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PAPER

An amyloid-like fibril-forming supramolecular cross-β-structure of a model peptide: a crystallographic insight†

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The peptide Boc-Val-Phe-OMe **1** bearing sequence similarity with the central hydrophobic cluster (CHC) of Alzheimer's Aβ[18-19] peptide self-assembles to produce amyloid-like straight unbranched fibrils as examined by atomic force microscopy and Congo red assay. Single crystal X-ray diffraction offers the atomic level structure of the supramolecular parallel β-sheet aggregation and antiparallel separation between layers (cross-β-structure).

Introduction

The conversion of a normally soluble protein into fibrillar aggregates is the key to a range of diseases including Alzheimer's disease,[1] Huntington's disease,[2] type II diabetes[3] and prion-related encephalopathies.[4] Irrespective of sequences, a number of proteins have a tendency to be misfolded and self-assembled to form elongated, unbranched fibers that must be deposited extracellularly and must share a common set of structural and biophysical properties, which define them as amyloid.[5] In addition, there are number of examples of amyloid material like Lewy bodies (α-synuclein)[6] and neurofibrillary tangles (tau-protein) that are deposited intracellularly.[7] Moreover these extracellular amyloid plaques are toxic and pathogenic.[8] For Alzheimer's disease, the Aβ peptides sequences are obtained from amyloid β precursor protein (APP) in normal metabolism.[9] The Aβ$^{1-42}$ has three specific regions: (a) residues 1–16 at hydrophilic N-terminus, (b) residues 17–21, the central hydrophobic cluster (CHC) and (c) residues 29–42 at hydrophobic C-terminus.[10] The resultant peptide fragments self-associate through strong intermolecular interactions to from amyloid fibrils, which may be the direct or indirect cause of the pathological conditions.[11] Although there is a large body of data on the conformation and β-sheet packing of amyloid fibrils, little is known about the basic building blocks and aggregation process at atomic level.[12]

Intensive investigations are going on to unravel the detailed molecular and structural principals behind the spontaneous formation of these fibrils.[13] Lansbury *et al.* have reported that the amyloid fibrils obtained from a self-assembling peptide Aβ$^{34-42}$ form a pleated antiparallel β-sheet structure (Fig. 1).[14] Banerjee and coworkers have reported the crystal structure of Aβ$^{40-42}$ and Aβ$^{9-11}$.[15] Tycko and coworkers have used ^{13}C solid state NMR spectroscopy to determine the antiparallel β-sheet arrangement in Aβ$^{16-22}$ model peptide.[16]

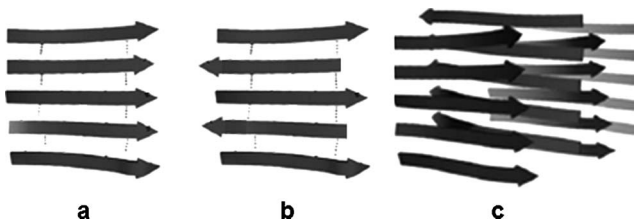

Fig. 1 (a) Parallel β-sheet, (b) antiparallel β-sheet and (c) cross-β-sheet model of amyloid β peptide.

Recent studies have established that amyloid fibrils formed from the residue Aβ$^{11-25}$ at different pHs also exhibit the antiparallel β-sheet structure.[17] In addition, there are multiple instances of amyloid models based on a parallel arrangement of β-sheets (Fig. 1).[18]

Previously, we have reported the formation of amyloid-like fibrils from L-Ala-modified analogues of amyloid β-peptide residue 17–20, that has been adopted from the β-sheet region of non-amyloidogenic proteins.[19] We also have discussed the intrinsic amyloidogenic behavior of terminally protected Alzheimer's Aβ$^{17-21}$ peptide.[20]

Herein we present the atomic level structure of amyloid formation. The terminally protected peptide **1**, bearing sequence similarity with the central hydrophobic cluster (CHC) of Alzheimer's Aβ[18-19], self-assembles to produce amyloid-like straight unbranched fibrils and examine by AFM and Congo red assay. The single crystal X-ray diffraction reveals that the peptide **1** self-associates to form a supramolecular parallel β-sheet layer structure and antiparallel separation between the layers.

