Morphology diversity of L-phenylalanine-based short peptide supramolecular aggregates and hydrogels

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Abstract: Supramolecular aggregates and hydrogels of diverse morphologies can be obtained by replacing the widely studied aromatic N-capping of phenylalanine derivatives by long alkyl chains. Simple changes on chain length and number of phenylalanine residues lead to a diversity of nanostructures including networks of fibers of different handedness and flat nanosheets. Moreover, additional morphologies could be achieved by a simple pathway selection. These results evidence the impact that small structural and methodological changes have on the self-assembly of small peptide fragments and recall its relevance for the understanding of protein aggregation as well as for the fine control of peptide material properties for applications.

Peptide based self-assembled materials have been widely studied in the last decade and have found cutting-edge applications in Nanotechnology and Biomaterials Science.[1] This is because an enormous variety of functional and biocompatible materials can be obtained by using the natural pool of amino acids. For instance, peptide self-assembled materials are being used as scaffolds for cell growth, as drug delivery vehicles in or in wound healing among other biomedical applications.[2] However, although automated synthesis gives access to a priori any peptidic sequence, long peptides and proteins may result expensive for bulk applications. Fortunately, short peptides and even single amino acid derivatives have also shown a rich supramolecular self-assembly behavior and are being currently explored as nanomaterials as well.[3]

In particular, phenylalanine-based short peptides have attracted great attention following pioneering work by Gazit who reported that slight structural variations of FF dipeptide may lead to a diversity of nanostructures.[4] Phenylalanine shows a high propensity to aggregate in aqueous media due to an inherent hydrophobicity combined with the potential for intermolecular \(\pi-\pi\) interactions. Indeed, FF fragments appear at the core of the sequence of amyloid peptides and clustering of F residues has been related to the formation of amyloid aggregates.[5] Starting from FF dipeptide shown to form hollow nanotubes by Gazit et al., molecular modifications have been introduced at N-terminus. The most studied N-capped FF derivative has been Fmoc-FF whose self-assembly landscape has been described in depth.[6] In difference from the parent FF, Fmoc-FF forms a fibrillar network in aqueous solutions leading to hydrogelation. The presence of \(\pi-\pi\) stacking interactions between Fmoc aromatic fragments has been proposed as the driving force for fiber formation. Following these pioneering results, a variety of aromatic N-capping groups have been introduced in order to modulate the self-assembly of FF and obtain new morphologies and functionalities. Those groups include naphthalene, pyrene, indole, carbazole, biphenyl or porphyrine fragments among others.[7] However, up to our knowledge, hydrogels and aggregates in water of alkyl-N-capped FF have not been reported. Here we study the self-assembly behavior in water of N-alkyl-FF dipeptides (C12FFOH and C16FFOH) and compare them with an aromatic N-capped analogue (ZFFOH) as well as with analogues with similar alkyl chain length bearing only one F residue (C12FOH and C16FOH) (Scheme 1). We explore the diversity of morphologies obtained using two different assembly protocols and try to understand the structure/morphology relationship.

Compound ZFFOH was commercially available and N-alkyl derivatives were easily prepared in gram scale by Schotten-Baumann reaction of F/FF with the corresponding acyl chloride (see SI for details). Self-assembly studies were carried out in water. Initially, a weighed amount of each compound was suspended in water, heated until dissolved and let to cool and stabilize at room temperature for 24h. Compounds ZFFOH, C16FFOH and C12FOH formed hydrogels with minimum gel concentrations of 1.4 mg/mL, 10 mg/mL and 1.5 mg/mL respectively. Rheology experiments showed that these hydrogels were very weak although still presented the viscoelastic behavior typical of a gel (G'>G'') (see SI for details). Compounds C12FFOH and C16FOH formed suspensions that were further studied at 2 mg/mL. Hydrogels and aggregates were studied by TEM, SEM and AFM in order to analyze their morphology. Compounds ZFFOH and C16FFOH formed entangled fibrillar networks typical of molecular hydrogels (Figure 1A,E and S5,7). However, C12FOH formed flat 2D sheets, an unusual morphology for hydrogels (Figure 1B). On the other hand, compound C12FFOH formed similar 2D aggregates but ineffective to immobilize the solvent (Figure 1C).

Scheme 1. Molecular structures of L-phenylalanine (F) derivatives.
Large flat aggregates of less than 3 nm in height were observed with the help of AFM (Figure 2).

