Chapter 2

Self-assembly of α,β-dehydrophenylalanine (ΔPhe) containing dipeptides into nanostructures.
2.1 Introduction

Molecular self-assembly, by which well-defined higher order structures result from spontaneous association of the components of the system by non-covalent forces, has emerged as an attractive tool for design and fabrication of nanostructures with novel properties \[2^{233,284}\]. Characterization of supramolecular assemblies involving small biomolecules has also generated a lot of interest for they offer a large variety of variations through chemical modifications. Numerous reports of organic and inorganic tubular assemblies of carbon \[2^{235,236}\], boron nitrite \[2^{237}\], zeolites \[2^{238,239}\], and carbohydrate based nanotubes \[2^{240}\], have catapulted the research in this vast area of material science research. Of particular interest have been the peptide based nanostructures because they offer easy but many opportunities for chemical variations, and hence control, in designing molecular assemblies which have been successfully demonstrated to be good models for ion channels and membrane pores \[2^{241,242,243,244}\]. Existence of pores filled with co-crystallized solvent molecules in the crystal structure of many dipeptides with two hydrophobic residues have been particularly well characterized and has opened possibilities for the development of such structures as biosensors, biocatalysts and specific molecular recognition platforms \[2^{245-248,111,113,122,125}\]. Recent demonstration of well-ordered and discrete peptide nanotubes by self-assembly of the diphenylalanine core recognition motif of Alzheimer’s β-amyloid polypeptide has further highlighted the potential use of peptide-based structures for design of folded and self-organized structures \[7^{6,87,111}\].

In the following work, the self-assembly process in dipeptides incorporating a noncoded, achiral amino acid i.e. α,β-dehydrophenylalanine residue (ΔPhe) has been investigated. ΔPhe is an analog of the naturally occurring phenylalanine amino acid, but with a double bond between Cα and Cγ atoms. Introduction of ΔPhe in peptide sequences has been known to induce conformational constraint, both in the peptide backbone as well as the side chain, and to provide the peptide with increased resistance to enzymatic degradation \[1^{56-161,186,187,192-226,230}\]. The studies describe the
synthesis, characterization and self-assembly of the dipeptide, Phe-ΔPhe, into distinct tubular structures which were stable at broad range of pH conditions and treatment of proteases. However, substitution of N-terminal Phe by other hydrophobic residues did not result in self-assembly of tubes under identical solvent conditions. The importance of aromatic interactions in the self-assembly of dipeptides into nanotubes was also investigated by the selective substitution of phenylalanine residue by a structurally analogous residue, cyclohexylalanine, that lacked aromaticity. The studies helped in deciphering few rules governing the rational design of dipeptide based nanostuctures.

2.2 Materials and Methods

2.2.1 Peptide synthesis: Dipeptides were synthesized as described below:

(a) H-Phe-ΔPhe-OH

Boc-Phe-OH (Novabiochem) (1.32g, 5mM) was dissolved in dry tetrahydrofuran (Sigma-Aldrich) and the resulting solution stirred in an ice-salt bath at -15°C. N-methyl morpholine (Sigma) (0.65ml, 5mM) was added to the solution followed by isobutyl chloro-formate (Sigma) (0.7ml, 5mM). After 10min, a pre-cooled aqueous solution of DL-threo-β-phenylserine (Sigma-Aldrich) (1g, 5.5 mM) and sodium hydroxide (0.22g, 5.5 mM) was added and mixture stirred overnight at room temperature. The reaction mixture was concentrated in vacuo, acidified with citric acid to pH 3.0 and extracted with ethyl acetate (Spectrochem) (3×20ml). The ethyl acetate layer was washed with water (2×15ml), with saturated sodium chloride (1×20ml), dried over anhydrous sodium sulfate and evaporated to yield Boc-Phe-DL-threo-β-phenylserine as an oily compound (2.2g, ~100%). The compound, Boc-Phe-DL-threo-β-phenylserine, was then mixed with anhydrous sodium acetate (0.53g, 6.5mM) in freshly distilled acetic anhydride (50ml) and stirred for 36hrs at room temperature. The thick
slurry obtained was poured over crushed ice and stirred till the oily suspension gave rise to a yellow colored solid. The precipitate was filtered, washed with 5% NaHCO₃, cold water and dried under vacuum. The resulting azalactone, Boc-Phe-ΔPhe-Azl (1.7g, 4.8mM), was dissolved in methanol, treated with 1.5 equivalents of 1N NaOH solution and stirred at room temperature for 3-4hrs. The mixture was then partially evaporated to remove methanol, acidified with citric acid to pH 3.0 and extracted with ethyl acetate (3x30ml), the combined ethyl acetate extract was washed with water (2x20ml), dried over anhydrous sodium sulphate and evaporated to yield Boc-Phe-ΔPhe-OH (1.8g, 4.5mM) as a white solid. Deprotection at the α-amino group was achieved by treatment with 98% formic acid (30ml) for 3hrs or 50% Trifluoro-acetic acid (TFA): Dichloromethane (DCM) for 1hr at room temperature. The reaction mixture was evaporated to dryness and the residue was precipitated with anhydrous diethyl ether (50ml). The resulting precipitate was filtered, washed several times with dry ether and subsequently lyophilized from 10% acetic acid-water (20ml) to yield the final compound H-Phe-ΔPhe-OH as white powder. Overall yield (1.2g, 82.6%); Rₜ = 0.18 (CHCl₃-MeOH, 9:1).

