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Heterochirality Restricts the Self- Assembly of Phenylalanine Dipeptides Capped with Highly Aromatic Groups

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Abstract

The influence of the stereochemistry in the self-assembly of phenylalanine (Phe) dipeptides bearing aromatic fluorenyl groups at both the *N*- and *C*-termini (Fmoc, OFm) has been investigated. For this purpose, Fmoc-*D*-Phe-*L*-Phe-OFm and Fmoc-*L*-Phe-*L*-Phe-OFm have been examined considering a wide variety of solvents, which differ in the dielectric constant and the volatility. Results reveal that replacement of *L*-Phe by *D*-Phe has a major impact on the self-assembly propensities, restricting drastically the structural diversity and polymorphism shown by the homochiral dipeptide. Thus, the analogous heterochiral dipeptide shows a great propensity to form micro-/nanofibers, independently of the environment properties. Theoretical calculations revealed that the stability of antiparallel disposition is much higher (a factor of *ca.* 15) for the Fmoc-*D*-Phe-*L*-Phe-OFm than for Fmoc-*L*-Phe-*L*-Phe-OFm, which has been attributed to the hydrophobic core formed in the former. Overall, results suggest that control on the backbone chirality is a potent and versatile strategy to drive and finely tune the self-assembly propensities of highly aromatic peptides.

INTRODUCTION

Much effort has been devoted to characterize the effect of increasing aromatic interactions on peptide self-assembly processes.¹⁻⁴ As has been proposed that aromatic interactions play a central role in early self-assembly recognition events, recent studies have been focused on small peptides with aromatic residues, such as *L*-phenylalanine (*L*-Phe).²⁻¹¹ In fact intermolecular π - π stacking involving aromatic side chains plays a key role in the assembly of the very simple *L*-phenylalanine dipeptide (*L*-Phe-*L*-Phe), which was found to form nanotubes.¹² The contribution of aromatic interactions increases when peptides are blocked at the ends with aromatic groups, such as the fluorenylmethoxycarbonyl (Fmoc) group.¹³⁻¹⁵ A well-known example is the Fmoc-*L*-Phe-*L*-Phe dipeptide which is a potent hydrogelator and, together with *L*-Phe-*L*-Phe, the most widely studied motif in this field.^{5,16,17}

Intermolecular hydrogen bonds and π - π stacking interactions between aromatic rings have been found to be crucial for uncapped di-, tri- and tetra-*L*-Phe, which spontaneously organize into nanotubes, nanofibers and many other supramolecular structures (*e.g.* nanospheres, microrods, helical ribbons and plate-like crystals).^{7-9,12} Based on the premise that large aromatic tricyclic fluorenyl moiety provides additional π - π stacking interactions for peptide self-assembly, we hypothesized that introduction of a fluorenyl group at the *C*-terminus should also have a strong impact on the self-assembly properties of such short *L*-Phe homopeptides. This possibility had not been explored, in high contrast to the well-known Fmoc-*L*-Phe-*L*-Phe (note that Fmoc is a typical protecting group in peptide synthesis). Accordingly, we undertook a systematic study aimed at evaluating the influence of fluorenyl-based groups blocking one or both peptide termini of short *L*-Phe

homopeptides.^{13,14} Our results evidenced that a single C-terminal fluorenyl group (incorporated as a fluorenylmethyl ester that we abbreviate as OFm) has a great impact on self-assembly^{13,14} when compared to the uncapped dipeptide *L*-Phe-*L*-Phe or Fmoc-*L*-Phe-*L*-Phe. The same holds true for the dipeptide bearing fluorenyl functionalities at both termini,¹⁵ Fmoc-*L*-Phe-*L*-Phe-OFm, and the effect increases with peptide length.¹⁵ Non unexpectedly, a C-terminal fluorenyl moiety proved to have a more pronounced effect than the smaller benzyl ester^{13,14} (phenyl vs fluorenyl). The effect of aromatic capping groups on the self-assembly of peptides has been recently reviewed and discussed by Martin and Thordarson.¹⁸

An interesting topic to explore within this context is the influence of stereochemistry (*i.e.* partial replacement of *L*-Phe by *D*-Phe) on the self-assembly of highly aromatic Phe homopeptides. Although some *D*-Phe containing peptides have been investigated,¹⁹⁻²¹ such investigations were mainly aimed to study their biological activity. Besides, a few recent works reported the self-assembly of *L*-/*D*-Phe-containing homopeptides.^{22,23} However, to the best of our knowledge, no study has addressed the effect produced by a change in backbone chirality in small Phe-containing peptides capped at both termini with highly aromatic groups.

