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Amyloid fibrils from organic solutions of amphiphilic dipeptide

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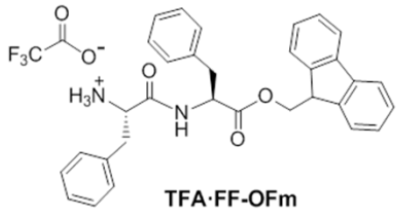
Non-hydrated organic solutions of a diphenylalanine amphiphile blocked at the C-terminus with a fluorenylmethyl ester and stabilized at the N-terminus with a trifluoroacetate have been used to prepare amyloid fibrils. The solvent used to prepare the stock solution together with the co-solvent added enable regulating the characteristics of the fibrils, which is important for their use in technological applications.

The diphenylalanine dipeptide L-Phe-L-Phe (FF), a key recognition motif of Alzheimer's β-amyloid polypeptides,[1] is among the simplest peptide-based building blocks because of its capacity of self-assembly into discrete and stiff nanotubes.[2] The FF core has been tuned enlarging the number of Phe-residues, introducing N- and/or C-terminal capping groups, and/or modifying the chemical structure of the own Phe-residue to regulate the hierarchy of the self-assembly process and achieve diverse supramolecular organizations (*e.g.* doughnut, toroid, stacked-braids, laminated helical-ribbons, microwires and dendritic structures).[3] Among the different structures obtained from small FF derivatives, those consisting of antiparallel β-sheets organized in amyloid-like fibrils are particularly relevant. This is because of their obvious relation with amyloid diseases[4] and, therefore, with the design of appropriate inhibitors for fibrils formation.[5] Furthermore, amyloid-like fibrils are utilized as biomaterials with advantageous applications not only in biomedicine but also in nanotechnology.[6-11]

The FF dipeptide capped in the N-terminus with a fluorenylmethoxycarbonyl (Fmoc) group, named Fmoc-FF, was found to form very stable hydrogels that were thought to arise from the stacking between Fmoc groups and between phenyl groups.[12] More recently, we reported that the TFA·FF-Fmoc peptide (Scheme 1), which corresponds to FF capped at the C-terminal with the Fmoc and stabilized with an ion-pair involving the protonated amino group and trifluoroacetate (TFA), self-associates into amyloid-like fibrils under very specific conditions.[13] This consisted in 1:9 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP)/water mixtures with low peptide concentrations (~0.5 mg/mL) and temperature (~4 ºC). However, straight microfibers were rapidly obtained when the peptide concentration increased and the water-content in the solution mixture decreased.



Scheme 1. Chemical structure of TFA·FF-Fmoc

Although addition of organic co-solvents to aqueous solutions for modulating solvent polarity has been used in the tuning of the amyloid-like protofibrils,[14] water is of key importance in the self-assembly of peptides and proteins, including amyloid fibril[15,16] formation. For example, FF dissolved in non-polar CH_2Cl_2 forms flower-like morphologies composed of short rods upon solvent evaporation, while addition of hydrogen bond forming co-solvents, as for example dimethylformamide (DMF) or ethanol induces FF to form fibre and ribbon structures but not amyloid fibrils.[17] Also, water is preferred to be not only the predominant solvent but also the unique solvent when amyloid fibrils are used as biomaterials for biomedical applications. However, controlled yield of amyloid fibrils exclusively using organic solvents becomes an interesting possibility when (nano)technological applications, such as filtration,[7] catalysis,[8] photonics,[9] detection,[10] and electronics[11] are considered. Among the most relevant technological

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advantages of amyloid fibrils are the degree of aggregation and orientation, the nucleation capacity and the number of binding sites, which are very high in comparison to conventional fibrils.[7-11] Within this context, Hamley and co-workers[18] reported that, FFKLVFF peptide, which is based on a fragment of the amyloid β (Aβ) peptide, forms amyloid-type fibrils from methanol (MeOH) solutions.

We envisaged the improved formation of TFA·FF-Fmoc amyloid fibrils similar to those achieved from diluted HFIP/water solutions using organic co-solvents. Initially, we considered the replacement of water by MeOH, which exhibits a dielectric constant that is less than a half that of water (32.7 vs 80.1) but retains some hydrogen bonding ability. More specifically, MeOH was added to a 5 mg/mL TFA·FF-Fmoc stock in HFIP until the peptide concentration decreased to 0.25, 0.5 and 1.0 mg/mL (1:19, 1:9 and 1:4 HFIP/MeOH, respectively), the latter being found as the most appropriated conditions for the formation of dense bundles of amyloid fibrils. Fig. 1 displays representative SEM micrographs and AFM images of the obtained structures. As it can be seen, dense branched supramolecular assemblies, which in turn are constituted by oriented and braided-like amyloid fibres, are observed. The birefringence associated to Congo red staining confirmed the amyloid structure (Fig. S1a).