The peptide was purified on a preparative reverse phase C₁₈ column (Deltapak, C₁₈, 15μ, I.D. 300×19mm) using acetonitrile-water linear gradient 5-45% acetonitrile (0.1%TFA)/water (0.1% TFA) at a flow rate of 4ml/min over 25min. The purified peptide was reinjected into an analytical reverse phase C₁₈ column (Phenomenex, C₁₈, 5μ, I.D. 250×4.6mm) using a acetonitrile-water linear gradient 5-45% acetonitrile (0.1%TFA)/water (0.1% TFA) at a flow rate of 1ml/min over 25min and was found to be 98% pure with retention time of 15min. The purified peptide was analyzed by mass spectroscopy (Applied Biosystems QStar (Q-TOF)) Observed Mass-310.32 Da, Expected Mass- 310 Da.
(b) **H-Gly-dPhe-OH**

The peptide was synthesized as described above starting with Boc-Gly-OH. Overall yield (0.88g, 40%); \( R_f = 0.1(\text{CHCl}_3-\text{MeOH}, 9:1) \); The peptide was purified as described above. The purified peptide was reinjected into an analytical reverse phase \( C_{18} \) column (Phenomenex, C18, 5μ, I.D. 250×4.6mm) using a acetonitrile-water linear gradient 5-45% acetonitrile (0.1%TFA)/water (0.1% TFA) at a flow rate of 1ml/min over 25min and analyzed by mass spectroscopy. Retention Time- 9min; Observed Mass-220.3 Da, Expected Mass-220 Da.

(c) **H-Ala-dPhe-OH**

The peptide was synthesized as described above starting with Boc-Ala-OH. Overall yield (1.35g, 58%); \( R_f = 0.1(\text{CHCl}_3-\text{MeOH}, 9:1) \); The peptide was purified as described above. The purified peptide was reinjected into an analytical reverse phase \( C_{18} \) column (Phenomenex, C18, 5μ, I.D. 250×4.6mm) using a acetonitrile-water linear gradient 5-45% acetonitrile (0.1%TFA)/water (0.1% TFA) at a flow rate of 1ml/min over 25min and analyzed by mass spectroscopy. Retention Time- 11min; Observed Mass-234.6 Da, Expected Mass-234 Da.

(d) **H-Val-dPhe-OH**

The peptide was synthesized as described above starting with Boc-Val-OH. Overall yield (1.51g, 58%); \( R_f = 0.1(\text{CHCl}_3-\text{MeOH}, 9:1) \); The peptide was purified as described above. The purified peptide was reinjected into an analytical reverse phase \( C_{18} \) column (Phenomenex, C18, 5μ, I.D. 250×4.6mm) using a acetonitrile-water linear gradient 5-45% acetonitrile (0.1%TFA)/water (0.1% TFA) at a flow rate of 1ml/min over 25min and
analyzed by mass spectroscopy. Retention Time- 12.5min; Observed Mass-262.4 Da, Expected Mass-262 Da.

e) **H-Leu-ΔPhe-OH**

The peptide was synthesized as described above starting with Boc-Leu-OH. Overall yield (1.07g, 39%); Rf = 0.1(CHCl3-MeOH, 9:1); The peptide was purified as described above. The purified peptide was reinjected into an analytical reverse phase C18 column (Phenomenex, C18, 5μ, I.D. 250x4.6mm) using a acetonitrile-water linear gradient 5-45% acetonitrile (0.1%TFA)/water (0.1% TFA) at a flow rate of 1ml/min over 25min and analyzed by mass spectroscopy. Retention Time- 14min; Observed Mass-276.23 Da, Expected Mass-276 Da.

(f) **H-Ile-ΔPhe-OH**

The peptide was synthesized as described above starting with Boc-Ile-OH. Overall yield (1.1g, 42%); Rf = 0.13(CHCl3-MeOH, 9:1); The peptide was purified as described above. The purified peptide was reinjected into an analytical reverse phase C18 column (Phenomenex, C18, 5μ, I.D. 250x4.6mm) using a acetonitrile-water linear gradient 5-45% acetonitrile (0.1%TFA)/water (0.1% TFA) at a flow rate of 1ml/min over 25min and analyzed by mass spectroscopy. Retention Time- 14min; Observed Mass-276.23 Da, Expected Mass-276 Da.