Herein, we report the self-assembly of a dipeptide blocked with two fluorenyl moieties that contains *D*-Phe and *L*-Phe, namely Fmoc-*D*-Phe-*L*-Phe-OFm (Scheme 1; abbreviated as *D*-Phe-*L*-Phe in the following), and compare it to the analogous homochiral dipeptide with two *L*-Phe residues, Fmoc-*L*-Phe-*L*-Phe-OFm (Scheme 1; *L*-Phe-*L*-Phe in the following). Although the latter peptide was preliminary studied,¹⁵ investigations have extended it in this work for comparison with *D*-Phe-*L*-Phe under identical experimental conditions. Results reveal that replacement of *L*-Phe by *D*-Phe has a major impact on the self-assembly

propensities, restricting drastically the structural diversity and polymorphism shown by the homochiral dipeptide.

Scheme 1. Chemical structure of *D*-Phe-*L*-Phe and *L*-Phe-*L*-Phe. Phe, Fmoc and Fm refer to phenylalanine, 9-fluorenylmethoxycarbonyl and 9-fluorenylmethyl, respectively.

RESULTS AND DISCUSSION

D-Phe-*L*-Phe was synthesized as described in the Supporting Information. *N*-Fmoc-protected *D*-Phe was coupled to a residue of *L*-Phe bearing the carboxylic acid derivatized as a 9-fluorenylmethyl ester (OFm) to give the corresponding dipeptide (Figures S1-S3). On the other hand, all experiments were performed using glass cover slips, glass being the most frequent substrate for peptide aggregation studies. Thus, although peptide self-assembly is known to be largely influenced by the properties of the substrate,²⁴ especially by the roughness and the hydrophilicity, the effect of the environment is usually the most crucial. By this reason, in this work we focus on the influence of the environment on the self-assembly of *D*-Phe-*L*-Phe and *L*-Phe-*L*-Phe by considering peptide solutions prepared using mixtures of solvents with very different vapor pressure and polarity.

Preliminary studies to explore the self-assembly of *D*-Phe-*L*-Phe were conducted by optical microscopy using 2 mg/mL peptide solutions in different 4:6 HFIP:co-solvent mixtures (HFIP: hexafluoro-2-propanol or hexafluoroisopropanol). Representative micrographs are displayed in Figure S4 and show a propensity to form fibrillar assemblies

in all the solvents tested. On this basis, deeper studies were performed for 5.0, 4.0, 2.0, 0.5, 0.25, 0.1, 0.05 and 0.01 mg/mL *D*-Phe-*L*-Phe concentrations in a variety of HFIP:co-solvent mixtures. The co-solvents used (water, methanol, isopropanol, acetone) differed in polarity, volatility and hydrogen bonding capacity. Identical experiments were carried out with the homochiral *L*-Phe-*L*-Phe dipeptide for comparative purposes (for details on sample preparation, see ESI).

D-Phe-*L*-Phe formed spherulitic-like structures when a drop of 20 μ L of the peptide HFIP stock solution (5 mg/mL) was placed on a clean glass cover slip and dried at 5°C until complete desiccation (Figure 1a). These pseudo-spherical structures, which consisted of a dense and disordered packing of poorly defined fibrous-like organizations, were surrounded and coated by a sparsely populated network of well-defined fibers with diameters ranging from 100 nm to 1 μ m. The addition of water as co-solvent strongly favoured the organization of the peptide molecules in fibrous mats, whereas spherulitic-like structures progressively disappeared. When the peptide concentration decreased to 4 mg/mL (4:1 HFIP:water, Figure 1b), cylindrical fibers with a diameter of $\sim$ 200 nm coexist with flat fibers similar to nanoplates (marked in red in Figure 1b). The organization in fibers was maintained with increasing water content until a peptide concentration of 0.5 mg/mL was reached (Figure S5), while no regular assembly was systematically detected below this threshold. Moreover, the fibers diameter decreased with peptide concentration, being of $\sim$ 150 and $\sim$ 100 nm for assemblies derived from 4:6 and 1:9 HFIP:water solutions (2 and 0.5 mg/mL, respectively). Instead, *L*-Phe-*L*-Phe formed stacked braid-like assemblies in HFIP:water for concentrations ranging from 0.5 to 2 mg/mL, whereas doughnut-like microstructures of different diameters (i.e. from $\sim$ 4 to $\sim$ 10 μ m) were observed for higher

concentrations (Figure S6). In no case were fibrous organizations detected from *L*-Phe-*L*-Phe HFIP:water solutions.

