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# Role of aromatic amino acids in amyloid self-assembly

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## Abstract

Amyloids are proteins of a cross- $\beta$  structure found as deposits in several diseases and also in normal tissues (nails, spider net, silk). Aromatic amino acids are frequently found in amyloid deposits. Although they are not indispensable, aromatic amino acids, phenylalanine, tyrosine and tryptophan, enhance significantly the kinetics of formation and thermodynamic stability, while tape or ribbon-like morphology is represented in systems with experimentally detected  $\pi$ - $\pi$  interactions between aromatic rings. Analysis of geometries and energies of the amyloid PDB structures indicate the prevalence of aromatic-nonaromatic interactions and confirm that aromatic-aromatic interactions are not crucial for the amyloid formation.

*Keywords:* amyloids, aromatic amino acids, self-assembly

## 1. Introduction

Amyloids are filamentous protein deposits varying in size from nanometers to microns and composed of aggregated peptide  $\beta$ -sheets [1]. Amyloid-forming proteins have attracted a great attention recently because of their association with over 30 diseases, namely neurodegenerative conditions like Alzheimer's, Huntington's, Parkinson's, Creutzfeldt-Jacob and prion disorders, and also systemic diseases such as amyotrophic lateral sclerosis (Lou Gehrig's disease) and type II diabetes. These diseases are all thought to involve important conformational changes in proteins, sometimes termed misfolding, that usually produce  $\beta$ -sheet structures with a strong tendency to self-assembly into fibrous deposits. There are a lot of studies about inhibition of amyloid- $\beta$  fibrillation in therapeutical purposes, [2, 3, 4, 5, 6, 7] but also a lot of evidences for physiological and even protective role of amyloid- $\beta$  deposits [8, 9, 10].

Soluble oligomers that are intermediates of the amyloid- $\beta$  peptide fibrillogenic pathway

are found to be toxic. Different aromatic small molecules are utilized to remodel soluble oligomers of the A $\beta$ 42 peptide into multiple conformers with reduced toxicity [11]. Experimental assays such as NMR spectroscopy, X-ray crystallography and peptide array measurements imply that an antibody fragment KW1 and a small dipeptide, d-tryptophan linked with  $\alpha$ -aminoisobutyric acid recognize oligomers through the aromatic rings of Phe19 and Phe20 of the A $\beta$  oligomer [12, 13, 14]. Also, computational studies suggested that BoDipy-oligomer was specific towards A $\beta$  oligomers through  $\pi$ - $\pi$  interactions of its aromatic rings [15].

There are non-pathological amyloidogenic proteins with physiological properties. Self-assembling peptides can be used as biocompatible nanoparticles, such as the water-insoluble photosensitizer Chlorin e6 for tumor delivery and photodynamic therapy [16]. An injectable and self-healing collagen-gold hybrid hydrogel is spontaneously formed by self-assembly and subsequent biomineralization. It is used as an injectable material for local delivery of therapeutic agents, showing enhanced antitumor efficacy [17]. Controlled phase transformation involving biomolecular organization is used to generate dynamic self-assembly systems and functional materials [18].

In spite of the fact that various amyloid-forming proteins and polypeptides do not show any simple sequence homology, all amyloid assemblies share similar ultrastructural and physiochemical properties [19]. All amyloid fibrils have a characteristic, long and unbranched fibrillar morphology with a diameter of 5–15 nm, a  $\beta$ -sheet-rich structure, and typical X-ray fiber diffraction with a reflection of 4.6–4.8 Å in the meridian. Another well-known characteristic of all amyloid fibrils is their interaction with specific dyes such as Congo red and thioflavin T, which results in definite optical behavior such as birefringence and fluorescence, respectively.

The common cross- $\beta$  structure observed in amyloid assemblies consists of multiple  $\beta$ -sheet peptides organized in antiparallel or parallel  $\beta$ -strands, stabilized by extensive hydrogen-bonding along the fibril axis with noncovalent ionic and hydrophobic interactions between amino acid side-chains occurring orthogonal to the fibril axis [20]. These interactions can be of different nature: electrostatic, van der Waals, hydrogen bonds, dispersion,  $\pi$ - $\pi$ , while the  $\pi$ - $\pi$  interactions are only present between aromatic residues.

The experimental and theoretical results showed that naturally occurring peptides form  $\alpha$ -helical intermediates during the process of aggregation. There is an evidence for a mechanistic role in amyloidogenesis of helical-containing intermediates, and several of the observations that support this are discussed below, in the Kinetics section.

The ability of polypeptide chains to form amyloid structures is not restricted to the relatively small number of proteins that are associated with recognized clinical disorders, but appears to represent a generic feature of aggregated polypeptide chains. The core structure of all amyloid fibrils seems to be primarily stabilized by hydrogen bonds, involving the polypeptide main chain. As the same chemical structure of the main chain is common to all polypeptides, this observation explains why fibrils formed from polypeptides of very different sequences seem to be so similar. Even though the ability to form amyloid fibrils appears to be generic, the propensity to do so under given conditions can vary markedly between different protein and peptide fragments. The relative aggregation rates for a wide range of peptides and proteins correlates with their physiochemical features, such as hydrophobicity, secondary-structure propensities, charge, and aromatic interactions [19].

The role of aromatic amino acid residues in amyloid formation is controversial and remains under debate. Aromatic residues frequently occur in natural amyloids leading to the concept that

aromatic amino acids play an important role in amyloid formation [21, 22, 23]. Propensity of aromatic amino acids to promote the self-assembly of peptide sequences into amyloids have been demonstrated. However, amyloids can be also formed by non-aromatic peptides, which indicates that aromatic amino acid residues do not play an essential role in amyloid formation [24, 25].

In last few years, numerous studies have been performed on different polypeptides using various experimental and computational methods to investigate role of aromatic amino acid residues in amyloid formation. Subject of studies were type 2 diabetes related Human islet amyloid polypeptide (IAPP or amylin) and its fragments [26, 27, 28, 29, 30, 31, 32, 33, 24, 34, 35, 36, 37] and Alzheimer's disease related amyloid- $\beta$  and its fragments [38, 39, 24]. Human calcitonin (hCT), is also well known as an amyloid forming peptide containing aromatics, associated with medullary carcinoma of the thyroid [40, 24]. Human muscle acylphosphatase (AcP) is not associated with any known human disease, however, it can form aggregates which show an extensive  $\beta$ -sheet structure [41].

In the study published recently [24] the role of aromatic amino acids in amyloid formation was studied by comparing behavior of aliphatic peptides with peptides containing aromatic amino acids. In the study several peptides presenting segments of core sequences within natural amyloid proteins were used; peptide from human amylin with sequence NFGAIL, two peptides from Alzheimer's amyloid- $\beta$  with sequences KLVFFAE and GGVVIA, and peptide from human calcitonin with sequence DFNKF that form toxic fibrils. The results were compared with the data of synthetic peptides with sequences LIVAGD, IVD and RADARADARADADA.

Synthetic amyloidogenic peptides containing aromatic residues have been designed in order to investigate the role of aromatic residues in amyloids [42, 24, 43, 44] or diphenylalanine motif proposed as the core motif for molecular recognition and self-assembly of amyloid- $\beta$  [23].

