A short water-soluble self-assembling peptide forms amyloid-like fibrils†

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A water-soluble tripeptide Val–Ile–Ala (VIA) 1, bearing sequence identity with the C-terminal portion of the Alzheimer Aβ-peptide ($A\beta_{40-42}$), self-assembles, in crystalline form, to produce an intermolecularly hydrogen bonded supramolecular β-sheet structure which self-associates to form straight, unbranched nanofibrils exhibiting amyloid-like behavior; in contrast, the synthetic tripeptide Ala–Val–Ile (AVI) 2 self-assembles to produce a β-sheet structure that forms branched nanofibrils which do not show any characteristic features of amyloid-like fibrils.

Alzheimer's disease (AD) is one of the most prevalent and well characterized fatal neurodegenerative diseases, in which self-aggregated β-sheet rich protein fragments deposit in specific regions of the human brain as amyloid fibrils.[1] The amyloid fibrils in AD generally consist of a β-amyloid protein ($A\beta_{1-42}$), which is produced from a larger amyloid precursor protein (APP)[2] by proteolytic cleavage. There are three specific regions in the $A\beta_{1-42}$ peptide: (a) a hydrophilic N-terminus region consisting of residues 1–16, (b) a central hydrophobic stretch consisting of residues 17–21 and (c) a long hydrophobic C-terminus consisting of residues 29–42.[3] It has been observed that the C-terminal sequence is critical for amyloid fibril formation.[4] The self-aggregated amyloid fibrils are generally formed from parallel[5a] or antiparallel[5b] cross β-sheet structures. Due to their low solubility and non-crystallinity, the structure determination of amyloid fibrils, obtained from the full length Aβ protein or its larger segment, becomes almost impossible using single crystal X-ray diffraction studies. Meticulous understanding of the self-association of amyloid fibrils is absolutely necessary to obtain a detailed knowledge concerning β-sheet aggregation and to develop a therapeutic agent against amyloid diseases in the future. Solid state NMR spectroscopy is also a powerful technique for elucidating the structures of non-crystalline fibrous materials like amyloid fibers. Earlier solid state NMR studies from Lansbury's group, regarding the amyloid fibrils obtained from a self-assembling peptide $A\beta_{34-42}$, indicate that the amyloid fibrils form a pleated antiparallel β-sheet structure.[6,7] Recent studies have established that amyloid fibrils formed from the residue $A\beta_{11-25}$ at different pHs also exhibit the antiparallel β-sheet structure.[8] Serrano and co-workers have made a pioneering contribution in investigating the residue-specific effect on the propensity of a given hexapeptide sequence to form amyloid fibrils.[9] Lynn *et al.* have also made important contributions to the structural characterization of amyloid fibrils using solid-state NMR techniques.[10]

Our group has been studying the self-association of terminally protected short model peptide structures into supramolecular β-sheets; these structures form amyloid-like fibrils upon further self-association.[11] However, we present here the self-association, in crystalline form, of a water-soluble tripeptide, comprised of the Aβ peptide residue 40–42 (VIA), peptide **1** (Fig. 1b), which forms an intermolecularly hydrogen bonded supramolecular β-sheet structure. This tripeptide also forms straight, unbranched nanofibrils which exhibit amyloid-like behavior. Another synthetic tripeptide **2** (AVI), having the same amino acid composition but a different sequence from that of the tripeptide **1** (Fig. 1c), has been synthesized, purified, characterized and studied to establish whether the amyloid-like fibrillation is sequence specific or not.

Preliminary investigations, regarding the secondary structures of peptides **1** and **2**, have been carried out using solid state FT-IR spectroscopy. FT-IR spectra of both peptides are characterized by well-defined CO-stretching bands at around 1633–1654 cm^{-1} and the NH-stretching band at around 3273–3303 cm^{-1}, typical for intermolecularly hydrogen bonded β-sheet structures in the solid state (ESI Fig. S5†).[12] Moreover, peptide **1** shows a shoulder at 1685 cm^{-1} indicating the formation of an antiparallel β-sheet structure. Furthermore, the NH-bending frequencies of peptides **1** and **2** appear at 1540 cm^{-1} and 1556 cm^{-1} respectively, suggesting the formation of a β-sheet structure.[13]