Figure 1. Self-assembly structures obtained at 5°C from *D*-Phe-*L*-Phe solutions in the solvents and concentrations indicated. (a) SEM images of the spherulitic-like structures formed in HFIP (5 mg/mL, stock solution). (b) Optical microscopy (top), SEM (bottom left) and AFM (bottom right) images of the nanofibers formed in 4:1 HFIP:water (4 mg/mL); red dashed circles indicate the coexisting nanoplates.

Substitution of water for methanol (MeOH) as co-solvent means not only a drastic reduction in polarity and hydrogen bonding capacity but also a significant increase in vapor pressure. Thus, the dielectric constant (ϵ) of water and MeOH is 78.3 and 32.6,

respectively, while their respective vapor pressures at 5°C ($P_{v,5}$) are 0.87 and 5.50 kPa. Due to these distinctive properties that affect the drying process, when MeOH dominates in the HFIP:MeOH mixtures (*i.e.* peptide concentration ≤ 2 mg/mL), *D*-Phe-*L*-Phe self-assembles into ultrathin nanofibers that align and fix together to form fibres of micrometric diameter (Figure 2a).

Figure 2. Self-assembly structures obtained at 5°C from *D*-Phe-*L*-Phe solutions in the solvents and concentrations indicated. (a) SEM and optical microscopy (inset) images of the nanofibers (grouped into microfibers) formed in 4:6 HFIP:MeOH (2 mg/mL). (b) SEM, optical microscopy (inset) and AFM images of the microfibers formed in 4:6 HFIP:*i*PrOH (2 mg/mL).

The use of isopropanol (*i*PrOH) as co-solvent, which is less polar and less volatile than both water and MeOH at 5°C ($\epsilon = 18$ and $P_{v,5} = 0.69$ kPa), triggers the assembly of *D*-Phe-*L*-Phe into smooth microfibers with an average diameter of 1.5 μm (Figure 2b). This

assembly is preserved with increased ⁱPrOH content and lower peptide concentration, but with reduced diameter (Figure S7).

The influence of alcohols on the self-assembly properties of *L*-Phe-*L*-Phe differ substantially from that described above for the heterochiral peptide. Stacked braid-like assemblies similar to those displayed in Figure S6 were reported¹⁵ for *L*-Phe-*L*-Phe in 1:49 and 1:99 HFIP:MeOH mixtures (0.1 and 0.05 mg/mL, respectively), no other regular and reproducible structure being detected for higher peptide concentrations. Interestingly, in this work, we have observed a new supramolecular structure when sonication was applied to 4:6 HFIP:MeOH *L*-Phe-*L*-Phe solutions (2 mg/mL) for 27 min (Figure 3a). It consists of almost perfect sheets of relatively wide micrometric surface and nanometric thickness that nucleate in compact clusters and, due to steric hindrance, fold over themselves giving rise to a morphology reminiscent of the mineral stone called "desert rose". These flower-like organizations, with the sheets grouped together as petals, grow on a bed of nanofibers, which also cover partially the sheets as spines. Moreover, *L*-Phe-*L*-Phe forms well-defined birefringent microfibers (Figure 3b) in 4:1 HFIP:ⁱPrOH (4 mg/mL), while poorly defined fibrous-like aggregates, stacked braids at the edges and irregular structures were observed in mixtures with higher ⁱPrOH content and lower peptide concentrations (Figure S8).