Among synthetic peptides, amphipathic peptides have an increased propensity to self-assemble into amyloid-like  $\beta$ -sheet fibrils when their primary sequence pattern consists of alternating hydrophobic and hydrophilic amino acids [45, 46, 43, 44]. These fibrils adopt a bilayer architecture composed of two  $\beta$ -sheets laminated to bury the hydrophobic side chains of the  $\beta$ -sheet in the bilayer interior, leaving the hydrophilic side chains exposed at the bilayer surface. One of the major applications of self-assembling amphiphilic peptides is the formation of hydrogels, networks of fibrils that induce order in the solvent medium. Peptides derived from  $(XKXE)_2$  and  $(XKXK)_2$ , where X is a hydrophobic residue, typically form hydrogels in media of high ionic strength in which the charge of the hydrophilic side-chains becomes masked, allowing noncovalent cross-linking between fibrils [45, 46].

The aim of this review is to systematize significance of aromatic amino acids to amyloid self-assembly from experimental and theoretical investigations, as this is frequently observed in the literature.

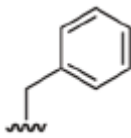
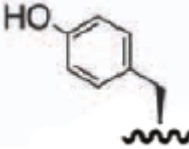
## 2. Aromatic amino acids: properties

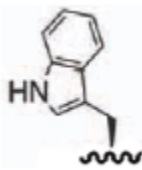




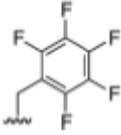
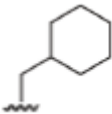
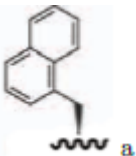
The natural aromatic amino acids, phenylalanine, tyrosine and tryptophan, are characterized by a high hydrophobicity and propensity to form  $\beta$ -sheet structure. They contain planar six-membered ring with a delocalized  $\pi$  system. This chemical characteristic, termed aromaticity, has been proposed to be responsible for the ability of these residues to promote amyloid fibrillation [41]. The  $\pi$ -stacking can provide two key elements that are highly

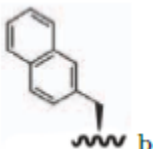
important for the formation of such structures: 1) an energetic contribution that stems from the stacking itself; such a contribution can thermodynamically drive the self-assembly process, and 2) specific directionality and orientation provided by the specific pattern of stacking. This is especially important since amyloid fibrils are well-defined supramolecular structures and a specific pattern of stacking should lead to a formation of an ordered structure [22]. It was suggested that  $\pi$ -stacking energy may be largely driven by entropy. Accordingly, ordered water molecules are being released from the aromatic rings upon intermolecular interaction. Therefore, despite the ordered structures formed by the stacking interactions, the overall entropy of the thermodynamic system, which includes bound water molecules, increases [22].

The exact role of aromatic amino acids in amyloids are probed by mutations of aromatic residues which provide separate observations of the mentioned properties: hydrophobicity,  $\beta$ -sheet propensity and ability to engage in  $\pi$ - $\pi$  interactions, Table 1. Of the known non-natural amino acids, cyclohexylalanine (Cha) is significantly more hydrophobic than Phe ( $\Pi_{\text{Cha}} = 2.72$ ,  $\Pi_{\text{Phe}} = 1.79$ ), and Cha lacks aromaticity while retaining a similar solvent accessible surface area [45, 46, 38, 32]. Phe to Cha mutations provide an interesting probe of aromatic vs. hydrophobic interactions in the context of peptide self-assembly by allowing the creation of variant peptides with increased hydrophobicity that lack the capability to engage in aromatic-aromatic interactions. Pentafluorophenylalanine, ( $F_5$ -Phe), another interesting non-natural amino acid, is intermediate in hydrophobicity relative to Phe and Cha ( $\Pi_{F_5\text{-Phe}} = 2.12$ ) while maintaining aromatic character [46, 38, 32, 35]. Also,  $\alpha$ -naphthylalanine (1-Nap) and  $\beta$ -naphthylalanine (2-Nap) are characterized by a higher hydrophobicity than the natural aromatic residues,  $\Pi_{1\text{-Nap}} = 3.08$ ,  $\Pi_{2\text{-Nap}} = 3.12$ , while maintaining aromaticity [32]. The steric effects are probed by mutations with non-planar Leu and Cha, larger residues Cha, 1-Nap, 2-Nap and  $F_5$ -Phe, and with 1-Nap whose naphthyl side chain is rotated toward the amide backbone relative to the 2-Nap side chain, in which the naphthyl group is extended into the intersheet space.

Table 1: Properties of aromatic amino acids and their analogs in amyloids [47, 48, 29, 38, 32]

Residue	Volume	Planar	Aromatic	Hydrophobicity	$\beta$ -sheet propensity	Structure
Phe	98.83 Å <sup>3</sup>	yes	yes	1.79	1.33	
Tyr	107.32 Å <sup>3</sup> ( $\approx$ Phe)	yes	yes	0.96	1.50	

Trp	125.55 Å (25% > Phe)	yes	yes	2.25	1.30	
Leu	96.22 Å (≈ Phe)	no	no	1.71	1.22	
Ala	61.00 Å (< Phe)	–	no	0.31	0.72	
Val	70.00 Å (< Phe)	–	no	1.22	1.86	
Ile	88.00 Å (< Phe)	–	no	1.80	1.67	
F <sub>5</sub> -Phe	123.33 Å (25% > Phe)	yes	yes	2.12	> Phe	
Cha	118.96 Å (20% > Phe)	no	no	2.72	> Phe	
1-Nap	142.20 Å (40% > Phe)	yes	yes	3.08	–	

2-Nap	142.00 Å (40% > Phe)	yes	yes	3.12	–	
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<sup>a</sup>rotated toward the amide backbone <sup>b</sup>extended into the intersheet space

### 3. Ability to aggregate

What influences the ability of an amyloid to aggregate was assessed by mutating important residues in natural occurring amyloid peptides, as well as by comparing different synthetic amyloidogenic peptides. This was done by detecting formed amyloid aggregates by electron microscopy, or by detecting quantity of formed protein  $\beta$ -structure by CD, infrared or Raman spectroscopy. Here, we review the role of aromatic amino acids in the capability of aggregation.

The general conclusion is that aromatic amino acids are not essential to amyloid aggregation. Many amyloids lacking aromatics manage to self-assemble. As already mentioned, the existence of non-aromatic amyloid sequences leads to the claim that aromatic amino acids are not indispensable for the amyloid self-assembly process [24, 25].

Human islet amyloid polypeptide (hIAPP) is a peptide with 37 residues containing three aromatic amino acid residues, Phe15, Phe23, and Tyr37. Formation of amyloids from IAPP polypeptides was studied using all mutants where one, two or three aromatic residues were substituted with leucine. Amyloid fibrils formed from each of the mutants were effective at aggregation at 16  $\mu$  M concentration and 12 h incubation time [34], also, at 0.53 mM concentration and 2 days of incubation [30], indicating that aromatic Phe is not essential for amyloid aggregation. The highly amyloidogenic hIAPP fragments containing one aromatic residue (Phe), hIAPP<sub>12-18</sub> [31], hIAPP<sub>21-29</sub>, hIAPP<sub>22-27</sub> and hIAPP<sub>10-19</sub> [29], also readily formed amyloids when Phe mutated to Leu and Ala.

In the case of amyloid- $\beta$ , there are aromatic rings at positions 19 and 20. Substituting Phe19 and Phe20 with Leu did not abolish aggregation at peptide concentration of 10  $\mu$  M and 5-7 days of incubation [39]. Human calcitonin (hCT) possesses three aromatics: Tyr12, Phe16 or Phe19. Peptides mutated to Leu residues at the position of 16, 19, and the triple mutant at 12, 16 and 19 all formed amyloids [40]. Human muscle acylphosphatase managed to form fibrils with Phe and Tyr residues substituted with a large hydrophobic (leucine), a small hydrophobic (alanine), a hydrophilic (serine or glutamine), and a charged (arginine) residue [41].