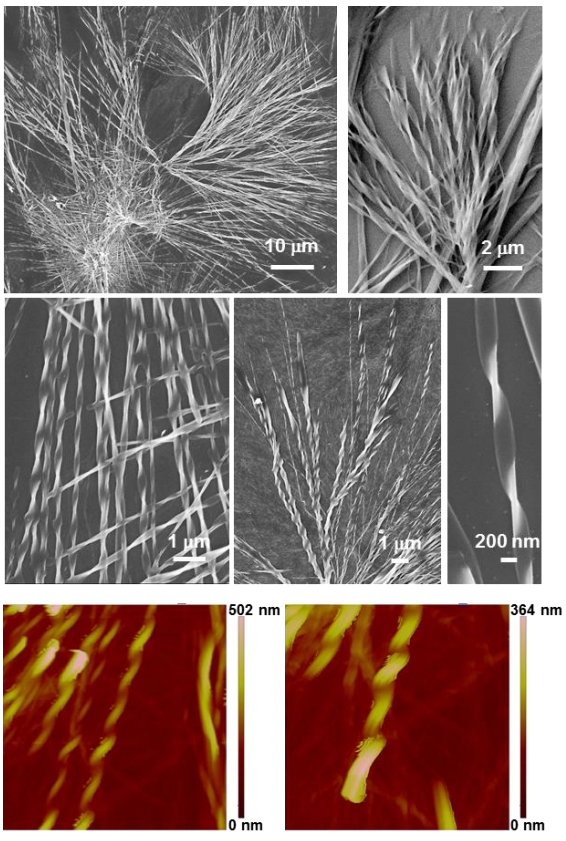

Fig 1. Amyloid-like fibrils obtained from 1 mg/mL TFA·FF-Fmoc solutions in 1:4 HFIP/MeOH at room temperature. Representative SEM micrographs and AFM images (5×5 μm^2) are shown.

At this point, we examined mixtures with DMF and/or dimethyl sulfoxide (DMSO). As the dielectric constant of HFIP, which is 16.7, is lower than that of DMF and DMSO (36.7 and 46.7, respectively), for a given amount of water co-solvent the HFIP/water mixture is expected to be less polar than the corresponding DMF/water and DMSO/water mixtures. Peptide molecules from diluted (≤ 0.5 mg/mL) DMF/water solutions organize in stacked braid-like microstructures at the glass coverslips edges (Fig. S2a), whereas a significant population of twisted microfibers located between straight microfibers was detected when the peptide concentration was higher than 1 mg/mL and, therefore, the content of DMF was closer to that of water (Fig. S2b). The substitution of water by a 50 mM KCl(aq) solution in the mixtures with DMF results in drastic morphological changes, which mainly consists in the apparition of branched-like structures (Fig. S3). Branches are constituted by irregular amorphous nodules for low peptide concentrations (i.e. high content of water) and by bundles of amyloid-like fibres for high peptide concentrations (i.e. low content of water). This interesting result suggests that DMF can promote the formation of amyloid fibrils.

This hypothesis was corroborated by self-assembled supramolecular structures obtained from 1 mg/mL TFA·FF-Fmoc solutions in 1:4 DMF/MeOH. Fig. 2 shows the formation of branched structures formed by straight microfibers of peptides, which in turn are connected by networks of thin amyloid fibrils. Congo red stain apple-green birefringence under polarized light confirms this diagnosis (Fig. S1b). The latter emerge from one microfiber, acting as individual connectors with other microfibers or merging with other fibrils forming branched-like fibrils. Comparison of these results with those achieved using the HFIP/MeOH solution indicates that the organic solvent used to prepare the stock peptide solution can be used to regulate the thickness and density of amyloid-like fibrils (Fig. S4).

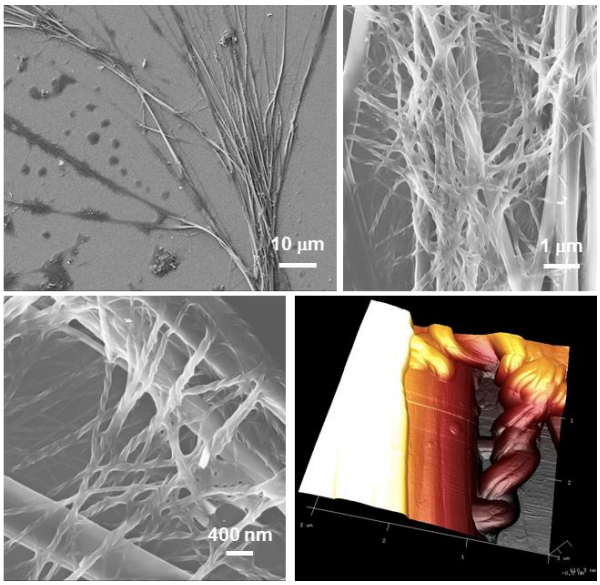

Fig 2. Amyloid-like fibrils obtained from 1 mg/mL TFA·FF-Fmoc solutions in 1:4 DMF/MeOH at room temperature. Representative SEM micrographs and 3D AFM topographic image (3×3 μm^2) are shown.

