Molecular Self-Assembly of Peptide Nanostructures: Mechanism of Association and Potential Uses

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Abstract: Molecular self-assembly offers unique directions for the fabrication of novel supramolecular structures and advanced materials. The inspiration for the development of such structures is often derived from self-assembling modules in biology, as natural systems form complex structures from simple building blocks such as amino acids, nucleic acids and lipids. Peptide-based nanostructures indicate an important route toward the production of ordered nanostructures as several studies had demonstrated their ability to form well organized assemblies. This includes cyclic peptides designed with alternating D- and L- amino acids, amphiphile peptides, peptide-conjugates and ionic self-complementary peptides. A naturally occurring self-assembly process of nano scale objects by polypeptides is that of amyloid fibril formation. These 7-10 nm fibrillar assemblies were already used for the formation of conductive nanowires. Short peptides have been used as model systems to study the molecular mechanism that leads to amyloid fibril formation. Based on the analysis of short amyloid forming fragments, it was recently suggested by our group and others that aromatic interactions may play a significant role in the process of amyloid fibrils formation in several cases. This hypothesis led to the discovery that the core recognition motif of the Alzheimer's β-amyloid polypeptide, the diphenylalanine element, has all the molecular information needed to self assemble into a novel class of peptide nanotubes. A highly similar analog and the simplest aromatic dipeptide, the diphenylglycine, forms spherical nanometric assemblies. Both designed and peptide fragment nanostructures were suggested to have many applications in various fields including molecular electronics, tissue engineering, and material science.

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Ehud Gazit received his B.Sc. (*summa cum laude*) in 1991, under the framework of Tel Aviv University Special Program for Outstanding Students. His Ph.D. (*with distinction*) was awarded in 1997 for his thesis performed under the guidance of Prof. Yechiel Shai at the Department of Membrane Research and Biophysics, Weizmann Institute of Science. In the years 1997-2000 he performed postdoctoral research under the guidance of Prof. Robert T. Sauer at the Massachusetts Institute of Technology (MIT). Ehud Gazit joint Tel Aviv University as a faculty member in October 2000 and he is currently an Associate Professor at the Department of Molecular Microbiology and Biotechnology. Ehud Gazit also holds a visit appointment at the Center of Biomedical Engineering of MIT. During his career Ehud Gazit was awarded with several awards including the John F. Kennedy Award and the Landau Research Award.

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BIOMOLECULAR SELF-ASSEMBLY

Nature forms complex multicomponent three-dimensional structures through spontaneous association of molecules termed "molecular self-assembly" [1]. The self- assembly process is mediated through weak intermolecular bonds, such as van der waals bonds, electrostatic interactions, hydrogen bonds and stacking interactions. These relatively low energy interactions are combined together to form intact and well-ordered supramolecular structures. In recent years, there is a great interest in the fabrication of new materials using natural building blocks such as nucleic acids, phospholipids, and amino acids. In order to accomplish this goal, a better understanding of the biochemical and biophysical nature of the molecular self-assembly process is needed. Such studies should lead to the ability to manipulate the self-assembly processes in artificial systems. In this review, we will focus on peptide-based self-assembled nanostructures with a special emphasis on amyloid-related

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peptide elements. The amino acids that constitute the peptide motifs have unique properties over other building blocks in nature as they represent a large variety of chemical moieties that enable facile chemical and biological modifications. We will describe the great effort invested in order to exploit these well-ordered nanostructures in various fields that include molecular electronics, drug delivery and material science.

Peptide-Based Nanostructures

Peptide building blocks had been used for the assembly of nano-ordered material already a decade ago when Ghadiri and co-workers were the first to describe a new class of organic nanotubes based on rationally designed cyclic polypeptides. These cyclic peptides were designed with an alternating even number of D- and L- amino acids, which interact through hydrogen bonding into an array of self-assembled nanotubes. The internal diameter of the nanotubes ranges between 7-8 Å and can be controlled by changing the number of the amino acids in the cyclic peptide sequence [2-3]. Various applications were offered for these tubular structures. One of the first application was based on their membrane interactions. As the cyclic peptide nanotubes are toxic to bacteria, they were demonstrated to serve as novel antibiotic agents [4]. Other potential applications include drug delivery, as these structures can serve as nanocontainers and application in material sciences, since new composite material can be formed by nucleation of inorganic materials onto the peptide structures [5].