In the self-assemble experiments of synthetic, amphipathic peptides of alternating hydrophobic and hydrophilic sequence, all the studied peptides self-assembled without phenylalanines [45]. The aromatic phenylalanine residues in (FKFE)<sub>2</sub> have been globally replaced with non-aromatic natural residues with lower hydrophobicity (Ala, Val and Leu) and a non-natural residue with greater hydrophobicity, Cha. Aromatic interactions are not strictly required for amyloid formation. While peptides of lower hydrophobicity (Val, Leu) are competent to self-assemble into fibrils, this does not ensure further supramolecular assembly into hydrogels. There is, therefore, an apparent relationship between side-chain hydrophobicity or aromaticity and hydrogelation propensity. The Cha-containing peptides formed gel-like materials, and were able to

hydrogelate with much greater efficiency. The non-aromatic peptides formed weak gels, whereas aromatic peptides formed rigid gels [46].

There are few cases in the literature where peptides failed to aggregate in the absence of aromatic residue. The role of aromaticity in amyloid formation of the hIAPP<sub>22-29</sub> fragment, was studied on peptides with substituents on the aromatic ring of Phe23 that contained electron donating groups or electron withdrawing groups. Results show that electron donating groups on the aromatic ring of Phe-23 prevent formation of amyloid while peptides with electron withdrawing groups on aromatic ring formed amyloid aggregates. Peptides of 1 mM concentration were incubated for a period of one week. The electron donating and electron withdrawing groups influence ability of peptide to form amyloids, but, do not influence peptide hydrophobicity, suggesting that it is not only the hydrophobic nature of aromatic residue that is relevant in the self-assembly of hIAPP<sub>22-29</sub> and indicating a special role of aromatic residues. Raman and Fourier transform infrared spectroscopy provided direct evidence of ring stacking in the aggregates derived from peptides containing electron poor phenylalanine analogs [35]. These analogs alter the electronic nature of a  $\pi$ -system, but do not influence peptide hydrophobicity or  $\beta$ -sheet propensity.

As these bulky external substituents on the aromatic ring still might introduce nonproductive steric interactions as they are tetrahedral or bent, peptide analogs which contain substituted ring with planar geometry of varying electron density were designed as an alternative [36]. Therefore, additional probes were undertaken with 4-formamido phenylalanine NHCOH-Phe, 4-nitrophenyl-alanine NO<sub>2</sub>-Phe, and 4-carboxyphenylalanine COOH-Phe. 4-formamido phenylalanine contains electron donating groups and the rest of analogues contain electron withdrawing groups. Again, peptide analogues with electron donating group did not aggregate within a period of 7 days. The effect of aryl substituent geometry on peptide self-assembly reveals that the electronic nature of substituents and not their steric profile is responsible for failure of the electron donating group peptides to aggregate. Rings with electron donating groups fail to form any type of  $\pi$ -stacking interactions as rings with an excess of electrons probably repulse each other.

The Tyr, Leu, and Trp 23 variants of hIAPP<sub>20-29</sub> failed to readily self-assemble at concentrations up to 1.5 mM within 16 h [32]. In the case of Tyr, failure to form amyloid can be explained by the dramatically lower hydrophobicity of this variant. Although more hydrophobic than the wildtype Phe variant, the Trp variant failed to self-assemble probably because of ability of the indole side chain to participate in hydrogen-bonding interactions, either with nearby side chains or water, which perturbs the requisite  $\beta$ -sheet conformation or inhibits the hydrophobic collapse that precedes nucleation events. In addition to this, Trp represents bigger steric profile that could disrupt  $\beta$ -sheet lamination. Tryptophans are not very frequent in amyloids, although there are cases of normally forming amyloids with Trp [49].

Substitution of the phenylalanine residue with other hydrophobic amino acids (valine, alanine, isoleucine, or leucine) completely prevented aggregation of the hIAPP<sub>22-29</sub> fragment, NFGAILSS [28]. The aggregation experiments were undertaken in 1 week time. There are also studies on co-aggregation of the full length hIAPP with its hIAPP<sub>22-29</sub> fragment. The fragment was mutated in the position of the phenylalanine residue in order to probe aromatic amino acid role in the molecular recognition. The substitution of the key Phe23 residue with the other natural aromatic amino acids still enabled the molecular recognition. On the other hand, substitution of



Phe23 with any of the other natural hydrophobic amino acids completely impeded the molecular recognition process.

A complete alanine scan (mutating one by one residue with Ala) was performed on the hIAPP<sub>22-27</sub> fragment and only the Phe23 to Ala mutant was incapable of forming ordered fibrils after 2 days incubated 0.01M sample [27]. The Phe-Phe stacking interactions were observed in MD simulations. The MD simulations showed that the main factor that determines the formation of regular fibrils is a coherent organization of the intersheet space. In particular, phenylalanine side chains cement the macromolecular assemblies due to their aromatic chemical character and restricted conformational flexibility observed in simulations.

Amyloid- $\beta$  16-22 fragment variants with Phe19 mutated to Ala and Tyr failed to undergo fibril formation at the peptide concentrations of 100  $\mu$ M, incubated for 2 weeks, but Cha variants, although non-aromatic, self-assembled at dramatically enhanced rates relative to wild-type [38].

In the case of the synthetic KFFEAAAKKFFE peptide, all of the Phe to Ala mutants completely abrogates assembly ability. This appears to indicate that all four of the phenylalanine residues are required for assembly. Peptide solutions were made up to a concentration of 5 mg/mL in water or phosphate-buffered saline (PBS), and incubated for 7 days [42]. The inability to aggregate may be the consequence of the charge repulsion between the K(Lys) residues at the insufficient ionic strength, or the lack of aromatic interactions, being that in all the aromatic mutants fluorescence emission spectra shifted to red wavelengths as indication of aromatic stacking interactions.

The mutation of Tyr 151 and 189 to Leu completely abrogated fibril formation of the pigment cell protein that forms physiological amyloid in melanosomes [50]. Also, the Trp 153 and 160 mutation to also aromatic Phe residue completely abrogated fibril formation showing that in this position, the aromaticity is not crucial for the aggregation but some other interactions typical for Trp. However, fibril formation was reduced for Phe149 to Leu mutant and double mutant Phe 207 and 215 to Leu. This suggests that aromaticity in positions 149, 207, and 215 is also preferred, although it is not essential. In the same peptide, the Phe207 to Leu and Phe215 to Leu single mutants gave no difference in fibril formation which suggests that position and other factors play role in the amyloid assembly, other than aromaticity.

In addition, the influence of hydrophobicity and  $\pi$ -stacking interactions was assessed by altering the sequence pattern of synthetic amyloidogenic peptides FDFD [44], and (FKFE)<sub>2</sub> [43]. For the best hydrogelators (lowest minimum gel concentrations and transparent), the presence of segregated blocks of aromatic and polar fragments was shown to be a determining factor [44]. Fluorescence redshift of 8 to 14 nm from the solution to a gel phase is observed in all cases, which suggests the presence of strong  $\pi$ -stacking interactions upon aggregation. The largest redshift (14 nm) corresponds to the least effective gelator, which suggests a strong involvement of aromatic residues in the structural rearrangement from solution to the gel state.

Among the (FKFE)<sub>2</sub> sequence variants, only the peptides with alternating aromatic/hydrophilic sequence formed hydrogels at concentrations of 1mM and 24 h incubation time [43], Figure 1A. All of these amyloidogenic peptides formed aromatic stacking interactions, probed by CD spectroscopic measurements. According to the model, Figure 1B, the remaining sequence-altered peptides cannot form  $\beta$ -sheets with exclusively hydrophobic and hydrophilic faces. These changes thus disrupt the potential of these variants to self-assemble into  $\beta$ -sheet bilayer fibrils.