Results and discussion

The peptide Boc-Val-Phe-OMe **1** used in this study has been adopted from the central hydrophobic cluster (CHC) Aβ[18-19] of

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† Electronic supplementary information (ESI) available: ^{1}H NMR, ^{13}C NMR, HRMS spectra. CCDC reference numbers 801305. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c0ob01033b

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the amyloid β-peptide, which is critical for fibril formation in Alzheimer's disease.[21] The aggregation behavior of the terminally protected peptide **1** was observed by atomic force microscope and DLS. DLS is a rapid screening method to define nanostructures by the presence of discrete peak intensity. From the DLS study, it was found that peptide **1** self-assembled in freshly prepared methanol solution into particles with an average diameter of 55 nm at a peptide concentration of 1 mM (Fig. 2).

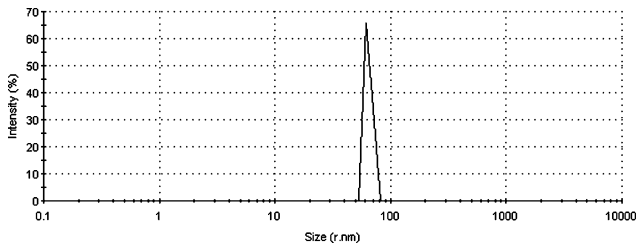

Fig. 2 DLS study of peptide **1** at 1 mM concentration showing particles with an average diameter of 55 nm.

A solution of the corresponding peptide in methanol–water (2 : 1) was incubated at 30 °C over 7 days and a small amount of that solution was drop cast on a clean microscopic cover slip and allowed to dry under vacuum at 30 °C for 2 days. Examination by atomic force microscopy revealed a fibrillar structure for the reported peptide. AFM micrographs (Fig. 3) clearly show that the peptide **1**, that has sequence similarity with the $A\beta^{18\text{-}19}$ peptide and only differs by terminal protecting groups, exhibits fibrillar morphology. The diameter of the non-branched fiber varied from 50 to 100 nm with lengths in several micrometre ranges.[22]

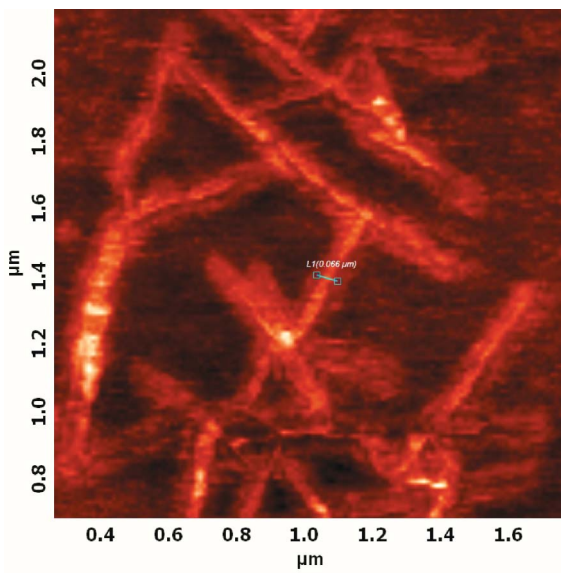

Fig. 3 AFM image showing fibrillar aggregates of peptide **1**.

The amyloid-like morphological property of the peptide fibrils were further studied by a Congo red (CR) binding assay.[23] Further, the aggregated fibrils obtained from the peptide were stained by Congo red and were observed through cross polarizers. Fig. 4 shows the typical green-gold birefringence of Congo red-bound fibrils of peptide **1** under cross polarizer. These results are consistent with Congo red binding to an amyloid β-sheet fibrillar framework with hydrogen bridges and hydrophobic exterior.[24]