(g) **H-Phe-Phe-OH**

Boc-Phe-OH (Novabiochem) (1.32g, 5mM) was dissolved in dry tetrahydrofuran (Sigma-Aldrich) and the resulting solution stirred in an ice-salt bath at -15°C. N-methyl morpholine (Sigma) (0.65ml, 5mM) was added to the solution followed by isobutyl chloro-formate (Sigma) (0.7ml, 5mM). After 10min, a pre-cooled aqueous solution of H-Phe-OH (Sigma-Aldrich)
(0.9g, 5.5mM) and sodium hydroxide (0.22g, 5.5mM) was added and mixture stirred overnight at room temperature. The reaction mixture was concentrated in vacuo, acidified with citric acid to pH 3.0 and extracted with ethyl acetate (Spectrochem) (3×20ml). The ethyl acetate layer was washed with water (2×15ml), with saturated sodium chloride (1×20ml), dried over anhydrous sodium sulfate and evaporated to yield Boc-Phe-Phe-OH dipeptide (1.9g, ~95%). Deprotection at the α-amino group was achieved by treatment with 98% formic acid (30ml) for 3hrs or 50% trifluoro-acetic acid (TFA): Dichloromethane (DCM) for 1hr at room temperature. The reaction mixture was evaporated to dryness and the residue was precipitated with anhydrous diethyl ether (50ml). The resulting precipitate was filtered, washed several times with dry ether and subsequently lyophilized from 10% acetic acid-water (20ml) to yield the final compound H-Phe-Phe-OH as white powder. Overall yield (1.38g, 89%); Rf = 0.17 (CHCl3-MeOH, 9:1).

The peptide was purified on a preparative reverse phase C18 column (Deltapak, C18, 15μ, I.D. 300×19mm) using acetonitrile-water linear gradient 5-45% acetonitrile (0.1% TFA)/water (0.1% TFA) at a flow rate of 4ml/min over 25min. The purified peptide was reinjected into an analytical reverse phase C18 column (Phenomenex, C18, 5μ, I.D. 250×4.6mm) using a acetonitrile-water linear gradient 5-45% acetonitrile (0.1% TFA)/water (0.1% TFA) at a flow rate of 1ml/min over 25min and was found to be 98% pure with retention time of 15min. The purified peptide was analyzed by mass spectroscopy (Applied Biosystems QStar (Q-TOF)) Observed Mass-312.2 Da, Expected Mass- 312 Da.

(h) H-Cha-Phe-OH

The peptide was synthesized as described above starting with Boc-Cha-OH (1.5g, 5mM) and coupling it to sodiated H-Phe-OH (0.9g, 5.5mM). Overall
yield (1.1g, 35%); \( R_f = 0.17(\text{CHCl}_3-\text{MeOH, 9:1}) \); The peptide was purified as described above. The purified peptide was reinjected into an analytical reverse phase C\(_{18}\) column (Phenomenex, C18, 5\(\mu\), I.D. 250\(\times\)4.6mm) using a acetonitrile-water linear gradient 5-45% acetonitrile (0.1%TFA)/water (0.1% TFA) at a flow rate of 1ml/min over 25min and analyzed by mass spectroscopy. Retention Time- 19min; Observed Mass- 318.23 Da, Expected Mass-318 Da.

(i) H-Cha-Cha-OH

The peptide was synthesized as described above by Boc-Cha-OH (1.5g, 5mM) and coupling it to sodiated H-Cha-OH (0.94g, 5.5mM). Overall yield (1.2g, 39%); \( R_f = 0.19(\text{CHCl}_3-\text{MeOH, 9:1}) \); The peptide was purified as described above. The purified peptide was reinjected into an analytical reverse phase C\(_{18}\) column (Phenomenex, C18, 5\(\mu\), I.D. 250\(\times\)4.6mm) using a acetonitrile-water linear gradient 5-45% acetonitrile (0.1%TFA)/water (0.1% TFA) at a flow rate of 1ml/min over 25min and analyzed by mass spectroscopy. Retention Time- 19min; Observed Mass- 324.8 Da, Expected Mass-324 Da.

(j) H-Phe-Cha-OH

The peptide was synthesized as described above starting with Boc-Phe-OH (1.32g, 5mM) and coupling it to sodiated H-Cha-OH (0.94g, 5.5mM). Overall yield (1.3g, 42%); \( R_f = 0.17(\text{CHCl}_3-\text{MeOH, 9:1}) \); The peptide was purified as described above. The purified peptide was reinjected into an analytical reverse phase C\(_{18}\) column (Phenomenex, C18, 5\(\mu\), I.D. 250\(\times\)4.6mm) using a acetonitrile-water linear gradient 5-45% acetonitrile (0.1%TFA)/water (0.1% TFA) at a flow rate of 1ml/min over 25min and analyzed by mass spectroscopy. Retention Time- 19min; Observed Mass- 318.23 Da, Expected Mass- 318 Da.
2.2.2 Assembly of dipeptides: A stock solution of the dipeptides was prepared by dissolving 1 mg of the peptides in 50μl of 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP). In some cases mild sonication or heating was necessary to dissolve the peptides. Assembly was initiated by addition of double distilled water (1ml) to the stock solution. The samples were aged 24-48 hrs before experiments.

2.2.3 Scanning Electron Microscopy: The peptide stock solution was diluted to a final concentration range of 0.5mg/ml in ddH₂O and aged for one day. A 20μL of the aged sample was dried at room temperature on a microscopic glass cover slip followed by gold coating in a gold chamber for a minute. The images were taken using a JSM JEOL 6300 SEM.

2.2.4 Transmission Electron microscopy: The peptides stock solutions were diluted to final concentration of 1 mg/ml in ddH₂O. The grid was stained with 1% uranyl acetate in water. Samples were viewed with a Phillips Technai 120KV low dose electron microscope.