Figure 1: 3D structural models for (FKFE)<sub>2</sub> peptides with varied sequences. A) forming one-side hydrophobic sheet, B) forming two-side hydrophobic sheet. Reprinted with permission from [43]. Copyright (2018) American Chemical Society.

As we can see, the role of aromatic residues in amyloid aggregation is a consequence of complex intertwining of different parameters such as peptide concentration, incubation time, 3D structure of a certain peptide and different properties of the aromatic residues including its position in the sequence. Even though in higher concentration and incubation time, some mutants lacking aromatics do not aggregate. The data indicate that, generally, hydrophobicity and sometimes the stacking interactions, are the properties of aromatic residues crucial for amyloid formation.

## 4. Morphology

In general, the aromatic phenylalanine-containing peptide displays different morphologies than the one not containing aromatic rings, suggesting that aromatic interactions, while not essential for self-assembly, may give rise to unique structural features. Tape or ribbon-like morphology is represented in systems with experimentally detected  $\pi$ - $\pi$  interaction between aromatic rings and this feature, the occurrence of tapes or ribbons, seems to increase with a peptide concentration.

### 4.1 Presence of the $\pi$ - $\pi$ interactions

In a transmission electron and atomic force microscopy study of the IAPP amyloid, the wild-type IAPP had noticeably more pronounced twisted ribbon morphology than the triple Phe to Leu mutant [30]. Also, the wild-type fibrils tend to occur mainly as pairs of fibrils and sometimes as triplets and multiplets of adjoining fibrillar structures. It is plausible that the aromatic residues, through favorable  $\pi$ -stacking interactions or aromatic hydrophobic interactions, may encourage formation of a hierarchical fibrillar architecture. Also, the Phe to F<sub>5</sub>-Phe substitution results in the formation of amyloid composed of what appears to be dense helical tapes [35]. There is a Raman spectroscopic evidence of  $\pi$ -stacking interactions between Phe residues and also between all pairs of Phe residues containing electron withdrawing groups, -NO<sub>2</sub>, -F, F<sub>5</sub> and -CN.

The aromatic peptides hIAPP<sub>22-27</sub>, amyloid- $\beta$ <sub>16-22</sub>, and calcitonin were compared to several natural and synthetic aliphatic peptides in the experiments of transition electron microscopy [24]. The peptides containing aromatic residues formed lamellar  $\beta$ -sheet aggregates made of flat nanotapes, while aliphatic peptides formed helical nanofibers of aliphatic peptides. There is experimental evidence for stacking interactions within aggregates of hIAPP<sub>22-27</sub> and amyloid- $\beta$ <sub>16-22</sub> [27, 38]. It is very likely that the FF motif has a role in the unique behavior of KLVFFAE fragment of the amyloid- $\beta$ <sub>16-22</sub> under all examined aspects. This is because the FF peptide alone forms nanotubes and extremely rigid structures rather than helical fibers, which are supported by the formation of a striking three-dimensional aromatic Phe-Phe stacking arrangement that serves as a glue between the hydrogen-bonded cylinders of peptide main chains [23]. It seems that a nanotube is actually formed via dense helical nanotubes or nanoribbons, as

observed in synthetic peptides experiments [25] and represented schematically in Figure 2.

Figure 2: Schematic representation of the hierarchical self-assembly process of  $KI_4K$ , consisting of three main steps, i.e., the formation of  $\beta$ -sheets, the lateral stacking of  $\beta$ -sheets, and the evolution of stacked  $\beta$ -sheets into nanofibrils or nanotubes because of inherently twisting of  $\beta$ -sheets.  $a$  is the thickness of  $\beta$ -sheets, which is equal to that of the lamellae arising from their stacking;  $b$  is the lamination of  $\beta$ -sheets. Adapted with permission from [25]. Copyright (2018) American Chemical Society.

Among the synthetic amphipathic amyloids, of the sequence  $(XKXX)_2$  where  $X$  is replaced by various hydrophobic residues, it is significant that only sequences where  $X$  is an aromatic residue, form helical tape-like structures. This indicates that aromatic amino acids exert a structural influence on self-assembly. In these structures, the aromatic stacking was observed by CD spectra. On the other hand, aliphatic peptides formed fibrils [45, 46]. Furthermore, it was observed that higher peptide concentrations of  $(FKFE)_2$  stabilized the helical tapes relative to the flat tapes [45], Figure 3. Microscopic analysis showed polymorphism between samples at 1 mM, with fibrillar structure, those at 2 mM, with mixed fibrillar and helical ribbon structure, and at 4 mM, with dominant helical ribbon structure.

Figure 3: Concentration dependent morphology of  $(FKFE)_2$  observed with transmission electron microscopy (left) and atomic force microscopy (right). The upper image: 1 mM, (A) and (B) 2 mM, (C) and (D) 4 mM. Adapted from [45] by permission of The Royal Society of Chemistry.

The more grouped phenylalanines exhibit wider tapes in morphology as reported in [43]. The  $(FKFE)_2$  and  $(FK)_2(FE)_2$  peptides effectively self-assembled into  $\beta$ -sheet nanoribbons that were 8 and 4 nm wide, respectively. The  $KEFFFFKE$  and  $FFKEKEFF$  self-assembled into distinct nanotapes that were  $\approx 20$  nm in width. In these peptides, there is also a CD spectroscopic evidence of stacking interactions.

It is important to stress that aliphatic peptides also exhibit ribbon and tube-like morphologies. Nanofibrils were formed from  $I_4K_2$  and nanotubes from  $KI_4K$ . The lateral stacking and twisting of the  $\beta$ -sheets were well-linked to the hydrophobic and electrostatic interactions between amino acid side chains and their interplay. For  $I_4K_2$ , the electrostatic repulsion acted to reduce the hydrophobic attraction between  $\beta$ -sheets, leading to their limited lateral stacking and more twisting, and final fibrillar structures; in contrast, the repulsive force had little influence in the case of  $KI_4K$ , resulting in wide ribbons that eventually developed into nanotubes [25].

#### 4.2. Absence of the $\pi$ - $\pi$ interactions

On the other hand, in all the systems where  $\pi$ - $\pi$  interaction was not confirmed

experimentally, or was confirmed its inexistence, there was no difference in morphology of aromatic and aliphatic peptides. For example, Phe and Leu variants of the full-length hIAPP [34], hIAPP<sub>20-29</sub> [32] and hIAPP<sub>22-27</sub> fragment [29] both revealed classic fibrillar morphology. The inexistence of aromatic stacking interactions was confirmed by solid-state NMR spectroscopy (PDBid 2KIB) for the hIAPP<sub>20-29</sub> fragment [32]. The Phe to Leu substitutions also do not interfere with the fibril morphology in the full-length amyloid- $\beta$  [39].

The double Cha mutant at position 19 and 20 in the amyloid- $\beta_{16-22}$  fragment formed stiff tubular architecture in contrast to twisted fibers of the aromatic mutants [38]. In the wild-type of this peptide,  $\pi$ - $\pi$  interactions were confirmed experimentally for Phe19.

## 5. Kinetics and Mechanism

The substitutions of aromatic amino acids in amyloid peptides by non-aromatic ones generally decrease the rate of fibril formation and alter the tendency of fibrils to aggregate. Thus, while aromatic residues are not an absolute requirement for amyloid formation, they do play a role in the fibril assembly process.

