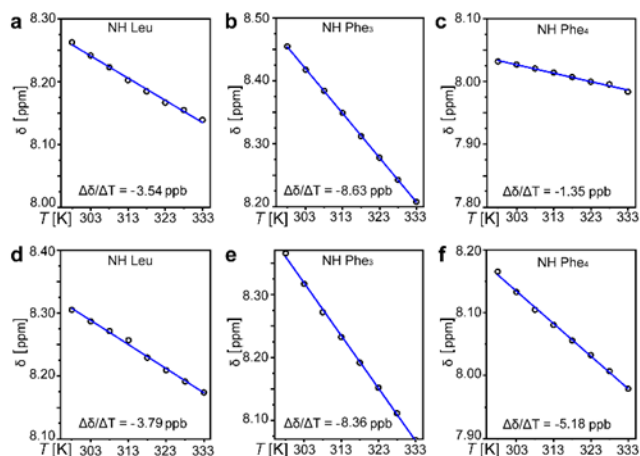


Figure 2. Intramolecular H-bonds in the heterochiral tetrapeptide. Variable-temperature $^1\text{H-NMR}$ analysis of CONH signals in DMSO (a-c) and with 20% water (d-f). Note: higher amounts of water led to precipitation.

$^1\text{H-NMR}$ spectra (see ESI Sections S2-S3) revealed $^3J_{\text{NH}\alpha\text{H}} \sim 4$ Hz that was compatible with $\Phi -60^\circ$, in contrast with the non-assembling homochiral L-tetrapeptide that displayed $^3J_{\text{NH}\alpha\text{H}} \sim 8$ Hz. The latter value is compatible with wider Φ values and an extended conformation,[13] as confirmed by MD simulations (see ESI Fig. S33). The presence of intramolecular H-bonds that could hold together the turn of the heterochiral tetrapeptide was verified by variable-temperature $^1\text{H-NMR}$ spectroscopy, firstly in DMSO as a non-aggregating solvent (Fig. 2a-c), and then in the presence of water (Fig. 2d-f). $^1\text{H-NMR}$ shifts of amide signals were visible and displayed a linear correlation with temperature from 298 K to 333 K, indicating no conformational loss. In particular, the NH chemical shifts of Leu and C-terminal Phe (Fig. 2a,c) displayed a temperature gradient $\Delta\delta/\Delta T > -4.6$ ppb/K, which is a strong indication of involvement in H-bonding.[14] Remarkably, the presence of water did not lead to loss of amide signals (Fig.2d-f), indicating they were not exchanging with the solvent, although only the NH of Leu maintained a temperature coefficient within the expected range for H-bonds (Fig.2d). Conversely, the same experiment carried out on the homochiral analog did not lead to any indication of H-bonds (see ESI section S6).

In silico data were in qualitative agreement with experiments in solution. Detailed analyses were performed on the trajectories extracted from MD simulations of a single homo- or hetero-chiral peptide in explicit water. For each peptide, we performed a MD simulation of 1 μs in length at 298 K, followed by heating to 363 K in 20 ns, and finally an equilibrium simulation of 1 μs at this temperature. We analyzed the secondary structural content and the Ramachandran plot (reported only for the 2nd and 3rd residues of the tetrapeptide, that is those for which both angles can be calculated), the preferred conformations, the end-to-end intramolecular distances, the frequency of formation of intramolecular H-bonds, and the peptide hydration. The heterochiral peptide displayed an intrinsic propensity towards turns (Table S1). Importantly, this feature seemed to be energy-driven and temperature-independent (i.e., there was no entropic gain with heating). The opposite was true for the homochiral analog, whose structural preference towards turns at rt was halved as compared to the heterochiral isomer (Table S1). The $\text{Ca}1$ -to- $\text{Ca}4$ distance (Fig. S34) was significantly longer for the homochiral peptide (visiting extended conformations) relative to the heterochiral one (adopting turns). MD confirmed the formation of an intramolecular salt bridge between termini, and

the engagement of Phe4 NH in intramolecular H-bonding, and of Phe3 and Leu NH to a lesser extent, in agreement with NMR data (Table S2).

A turn conformation stabilized by the intramolecular salt bridge between the charged termini was confirmed for the heterochiral tetrapeptide by single-crystal X-ray diffraction (XRD) (Figure 3). Surprisingly, the NH of Leu and Phe3 were engaged in H-bonding, albeit intermolecularly with the CO of the same residues of an adjacent tetrapeptide molecule, as a distinctive feature of the solid phase, as opposed to the intramolecular H-bond of the peptide in solution. The dihedral angles of the crystal structures are compatible with one of the visited conformations observed by MD, albeit not the most representative one (Fig. S43).