2.2.5 Circular Dichroism Spectrometry: All spectra were recorded on a JASCO-810 polarimeter equipped with a Peltier type thermostat and purged continuously with dry N₂ gas at 10 LPM during data acquisition. Data was collected in a quartz cuvette with path length of 1cm between 190nm to 300nm at scan speed of 25nm/min and response time of 16sec. The average of 50 scans was used for analysis of the spectrum. The CD spectra were converted to molar ellipticity. The concentration of the peptide was estimated by UV spectrometry with ε₂₀₅ = 5167 per peptide bond [256].

2.2.6 Fourier Transform Infra Red Spectrometry: Spectra were collected on a Perkin Elmer Spectrum BX-II FTIR spectrometer. The assembled peptide samples were spotted on a CaF₂ window and air-dried at 99% relative humidity. The samples were then rehydrated with D₂O for 30min before collecting the spectra. Each spectrum was average of 1000 collections at a resolution of 4cm⁻¹ in the spectral range of
1400cm⁻¹ to 1900cm⁻¹. The FTIR spectra were smoothed with smoothing length of 20. Subsequently, the second order derivative spectra were calculated with 13 data points. The spectral processing was done with Spectrum™ supplied by Perkin Elmer. To monitor the HD exchange, the tubes were collected by centrifugation and washed with water twice. After drying under vacuum, the tubes were rehydrated with D₂O and the spectra were collected at intervals of 1 min. Each spectrum was average of 10 collections at a resolution of 4cm⁻¹ in the spectral range of 1400cm⁻¹ to 1900cm⁻¹. The area under the Amide II peak was plotted against time to observe the kinetics of deuteration.

2.2.7 X-ray crystallography: The peptide, Phe-ΔPhe, was crystallized by controlled slow evaporation of peptide in acetic acid-water mixture. Plate like crystals, suitable for x-ray diffraction, appeared within 3-4 days. X-ray diffraction data was collected on a Bruker AXS SMART APEX CCD diffractometer with MoKα radiation (α=0.71073Å). The structure solution was obtained using direct methods employed in SHELXS. The structure was refined to an R-factor of 3.74%. Acetic acid molecules were located in the electron density map. (CCDC 298818).

2.3 Results and Discussion

2.3.1 Self-assembly of diphenylalanine dipeptide

Alzheimer’s disease has been characterized by heavy deposition of insoluble plaques composed primarily of Aβ₁₋₄₂. Fragmentation based approach to delineation of the core sequence responsible for aggregation resulted in the identification of a hepta peptide sequence, KLVFFAE, as the core determinant for fibrillogenesis. The fibril formed by the peptide showed all characteristics features of amyloid fibrils formed by Aβ₁₋₄₂. The peptide assembled into tubular structure with a β-sheet signature as evidenced from TEM and FTIR studies.

Interestingly, the central portion of this sequence, i.e. the diphenylalanine motif was observed to be sufficient for assembly into nanotubular structures as in amyloids
(Figure 2-1). This resulted in the first report of dipeptide based self assembled nanotubes. TEM images revealed a light shell and the dark center suggesting hollow tubular structures filled with the negative stain, uranyl acetate. Energy-dispersive x-ray analysis (EDX) indicated the presence of uranium within the assembled structures. The nano-tubular structures had average diameter between 100-150nm with length over microns (Figure 2-2). The nanotubes were highly ordered and without the usual branching and curving typical of amyloid fibrils. The tubular structures formed by the dipeptides also showed green-gold birefringence upon staining with Congo red dye, which was consistent with an organization that may be similar to that of amyloid structures. FTIR studies suggested a \( \alpha \)-sheet structure due to the appearance of 1630 cm\(^{-1}\) peak. However, the CD spectrum did not show any existence of \( \beta \)-sheet.

Figure 2-1: Self-assembly of well-ordered and elongated peptide nanotubes by a molecular recognition motif derived from the \( \beta \)-amyloid polypeptide. (A) The central aromatic core of the \( \beta \)-amyloid polypeptide is involved in the molecular recognition process that leads to the formation of amyloid fibrils. Various fragments of the core form amyloid fibrils or inhibit their formation. (B) TEM images of the negatively stained nanotubes formed by the diphenylalanine peptide. (C) HR-TEM images of negatively stained peptide nanotubes, visualized by field emission gun microscope.

Deeper insight into the mode of assembly of the diphenylalanine monomers occurring in the assembly has been obtained from the crystal structures of the dipeptide obtained by evaporation of an aqueous solution of Phe-Phe at 80°C. The crystal structure of the diphenylalanine dipeptide exhibited the occurrence of nearly circular channels formed by the translation of six peptide molecules. The molecular diameter of the channels was 24 Å and the van der Waals' diameter was 10 Å. The monomers were stabilized by extensive π-stacking interaction between the phenyl rings. An extensive network of intermolecular hydrogen bonding between the N- and C-terminus as well as between the amide bond provided additional stability to the structure. Subsequent fibre diffraction studies of the dipeptide in the crystalline and in the nanotubular assembly showed that the molecular arrangement of the monomers was similar in both the cases. This was an interesting...
observation as it was previously speculated that the dipeptide molecules arranged themselves with different packing arrangements in the crystals and the self-assembled nanotubes. Further, due to the hollow nature of the nanotubes formed by the diphenylalanine dipeptide, the assembled structures were used as casts for generating metallic nanowires. The organic cast was subsequently proteolytically digested to yield the free nanowires. The studies brought out the ability of short aromatic peptides to self-assemble into distinct nanostructures with potential applications. However, the tunability of the assembling behavior, morphology of the final assembly and the stability of the resultant structures to various conditions are believed to be important determinant for their biomedical applications.