The rate of amyloid formation was different for three single IAPP mutants; the rate was decreasing in the order F15L mutant, wild type, F23L mutant, and the F37L mutant [34]. The triple leucine mutant and the F23L/Y37L double mutant had the slowest kinetics [30, 34], Figure 4.

Figure 4: Triple mutant of IAPP forms amyloid significantly more slowly than wild-type IAPP. ThT fluorescence monitored kinetic assays of fibril formation by wild-type IAPP (squares) and IAPP-3XL (triangles). The solid line represents the best fit to a sigmoidal curve. Reprinted with permission from [30]. Copyright (2018) American Chemical Society.

Salmon calcitonin (sCT), having Leu residues (Leu12, Leu16 and Leu19) instead of Tyr12, Phe16 or Phe19 for human calcitonin (hCT), is known to form the fibrils much slower than hCT. In that context, hCT was mutated to Leu residues at the position of 16 (F16L), 19 (F19L), and 12, 16 and 19 (TL) to reveal the role of aromatic side-chains on amyloid fibrillation using solid-state <sup>13</sup>C NMR. The kinetics of hCT mutants were significantly slower than that of hCT. Therefore, it is evident that aromatic residues of Phe16, Phe19 and Tyr12 play important roles in fibril formation [40].

Self-assembly of peptides is a hierarchical set of events at multiple time and length scales driven by different molecular interactions at different stages [51, 52]. For example, in the study of nucleation and growth of supramolecular polypeptides through liquid-liquid phase separation observed in time by cryogenic transmission electron microscopy and Raman spectroscopy for detecting the  $\pi$ - $\pi$  interactions [53], it was concluded that hydrophobic interactions govern the formation of disordered droplets, while hydrogen bonding and  $\pi$ - $\pi$  stacking interactions predominate in the dynamic evolution of droplets to nanofibrils.

Mechanism and specific events are observed during the amyloid aggregation by MD simulations. In the process of hIAPP dimerization probed by REMD, transient  $\alpha$ -helical intermediates were detected, followed by conversion to  $\beta$ -sheet-rich structure. Simulations

showed that  $\alpha$ -helix-to- $\beta$ -sheet transition was accompanied by an increase of interpeptide contacts. The highest contact probabilities come from A13-F23 and F15-N22 contacts, while Phe-Phe interactions were limited, indicating that hydrophobicity is more important than direct aromatic stacking [37].

The study of the second core peptide from Alzheimer's amyloid- $\beta$  [24] with sequence KLVFFAE, with two phenylalanines, showed interesting results, very different from all other sequences examined in this study which did not contain subsequent phenylalanines (LIVAGD, IVD, NFGAIL, GGVVIA, DFNKF and RADARADARADARADA). This results are especially significant since the FF motif has been considered for a long time as the key motif for aggregation of amyloid- $\beta$  [23]. The results showed that naturally occurring peptides with one aromatic residue with sequences NFGAIL and DFNKF form amyloids via the same mechanism as designed synthetic aliphatic peptides with sequences LIVAGD and IVD. All these peptides form  $\alpha$ -helical intermediates during the process of aggregation. The KLVFFAE sequence did not form any  $\alpha$ -helical intermediates during the process of aggregation; it formed  $\beta$ -sheet aggregates without intermediate step. Also, it had the fastest cluster formation rate observed in simulations.

Also, aromatic-aromatic interactions were found to promote the pentapeptides connected to an aromatic motif (i.e., pyrene) transform from  $\alpha$ -helix to  $\beta$ -sheet conformation prior to self-assembly [54].

The  $\alpha$ -helix to  $\beta$ -strand transition occurs in the region which includes all the aromatic residues of the calcitonin sequence, Phe16, Phe19 and Phe22, the region Gly10-Phe22 [40]. The  $^{13}\text{C}$  chemical shifts of the labeled sites showed that the conformations of monomeric human calcitonin (hCT) mutants take  $\alpha$ -helices as viewed from the Gly10 moiety. The hCT mutants formed fibrils and during the fibril formation, the  $\alpha$ -helix around Gly10-Phe22 changed to the  $\beta$ -sheet, and the major structures around Ala26-Ala31 were random coil in the fibrils. Molecular dynamics simulation was performed for the  $\beta$ -sheet system of hCT9-23 and its mutants F16L, F19L and the triple mutant. In one of the stable fibril structures, Phe16 interacts with Phe19 of the next strand alternatively. In the hCT mutants, lack of Phe16-Phe19 interaction causes significant instability as compared with the hCT fibril, leading to the reduction of the rate aggregation constant value, as observed experimentally in the hCT mutants. Furthermore, the simulations show that the Phe16-Phe19 intersheet interactions are more stable than the electrostatic interactions between Asp15 and Lys18, even though electrostatic interactions are stronger than  $\pi-\pi$  interactions. This is because Asp and Lys side chains are more mobile as shown in the simulations.

One study [55] of the labeled DFNKF peptide and related peptides from calcitonin found that the conformational change from random coil to  $\beta$ -sheet occurred at the vicinity of the Phe2 residue in pentapeptide DFNKF. In the fibril structure formed by the full-length calcitonin, there was a head-to-tail anti-parallel  $\beta$ -sheet arrangement of the peptide chains. This arrangement had the phenyl rings of two strands (Phe16) facing each other on the same side of the  $\beta$ -sheet, located in such a manner that they could stack in parallel and make  $\pi-\pi$  interactions, thus helping to stabilize the fibril structure.

## 6. Thermodynamics

In the literature, the thermodynamic stability of amyloid aggregates is determined by the Gibb's free energy calculations. It is derived from the equilibrium monomer concentration through

the association equilibrium constant. The equilibrium monomer concentration is determined by various spectroscopic measurements after sedimentation or ultracentrifugation, like HPLC [38, 32, 43] or NMR techniques [46].

The  $\pi$ -stacking seems to exert a great influence in the thermodynamic stability. According to the experimental data, Phe19 residue in the A  $\beta_{16-22}$  fragment interacts with Phe19 of the neighbouring strand in the sheet, and Phe20 interacts only with aliphatic amino acids in its surrounding [38]. At the position Phe19, mutation with an aromatic and more hydrophobic residue F<sub>5</sub>-Phe stabilizes the peptide, and a non-aromatic Cha destabilizes it. The aromaticity has no effect in the position Phe20, as both aromatic and non-aromatic mutants exhibit increase in stability, probably because they are more hydrophobic than the wild-type peptide. The stability was assessed by Gibb's free energy measurements through a HPLC determined concentration of monomers at endpoint. The double Cha and F<sub>5</sub>-Phe mutants showed that the thermodynamic effects were additive.

Another peptide with the  $\pi$ - $\pi$  interaction present, shows the importance of these interactions in the thermodynamic stability [46]. The minimum at 204 nm in the CD spectra has been previously observed in cross- $\beta$  assemblies of (FKFE)<sub>n</sub> amphipathic peptides and has been attributed to  $\beta$ -stacking of the aromatic side chains. The amount of monomer remaining unincorporated into fibrils is significantly higher for (IKIK)<sub>2</sub> than for (FKFK)<sub>2</sub> at minimal assembly-inducing salt concentrations. This indicates that the equilibrium between fibril and monomer is more favorable for the Phe peptide, suggesting that aromatic amino acids exert a thermodynamic stabilizing effect on cross- $\beta$  fibril assemblies relative to nonaromatic counterparts. The role of Phe here is complex, as other properties like hydrophobicity and planarity must be taken into account.