Another cyclic peptide that was later reported to self assemble into tubular structures is the Lanreotide octapeptide, which is synthesized as a growth hormone inhibitor. It was found that Lanreotide can self-assemble into tubular structures with nanometric dimensions in aqueous solution. These nanotubes are suggested to self-assemble through segregation of aliphatic/aromatic residues that contribute to the conversion into β-sheet fibrils [6].

Peptide-based tubular structures can also self-assemble by linear peptides as was demonstrated by a family of bolaamphiphiles peptides by Matsui and co-workers (Fig. **1A**, **2A**). In these structures, two hydrophilic peptides are conjugated through hydrophobic peptide linker. The driving force for the formation of these structures is most likely the intermolecular association of the hydrophobic moieties in the aqueous solution to form ordered structures similar to a micellization process.

Tubular structures self-assembled by one of these peptides, bis(N-α-amido-glycylglycine)-1, 7-heptane dicarboxylate, were used as a template to form metal wires [7]. These nanotubes were also immobilized *via* hydrogen bonds onto Au substrates functionalized with self-assembled monolayer of 4-mercaptobenzoic acid. Another approach for such immobilization was performed by coating the peptide nanotubes with avidin and binding them to gold surfaces that were treated with biotinylated self-assembled monolayer [8]. The immobilization of the nanotubes onto Au electrodes and metallization by electroless deposition may enable their use in nano electronics [9]. Moreover, these tubular structures were used as an anchor for 12 amino acids peptide with high content of histidine residues and therefore high affinity to nickel ions. In this manner, nickel nanocrystals were grown