2.3.2 Self assembly of designed dipeptide Phe-ΔPhe into nanotubes.

In this study, the role of conformational constraint in the monomeric dipeptide and its influence on the assembly behavior was investigated as a novel strategy for the generation of peptide based self-assembling systems with greater structural stability as well as resistance to proteolytic degradation. Based on the observation that diphenylalanine dipeptide assembled into nanotubes, we synthesized and characterized the self-assembly of the dipeptide H-Phe-ΔPhe-OH.

2.3.2.1 Microscopic investigation

Optical micrographs of the aggregates were recorded using Differential Interference Contrast microscope, which revealed the presence of distinct tube like assemblies of over a micrometer in length (Figure 2-3a). The TEM analysis with negative staining of the dipeptide showed ordered and tubular assemblies with length in the micrometer range (Figure 2-3b). The width of the independent tubes ranged between 25-30 nm (Figure 2-4). No branching or curving of the tubes was observed in any of the field scans. It was interesting to note the absence of any amorphous aggregates. The process of assembly was kinetically fast, as micrographs taken after a few minutes and after 24 hrs of incubation did not reveal any significant difference in the
morphology of the tubes. The morphology of the tubular structures was also analyzed by Scanning Electron Microscopy (SEM) and Environment Scanning Electron Microscopy (E-SEM) (Figure 2-5), which further suggested that the tubular morphology was not an artifact of sample preparation. The occurrence of relatively uniform and thin size of the tubes formed by Phe-ΔPhe (Figure 2-4) was in contrast to the wider and more dispersed size of tubes formed by the dipeptide, Phe-Phe, the saturated analogue of the dehydro-dipeptide. This suggested the impact of conformational constraint induced by the -ΔPhe- residue leading to the observed difference. It was quite likely that conformational restrictions both in the dipeptide backbone and the side chain of the C-terminal Phe limited the range in size distribution and also led to a more compact assembly. It may therefore be expected that the introduction of conformational constraint could be used as a tool to fine-tune the nature of the existing assemblies particularly where it may be possible to introduce such changes with ease.

2.3.2.2 Stability under different conditions

We next investigated the stability of the nanotubes under acidic (0.1N HCl), neutral and basic (0.1N NaOH) conditions by varying the pH of the medium after tube formation. Interestingly, the morphology of the fundamental tubular unit remained unchanged (27-30 nm,) over the range of pH used though there were changes in the staining features (Figure 2-6) and the average number of tubes in a field. However, at very high concentrations of the acid or alkali (1N and above), we observed disruption of the assembled structure. Thus, it was clear that at physiologically relevant conditions, the tubes would be stable and could be used for potential biomedical applications.
Figure 2-3: Figure showing the (a) differential interference contrast (DIC) and (b) TEM images of self-assembled Phe-$\Delta$Phe showing the occurrence of ordered and tubular assemblies with length in the micrometer range.

Figure 2-4: The size distribution profile of the nanotubes calculated from the TEM images. The average width of the independent tubes ranged between 25-30 nm. The tube dimensions were smaller to those obtained by the self-assembly of Phe-Phe dipeptide.
Figure 2-5: (a) Scanning electron microscopy (SEM) and (b) Environment scanning electron microscopy (E-SEM) images of the Phe-ΔPhe nanotubes. The images suggested that the tubular morphology was not an artifact of sample preparation. The tube dimensions were comparable to those obtain under TEM.

Susceptibility to proteolysis has been a major drawback of peptides and peptide-based assemblies especially for in vivo delivery applications. However, the introduction of modified or non-protein amino acid could confer a high degree of resistance to enzymatic degradation to the building blocks of the assembling systems. We found that the tubular structures formed by Phe-ΔPhe were intact without any change in morphology when the peptide was left for incubation with proteinase K for more than 36 hours (Figure 2-6). The tubes were also found to be stable to treatment of trypsin, chymotrypsin and cell culture supernatants.

The high stability of the self-assembled tubes over a broad range of pH conditions and to a highly non-specific proteolytic enzyme like Proteinase K could make these tubes interesting candidates for future applications.
Figure 2-6: TEM images demonstrating the stability of the tubes under different pH conditions (a) acidic (0.1N HCl) (Scale Bar – 200nm), (b) alkaline pH (0.1N NaOH) (Scale Bar – 100nm), proteinase K treatment in 50mM Tris-HCl pH 7.2. (Scale Bar – 100nm).
2.3.2.3 Crystallization studies

Extensive crystallization studies have been conducted on hydrophobic dipeptides in order to investigate the conformational properties of the small peptides\textsuperscript{107-135}. Of special interest have been the FF and VA class of dipeptides that crystallized with the occurrence of tubular channels with trapped solvent molecules\textsuperscript{122,125,132}. Therefore, in order to investigate the molecular structure of the assembly, the dipeptide Phe-\(\Delta\)Phe was crystallized by controlled slow evaporation of the peptide in acetic acid-water mixture. The crystal structure was solved by our collaborators at Department of Physics, IISc, Bangalore.