The gelator was first dissolved in alkaline phosphate buffer, thanks to repulsion between negative charges in its anionic form. Subsequent pH lowering to neutral triggered amyloid fibrillation of the zwitterion in samples as diluted as 0.050 % wt. When the concentration was increased to 0.67 % wt. a self-supportive hydrogel was obtained, in contrast with the L-analog (see ESI Sections S8-S9). Interestingly, no cytotoxicity was found for the heterochiral peptide gel in live/dead assays on fibroblast cells (see ESI Section S10). Furthermore, the gel was highly resistant to protease digestion. Despite the presence of one natural peptide bond in the building block, the hydrogel was nearly unaltered after 24h of treatment with a large excess of enzyme (see ESI Section S11). Interestingly, the amyloid assembly contributed to such resistance, since the majority of the tetrapeptide in solution was digested within a few hours. We infer that the amyloid structure displays dry regions of phenylalanine zippers[5b, 15] that reduce contact with water and thus provide protection against enzymatic hydrolysis.

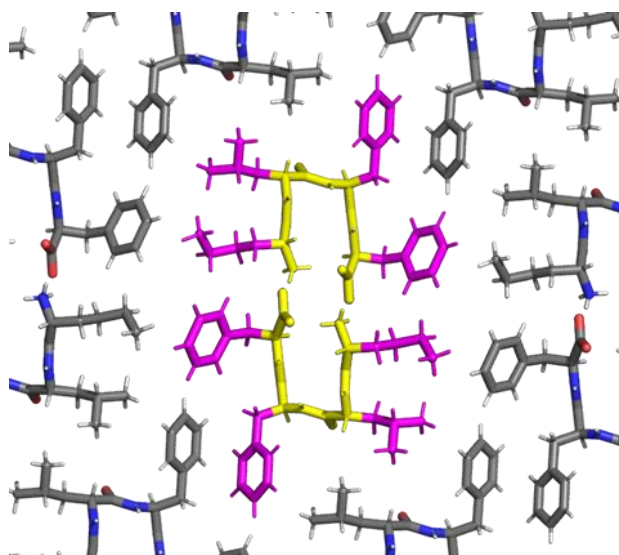


Figure 3. Single-crystal XRD data (CCDC2347110) for the heterochiral tetrapeptide confirmed the turn conformation stabilized by the salt bridge between termini. The bent backbone is highlighted in yellow, while the hydrophobic sidechains of the central molecules in magenta.

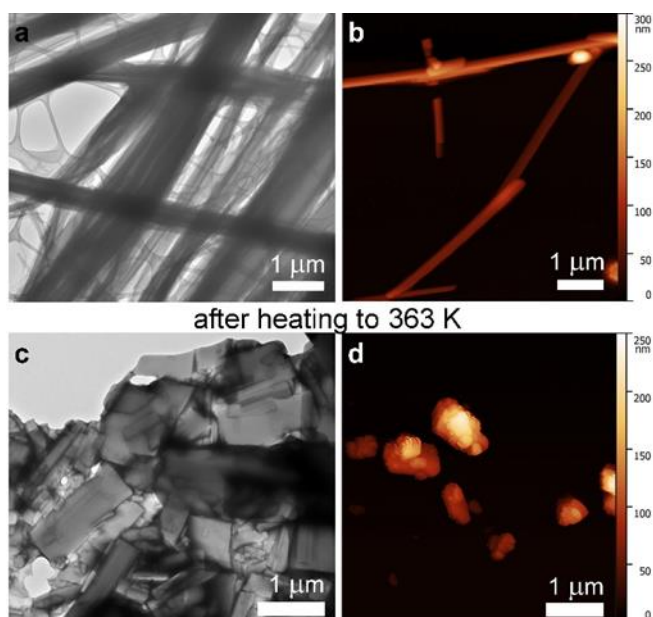


Figure 4. TEM (a,c) and AFM (b,d) of the heterochiral tetrapeptide forming fibrils at rt (a,b) which irreversibly converted into plates upon heating to 363 K (c,d).

Transmission Electron Microscopy (TEM) and Atomic Force Microscopy (AFM) investigations revealed bundles of fibrils spanning the microscale in length (Fig. 4a,b). Heating up to 363 K was required to disassemble the stable supramolecular arrangement. Instead of dissolution, as typically observed for short peptide gels, an irreversible transition to plates occurred (Fig. 4c, d). Differential scanning calorimetry (DSC) confirmed the stability of the sample up to an endothermic transition at $T_m = 362$ K (see ESI Fig. S36).