Preliminary analysis of the conformational features of peptide **1** has been further supported by single-crystal X-ray diffraction studies.[14] Colorless needle-shaped single crystals, suitable for X-ray analysis, have been obtained from slow evaporation, at room

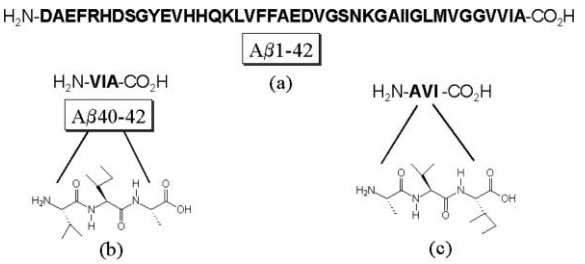

Fig. 1 (a) Amino acid sequence of $A\beta_{1-42}$ (isolated from an amyloid plaque core from an AD brain), (b) sequence and chemical structure of model amyloid peptide $A\beta_{40-42}$ (peptide **1**) and (c) sequence and chemical structure of peptide **2**, which has a different sequence.

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temperature, of a water–ethanol (98 : 2) solution of peptide **1** (200 mg per 30 mL). The molecular conformation of peptide **1** (ESI Fig. S6†) illustrates that there are four molecules (designated as A, B, C and D) in the asymmetric unit. The molecules A and B are joined together by four intermolecular hydrogen bonds and the other two molecules, C and D, are joined together by two intermolecular hydrogen bonds between amide C=O and NH functionalities to form two stable molecular dimers in the asymmetric unit. Backbone torsion angles of each molecule (A, B, C and D) of peptide **1** fall mostly within the extended region of the Ramachandran diagram[15] (ESI Table 1†). Thus, peptide **1** provides an overall extended backbone structure for each molecule present in the asymmetric unit. There are four intermolecular hydrogen bonds, namely N10B–H···O2A, N5A–H···O9B, N5B–H···O9A and N10A–H···O2B, that are responsible for connecting the individual molecules (A and B) to form the dimer of peptide **1** along the crystallographic *a* axis (Fig. 2a). The molecular duplex formed by the molecules A and B self-assembles, *via* intermolecular hydrogen bonds and other non-covalent interactions, to form an infinite antiparallel β-sheet assemblage in the crystal along the crystallographic *a* direction. Another two intermolecular hydrogen bonds, N8A–H···O6B and N8B–H···O6A, are involved in joining the individual dimers of peptide **1** to form the monolayer β-sheet structure along the crystallographic *a* axis. These individual β-sheet columns are themselves regularly stacked, *via* van der Waals interactions, to form a complex quaternary supramolecular β-sheet structure. The hydrogen bonding parameters of peptide **1** are listed in the ESI Table 2†. There are four intermolecular hydrogen bonds, namely N10A–H···O2D, N10A–H···O1D, N10B–H···O2C and N10B–H···O1C, that are responsible for joining the other molecules, C and D, of peptide **1** to form the pleated β-sheet structure. The molecular duplex formed by the molecules C and D also self-assembles, *via* intermolecular hydrogen bonding and other non-covalent interactions, to form an infinite antiparallel β-sheet assemblage in the crystal along the crystallographic *a* direction (Fig. 2a).

These individual β-sheet columns are themselves regularly stacked, *via* van der Waals interactions, to form a complex quaternary supramolecular β-sheet structure. Electrostatic interactions and hydrogen bonding of the ammonium groups and carboxylic anions play an important role in forming the supramolecular β-sheet structure in the solid state without solvent. FT-IR data also support the zwitterionic nature of the peptide **1** in the solid state (ESI Fig. S5).† There are two intermolecular hydrogen bonds, namely N8C–H···O6D and N8D–H···O6C, that are responsible for connecting the individual molecules (C and D) to form the dimer of peptide **1** along the crystallographic *a* axis (Fig. 2a). Four intermolecular hydrogen bonds, namely N5C–H···O9D, N5D–H···O9C, N10D–H···O2C and N10C–H···O2D, are involved in joining the individual dimers of peptide **1** to form the monolayer β-sheet structure along the crystallographic *a* axis. There are four intermolecular hydrogen bonds, namely N10C–H···O1B, N10C–H···O2B, N10D–H···O1A and N10D–H···O2A, that are responsible for connecting to the other molecules, A and B, of peptide **1** to form a corrugated β-sheet structure (Fig. 2b).