Finally, C16FOH which formed a viscous suspension showed thin fibrils (Figure 1D). A closer look at the fibers displayed by C16 analogues revealed the presence of helical morphologies (Figures 1D,E and S7,8). AFM was very helpful to analyze the expression of chirality on the thin fibrils of those compounds. As can be seen in Figure 3, C16FFOH was formed by coiled fibrils of ca. 20 nm of diameter and several micrometers of length that displayed left handedness with a pitch of ca. 250 nm. On the other hand, C16FOH showed larger fibrils of ca. 100-200 nm of diameter and opposite right handedness with a regular pitch of ca. 600 nm (see SI for additional details).

It has been reported that the method used to prepare self-assembled materials can have an influence on the final morphology of the aggregates and gels.[8] Self-assembly pathways may be diverged by different heating-cooling protocols, use of different external stimuli such as light, pH, addition of co-solvents and seeds as well as the way in which those stimuli are applied. It has been reported that self-assembly of diphenylalanine and its derivatives can lead to a wide diversity of nanostructures. It has been shown that for molecules as simple as FF, kinetics and thermodynamics of self-assembly can be tuned by changing pH, ionic strength and preparation protocol among others.[9] For instance, Gazit et al. have studied in detail the assembly pathways of Boc-FF, have shown how different metastable morphologies evolve with time from monomers to spheres, then fibrils to end up with stable tubes and have...
estimated the kinetic and thermodynamic determinants of the process.[10] Here we aimed to explore the morphology landscape by playing with aging temperature and time. We initially prepared the aggregates by heating until dissolution followed by immediate cooling to room temperature and 24 h of aging before study. Then a new set of experiments was performed in which hot solutions were immediately immersed in a bath at 50°C and aged for 6 h and then stabilized at room temperature for 18 h. Following this methodology aggregated suspensions and very weak gels were obtained (see SI). In this way, we planned to study two different kinetics of assembly. Morphology analysis was performed by electron microscopy and revealed important changes in the case of C12 derivatives in which 2D aggregates were replaced by one dimensional ones. C12FFOH shows a dense network of fibers and C12FOH appears as a mixture of rods of different lengths and widths (Figure 4 and S9,11).

Figure 4. Electron micrographs of aggregates formed by C12FFOH (A,B) and C12FOH (C,D) by stabilization at 50 ºC.

In the case of C16 derivatives, changes were also evident. C16FOH appears now as a mixture of tapes and stiff rods with a high aspect ratio (Figure 5A,B and S11). Some tapes are micrometers of width and more than 50 µm of length whereas rods are ca. 50 nm of width and very long as well. In addition, these rods are straight and without any sign of chirality. On the other hand, C16FFOH maintains its fibrillar aspect and, although some coiled fibrils of ca. 30 nm width are visible, it has lost the helicity of fibers clearly evident at r.t. (Figure 5C,D).

In order to get insight into the molecular structure/morphology relationship aggregated samples were lyophilized and studied by FTIR and WAXD. FTIR of peptides and proteins offers valuable information about H-bonding interactions involving amide groups. In the current case, on top of that, terminal carboxylic acid groups can introduce additional H-bonding interactions. All aggregates present amide I stretching bands in the region of 1600-1650 cm⁻¹, carboxylate C=O stretching bands in the range of 1700-1730 cm⁻¹ and N-H and O-H stretching vibrations in the region of 3300-3500 cm⁻¹ that are informative about H-bonding (see SI). In addition, compounds bearing alkyl chains present C=H stretching bands in the 2800-2900 cm⁻¹ region (see SI for a full list of bands). Aggregates of C12FFOH, C12FOH, C16FFOH and C16FOH prepared at room temperature presented an amide I band slightly above 1640 cm⁻¹ which is the upper limit reported for β-sheet secondary structures meaning that H-bonding is not as intense as in a pure β-sheet aggregate. In the case of ZFFOH this band appeared shifted to 1660 cm⁻¹ due to the overlap of the carbamate C=O stretching band. Carboxylic acid C=O vibrations appeared as broad bands between 1697-1730 cm⁻¹ meaning that different H-bonding motifs are coexisting (free C=O, acyclic and cyclic intermolecular H-bonds).[11] On the other hand, C-H stretching vibrations (antisymmetric and symmetric) appeared at ca. 2850 and 2920 cm⁻¹ being characteristic of all-trans chain conformations (except for ZFFOH).[12] N-H stretching vibration bands appeared at 3294-3313 cm⁻¹ being typical of H-bonded amides. Therefore, H-bonding appears as an important intermolecular interaction in addition to dispersive interactions between alkyl chains and the hydrophobic effect. Moreover, the amount of water molecules available for H-bonding can be determinant to obtain one nanostructure or another. Kim and Ihee et al. have shown that FF self-assembly into nanotubes or nanowires depending on free-water content, namely the concentration of FF relative to available water.[13] A similar effect has been also observed by Yan et al. for FF in different organic solvents and ionic liquids where trace water molecules are determinant for the observed morphologies.[14] In the current case, the appearance of broad bands in FTIR associated to C=O vibrations (amide and carboxylic acid) suggests a variety of H-bonding interactions that most likely involved water molecules.