All-atom MD simulations of heterochiral peptides' self-assembly revealed a general rigidification of the system after heating that persisted upon subsequent cooling to rt, with peptides sampling a significantly smaller range of conformations (see ESI Section S7). Initially (Fig. 5a), the supramolecular assemblies at rt gave rise to a 3D network containing water channels along three directions and compatible with the observed fibrillar hydrogel. During heating, solubility decreased, the hydrophobic peptides aggregated, and 5-to-9 water molecules per peptide molecule were released from solvation shells, leading to an increase in entropy and the formation of aggregates that are fully separated from each other along one direction (Fig. 5b). These aggregates could serve as seeds for the onset of plates, that is structures likely corresponding to a thermodynamic sink and thus leading to an irreversible transition. Re-assembly was driven by the entropic gain of the system due to the release of water molecules during the heating phase. Due to the temperature dependence of this term ($T\Delta S$) in the overall free energy balance of the process, this gain increases with increasing temperatures, leading the system to a deeper free energy minimum and rendering the process virtually irreversible.[16] This picture is corroborated by several analyses including the calculation of the Solvent Accessible Surface Area (SASA), which decreases in the morphological transition, and the number of water molecules set free in the bulk phase, correlated with that reduction. The reduction in SASA (Table S4), leading also to a reduction in the number of water-peptide H-bonds is, however, partly compensated by the increase in the number of such bonds involving only the peptides (Table S5). Moreover, the overall number of bridging waters did not change before and after the heating/cooling steps. This is likely because

virtually all the possible H-bonds of the termini and backbone were saturated in the MD simulation before the heating of the system, a picture consistent with the very minor changes in the nature of the surface exposed to the solvent (Figure 5).

Interestingly, the β -structure content was reduced, but no dramatic change to a different conformation was seen, as confirmed by MD simulations, FT-IR, circular dichroism (CD), and Thioflavin T fluorescence analyses (Fig. 6 and S40). An increase of the temperature up to 363 K leads the peptide to sample different conformations, some corresponding to L- α -helices. However, this difference almost vanishes when simulating self-assembly of hundreds of tetrapeptides in explicit solvent (Fig. S35), thus supporting the role of water thermodynamics in the process and the high stability of β -structures. Layered β -structures have been predicted to be the most stable secondary conformation for amyloid superstructures, due to the preference of backbones to engage in extended H-bonding to form sheets.[17]

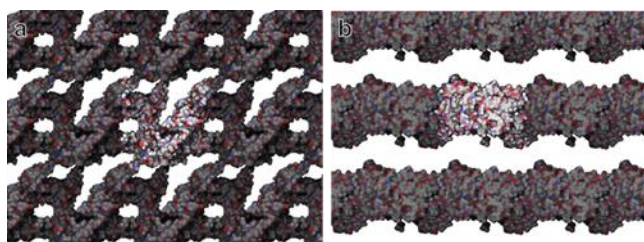


Figure 5. Representative conformations extracted from MD simulations of heterochiral tetrapeptide assemblies at rt before (a) and after heating to 363 K and cooling to 298 K (b). Surfaces are colored by atom type (grey, white, blue, and red for C, H, N, and O respectively).

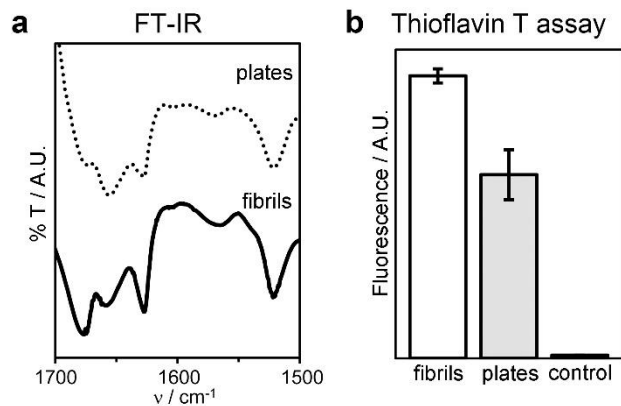


Figure 6. Conformation of fibrils and plates: (a) FT-IR spectra of the amide I-II regions, (b) Thioflavin T fluorescence assay.