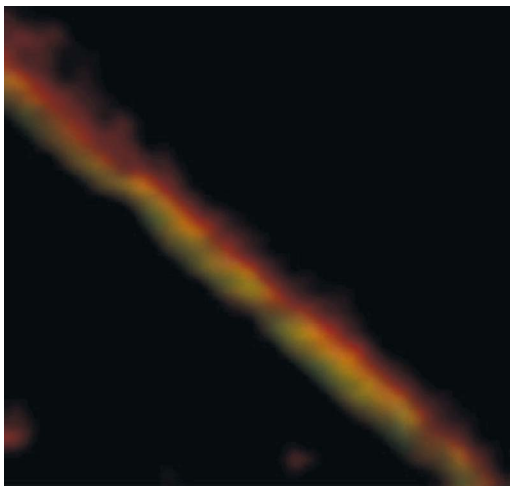

Fig. 4 Congo red assay of peptide **1** fibrillar aggregates showing green-gold birefringence.

Solid-state FTIR spectroscopy was performed to study the secondary structure of the peptide **1** in fibrils. The region 1800–1500 cm^{-1} is important for the stretching band of amide I and bending peak of amide II and hydrogen-bonded urethane groups.[25] Another informative frequency range is 3500–3200 cm^{-1}, corresponding to the N–H stretching vibrations of the peptide.[25] An intense band at 3341 cm^{-1} indicates the presence of strongly hydrogen-bonded NH groups. No band has been observed at around 3400 cm^{-1}, indicating that all NH groups are involved in intermolecular hydrogen bonding.[26] The amide I band at 1667 cm^{-1} and amide II band at 1522 cm^{-1} suggest that the peptide adopt extensively hydrogen-bonded network in fibrils (Fig. 5).[27]

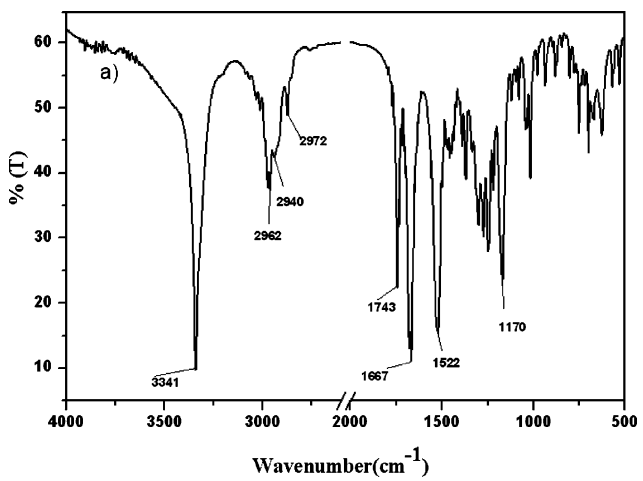

Fig. 5 FTIR spectra of peptide **1** fibrillar aggregates.

The structure and self-assembly of the peptide **1** at atomic level was further studied by single crystal X-ray diffraction.[28] The solid state conformation of peptide **1** shows that the peptide adopts an extended backbone conformation (Fig. S1, ESI†). Most of

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Table 1 Selected backbone torsion angles (°) for peptide **1**

O1–C3–N2–C4	174.9 ω1	C4–C5–N1–C6	179.7 ω2
C3–N2–C4–C5	–93.2 ϕ1	C5–N1–C6–C16	–121.3 ϕ2
N2–C4–C5–N1	–136.1 ψ1	N1–C6–C16–O6	29.2 ψ2

the backbone torsion angles (Table 1) of peptide **1** are in the β-sheet region of Ramachandran diagram [except ψ2 (29.2°)]. The individual sub-units of peptide **1** are themselves regularly interlinked through two intermolecular hydrogen-bonding interactions N1–H1^{a} ··· O4 (2.18 Å; 2.86 Å; 135°; $a = 1 + x, y, z$) and N2–H2^{b} ··· O3 (2.15 Å; 2.99 Å; 169°; $b = -1 + x, y, z$) and thereby form a supramolecular parallel β-sheet along the crystallographic *a* direction (Fig. 6).