The solvent properties become seriously altered when acetone ($\epsilon = 20.7$ and $P_{v,5} = 12.62$ kPa) was used as the co-solvent. Acetone cannot act as hydrogen bond donor and is much more volatile than water, MeOH or ⁱPrOH. Figure 4 shows representative optical microscopy and SEM images of the fibrous structures obtained at 5 °C from *D*-Phe-*L*-Phe solutions in HFIP:acetone at different concentrations. The random distribution of fibers formed in 4:6 HFIP:acetone by the 2 mg/mL solution (Figure 4a) results in the nucleation

of fibrillar branched-like structures for 1 mg/mL peptide concentration (Figure 4b). The branched structure is fully consolidated in 1:99 HFIP:acetone for a peptide concentration of 0.05 mg/mL (Figure 4c). Below this threshold, only twisted fibers spread onto the glass surface were detected (1:499 HFIP:acetone, Figure 4d).

Figure 3. SEM images of the self-assemblies formed at 5°C by *L*-Phe-*L*-Phe solutions in: (a) 4:6 HFIP:MeOH (2 mg/mL); (b) 4:1 HFIP:*i*PrOH (4 mg/mL), optical micrograph showing birefringent microfibers; (c) 4:1 HFIP:acetone (4 mg/mL).

In comparison, solutions of *L*-Phe-*L*-Phe in 4:1 HFIP:acetone (4 mg/mL) form bundled arrays of nanofibers (diameter below 100 nm) that are hierarchically organized in mats of randomly oriented microfibers (Figure 3c), whereas increased acetone content precluded the formation of stable self-assembled structures. This result is in high contrast with the

great tendency shown by *D*-Phe-*L*-Phe to adopt fibrillar assemblies from acetone-based solutions even at very low concentrations.

Figure 4. Microstructures obtained by self-assembly from *D*-Phe-*L*-Phe solutions in HFIP:acetone: (a) 4:6 (2 mg/mL); (b) 1:4 (1 mg/mL); (c) 1:99 (0.05 mg/mL); and 1:499 (0.01 mg/mL).

The energy landscape of *D*-Phe-*L*-Phe was examined using density functional theory (DFT) calculations with the M06L functional,²⁵ which has broad accuracy for non-covalent interactions^{26,27} including π - π stacking, and the 6-31G(d,p) basis set. Calculations were performed in vacuum to compare the intrinsic stability of the different pre-formed structures with the experimental results on the dry state and also with our previous theoretical study of *L*-Phe-*L*-Phe.¹⁵ The most stable conformation of *D*-Phe-*L*-Phe (Figure 5a) corresponds to the fully-extended arrangement, which is stabilized by two intramolecular hydrogen bonds closing 5-membered cycles (C_5) and two π - π interactions involving the Fmoc $\cdots$ *D*-Phe and *L*-Phe $\cdots$ OFm aromatic rings. The second minimum also shows an extended conformation, even though in this case the Fmoc $\cdots$ *D*-Phe π - π stacking is replaced by an N-H $\cdots$ π interaction between the *L*-Phe amide and the phenyl ring of *D*-Phe (Figure S9). This structure is 1.7 kcal/mol less stable than the global minimum, indicating that the formation of two π - π stacking interactions is preferred.

The stability of the parallel and antiparallel β -sheet alignments was calculated considering three *D*-Phe-*L*-Phe molecules (Figure 5b), and the two fully-extended conformations mentioned above. The antiparallel disposition was significantly more favoured than the parallel β -sheet in both cases ($\Delta E > 27$ kcal/mol), which is consistent with the FTIR band observed at 1694 cm^{-1} (Figure S3). The former is stabilized by multiple π - π stacking interactions, in addition to the intermolecular N-H $\cdots$ O H-bonds typical of β -sheets. These aromatic interactions involve all six terminal fluorenyl groups as well as most of the side chains of *D*-Phe and *L*-Phe, which mainly interact with fluorenyl moieties (Figure 5c). Our study of *L*-Phe-*L*-Phe¹⁵ showed that the antiparallel sheet is preferred over the parallel one by only 1.8 kcal/mol. Thus, the disposition of aromatic rings, which is

dictated by the backbone stereochemistry, is much more effective to determine antiparallel β -sheet preference in *D*-Phe-*L*-Phe.