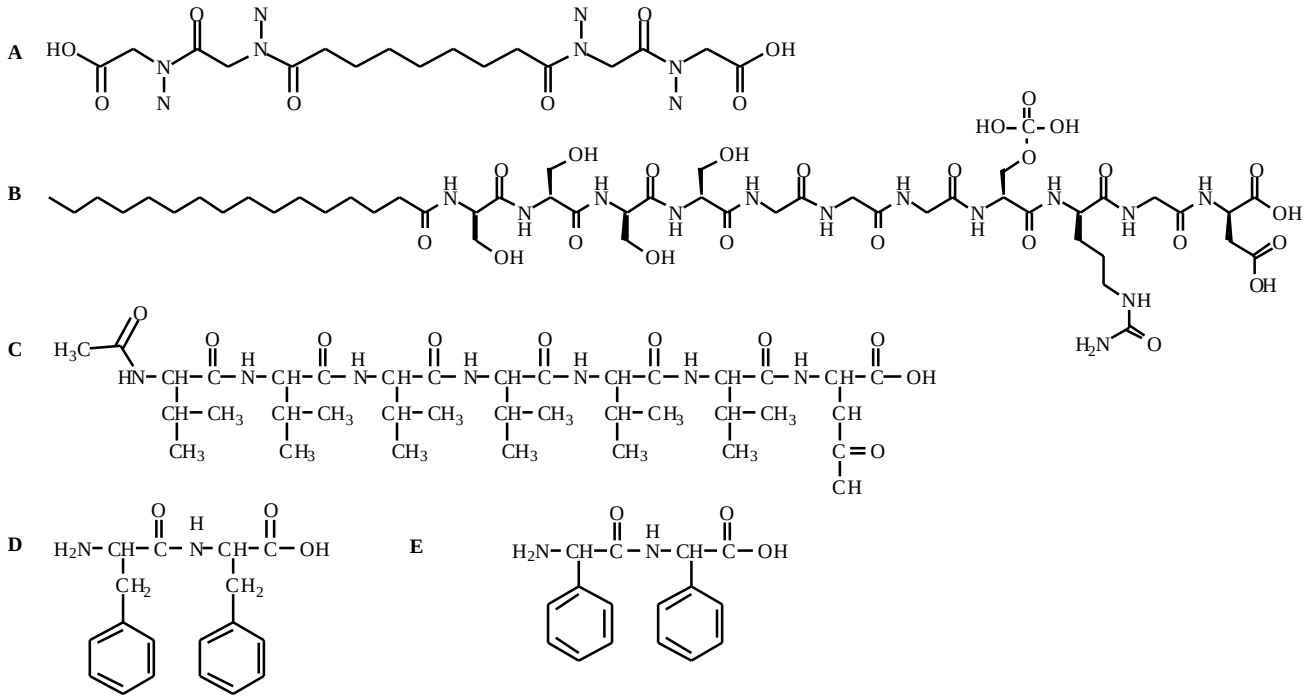

Fig. (1). Chemical structures of the various self-assembling peptides and peptides-conjugates. A. The linear structure of the bolaamphiphiles peptide-conjugate, bis(N-α-amido-glycylglycine)-1,7-heptane dicarboxylate. **B**. Peptide-conjugate amphiphile composed of hydrophilic peptide with hydrophobic alkyl tail **C.** The V6D1 surfactant-like linear peptides. **D.** the diphenylalanine peptide **E.** The diphenylglycine peptide.

onto the nanotube surface resulting in magnetic tubes that can be used as building blocks for magnetic devices and recording media [10]. For other applications such as microelectronic and sensors, Cu nanocrystals can be nucleated onto the nanotubes using the same approach [11].

A parallel molecular direction for the assembly of nano scale peptide structures that was developed independently by Stupp and co-workers is based on the conjugation of single hydrophilic peptide motif with hydrophobic alkyl tail to form amphiphile peptide (Fig. **1B**, **2A**). As the bolaamphiphiles peptide-conjugates, these peptide-conjugates self-assemble into well-ordered nanofibers. The nanofibers can nucleate calcium ions resulting in mineralization of hydroxyapatite as it contains phosphorylated serine residues. Cysteine residues and RGD (Arg-Gly-Asp) were also incorporated in the sequence; Cysteine residues allow covalent bonding between adjacent molecules and the RGD sequence promotes adhesion and growth of cells. The resulted nanofibers resemble the lowest level of hierarchical organization of bone in which collagen and hydroxyapatite interact together [12]. These peptide-amphiphiles can be used as artificial three-dimensional scaffolds as the self-assembled nanofibers form fibrillar network. In order to encourage cell differentiation, adequate peptide sequences were incorporated [13].

The use of short peptides with high affinity to inorganic material was also demonstrated by using phage-display technology to create magnetic, semi-conductive and conductive nanowires. Short peptides were displayed on the phage pIII or pVIII coat proteins and were selected for their affinity to a specific material with the desired properties. Nanowires were formed by incubation of the phages displaying the selected peptide with the specific material it nucleates [14].

Later studies by Zhang and co-workers have demonstrated the ability to use non-conjugated peptide as building blocks to form ordered nano scale structures. These surfactant-like linear peptides are characterized by well-defined hydrophilic and hydrophobic residues (Fig. **1C**, **2B**). These peptides are composed of 7-8 residues and have similar properties to other biological surfactant. The peptides are characterized by a hydrophilic head composed of aspartic acid and a tail composed of hydrophobic amino acids such as alanine, valine, or leucine. When dissolved in water, these peptides self-assemble by hydrophobic interactions into well-ordered nanotubes and nanovesicles [15].

Earlier approach by Zhang and co-workers for the assembly of non-conjugated peptide, was based not only on hydrophobicity, but mainly on self-complementary ionic peptides (Fig. **2C**). These linear peptides are highly (>50%)