In the crystal structure, Phe-\(\Delta\)Phe existed as a monomer in the crystal asymmetric unit. The tubular structure was formed by four dipeptide molecules (Figure 2-7) resulting in a rectangular channel having Vander Waals dimension of 6.0x4.5Å. As discussed earlier, the saturated analogue, Phe-Phe, had exhibited nearly circular channels formed by the translation of six peptide molecules \textsuperscript{125}, with a diameter of 24Å. It was interesting to note that by the introduction of a conformational constraint in the molecule, the channel shape and surface area could be modulated to some extent.

According to Gorbitz, as dipeptides contain only one peptide bond and the dihedral angles cannot be defined by classical secondary structural elements, a simplified description of a dipeptide conformation could be made by calculating a torsion angle \(\theta = C_1\beta-C_1\alpha\cdot\cdot\cdot C_2\alpha-C_2\beta\) \textsuperscript{17}. This angle defined the relative position of the two side chains with respect to the peptide plane. It has been demonstrated that for zwitterionic \(L\)-Xaa-\(L\)-Xaa dipeptides (Xaa is neither Gly nor Pro), the side chains usually pointed in almost opposite direction with \(|\theta|\) usually being > 135° \textsuperscript{122,125}. According to this torsion angle description, Phe-Phe, occurred in an unusual conformation with \(\theta\) being 40.2°. The side chains were thus located on the same side of the peptide bond plane and appeared to emanate out from the channel core.
However, for Phe-ΔPhe this torsion angle ($|\theta|$) had a value of 149.70°. This implied that the side chains were present on both side of the peptide bond plane like the other members of the FF class of saturated dipeptides. It might have probably occurred due to the existence of conformational constraint in the molecule induced by the -ΔPhe residue.

![Figure 2-7](image)

*Figure 2-7: View of the crystal packing (left) reveals a tubular structure formed by the assembly of four dipeptide molecules of Phe-ΔPhe. Enlarged view of the tubular structure (right) formed by the aggregation of four dipeptide molecules of Phe-ΔPhe with the acetic acid molecule being trapped inside the tube. The figure also shows the head-to-tail hydrogen bonding seen in the dipeptide molecule. *(The side chains not involved in the channel core formation has been omitted for clarity.*)
The crystal structure of Phe-ΔPhe exhibited molecules of acetic acid trapped in the rectangular channels. The acetic acid molecules were found to be hydrogen bonded to the scaffold suggesting the important role of the solvent molecules in stabilizing the scaffold structure. Similar features had been previously reported in the self-assembly of the dipeptide (R)-Phenylglycine-(R)-Phenylglycine and (R)-(1-Naphthyl) glycyI-(R)-phenylglycine, where the dimensions of the self-assembled structure was shown to be modulated by the nature of the solvate. Interestingly, in these structures, different solvate molecules modulated the overall conformation of the self-assembled structure [245-248]. Though, the crystal structure of the Phe-Phe dipeptide also exhibited the occurrence channels filled with the solvent water molecules, the solvent molecules did not hydrogen bond with the scaffold. The crystal structure of the Phe-ΔPhe dipeptide exhibited a C(8) pattern of head-to-tail hydrogen bonding. Similar pattern of hydrogen bonding have been previously reported in the crystal structure of other hydrophobic saturated dipeptides [122, 125]. The stacked aromatic rings in the dehydro-dipeptide were held by intermolecular C-H···π interactions, giving rise to overall stability to the assembled structure. Stabilization of assemblies by multitude of such weak interactions has been very well known in literature and one of the fundamental requirements in the design of self-assembled nanoparticles.

2.3.2.4 Spectroscopic studies

The molecular structure of the aggregates was also investigated by circular dichroism (CD) and FT-IR spectroscopy. The CD signature of the dipeptide nanotube (Figure 2-8a) was characterized by a strong positive band at 197nm ([θ] ~ 10000; Π-Π* transition), a second positive band near 220nm ([θ] ~ 8000; n-Π* transition) suggestive of a probable turn like structures in solution. The ΔPhe ring contributed the broad negative band with peak near 280nm ([θ ~ 4500) due to charge-transfer transition. The FT-IR spectrum of the tubular assembly was characterized by strong peaks at 1687cm⁻¹ and 1647cm⁻¹ (Fig 2-8b). The peak at 1647cm⁻¹ was assigned to
aperiodic secondary structures involving type I, II, VIa and VIII turns. The peak at 1686 cm\(^{-1}\) could be assigned as marker band for turn conformation adopted by the molecule \(^{250-253}\). As discussed earlier, the conformation of dipeptides cannot be defined in terms of classical secondary structure elements. However, the FTIR and CD spectrum of many homo-aromatic self-assembling dipeptides exhibited spectroscopic features of helices, sheets and turns. Though the exact origin of the spectral features are intriguing, occurrence of such bands do suggest that there might exist similarity between the structural organization of the dipeptides and the classically defined secondary structures \(^{[88]}\).