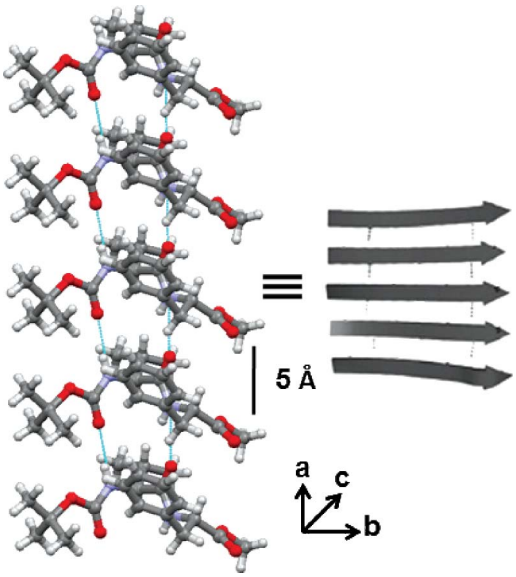

Fig. 6 Intermolecular hydrogen-bonded supramolecular parallel β-sheet arrangement of peptide **1** along the crystallographic *a* direction.

In higher order aggregation, the peptide **1** molecules organize into a multi-layer β-sheet assembly with both the meridional 5.0 Å and the equatorial 9.0 Å distances, or reflections, observed in the diffraction patterns of many amyloid fibers (Fig. 7).[29] The first distance is associated with the backbone separation within one β-sheet (or layer) and the second with the backbone separation between layers. The crystal packing is different from the Görbitz NH_2-Val-Phe-COOH hydrogen-bonded cage formation[30] or other self-assembled dipeptides.[31]

Conclusions

In conclusion, the report presents the atomic details of the self-assembly of peptide to form amyloid-like fibril. The peptide **1** bearing sequence similarity with the central hydrophobic cluster (CHC) of Alzheimer's Aβ[18-19] peptide self-assembles to produce amyloid-like straight unbranched fibrils. The X-ray crystallography reveals that the peptide **1** forms a parallel supramolecular β-sheet layer structure and antiparallel separation between layers in higher order aggregation. Thus the backbone amide groups form an extensive network of hydrogen bonds that run parallel to the fibril long axis. The result provides important insights into the

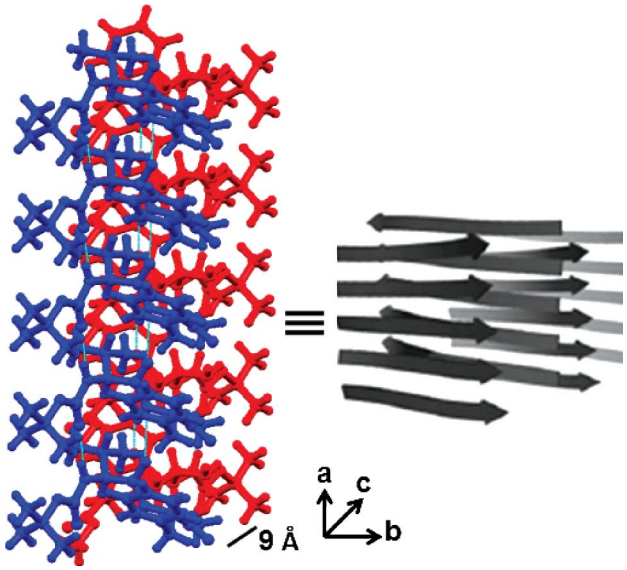

Fig. 7 Supramolecular cross-β-structure of peptide **1** at higher order assembly along *a* axis.

general properties of these assemblies and validates the proposed cross-β-model[29] to define the fibril structure.

Experimental

General

All L-amino acids were purchased from Sigma chemicals. HOBt (1-hydroxybenzotriazole) and DCC (dicyclohexylcarbodiimide) were purchased from SRL.

Peptide synthesis

The peptides were synthesized by conventional solution-phase methods using racemization free fragment condensation strategy. The Boc group was used for N-terminal protection and the C-terminus was protected as a methyl ester. Coupling was mediated by dicyclohexylcarbodiimide/1-hydroxyl benzotriazole (DCC/HOBt). The intermediates were characterized by 500 MHz ^{1}H & ^{13}CNMR and FT-IR spectroscopy. The final compound was fully characterized by 500 MHz ^{1}H NMR spectroscopy, ^{13}C NMR spectroscopy, mass spectrometry, and IR spectroscopy. The products were purified by column chromatography using silica (100–200-mesh size) gel as stationary phase and n-hexane–ethyl acetate mixture as eluent.