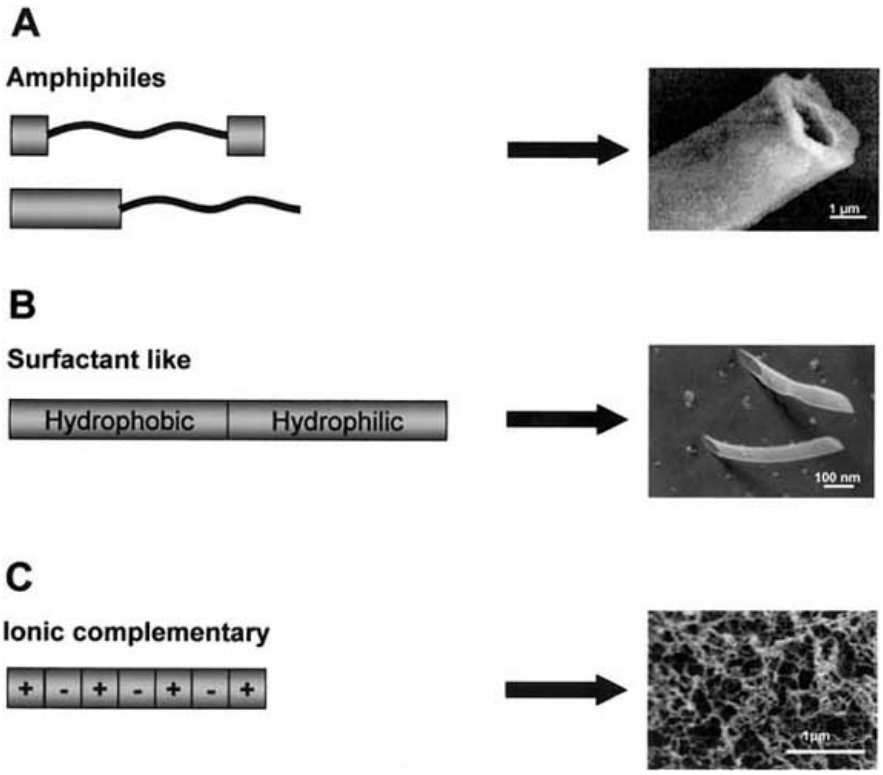

Fig. (2). Self-assembly of nano-scale object by peptides and peptide-conjugates. A. On the right, schematic representation of two families of amphipile peptide conjugates, the bolaamphiphiles peptides and peptide conjugated with hydrophobic alkyl tail. On the left, scanning electron micrograph of bolaamphiphile tubule decorated with gold nanocrystal. (Reproduced with permission from [7] Copyright (2000) American Chemical Society, U.S.A.); **B.** On the right, schematic representation of surfactant like peptide, on the left, quick-freeze deep-etch TEM image of self assembled tubular structures formed by A6D and V6D, which belong to the surfactant-like peptides (Reproduced permission from [15] Copyright (2002) National Academy of Sciences, U.S.A.); **C.** On the right, schematic representation of ionic self-complementary peptides. On the left, scanning electron micrograph of self assembled fibrillar scaffold form by ionic self-complementary peptides (Reproduced permission from [16] Copyright (2000) National Academy of Sciences, U.S.A.).

charged with opposing charges using alternately basic and acidic amino acids, based on molecular organization of a segment within the Zuotin yeast protein. Moreover, the peptides are arranged to contain two distinct surfaces namely the hydrophilic and the hydrophobic. These peptides adopt β-sheet conformation and self-assemble into nanofibers. In some cases, these nanofibers further form a hydrogel, which allows extensive neurite outgrowth and therefore can be used as a scaffold for tissue engineering [16].

Amyloid Fibril Formation and Their Applications as Nanostructures

Amyloid fibrils are a key example of protein self-assembly into ordered nano-scale fibrillar structures in nature (Fig. **3A**). The accumulation of amyloid fibrils is a common characteristic of several unrelated diseases including Alzheimer's disease, Type II diabetes and prion diseases [17-23]. All these diseases are characterized by the trans-formation of soluble proteins into aggregated fibrillar deposits in different organs and tissues. The pathological significance of amyloid fibril formation is not completely understood in all cases. One such example is aortic medial amyloid deposits that occur virtually in all individuals older than 60 years, but its medical consequence is not known yet [24]. Although different amyloid-forming proteins do not share clear sequence homology, the fibrillar structures that are being formed have very similar physicochemical and ultrastructural characteristics as determined by transmission electron microscopy, x-ray fiber diffraction, and other biophysical techniques [17-23]. Furthermore, amyloid fibrils are formed *in vitro* also by disease-unrelated proteins [25-29]. This finding suggests that amyloid fibrils may serve as a generic structural form of aggregated proteins [19, 22]. Nevertheless, despite the significant medical importance of the amyloid fibril formation process and its consideration as a universal structural form, the exact mechanism that leads to the self-assembly of polypeptides into ordered fibrils is not fully understood.

Amyloid fibrils were suggested as building blocks for nano electronics as the N-terminal and middle region (NM) of yeast *saccharomyces cerevisiae* Sup35 were used as a template for the production of conductive nanowires. NM forms β-sheet-rich amyloid fibrils with a diameter of about 10 nm. Their small diameter together with their chemical stability upon proteases and denaturizing agents make them favorable for their use as templates for conducting nanowires. By genetic engineering, the NM sequence was modified to contain cysteine residues. These residues were used to bind gold nanoparticles along the self-assembled NM fibrils. Enhancement protocol was later performed in order to get continous conductive nanowires [30].