Also,  $\pi$ - $\pi$  interactions are present in all the (FKFE)<sub>2</sub> peptides with varied amino acid sequence patterning, but here hydrophobicity seems to exert bigger influence than the aromaticity itself [43]. The most stable were the peptides with alternating aromatic/hydrophilic sequence patterning, FKFEFKFE and FKFKFEFE, relative to the sequence patterns that feature Phe-Phe dipeptide motifs separated by hydrophilic amino acid. Models based on previous experimental data shows that the peptides with the alternating sequence patterning possess exclusively hydrophobic and hydrophilic faces that result in bilayer assembly.

In the context of the hIAPP<sub>20-29</sub> without  $\pi$ - $\pi$  interactions, the only Phe residue was mutated by Tyr, Leu, Phe, F<sub>5</sub>-Phe, Trp, Cha, 1-Nap and 2-Nap. The Tyr, Leu and Trp mutants did not aggregate, Cha mutant fibrillized with similar thermodynamic stability relative to the wild-type, and the F<sub>5</sub>-Phe, 1-Nap, and 2-Nap variants self-assembled forming fibrils with greater thermodynamic stability than the wild-type peptide. Even though Tyr and Trp are aromatic, they are less hydrophobic than Phe. F<sub>5</sub>-Phe, 1-Nap and 2-Nap are planar and with greater hydrophobicity than Phe. These results indicate that the high amyloidogenicity of aromatic amino acids is a function of hydrophobicity,  $\beta$ -sheet propensity and planar geometry and not the ability to form stabilizing or directing  $\pi$ - $\pi$  bonds [32].

## 7. Interactions in molecular structures of amyloid peptides in Protein Data Bank

There are three broad classes that contribute to the stabilization of the native structure of proteins in general: hydrogen bonds, electrostatic interactions and van der Waals interactions. Aromatic-aromatic interactions form a fourth, and important, class [56]. These energetically favorable nonbonded interactions can be defined as pairs with the distances between ring centroids between 4.5 and 7 Å, angle between rings 30° to 90°, and free energies of formation between -0.6 and -1.3 kcal/mol. Although these energies are small contribution to the stability of the protein, they are comparable to the free energy of protein folding. The aromatic-aromatic interactions can also be important in amyloids, as special case of proteins.

By analyzing the aromatic-aromatic interactions in all amyloid structures published in the Protein Data Bank [57], it was established that aromatic amino acids are not present in every amyloid sequence, and thus they are not essential for amyloid self-assembly [58]. The aromatic-aromatic interactions in amyloids are less frequent than the aromatic-nonaromatic interactions.

Furthermore, there is a difference in the interactions between adjacent  $\beta$ -strands within the same  $\beta$ -sheet (intrasheet) and between opposite  $\beta$ -sheets (intersheet), Figure 5. The intersheet interactions are much less frequent and are never parallel: they represent inter-ring angle in the range of 30-40°. On the other hand, the intrasheet interactions are far more frequent and always parallel, however, they are probably the consequence of the steric condition inside a protein  $\beta$ -sheet built up by backbone hydrogen bonds.

Since our data indicate more frequent aromatic-nonaromatic than aromatic-aromatic interactions in amyloids, it seems that aromatic-aromatic  $\pi$ - $\pi$  interactions are not crucial for the amyloid formation.

Figure 5: Intersheet (left) and intrasheet (right) interaction within amyloid PDB structures. Adapted with permission from [58]. Copyright (2018) American Chemical Society.

Another interesting aspect is that interactions between Phe and Tyr or Trp rarely occur in amyloid structures though they are found in proteins. One should recognize that in amyloids aromatic-aromatic interactions are not very frequent. In addition the low possibility of Phe/Tyr interactions is probably a consequence of large tendency of Tyr to interact with the opposite sheet backbone by forming hydrogen bonds through its -OH group [58]. These interactions are quite strong, stronger than interactions of Tyr with the Phe, hence they determine the interactions of Tyr in amyloids. On the other hand there is quite small number of amyloid sequences containing Trp together with Phe or Tyr, making Trp interactions with Phe or Tyr not very likely.

## 8. Quantum chemical calculations on interaction energies

Theoretical studies explain experimentally observed data about importance of aromatic amino acids in amyloid structures using DFT calculations on interaction energies in amyloids. Our recent quantum chemical calculations [59] indicate that the interaction energies between two amyloid  $\beta$ -sheets are similar for amyloids with aromatic residues and nonaromatic amyloids (amyloids without aromatic residues), Table 2 and 3. These results support experimental findings that amyloid  $\beta$ -structures can be formed by peptides without aromatic amino acids.

Table 2: Evaluated interaction energies\* (in kcal mol<sup>-1</sup>) for different types of interactions between two tetramer  $\beta$ -sheets in model systems of amyloids containing aromatic amino acids. Interactions: Ar-Ar - aromatic-aromatic, Ar-nAr - aromatic-nonaromatic, nAr-nAr - nonaromatic-nonaromatic, nAr-B - nonaromatic-backbone, Ar-B - aromatic-backbone, B-B - backbone-backbone. Adapted from [59].

Structure	Sequence	Ar-Ar	Ar-nAr	nAr-nAr	nAr-B	Ar-B	B-B	total
2Y2A	KLVFFA	-11.69	-14.18	-4.72	-5.43	-5.08	-1.02	-42.12
2Y29	KLVFFA	-10.22	-15.23	-5.46	-5.00	-5.63	-0.87	-42.41
3OW9	KLVFFA	-9.16	-26.94	-4.53	-7.54	-5.85	1.36	-55.38
5E5V	NFGAILS	-2.76	-13.06	-9.55	-22.5	-38.89	-7.65	-94.41

\*Energies calculated at B3LYP-D3/6-31G\* level.

Table 3: Evaluated interaction energies\* (in kcal mol<sup>-1</sup>) for different types of interactions between two tetramer  $\beta$ -sheets in model systems of amyloids containing aromatic amino acids. Interactions: nAr-nAr - nonaromatic-nonaromatic, nAr-B - nonaromatic-backbone, B-B - backbone-backbone. 3Q2X<sub>mod</sub> is a 3Q2X model system modified to remove the influence of the hydrogen bonds, the Lys amino group was replaced by hydrogen atom. Adapted from [59].

Structure	Sequence	nAr-nAr	nAr-B	B-B	total
2Y3J	AIIGLM	6.54	-59.63	-3.56	-56.65
2Y3L	MVGGVVIA	3.78	-43.79	-5.50	-45.51
3Q2X	NKGAI	-19.14	-106.58	-8.35	-134.07
3Q2X <sub>mod</sub>	NK <sub>mod</sub> GAI	-16.49	-58.20	-8.35	-83.04

\*Energies calculated at B3LYP-D3/6-31G\* level.

In spite of similar interaction energies, different types of interactions are responsible for stability of amyloids with and without aromatic residues; the predominant interactions are aromatic-nonaromatic in the case of amyloids with aromatic amino acids, while the predominant interactions are nonaromatic-backbone in the case of nonaromatic amyloids.

Model systems were obtained by computational mutation of specific amino acids in a way that different side chains were depicted in the crystal structures such as an aromatic residue in aromatic or nonaromatic surrounding, or a nonaromatic residue in aromatic or nonaromatic surrounding. The possible contributions to the overall interaction energy between two  $\beta$ -sheets are schematically represented in the Figure 2 from the reference [59]. The density functional theory at the B3LYP-D3/6-31G\* level of theory was employed in calculations.

The results of the amyloids containing aromatic amino acids indicate that aromatic-nonaromatic interactions between  $\beta$ -sheets are in all investigated cases stronger than aromatic-aromatic interactions, Table 2. Relatively large aromatic-nonaromatic interaction energies are consequence of large number of nonaromatic groups surrounding aromatic residues. Moreover, it was shown that aromatic-nonaromatic interactions between benzene and cyclohexane can be quite strong [60].