WAXD of lyophilized xerogels was used in order to get insight into the actual packing of molecules within the aggregates (see SI). Xerogels of ZFFOH were amorphous which is typical for dynamic soft gel networks. The rest of compounds, all of them bearing alkyl tails, showed several diffraction peaks revealing the internal organization of aggregates. Therefore, changes on diffraction patterns could be correlated with the observed morphology changes. In general, patterns of aggregates formed at r.t. were more complex and showed more polymorphs than...
the hydrophobic effect will be manifested in both aromatic and alkyl fragments. These two regions will therefore respond in different ways to environmental changes and their balance will determine the final energetic state of the system. H-bonding, π-π stacking and van der Waals are enthalpy-driven interactions and therefore will be more sensitive to temperature changes than the hydrophobic effect which is mainly dominated by entropy. Although it is difficult to draw a deconvolution of the contribution of each interaction, it seems clear that ageing at 50 °C will affect particularly to H-bonding whereas the hydrophobic effect will be fully operative under those conditions. Thus, hydrophobicity dominates the self-assembly of C16FFOH, that is organized in a similar manner within the fibers under the two experimental conditions. Slight differences appear only at the microscopic length scale related to bundling of fibers (see Figures 1E and 5C,D). In the case of C12FFOH, the decrease of four methylene units leads to two different polymorphs (Figure S13, A and B) at r.t. and to a single one after thermodynamic equilibration at 50 °C (Figure S13, B). Polymorph A is formed under kinetic control whereas polymorph B is the thermodynamically most stable of the pair. Taking into account these last results it is likely that longer tails and thermodynamic control lead to homogeneous fibrillar networks. In other words, ageing at 50 °C weakens the H-bonds of the peptide fragments and hydrophobicity of the tails drives the system towards the most stable polymorph. Finally, in the case of C16FOH and C12FOH the number of potential H-bonding sites and aromatic units is reduced and therefore the directionality of intermolecular interactions. As a consequence, the degree of organization is reduced as shown by WAXD, especially at r.t., where many broad peaks appear. Ageing at 50 °C again favours the most stable polymorphs. To sum up, it seems that an alkyl chain of 16 C atoms a two phenylalanine residues are the right balance to obtain a uniform fibrillar network formed by a single polymorph.

As a conclusion, we have shown that morphology diversity of small phenylalanine derivatives can be expanded by a simple chemical modification as well as by self-assembly pathway selection – controlled selection of preparation protocols. Replacement of benzoyloxycarbonyl (Z) N-capping by an alkyl chain converts a soft fibrillar hydrogel network into well-organized aggregates. Moreover, by a small change of preparation procedure an evident polymorphism appears that is further expressed into a variety of nanostructures. These results can be extended in the future by applying diverse pathway selection stimuli such as pH changes and ultrasounds in order to map the rich self-assembly behavior of alkyl-phenylalanine derivatives in aqueous solutions.
Experimental Section

Self-assembly experiments. Desired amounts of compounds were weighed in cylindrical screw capped vials with internal diameter of 1.2 cm and 4.5 cm of height. Then, 1mL of water was added and compounds were dissolved by gentle heating with a heat gun. The formation of gels and aggregates was accomplished following two cooling protocols: a) standing at room temperature (23 °C) for 24 h and b) standing at 50 °C for 6h and then 18h at r.t. The minimum gel concentration was determined using the tube inversion methodology at room temperature. Samples for TEM and AFM were prepared by adding a drop of gel/suspension on top of a copper grid or a mica disk respectively. Samples for SEM, FTIR and WAXD were lyophilised prior to deposition on top of the respective sample holders.

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[1] a) S. Zhang, Nat. Biotechnol. 2003, 21, 1171. b) E. Gazit, Chem. Soc. Rev. 2007, 36, 1263. c) S. Cavalli, F. Albericio, A. Kros, Chem. Soc. Rev. 2010, 39, 241. d) H. Cui, M. J. Webber, S. I. Slup, Biopolymers 2010, 94, 1. e) I. W. Hamley, Soft Mater. 2011, 7, 4122. f) C. J. Bowerman, B. L. Nilsson, Biopolymers 2012, 98, 169. g) N. Singh, M. Kumar, J. F. Miravet, R. V. Uljijn, B. Escuder, Chem. Eur. J. 2017, 23, 981.

[2] a) A. N. Moore, J. D. Hartgerink, Acc. Chem. Res., 2017, 50, 714.