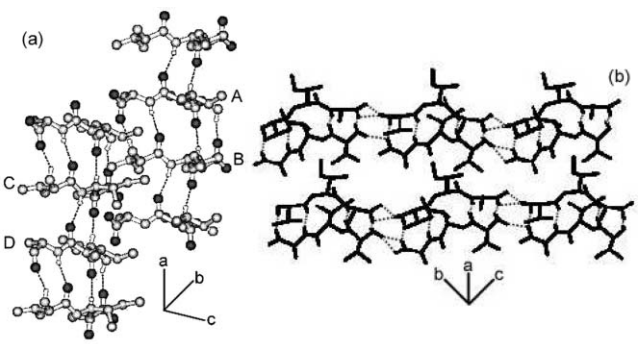

Fig. 2 (a) Packing diagram of peptide **1** along the crystallographic *a* direction, illustrating intermolecular hydrogen bonding in the solid state and the formation of continuous antiparallel β-sheet columns. (b) Higher order packing of peptide **1** forming quaternary β-sheet structures. Hydrogen bonds are shown as dotted lines. Only hydrogen bonded H atoms are shown for clarity.

Both peptides, **1** and **2**, self-assemble to form nanofibrillar structures (Fig. 3). However, their morphologies are different. Transmission electron microscopic (TEM) studies of peptide **1** reveal that it has a straight, unbranched nanofibrillar morphology with a diameter of 5–10 nm (Fig. 3a), while the fibrils formed by peptide **2** are small, branched fibrils with a diameter of 10–20 nm (Fig. 3b), indicating their non-amyloidogenic fibrillar nature.

Nanofibrils obtained from these reported peptides, **1** and **2**, have been examined for staining with a physiological dye, Congo red, and viewed through a cross polarizer, under a polarized microscope, to look for the typical birefringence of amyloid fibrils. Peptide **1** binds with Congo red and exhibits a typical green gold birefringence when viewed through the cross polarizer, indicating amyloid-like behavior (ESI Fig. S7a†). However, the fibrils obtained from peptide **2** do not show any characterized birefringence typical for amyloid fibrils, after being stained with the dye Congo red and viewed through the cross polarizer (ESI Fig. S7b†).

The aged solution of tripeptide **1** fibrils exhibited double minima at 1633 and 1650 cm^{-1} (ESI Fig. S9†). This amide I FT-IR spectrum is consistent with an antiparallel β-sheet structure.[9,16] The amide I band at 1634 cm^{-1}, observed with the aged solution of tripeptide **2** fibrils, is typical for the formation of a β-sheet structure.[9,16] From the CD spectral data (ESI Fig. S10†) it may be concluded that, at room temperature, the aged solution of peptide **2** fibrils is dominated more by some type of unstructured or random structure than by that of the aged solution of peptide **1** fibrils.

Like Congo red dye, thioflavin T (ThT) is another amyloid-specific dye. Fig. 4 shows the enhancement of the fluorescence of

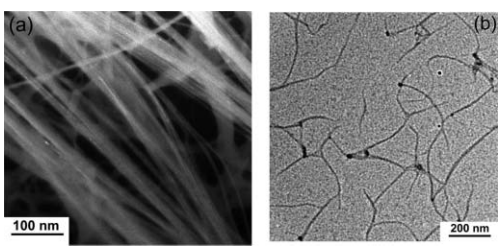

Fig. 3 TEM images of the fibril formation of (a) peptide **1** and (b) peptide **2**. The peptides were dissolved in distilled water at pH 7 (c = 1 mg mL^{-1}) and incubated at 37 °C for 7 days.