To assess the solvent accessibility of the tubes as a probe for the hollow architecture of the tubes, the technique of H-D exchange was utilized \(^{[254, 255]}\). The ratio of the area under the peak in the amide I peak (1714 cm\(^{-1}\) to 1626 cm\(^{-1}\)) to the amide II region (1626 cm\(^{-1}\) to 1543 cm\(^{-1}\)) was plotted against time to characterize the exchange kinetics. The results indicated that (Figure 2-9) the ratio decreased with time and saturation was reached within 6 minutes. This suggested that the monomers in the self-assembled structures were highly solvent accessible probably due to the hollow nature of the tube (also suggested by the crystal structure) and thus allowed for the rapid HD exchange. This observation was in contrast to the exchange rates observed in case of \(\beta\)-sheet based fibrillar assemblies that exhibited slower exchange rates. However, it did suggest that the tubes were porous and could thus be used to entrap low molecular weight molecules for potential biomedical applications.

It was thus evident that the incorporation of conformational constraint altered the assembly behavior and the properties of the nanotubes in multiple ways.
Figure 2-8: (a) Circular dichroism and (b) fourier transform infra red spectrum of the Phe-ΔPhe nanotubes assemblies. Though, the conformation of dipeptides cannot be defined in terms of classical secondary structure elements, the FTIR and CD spectrum of the self-assembling Phe-ΔPhe dipeptide exhibited spectroscopic features of turns.

Figure 2-9: Figure showing the ratio of decrease in Amide I peak area with reference to Amide II peak area as an index of hydrogen deuterium exchange.
2.3.3 Role of N-terminal side chain on the self-assembly of nanotubes

In order to probe the role of the side chain of the N-terminal amino acid in the H-Xaa-ΔPhe-OH motif, the self-assembly of the peptides with Xaa from the different groups of the amino acids was investigated.

Electron micrographs of aged aqueous solutions of Gly-ΔPhe and Ala-ΔPhe showed no self-assembly even at very high concentrations of the peptide (~10mg/ml). The peptide Leu-ΔPhe assembled into tightly packed crystalline tubular structures with tube diameter in the range of 100-150nm. Val-ΔPhe, however, assembled into thin flaky elongated structures (Figure 2-10). Similar two dimensional assemblies have been formed by another dipeptide derivative \[\text{[88]}\]. The peptide Ile-ΔPhe assembled into long (over many microns) tubular structures with very large (500 nm) diameter (Figure 2-10) that were heavily stained with uranyl acetate probably suggesting a hollow architecture. With Xaa as Glu or Lys, the dipeptides assembled into distinct vesicular structures in aqueous medium. The self-assembly behavior of this class of structures has been discussed in detail in Chapter 4. Dipeptide containing hydroxyl groups like Ser at Xaa position did not form assemblies under the conditions tested. Also amide group containing dipeptide like Gln-ΔPhe did not assemble into nanostructures under conditions tested.

The observations indicate that a minimum level of bulk was necessary to initiate the self-assembly behavior in the dipeptides scaffold with Xaa belonging to the hydrophobic group of amino acids. Moreover, the nature of the functional group at the N-terminus influenced the propensity as well as the nature of the self-assembly in the H-Xaa-ΔPhe-OH dipeptide motif. These results could be utilized for the rational design of dipeptide based nanostructures.
Figure 2-10: Transmission electron micrographs (TEM) of ΔPhe containing free dipeptides. Leu-ΔPhe assembled into tightly packed crystalline tubular structures whereas Val-ΔPhe resulted in thin flaky elongated self-assembled structures. The peptide Ile-ΔPhe assembled into long and wide (~200nm) tubular structures.
2.3.4 Role of aromaticity in the self-assembly of nanotubes

Having established that conformational constraint could be used as a potential strategy to modulate features of self-assembled structures, we investigated the role of peptide Ile-ΔPhe assembled into long (over many microns) tubular structures with large (500 nm) diameter. This suggested that a minimum level of bulk was necessary to initiate the self-assembly behavior in the dipeptides scaffold H-Xaa-ΔPhe-OH with Xaa belonging to the hydrophobic group of amino acids.

aromaticity in the self-assembly of dipeptides into nanotubes. The pi-stacking interactions have been previously proposed to stabilize tubular architectures [74-78, 122, 125]. To probe the necessity of pi-stacking interactions for structures to assemble into tubular architectures, we designed, synthesized and studied for their self-assembling behavior of H-Phe-Phe-OH (Phe-Phe), H-Cha-Phe-OH (Cha-Phe), H-Phe-Cha-OH (Phe-Cha) and H-Cha-Cha-OH (Cha-Cha) where, Cha stands for Cyclohexylalanine, a modified amino acid similar to phenylalanine but with an aliphatic ring.