In conclusion, we describe the first uncapped D,L-tetrapeptide self-assembly into a hydrogel that, upon heating, undergoes an irreversible morphological transition from fibrils to plates, in marked contrast with all the short peptide assemblies described thus far. This investigation reveals that the peptide undergoes only minor conformational changes, while water plays a key role in the transition, whereby water-peptide interactions are replaced with peptide-peptide interactions, and the entropic gain of the water molecules that are set free locates the system in an energetic sink. These results shed new light on the modulation of peptide assemblies and their morphology.

Experimental Section

2-chlorotrytil resin, O-Benzotriazole-N,N,N,N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU), and Fmoc-protected amino acids were purchased from GL Biochem (Shanghai) Ltd. All solvents were purchased of analytical grade from Merck. All the other chemicals were from Sigma. High purity Milli-Q-water (MQ water) with a resistivity greater than 18 M Ω cm was obtained from an in-line Millipore RiOs/Origin system. ^1H -NMR spectra were recorded at 400 MHz and ^{13}C -NMR spectra were recorded at 101 MHz on a Varian Innova Instrument with chemical shift reported as ppm (in DMSO with tetramethylsilane as internal standard). ESI-MS spectra were recorded on an Agilent 6120 single quadrupole LC-MS system. Peptides were synthesized in solid phase using standard protocols and Fmoc protection (more details are provided in the ESI). Each peptide was dissolved by 10 min of ultrasonication and heating in sodium phosphate buffer (0.1 M pH 12.0) to a final concentration of 30 mM and then an equal volume of sodium phosphate buffer (0.1 M pH 5.8) was added to get a final pH of 7.2 ± 0.1 and a final concentration of 15 mM.

All-atom MD simulations and analyses were performed with the AMBER22 and AmberTools23 software packages.[18] The parameters for D/L N-terminal norleucine were taken from the literature,[19] and the ff19SB AMBER force field[20] was employed together with an OPC model for water.[21] Simulations of single peptides in solution. First, we performed MD simulations of a single homo- (Nle-Leu-Phe-Phe) and hetero- (DNle-DLeu-Phe-Phe) chiral peptide in explicit water as follows. The initial structure of the homochiral peptide was generated using the sequence command from the xleap tool of AMBER22 and relaxing the structure. The heterochiral peptide was built from the homochiral structure using VMD1.9.3.[22] Starting from these structures, three consecutive restrained structural optimizations (up to 25,000 steps) were performed in the presence of harmonic restraints ($k=1$ kcal/mol $\cdot\text{\AA}$) applied to: a) all non-hydrogenous atoms of the system; b) backbone atoms; c) $\text{C}\alpha$ atoms. Reference structures at steps b) and c) were the final ones from the previous step. Next, up to 50,000 cycles of unrestrained optimization were performed. Each system was then heated to 298 K in 1 ns via constant-pressure-temperature (NTP) MD simulations (using the isotropic Berendsen barostat and the Langevin thermostat) followed by an equilibration phase of 10 ns. Starting from the equilibrated structure, a MD simulation in the NVT ensemble of 1 μs in length was performed using a time step of 2 fs. Next, heating to 363K in was simulated in 20 ns, followed by another equilibrium simulation of 1 μs in length at this temperature. Periodic boundary conditions were employed, and electrostatic interactions were estimated using the Particle Mesh Ewald scheme with a cutoff of 9.0 \AA for the short-range evaluation in direct space and for Lennard-Jones interactions (with a continuum model correction for energy and pressure). Self-assembly. 5 independent MD simulations of the self-assembly of 512 heterochiral tetrapeptides in water solution were performed following published protocols.[23] Briefly, the initial conformation of the assembling peptides was generated by placing their centers of mass on a 8x8x8 grid of 17.5 \AA -spaced points. Initial orientations of peptides were randomized, and the systems were solvated with water molecules, for a total number of atoms around 235,000. Initial structures of the 5 independent simulations were taken from the optimized structure of the corresponding peptides (simulation 1) and from the most populated conformations extracted (simulations 2 to 5) from a cluster analysis performed on the 1 μs -long MD simulation described in the previous paragraph. Hierarchical agglomerative clustering was performed, setting the number of clusters to 4 and using the average distance (average linkage keyword in cpptraj) and symmetric RMSD as metric (srmsd keyword). Each simulation was performed as described in the previous paragraph regarding the structural optimization and initial heating steps. Then, a MD simulation of 100 ns in length at 298 K (NVT ensemble) was performed, followed by: heating (in 20 ns) and equilibrium simulation (1 μs) at 363 K, cooling to 298 K in 100 ns, and finally another equilibrium simulation of 1 μs in length at this temperature.

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Keywords: peptides • self-assembly • gels • phenylalanine • supramolecular

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