Diluted TFA·FF-Fmoc DMSO/water solutions systematically form large branched supramolecular structures (i.e. several hundreds of μm), resembling spherulitic-like organizations that

result from the compact assembly of much smaller fibers (Fig. S5). However, no well-defined structure is obtained from solutions with a peptide concentration higher than 0.5 mg/mL. Besides, irregular branched structures, which look like spherulites formed on drying, were obtained when a very small amount of 50 mM KCl(aq) was added to the DMSO stock solution (Fig. S6), while no organized self-assembly occurred when the content of KCl (aq) was increased. Also, amyloid-like assemblies were not obtained from DMSO/MeOH mixtures (Fig. S7), which could be attributed to the high polarity of DMSO used to prepare the stock TFA·FF-OFm solutions or to the ability of both DMSO and MeOH to interact with the peptide molecules. In any case, the polarity of the solvent used to prepare peptide stock solutions seems to be crucial for the formation of amyloid-like fibrils, appropriated pre-assemblies being formed when the polarity is relatively low.

Amyloid assemblies show a predominant β-sheet structure, as is typically observed by circular dichroism (CD). Interestingly, CD measurements of 0.1 mg/mL TFA·FF-Fmoc 1:49 HFIP/MeOH solution reveals the formation of a well-arranged antiparallel β-sheet of self-assembled peptides through the peaks of 196 nm and 215 nm (Fig 3a). The positive maximum at 224 nm indicates the presence of π-π stacking of aromatic units in dilute HFIP:MeOH solutions. This interaction is frequently detected in the CD profiles for biomolecular self-assembly with β-turn conformation.[19] A less uniform β-sheet is interpreted from the CD spectrum recorded from a 1:49 HFIP/water 0.1 mg/mL peptide solution with a positive maximum at 197 nm and a negative shoulder and peak appear at 208 and 222 nm (Fig 3b), respectively. The latter two bands have been attributed to folded helical-like backbone conformations, even though the peptide under study is too short for forming the intramolecular hydrogen bonds typically found in helical structures. This observation suggests that the stability of the β-sheet increases with decreasing environmental polarity and hydrogen bonding capacity. In contrast, the self-assembly in dilute DMF/MeOH solution is apparently driven by stacking interactions since the positive maximum at 208 nm is usually attributed to the (pseudo)extended conformation (Fig 3c).[20]

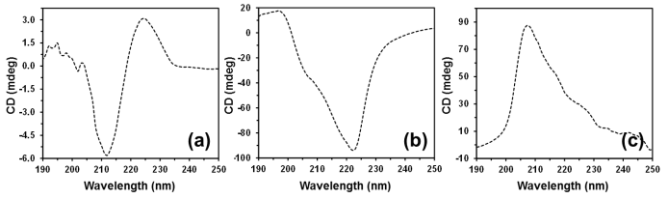

Fig. 3. CD spectra of 0.1 mg/mL TFA·FF-Fmoc in (a) 1:49 HFIP/MeOH, (b) 1:49 HFIP/water and (c) 1:49 DMF/MeOH solutions.

Further study on the self-assembly of TFA·FF-Fmoc was performed by DFT calculations in a solvent-free environment using the Minnesota 2006 local functional[21] (M06L) combined with 6-31G(d,p) basis set. It is worth noting that M06L is a local meta-generalized gradient approximation that was parameterized against several molecular databases and has broad accuracy for non-covalent interactions.[22] As the focus of DFT calculations is to determine the intrinsic stability of different pre-formed structures on the drying state to find a correlation with the microscopy studies, all them were performed in vacuum. Analysis of the energy landscape of a single TFA·FF-Fmoc ion-pair led to two significant conformations, A and B, which are depicted in Fig. 4a. The former corresponds to the pseudo-extended conformation typically found in β-sheet structures, while the latter is a folded conformation stabilized by an intramolecular N–H···π interaction. The latter favours B over A by 4.1 kcal/mol.