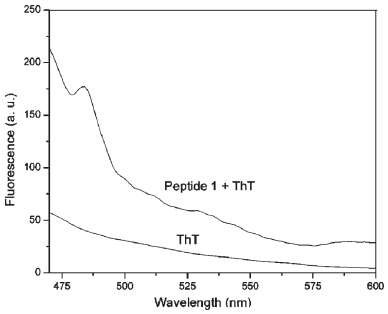

Fig. 4 Thioflavin T (ThT) binding assay for peptide **1**.

ThT after binding with the nanofibrils obtained from peptide **1**. This enhancement is more than three times higher for a solution of peptide **1** (0.4 mg mL^{-1}) fibrils containing ThT and incubated for one week than that observed for ThT alone. Peptide **2** does not show any significant binding and fluorescence enhancement with ThT, indicating its non-amyloid character.

FT-IR studies clearly suggest that both peptides self-assemble to form hydrogen bonded supramolecular β-sheet structures in the solid state and in fibrils. The crystal structure of peptide **1** further supports the formation of an antiparallel β-sheet structure using multiple hydrogen bonds. Interestingly, peptide **1** forms amyloid-like fibrils as is evident from a typical green gold birefringence after staining with the physiological dye Congo red and the enhancement of fluorescence upon binding with another specific dye, thioflavin T. The peptide **1** also forms straight, unbranched nanofibrils. Both peptides **1** and **2** have the same amino acid compositions; however, peptide **2** has an amino acid sequence that is different from peptide **1**. Peptide **2** also self-assembles to form nanofibrillar structures but its morphology is entirely different (branched and small) from that observed for peptide **1**. Fibrils obtained from peptide **2** fail to exhibit amyloid-like behavior. Previous reports have shown the formation of amyloid fibrils from short water-soluble peptides like pentapeptide or tetrapeptide segments of real amyloidogenic proteins/peptides[16] and also from *de novo* designed hexapeptides.[9] Herein, our present study clearly demonstrates the formation of amyloid-like fibrils from a water-soluble tripeptide segment, comprised of the C-terminal part of $A\beta_{1-42}$ (*i.e.* $A\beta_{40-42}$). To the best of our knowledge, this is the shortest peptide segment that not only forms amyloid-like fibrils, but also produces needle-shaped crystals perfectly suitable for single crystal X-ray diffraction studies in order to determine its structure and intermolecular arrangements at atomic resolution.

This study clearly demonstrates that a short water-soluble tripeptide, comprised of the three C-terminal amino acids of $A\beta_{1-42}$ (*i.e.* $A\beta_{40-42}$), self-assembles to form a supramolecular β-sheet structure in crystals and that this tripeptide also forms amyloid-like fibrils from an aqueous solution under the proper conditions. The molecular arrangement of the self-association of this fibril forming peptide, is clearly understood using single crystal X-ray diffraction studies. This can help to understand how self-association occurs in amyloid-like fibrillation using short peptide segments. Interestingly, peptide **2**, which has a different amino acid sequence but the same amino acid composition as peptide **1**, fails to form amyloid-like fibrils, indicating the sequence specific nature of a peptide in amyloid-like fibril formation. The effect of temperature and the pH stability of the fibres will be explored in the near future.

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14 **Crystal data for peptide 1**: $4(C_{14}H_{27}N_3O_4)\cdot CF_3CO_2H\cdot 0.5C_2H_5OH$, ($C_{59}H_{112}F_3N_{12}O_{18.5}$), FW = 1342.61, triclinic, space group P_1, a = 9.6224(5), b = 14.0220(8), c = 16.2566(9) Å, α = 101.369(5), β = 105.131(5), γ = 90.220(4)°, Z = 1, d_{calc} = 1.076 g cm^{-3}, μ = 0.084 mm^{-1}, intensity data for peptide **1** were collected with CuKα radiation at 100 K. R_{int} = 0.0348. The final R values were $R1$ = 0.1266 and w$R2$ = 0.3327 for 7352 data ($I > 2\sigma(I)$) for peptide **1**. The high R value was due to the fact that the crystal was twinned. The asymmetric unit contains 4 molecules of peptide **1** together with one molecule of trifluoroacetic acid and 0.5 molecules of ethanol. CCDC 609564. For crystallographic data in CIF or other electronic format see DOI: 10.1039/b607657b.
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