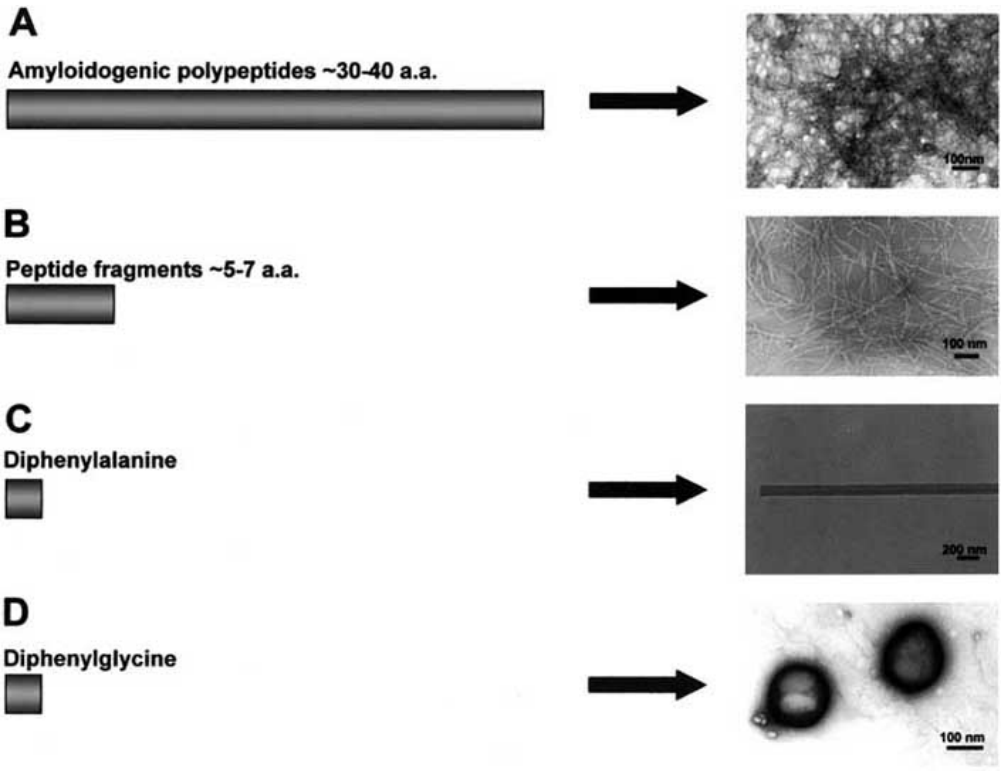

Fig. (3). Self-assembly of amyloid polypeptides, amyloid fragments, and core dipeptides into nanoscale objects. A. Transmission Electron micrographs of amyloid fibrils form by the full length Islet Amyloid Polypeptide (IAPP), which form fibrillar deposits in the pancreas of type 2 diabetes patients. This morphology is a common characteristic for all amyloid fibrils formed by different polypeptides; **B.** Schematic representation of the relative size of amyloid forming peptide fragments. Transmission Electron micrographs of fibrils formed by the NFGSVQ peptide fragment from Medin; **C.** Schematic representation of the relative size of nano-objects forming dipeptide fragments. Transmission electron micrographs of peptide nanotubes that are form by the diphenylalanine peptide; **D.** Further simplification of the aromatic dipeptide motif. Transmission electron micrographs of peptide nanospheres that are formed by the diphenylglycine peptide.

Short Peptide as a Model for Amyloid Fibril Formation

New insights regarding the determinants that mediate the self-assembly of the various polypeptides into amyloid fibrils have a key impact both from the medical point of view as well as the technological one. Thus, much attention is devoted to the study of minimal amyloid forming fragments (Fig. **3B**). As short peptides are easy to design and synthesize, they serve as an excellent model system for studying amyloid fibrils formation in particular and biological self-assembly processes in general.