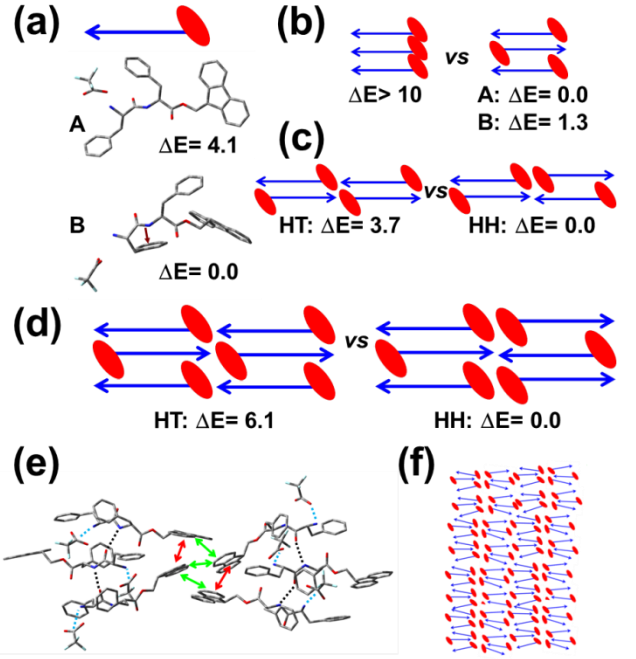

Fig. 4. (a) Sketch of the TFA·FF-Fmoc complex and both pseudo-extended (A) and folded (B) minimum energy conformations. (b) Calculated parallel (left) and antiparallel (right) β-sheets. (c,d) Calculated HT and HH packing models with (c) two and (d) three strands per antiparallel sheet. (e) Minimum energy structure for the HH packing model. (f) Sketch illustrating the formation of aromatic cores in fibrils. Electrostatic TFA···NH_3^+, N–H···O=C hydrogen bonds, intra-sheet π-π stacking and inter-sheet π-π stacking are represented by light blue dashed lines, black dashed lines, red arrows and green arrows, respectively. Relative energies (ΔE, in kcal/mol) are displayed in (a-d).

In order to investigate the most stable β-sheet alignment of TFA·FF-Fmoc strands, complexes involving three molecules disposed in parallel and antiparallel were calculated considering both A and B conformations. The antiparallel assembly was the most favoured disposition, independently of the conformation, the instability of the parallel disposition being higher than 10 kcal/mol (Fig. 4b). This result is consistent with FTIR observations (Fig. S8). Moreover, the alignment of strands arranged in the pseudo-extended conformation was the most stable. This must be attributed to the formation of networks of attractive intermolecular π-π stacking interactions, involving the phenyl side group of the first Phe residue and the Fmoc blocking group of the adjacent strands (Fig. S9a). These interactions predominate over the N–H···O hydrogen bonds. In contrast, weak CF_3···π are the predominant intermolecular interactions when the folded conformation B is considered (Fig. S9b). Obviously, cooperative intermolecular π-π stacking interactions are energetically favoured with respect to CF_3···π interactions. In all cases, the charged N-terminal group is stabilized by the TFA counter-anion, which remains very close.

Pre-assembled antiparallel β-sheets made of two and three TFA·FF-Fmoc strands, which were arranged in conformation A, were constructed to investigate the packing between neighbouring sheets. Two packed models were considered: (i) the head-tail (HT); and (ii) the head-head (HH). Calculations on models with two strands per β-sheet indicated that the HH is favoured over the HT by 3.7 kcal/mol (Fig. 4c). This energy difference increases to 6.1 kcal/mol by adding one more strand to each β-sheet (Fig. 4d). In addition of well-defined inter-strand hydrogen bonds and stabilizing electrostatic interactions between TFA counteranions and the N-terminus of each strand, the HH packing model exhibits a hydrophobic core with both intra- and inter-sheet π-π stacking interactions between neighbouring Fmoc groups (Fig. 4e). The nucleation of such π-π stacked cores and the lack of water mediated interactions produce small shifts among neighbouring strands, giving place to the amyloid fibril formation (Fig. 4f).

To conclude, TFA·FF-Fmoc amyloid fibrils have been obtained using non-aqueous organic environments. In contrast to the published literature, where the importance of water in the formation of amyloid fibrils has been emphasized, the amphiphilic dipeptide studied in this work has been demonstrated to self-assemble in amyloid structures when HFIP or DMF is used as solvent and MeOH as co-solvent. Moreover, the polarity of the organic solvent allows regulating the dimensions and density of the fibrils. Overall, results suggest that the drying process, which obviously depends on the properties of the solvent and co-solvent (e.g. the vapour pressure, the dielectric constant and the existence of hydrogen bonding groups), plays a decisive role in the formation of the amyloid-like fibrils, even though this hypothesis is not directly supports by experimental data. The preparation of amyloid structures using small synthetic peptides anticipates promising technological applications in fields like filtration, catalysis, photonics, detection, and electronics.

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Conflicts of interest

There are no conflicts to declare.

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