(a) Boc-Val-OH(2). A solution of L-valine (1.17 g, 10 mmol) in a mixture of dioxane (20 mL), water (10 mL) and 1 M NaOH (10 mL) was stirred and cooled in an ice-water bath. Di-*tert*-butylpyrocarbonate (2.4 g, 11 mmol) was added and stirring was continued at room temperature for 6 h. Then the solution was concentrated in vacuum to about 10–15 ml, cooled in an ice-water bath, covered with a layer of ethyl acetate (about 50 ml) and acidified with a dilute solution of $KHSO_4$ to pH 2–3 (Congo red). The aqueous phase was extracted with ethyl acetate and this operation was done repeatedly. The ethyl acetate extracts were pooled, washed with water and dried over anhydrous Na_2SO_4 and

evaporated in a vacuum. The pure material was obtained as a waxy solid. Yield 2.02 g (9.30 mmol, 93.0%).

^{1}H NMR (DMSO-d_6, 500 MHz, δ_{ppm}, 1 mmol in 0.5 mL): 12.426 (b, 1H, COOH), 6.893–6.875 (d, 1H, J = 9 Hz, NH Boc), 3.800–3.783 (m, 1H, C^{α} Val), 2.021–1.941 (m, 1H, C^{β} Val), 1.379 (s, 9H, BOC), 0.877–0.848 (m, 6H, C^{δ} Val). ^{13}C NMR (DMSO-d_6, 125 MHz, δ_{ppm} 10 mmol in 0.5 mL): 173.51, 155.78, 77.96, 59.09, 29.52, 28.20, 19.14, 18.13, FT-IR (KBr): 3328, 2975, 2936, 1718, 1508, 1396, 1369, 1255, 1163 cm^{-1}. Anal. calcd for $C_{10}H_{19}NO_4$ (217.26): C, 55.28; H, 8.81; N, 6.45%; Found: C, 55.31; H, 8.83; N, 6.42%.

(b) Boc-Val-Phe-OMe (1). 2.0 g (9.20 mmol) of Boc-Val-OH was dissolved in 25 ml dry DCM in an ice-water bath. H-Phe-OMe was isolated from 3.957 g (18.4 mmol) of the corresponding methyl ester hydrochloride by neutralization, subsequent extraction with ethyl acetate and ethyl acetate extract was concentrated to 10 ml. It was than added to the reaction mixture, followed immediately by 1.898 g (9.20 mmol) dicyclohexylcarbodiimide (DCC) and 1.408 g (9.20 mmol) of HOBt. The reaction mixture was allowed to come to room temperature and stirred for 48 h. DCM was evaporated and the residue was dissolved in ethyl acetate (60 ml) and dicyclohexylurea (DCU) was filtered off. The organic layer was washed with 2 M HCl (3 × 50 ml), brine (2 × 50 ml), 1 M sodium carbonate (3 × 50 ml) and brine (2 × 50 ml) and dried over anhydrous sodium sulfate; and evaporated in a vacuum to yield Boc-Val-Phe-OMe as a white solid. The product was purified by silica gel (100–200 mesh) using n hexane – ethyl acetate (3 : 1) as eluent. Yield: 2.61 g (6.89 mmol, 74.89%).