It is interesting that for the 5E5V model system, that has longer sequence (NFGAILS) with



only one aromatic amino acid in the sequence, Ar/nAr interactions are still stronger than other interactions between side chains. The side chain-backbone interaction energies are significantly stronger for the 5E5V than in the other models probably because 5E5V has smaller inter-sheet distance comparing to the inter-sheet distance in other amyloids in Table 2.

Furthermore, in spite of polar amino acids present in the sequences of all studied peptides, practically all nonaromatic interactions are interactions of aliphatic side chains. Similar conclusion arises from the PDB search [58]: in aromatic containing amyloids, the aromatic-nonaromatic interactions, and especially aromatic-aliphatic ones, are more frequent than the aromatic-aromatic interactions.

The calculations on decomposition of surrounding energies in the nonaromatic systems (Table 3), show that the predominant interactions are nonaromatic-backbone interactions.

## 9. Role of aromatic amino acids in amyloid aggregation inhibition

A principal approach for the inhibition of amyloid formation is based on the use of modified molecular recognition elements. Therefore, a mutated amyloid with lower amyloidogenicity can be successful in inhibition. The mechanism underlying the inhibition could be based on the low amyloidogenic affinity of a peptide which still recognizes the parent peptide and therefore blocks further assembly [28, 32, 36], or increasing the amyloid solubility by introducing an inhibitor with hydrophilic groups [61].

Also, inhibitors containing aromatic groups have been synthesized to prevent amyloid- $\beta$  aggregation [62, 63]. The inhibitory action of short peptides or compounds containing aromatic groups is suggested to rely on  $\pi$ -stacking between the aromatic groups [39]. The small molecule inhibitors approach was initially based on early findings that demonstrated that small aromatic molecules such as Congo red and thioflavin T interact specifically with amyloid fibrils and inhibit their formation [19]. Structural analysis of the co-crystallization of the amyloidogenic insulin and Congo red showed that the phenyl ring in Congo red interacts with the aromatic moiety Phe24 of the insulin dimer, by specific aromatic interactions [64]. Curcumin, a biphenolic compound, is an inhibitor of  $\beta$ -sheet formation of the full-length IAPP [33]. Resveratrol, a natural polyphenol, has been reported to inhibit amyloid formation by IAPP and by amyloid- $\beta$  peptide. IAPP mutations of Phe15, or Tyr37 to Leu weakens the ability of resveratrol to inhibit amyloid formation by IAPP [65], pointing to the importance of the aromatic residues in inhibition. Phenol red compound has been shown to have concentration-dependent inhibition towards the full-length hIAPP. The ThT fluorescence assay demonstrated that higher phenol red concentrations (more than 4-fold greater than that of hIAPP) resulted in constant low fluorescence levels even after incubation for 1 week and an inhibition level of 90% was achieved [28]. The binding of phenol red molecules to the protofibrils of an amyloidogenic fragment (NFGAIL) of hIAPP has been investigated by molecular dynamics simulations. Through its three aromatic rings, the phenol red molecule preferentially interacted with the hydrophobic side chains of Phe, Leu, and Ile; and the polar sulfone and hydroxyl groups were mainly exposed in solvent. Thus, phenol red improves the solubility of the early protofibrils and represses further fibril growth [61].

The idea of using a non-amyloidogenic peptide that would be recognized by the original amyloidogenic peptide led to the aggregation assays with mutated IAPP fragments [28, 36, 32]. A molecular recognition assay using peptide array analysis suggested that molecular recognition between the full-length hIAPP and its 22-29 fragment (NFGAILSS), in which phenylalanine was substituted with tyrosine (NYGAILSS), is mediated by aromatic rather than hydrophobic

interactions [28]. On the basis of the molecular arrangement of the Phe-Phe interaction, it was suggested that the inhibition stems from the geometrical constraints of the benzene-phenol interaction. It was demonstrated in MD simulations of Tyr and Phe amino acids, that phenylalanine pairs are preferentially arranged in a parallel displaced geometry that is consistent with aromatic stacking. The simulation revealed one predominant (91% of the population) Phe-Phe energetically favored stacked state, with 4.5 Å distance between the two ring centroids, which is consistent with  $\beta$ -sheet stacking. On the other hand, the Phe-Tyr pair had 67% states with the angle between rings of 31° [66].

Our recent results of the crystallographic amyloid structures survey are in accordance with MD simulations showing extremely low number of Phe-Tyr (4) interactions in comparison to Phe-Phe (1328) [58]. The angle values of the Phe-Phe interactions were mostly planar and up to 30°, and the Phe-Tyr interactions were of angle 10-70°.

Peptides derived from the hIAPP22-29 fragment that contain phenylalanine analogs at position 23 with a variety of electron donating and withdrawing groups were studied [36]. Non-aggregating peptides were found to inhibit the self-assembly of the full-length hIAPP. These single electron rich substitution, p-amino- and p-formamido-phenylalanine peptides are more potent inhibitors than the previously reported NYGAILSS sequence [28]. A potential mechanism of inhibition was proposed to be the prevention of a flexible 20-29 loop formation required for assembly. Namely, assembly in the absence of inhibitory peptides is conducted via U-shaped structures, where the 20-29 region encompass a loop which connects each  $\beta$ -strand. Electron rich peptides bind to full-length amylin and enforce localized  $\beta$ -sheet structure in the 20-29 region. This localized structure limits flexibility and prevents the formation of the loop required for the conversion to U-shaped monomers.

Tryptophan also exhibits inhibitory effects. Trp variant of the hIAPP20-29 fragment interferes with the ability of IAPP20-29 to form fibrils either by binding to exposed fibril ends or keeping the wild-type peptide in the native state and thereby perturbing the equilibrium between fibril and monomer [32]. Although more hydrophobic than the wild-type Phe variant, the Trp mutant itself did not assemble probably because of ability of the indole side chain to participate in hydrogen-bonding interactions, either with nearby side chains or water, which perturbs the requisite  $\beta$ -sheet conformation or inhibits the hydrophobic collapse that precedes nucleation events. Larger steric profile of Trp relative to Phe might also be the molecular cause of self-assembly inhibition.

The importance of aromatic residues is observed in the fact that graphene could inhibit more efficiently the aromatic containing amyloids, than the aliphatic ones, based on our recent DFT study [67]. The interaction in amyloid-graphene aggregates can be stronger than the interactions for respective amyloid-amyloid aggregates. This is in accordance with the experimental observations that graphene and its modifications interact strongly with the aromatic amino acids in amyloid side-chains, and inhibit the aggregation of amyloid  $\beta$  fibrils [68, 69, 70, 71]. Aromaticity itself could not be considered the decisive factor that influences the interaction energies. A favorable conformation of the side-chains, leading to a larger number of contacts between the amyloids and graphene seems to exert even more decisive impact. In addition, the correlation of interaction energies with the number of interactions, and with the distance between the amyloid  $\beta$ -sheet and the graphene flake, indicate stronger interaction of aromatic rings with the graphene. The interactions in question are CH/ $\pi$  and NH/ $\pi$ , and close-to-stacking interactions with the interring angle of between 20° and 40°.

## 10 Conclusion

Aromatic amino acids are nonessential for amyloid formation, however, they enhance significantly the kinetics of formation, thermodynamic stability, and can influence the structure and morphology of amyloids. Peptides without aromatic amino acid residues, containing only aliphatic residues can also form amyloids.