[3] a) D. M. Ryan, B. L. Nilsson, Polym. Chem., 2012, 3, 18. b) L. Adler-Abramovich, E. Gazit, Chem. Soc. Rev., 2014, 43, 6881.

[4] a) M. Reches, E. Gazit, Science, 2003, 300, 625. b) X. Yan, P. Zhu, J. Li, Chem. Soc. Rev., 2010, 39, 1877.

[5] G. Wei, Z. Su, N. P. Reynolds, P. Arosio, I. W. Hamley, E. Gazit, R. Mezzenga, Chem. Soc. Rev., 2016, 46, 4661.

[6] a) A. Smith, R. Williams, C. Tang, P. Coppo, R. Collins, M. Turner, A. Saiani, R. V. Uljijn, Adv. Mater., 2008, 20, 37. b) A. Mahler, M. Reches, M. Rechter, S. Cohen, E. Gazit, Adv. Mater., 2006, 18, 1365. c) N. A. Dudukovic, B. C. Hudson, A. K. Paravastu, C. Zukoski, Nanoscale, 2018, 10, 1508. d) R. Xing, C. Yuan, S. Li, J. Song, J. Li, X. Yan, Angew. Chem. Int. Ed. 2018, 57, 1537.

[7] a) A. D. Martin, A. B. Robinson, A. F. Mason, J. P. Wojciechowksi, P. Thordarson, Chem. Commun., 2014, 50, 15541. b) A. D. Martin, A. B. Robinson, P. Thordarson, J. Mater. Chem. B, 2015, 3, 2277. c) S. Bian, H. Cai, Y. Cui, M. He, W. Gao, X. Chen, Y. Sun, J. Liang, Y. Fan, X. Zhang, J. Mater. Chem. B, 2017, 5, 3667. d) K. Tao, B. Xue, S. Freire, I. Slutsky, Y. Cao, W. Wang, E. Gazit, Chem. Mater., 2017, 29, 4454. e) A. D. Martin, J. P. Wojciechowski, A. B. Robinson, C. Heu, C. J. Garvey, J. Ratcliffe, L. J. Waddington, J. Gardiner, P. Thordarson, Sci. Rep. 2017, 7, 43947. f) M. Johny, K. Vijayalakshmi, A. Das, P. Roy, A. Mishra, J. Dasgupta, Chem. Commun., 2017, 53, 9348. g) M. T. Jeena, L. Palanikumar, E. M. Go, I. Kim, M. G. Kang, S. Lee, S. Park, H. Choi, C. Kim, S.-M. Jin, S. C. Bae, H. W. Rhee, E. Lee, S. K. Kwak, J.-H. Ryu, Nat. Commun., 2017, 8, 26.

[8] a) J. Raeburn, A. Z. Cardoso, D. J. Adams, Chem. Soc. Rev. 2013, 42, 5143. b) V. J. Nebot, S. Díaz-Oltra, J. Smets, S. Fernández Prieto, Juan F. Miravet, B. Escuder, Chem. Eur. J., 2014, 20, 5762. c) S. Díaz-Oltra, C. Berdugo, J. F. Miravet B. Escuder, New J. Chem. 2015, 39, 3765. d) E. R. Draper, H. Su, C. Brasnett, R. J. Poole, S. Rogers, H. Cui, A. Seddon, D. J. Adams, Angew. Chem. Int. Ed. 2017, 56, 10467.

[9] J. Wang, K. Liu, R. Xing, X. Yan, Chem. Soc. Rev. 2016, 45, 5589.

[10] A. Levin, T. O. Mason, L. Adler-Abramovich, A. K. Buell, G. Meisl, C. Galvagnios, Y. Bram, S. A. Stratford, C. M. Dobson, T. P. J. Knowles, E. Gazit, Natl. Commun. 2014, 5, 5219.

[11] a) S. Abraham, Y. Lan, R. S. H. Lam, D. A. S. Grahame, J. J. H. Kim, R. G. Weiss, M. A. Rogers, Langmuir 2012, 28, 4955.

[12] M. Masuda, V. Vill, T. Shimizu, J. Am. Chem. Soc. 2000, 122, 12327.

[13] a) J. Kim, T. H. Han, Y. Kim, J. S. Park, J. Choi, D. G. Churchill, S. D. Kim, H. Ihee, Adv. Mater., 2010, 22, 583.

[14] a) J. Wang, K. Liu, L. Yan, A. Wang, S. Bai, X. Yan, ACS Nano 2016, 10, 2138. b) J. Wang, C. Yuan, Y. Han, Y. Wang, X. Liu, S. Zhang, X. Yan, Small, 2017, 13, 1702175.

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