^{1}H NMR ($CDCl_3$,500 MHz, δ_{ppm} 1 mmol in 0.5 mL): 7.299–7.101 (m, 5H, aromatic protons), 6.317–6.306 (d, 1H, J = 5.5 Hz, NH Phe), 5.006–4.991 (d, 1H, J = 7.5 Hz, NH Boc), 4.888–4.849 (m, 1H, C^{α} Phe), 3.902–3.874 (m, 1H, C^{α} Val), 3.709 (s, 3H, OMe), 3.159–3.086 (m, 2H,C^{β} Phe), 2.101–2.062 (m, 1H, C^{β} Val), 1.443 (s, 9H, BOC), 0.963–0.910 (m, 6H, C^{δ} Val).^{13}C NMR (125 MHz, 10 mmol in 0.5 mL $CDCl_3$): 171.64, 171.24, 155.65, 135.64, 129.15, 128.52, 127.06, 79.71, 59.78, 53.06, 52.18, 37.89, 30.81, 28.22, 19.06. M.P: 82–83 °C. Mass spectra: [M + Na^+]: 401.4155. [M + K]$^+$: 416.4383. FT-IR (KBr): 3341, 2972, 2962, 2940, 1743, 1733, 1677, 1667, 1522, 1300, 1273, 1248, 1170 cm^{-1}. Anal. calcd for $C_{20}H_{30}N_2O_5$ (378.46): C, 63.47; H, 7.99; N, 7.40%; Found: C, 63.48; H, 8.10; N, 7.42%.

NMR experiments

All NMR studies were carried out on a Brüker AVANCE 500 MHz spectrometer at 278 K. Compound concentrations were in the range 1–10 mmol in $CDCl_3$ and $(CD_3)_2SO$.

FT-IR spectroscopy

All reported solid-state and fibril FT-IR spectra were obtained with a Perkin Elmer Spectrum RX1 spectrophotometer with the KBr disk technique.

Mass spectrometry

Mass spectra were recorded on a Q-Tof Micro YA263 high-resolution (Waters Corporation) mass spectrometer by positive-mode electrospray ionization.

Dynamic light scattering

The particles sizes of the peptide **1** aggregates were determined by DLS instrument (model ZETASIZER nano series nano zs) with 1 mM peptide solution in methanol.

X-Ray crystallography

Single crystal X-ray analysis of peptide **1** was recorded on a Bruker high resolution X-ray diffractometer instruments.

Morphological studies

The morphology of the reported compound was investigated by atomic force microscopy (AFM). A small amount of solution (1 mg mL^{-1} MeOH: H_2O 2 : 1 v/v) of the corresponding compounds was incubated at 30 °C over 7 days and placed on a clean microscope cover glass and then dried by slow evaporation. The material was then allowed to dry under vacuum at 30 °C for two days. Images were taken with an NTMDT instrument, model no. AP-0100 in semicontact-mode.

Congo red assay

An alkaline saturated Congo red solution was prepared. The dried peptide fibrils from methanol–water were stained by alkaline Congo red solution (80% methanol/20% glass distilled water containing 10 ml of 1% NaOH) for 2 min and then the excess stain (Congo red) was removed by rinsing the stained fibril with 80% methanol/20% glass distilled water solution for several times. The stained fibrils were dried under vacuum at room temperature for 24 h, then visualized at 40× magnification and birefringence was observed between crossed polarizers (Olympus optical microscope equipped with polarizer and CCD camera).

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28 Crystal data for peptide **1**: $C_{20}H_{30}N_2O_5$, Mw = 378.46, orthorhombic, space group $P2_12_12_1$, $a = 5.105(5)$, $b = 10.205(12)$, $c = 40.956(4)$ Å, $U = 2134$ Å^3, $Z = 4$, dm = 1.178 Mg m^{-3}. Intensity data were collected with Mo-Kα radiation at room temperature using Bruker APEX-2 CCD diffractometer. The crystal was positioned at 70 mm from the Image Plate. 100 frames were measured at 2° intervals with a counting time of 5 min to give 14 620 independent reflections. Data were processed using the Bruker SAINT package and the structure solution and refinement procedures were performed using SHELX97.[32] The non-hydrogen atoms were refined with anisotropic thermal parameters. The hydrogen atoms were included in geometric positions and given thermal parameters equivalent to 1.2 times those of the atom to which they were attached. The final R values were R_1 0.0453 and wR_2 0.1315 for 2070 data with $I > 2\sigma(I)$. The largest peak and hole in the final difference Fourier were –0.15 and 0.17 e Å^{-3}. The data have been deposited at the Cambridge Crystallographic Data Center with reference number CCDC 801305.
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