A very short peptide fragment that can form well-ordered amyloid fibrils *in vitro* is the NFGAIL hexapeptide fragment of the islet amyloid polypeptide (IAPP). The morphology of the fibrils formed by this peptide is similar to that formed by its correspondent full-length parent molecule. A shorter fragment, FGAIL, also formed fibrillar structures, but those structures had different morphology as compared to longer amyloidogenic peptides [31]. Amyloid-fibrils composed of the IAPP are found in the postmortem of nearly all patients with Type II diabetes [32-34]. The importance of the phenylalanine residue in amyloid fibrils formation by a minimal amyloid-forming fragment of IAPP was previously demonstrated, using an alanine-scan [35]. The substitution of the phenylalanine, but not of any other residue by an alanine, abolished the ability of the fragment to form amyloid fibrils *in vitro* [35]. Based on this observation, the remarkable occurrence of aromatic residues in other short amyloid related sequences, and the well-known role of stacking interactions in processes of self-assembly, we suggested that stacking of aromatic residues may play a role in the acceleration of the process of amyloid fibrils formation [35-37].

Based on this hypothesis, we later discovered two minimal active amyloidogenic peptides; The NFLVH fragment [38] of the hIAPP and the NFGSVQ fragment, which derived from a peptide component of the aortic medial amyloid, a form of localized amyloid that occurs in virtually all individuals older than 60 years [32,39]. These short sequences have remarkable similarly to the NFGAIL fragment of the hIAPP. When our research was extended to learn about the fertilization mechanism of the human hormone, calcitonin, we identified a pentapeptide fragment of human calcitonin, NH_2-DFNKF-COOH, that can form well-ordered amyloid fibrils, similar to those formed by the full length human calcitonin [40]. This fragment was identified based on our hypothesis regarding the central role of aromatic residues in amyloid fibrils formation, the effect of pH on the fibrillization of human calcitonin and the analysis of conformationally constrained analogues of the hormone [41]. This was the first reported case of a peptide as short as a pentapeptide that can form amyloid fibrils with the same ultrastructural properties as much longer polypeptides. A shorter truncated tetrapeptide, NH_2-DFNK-COOH, also formed fibrils albeit less ordered than those formed by the pentapeptide. As these short amyloidogenic fragments are relatively hydrophilic, we showed that there is no correlation between the hydrophobicity of a peptide and its amyloidogenic potential [40]. These results got further support when Johansson *et al.* studied the ability of charged tetrapeptides to form amyloid fibrils and suggested that hydrophobic interactions are not sufficient for fibril formation [42].

Modification of short peptide fragments resulted in the formation of ordered structures in the nano scale. Recent research on the ability of tripeptides to form amyloid fibrils