Some peptides did not self-assembled without aromatic residues. However, using the right experimental conditions, the question is whether any protein sequence and of any length would form amyloid fibrils without aromatic residues. In the Morphology section we discussed that aromatic sequences form tape or ribbon-like fibrils, in contrast to the classical fibrillar morphology found in nonaromatic sequences. The remaining question for the future is the possibility of prediction of formation and tuning morphology in the light of aromatic amino acids role in amyloids.

Aromatic residues appear to be highly amyloidogenic due to an ideal combination of several properties: high hydrophobicity, low chain flexibility and  $\beta$ -sheet propensity in addition to a planar steric profile that is readily accommodated in 'steric zipper' structures in laminated  $\beta$ -sheets. Aromaticity is also important in the context of directional  $\pi$ -stacking interactions that could contribute entropically and energetically to the stability of amyloid structures.

The analysis of geometries and energies of the amyloid PDB structures indicate the importance of aromatic-nonaromatic interactions and confirm that presence of aromatic amino acids is driving force for the amyloid formation. However, aromatic amino acids are not crucial for amyloid formation, since sequences without aromatic amino acids can form amyloids. On the other hand it is still not clear which nonaromatic residues are responsible for the stability of the amyloids and it should be the resolved in future research.

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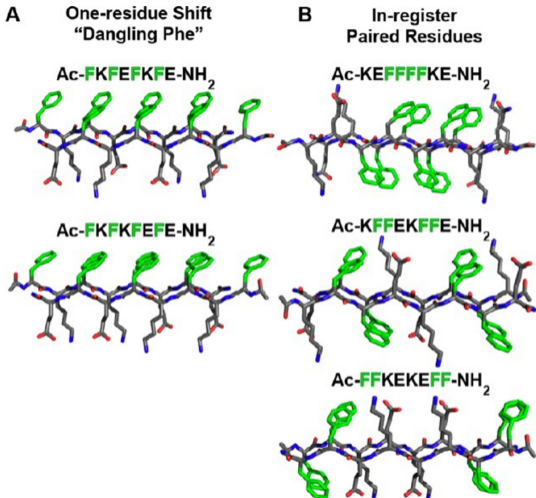


Figure 1

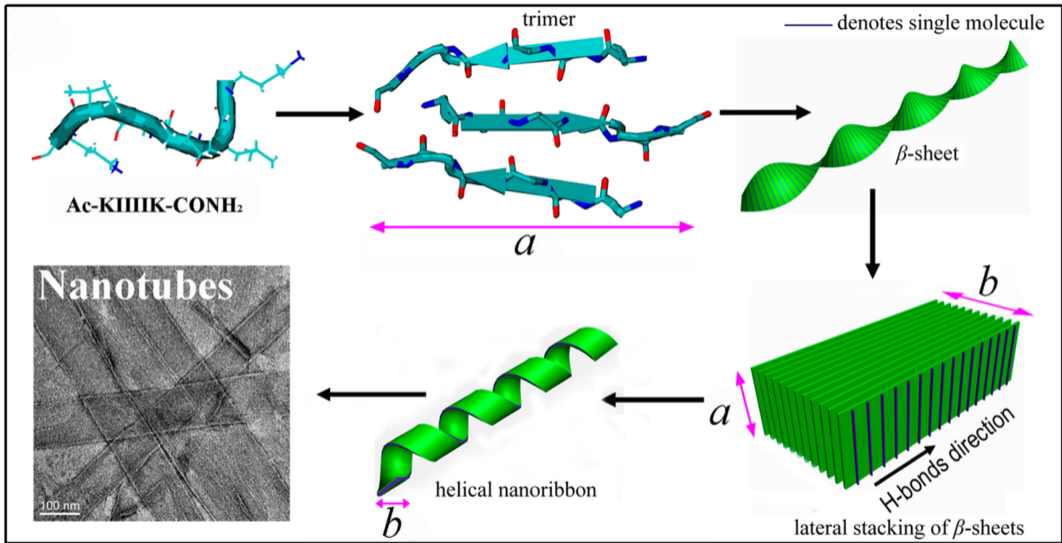


Figure 2

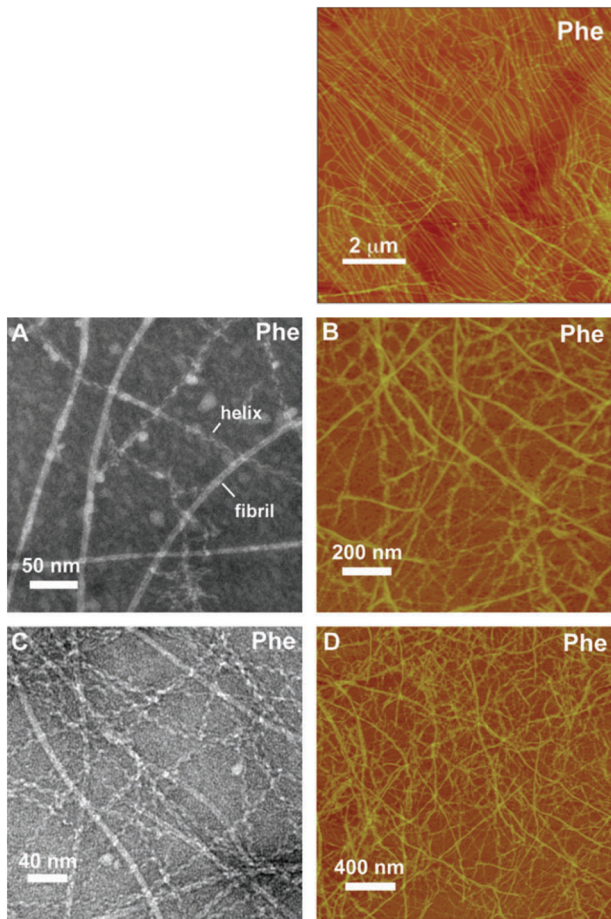


Figure 3

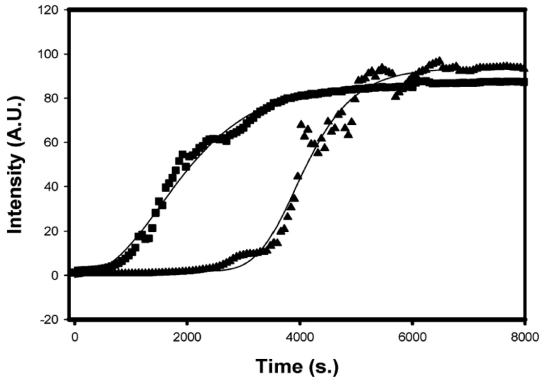


Figure 4



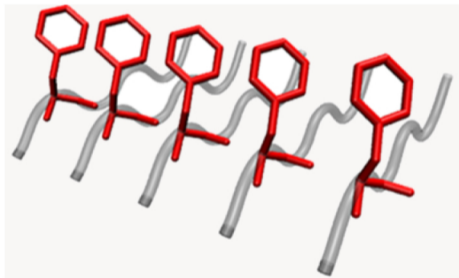
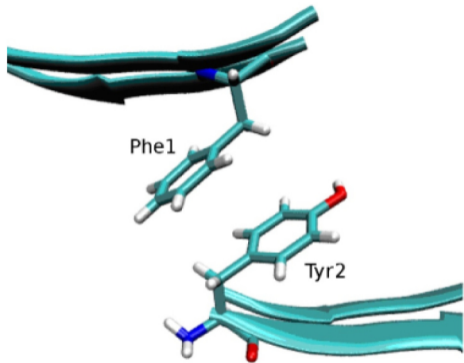


Figure 5