Figure 5. (a) Minimum energy conformation of *D*-Phe-*L*-Phe stabilized by two intramolecular C₅ H-bonds (red dashed lines) and two π - π stacking interactions (light green arrows). (b) Calculated parallel (left) and antiparallel (right) β -sheet alignments of three peptide molecules. (c) Minimum energy structure of the antiparallel β -sheet, indicating π - π stacking interactions: Fmoc...OFm (dark green arrows); Fmoc...Phe and Phe...OFm (light green arrows). (d) Calculated head-tail (HT) and head-head (HH) packing models for two antiparallel β -sheets (each formed by three peptide molecules). (e) Minimum energy structure of the most stable HT packing model, showing the aromatic core formed by Fmoc and OFm groups of adjacent sheets (dashed red circle). Relative energies (ΔE) in (b) and (d) are given in kcal/mol.

To investigate the packing between neighbouring sheets, pre-formed antiparallel β -sheets made of three *D*-Phe-*L*-Phe strands were constructed considering the head-tail (HT) and head-head (HH) models (Figure 5d). The HT assembly is favoured by 5.0 kcal/mol. In addition to the intra-sheet stabilizing forces described for Figure 5c, this HT packing exhibits a hydrophobic core with Fmoc...OFm π - π stacking interactions involving molecules of adjacent sheets (red circle in Figure 5e). The nucleation of such π - π stacked cores is consistent with the remarkable ability shown by *D*-Phe-*L*-Phe to form fibrillar assemblies even in the absence of water or other solvents able to act as H-bond donors, and at very low concentrations. Thus, the formation of aromatic cores due to the inability to interact with solvent molecules is the driving force of the aggregation process, leading to a decrease in the entropy of the system as it occurs in the hydrophobic collapse effect in protein folding processes.

Actually, the large energy gap between the antiparallel and parallel β -sheets calculated in this work ($\Delta E > 27$ kcal/mol, Figure 5b) deserves special attention. In our previous work¹⁵ on *L*-Phe-homopeptides blocked with Fmoc and OFm groups, such energy difference increased with peptide length, being of 1.8, 3.3 and 9.8 kcal/mol for the di-, tri- and tetrapeptide, respectively. These stability differences correlated with the distinct self-assembly propensities observed experimentally for these Fmoc-(*L*-Phe)_n-OFm peptides, allowing us to conclude that additional π - π stacking interactions associated to increased peptide length favoured antiparallel β -sheet formation with concomitant stabilization of the nanostructures formed and reduction of polymorphism.¹⁵ Such a conclusion was also in perfect agreement with previous works by us and other authors on *L*-Phe homopeptides

uncapped or containing an *N*-Fmoc group only.²⁸ On the other hand, the preference towards the antiparallel β -sheets found for *D*-Phe-*L*-Phe and *L*-Phe-*L*-Phe is fully consistent with that reported for other highly aromatic Phe-based peptides. For example, Accardo and co-workers observed similar features for the different hexaphenylalanine, (*L*-Phe)₆, variants using molecular dynamics simulations.²⁹

Therefore, the strong preference for antiparallel β -sheet formation calculated for the heterochiral dipeptide studied in the present work (Fmoc-*D*-Phe-*L*-Phe-OFm) may be at the basis of the singular behavior observed for such a small peptide in comparison to its homochiral analogue, both from quantitative and qualitative viewpoints: higher tendency to self-assembly and much more restricted structural diversity.

CONCLUSIONS

In conclusion, the self-assembly of Phe-dipeptides blocked with two fluorenyl functionalities is governed by the backbone stereochemistry. The heterochiral *D*-Phe-*L*-Phe dipeptide exhibits a high propensity to form fibrillar structures independently of the solvent composition, whereas the morphology of the *L*-Phe-*L*-Phe assemblies changes with the environment. Moreover, the heterochiral compound retains the ability to self-assembly under conditions in which its homochiral counterpart does not adopt any kind of stable supramolecular organization. The outstanding capacity of Fmoc-*D*-Phe-*L*-Phe-OFm to adopt fibrillar assemblies even in non-aqueous media is essential for the potential development of nanotechnological applications. The results in this work, together with our previous reports on *L*-Phe-dipeptides capped with one *C*-terminal^{13,14} or two¹⁵ fluorenyl groups and the numerous studies on Fmoc-*L*-Phe-*L*-Phe,^{5,16,17} evidence that the adequate combination of

terminal fluorenyl moieties with controlled peptide backbone chirality (which dictates the orientation of all aromatic groups in the molecule) is a potent and versatile strategy to drive and finely tune the self-assembly propensities of Phe-based peptides.

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