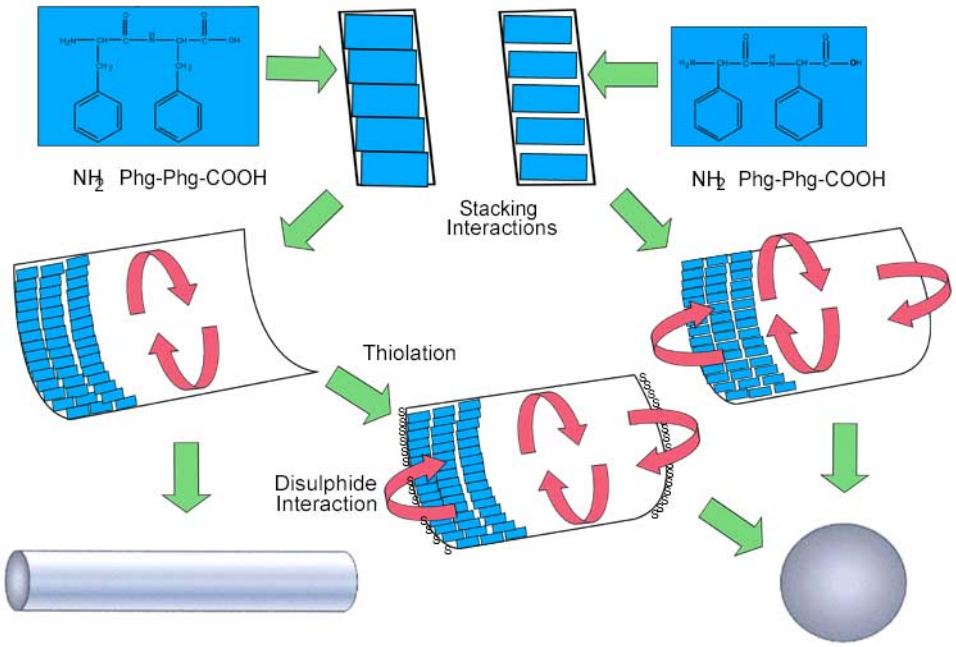

Fig. (4). Schematic illustration of the self-assembly mechanism of the diphenylalanine and diphenylglycine peptides into nanotubes and nanospheres. A stacking interaction between aromatic moieties of the peptides is suggested to provide energetic contribution as well as order and directionality for the initial interaction to form extended pleated sheet that is stabilized by hydrogen bonds and aromatic stacking interactions. The formation of the tubular structures may occur by a closure of the extended sheet along one axis of the two dimensional layer. Alternatively, the formation of spherical structure may result from a closure of the sheet along two axes. The introduction of a thiol group may assist the closure at the second axis. (Reproduced permission from [53] Copyright 2004, American Chemical Society).

revealed that three tripeptides: Boc-Ala-Aib-Val-OMe, Boc-Ala-Aib-Ile-OMe and Boc-Ala-Gly-Val-OMe can self-associate to form supermolecular β-sheet structures and further aggregates into amyloid-like fibrils [43]. Other studies had indicated the ability of bis scaffold of pentapeptide, derived from the prion repeat, to form well-ordered fibrils in the nano-scale [44].

From Amyloid Structures to Peptide Nanotubes

Motivated to find the minimal amyloidogenic fragment and based on our insights into the role of aromatic residues in the process of amyloid formation, we became highly interested in the molecular properties of a peptide fragment corresponding to the core recognition motif of the Alzheimer's β-amyloid polypeptide, the diphenylalanine aromatic module (Fig. **1D**, **3C**). This motif is of special interest since several studies identified the ability of larger peptides and conjugated organic molecules that contain this motif to inhibit fibrils formation by Aβ [45-46]. Some of those inhibitors are currently under clinical trails as potential drugs to treat Alzheimer's disease. We found that two amino acid peptide, the diphenylalanine motif, is sufficient to self-assemble into a novel class of peptide nanotubes (Fig. **3C**) [47]. These peptide nanotubes are formed as individual entities, have a long persistence length and their proteolytic liability allows their use as nanoscale casting mold for metal nanowires [47]. It was also found that these peptide nanotubes are remarkably rigid with a Young modulus of 19 GPa [48]. The discovery of these peptide nanotubes was followed by the fabrication of peptide-nanotubes platinum-nanoparticle composites from the enzymatically stable tubes [47] self-assembled by the $_D$-Phe-$_D$-Phe dipeptide [49]. Other studies had indicated the potential use of the peptide nanotubes in electrochemical biosensing [50]. It was also demonstrated in an independent study that a large fragment of β-amyloid can form similar nanotubular assemblies [51]. We later revealed that modification of the charged termini of the diphenylalanine peptides, by acetylation of the N-terminal amine and amidation of the C-terminal carboxyl, results in the formation of peptide nanotubes of similar ultrastructural properties as compared to the non-modified dipeptide. This further supports the role of the aromatic moieties, rather than the charged one, in the formation of the peptide nanotubes [52]. Other amine modified diphenylalanine peptides, such as Boc-Phe-Phe, self-assembled into amyloid-like structures. This finding may have implication in the field of material science as these peptides can be used as a low cost source for the fabrication of amyloid-like fibrils that can later be used as a template for conductive nanowires. [52]

When extended our studies on aromatic dipeptides, we discovered that diphenylglycine, a highly similar analogue and the simplest aromatic peptide, forms spherical nanometric assemblies (Fig. **1E**, **3D**). As the nanotubes, the nanospheres assemble efficiently and have remarkable stability. Nanosphere structures were also self-assembled when a thiol group was introduced into the diphenylalanine peptide. This modification alters the diphenylalanine assembly from tubular to spherical particles similar to those formed by diphenylglycine [53].

SUMMARY

Recent studies had brought new challenges and directions towards the understanding of peptide self-assembly at the molecular level. It was demonstrated that peptides could be used in various ways to engineer complex nanostructures for different applications such as molecular electronics, tissue engineering, and drug delivery. Peptides hold a great promise in the "bottom up" approach due to their low cost, their simplicity and the ability to easily decorate them with chemical and biological elements.

There is yet a great challenge to figure out the consistency of peptide self assembly process. Once this question is answered, it will open numerous possibilities for nanotechnological applications.

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