2.3.4.1 Microscopic Investigations

The electron micrograph of the dipeptide Phe-Phe exhibited large tubular structures with a diameter of 200-400nm and length over 1μm (Figure 2-11). The Cha-Phe dipeptide was characterized by thin fibrils along with a large number of vesicular structures (Figure 2-11). The fibrils were roughly 20nm in diameter and exhibited twisting and curving along their length. The vesicles exhibited an average diameter of 50nm with a tendency to fuse with each other. This suggested that the vesicles were probably the structural precursors of the fibrils. Moreover, on longer incubation (~15 days), the field scans did not show any vesicles suggesting that they were the kinetically trapped intermediates in the pathway of the assembly of the dipeptide fibrils. The electron micrographs of Phe-Cha peptide showed a large number of pleomorphic vesicular structures in a large size range of 10-80nm (Figure 2-11) indicating that the position of the aromatic residue in the dipeptide also affected the
morphology of the assembly. The peptide Cha-Cha also assembled into vesicular structures with an average diameter of 20nm and low poly-dispersity (Figure 2-11). The above observations clearly highlighted the crucial role of aromatic stacking interactions in the self-assembly of nanotubes. It was interesting to note that not only the presence of aromatic residues dictated the assembly but also its position in the dipeptide. However, it appeared that it was not always necessary to have aromatic residues to initiate self-assembly. Thus, appropriately chosen aliphatic ring structures could also act as motifs for self-assembly. This observation was in contrast to the predominant belief that aromatic homo-dipeptides were the central motif for the formation of ordered self-assembled structures.

2.3.4.2 Spectroscopic Studies

To delineate the role of molecular conformation on the assembly behaviour of the dipeptide we compared their CD and FTIR spectra. The CD spectra of the assemblies was characterized by the π-π* transition band at 197-198nm and an n-π* transition band between 203-215nm (Table 2-1). The position of n-π* transition band blue shifted with decreasing aromaticity. The FTIR spectra of the dipeptide assemblies were characterized by ν_{C=O} (backbone; Amide I) band at 1615 cm⁻¹ (for Phe-Phe), 1667 cm⁻¹ (for Cha-Phe), 1665 cm⁻¹ (for Phe-Cha) and 1670 cm⁻¹ (for Cha-Cha) (Table 2-1). However, we did not observe any correlation between the nature of assembly and its molecular conformation. This was not surprising as self-assembled dipeptides have been shown exhibit FTIR spectra corresponding to all major classes of secondary structure and there have been no correlations between the morphology and molecular conformations of the peptide based nano-assemblies.

The above spectroscopic evidences along with the electron micrographs clearly suggested that appropriately placed aromatic moieties were very crucial in the design of dipeptide based nanotubes. Moreover, aromaticity was not the core driving factor for self-assembly in dipeptides and amino acids containing aliphatic ring side-chains could also be used.
Figure 2-11: TEM images of the nano-structures formed by (a) Phe-Phe, (b) Cha-Phe, (c) Phe-Cha and (d) Cha-Cha. The electron micrograph of the dipeptide Phe-Phe exhibited large tubular structures with a diameter of 200-400nm and length over 1μm (*Excerpted from *Science* 25 April 2003:Vol. 300. no. 5619, pp. 625 – 627). The Cha-Phe dipeptide was characterized by thin fibrils along with a large number of vesicular structures. The fibrils were roughly 20nm in diameter and exhibited twisting and curving along their length. The vesicles exhibited an average diameter of 50nm with a tendency to fuse with each other. The Phe-Cha peptide showed a large number of pleomorphic vesicular structures in a large size range of 10-80nm. The peptide Cha-Cha also assembled into vesicular structures with an average diameter of 20nm and low poly-dispersity.
Table 2-1: FTIR peaks and CD spectra of the nano-structures formed by (a) Phe-Phe, (b) Cha-Phe, (c) Phe-Cha and (d) Cha-Cha.

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<thead>
<tr>
<th>Peptide</th>
<th>CD</th>
<th>FTIR</th>
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<tr>
<td></td>
<td>$\pi-\pi^*$ Transition (nm)</td>
<td>n-$\pi^*$ Transition (nm)</td>
</tr>
<tr>
<td>Phe-Phe</td>
<td>197</td>
<td>215</td>
</tr>
<tr>
<td>Cha-Phe</td>
<td>197</td>
<td>211</td>
</tr>
<tr>
<td>Phe-Cha</td>
<td>197</td>
<td>213</td>
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<td>Cha-Cha</td>
<td>198</td>
<td>203</td>
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2.5 Conclusion

Although biological scaffolds, including short peptides, offer a myriad of potential applications to nanotechnology, their relative instability may be a major concern in realizing their potential application. The main aim of the present work was to explore the possibility of using non-protein amino acids, capable of providing well-defined conformational characteristics and higher stability, particularly to enzymatic degradation. The introduction of $\Delta$Phe in the dipeptide affected the pattern of peptide assembly resulting in longer and thinner nano-tubes than previously reported peptide based tubular structures. Also, their stability to different pH conditions and proteases could make them potentially useful for various applications. However, the studies also brought out the crucial role of aromatic moieties in the design of dipeptide based self-assembling nanotubes. It was evident that the nature of the side-chain and their position in the dipeptide scaffold also dictated the nature of assembly. Moreover, aromaticity was not the core driving factor for self-assembly in dipeptides; peptide based nanostructures could also be
designed with amino acids containing aliphatic ring side-chains. Thus, it was evident that dipeptides could be rationally designed with chosen kind of functional groups at appropriate position to generate nano-particles with desired properties.