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Formation of Closed-Cage Nanostructures by Self-Assembly of Aromatic Dipeptides

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ABSTRACT

We recently demonstrated that the diphenylalanine recognition motif of the Alzheimer's β-amyloid polypeptide self-assembles into ordered and discrete nanotubes. Here, we reveal that diphenylglycine, a highly similar analogue and the simplest aromatic peptide, forms spherical nanometric assemblies. As the nanotubes, the nanospheres assemble efficiently and have remarkable stability. The introduction of a thiol group into the diphenylalanine peptide alters its assembly from tubular to spherical particles similar to those formed by diphenylglycine. The formation of either nanotubes or closed-cages by fundamentally similar peptides is consistent with a two-dimensional layer closure, as described both for carbon and inorganic nanotubes and their corresponding buckminsterfullerene and fullerene-like structures.

The discovery in 1985 of the novel nanometric closed-caged form of carbon, known as fullerenes or buckminsterfullerene, marks a key finding in physics, material science, and nanotechnology.[1] This finding was followed six years later by the discovery in 1991 of the highly related carbon tubular nanostructures that were denoted as carbon nanotubes.[2] Other studies revealed also that inorganic material, including MoS_2, WS_2, $CdCl_2$, and $TlCl_3$, could alternatively assemble into either nanotubular or fullerene-like nanoscale structures.[3−6] These various organic and inorganic self-assembled nanostructures were suggested to have key potential in nanotechnological devices and applications.[5−10] Other studies have shown the ability of much larger bioorganic molecules, including peptides, lipids, nucleic acids, and even phage particles, to self-assemble into uniform and well-ordered structures of nanometric dimensions.[11−20] Biomolecular nanostructures are an especially intriguing group of supramolecular assemblies as they provide a large range of chemical modifications. Moreover, these nanostructures allow the utilization of the specificity of biological systems for biosensing, catalytic activity, and highly specific molecular recognition processes. Several studies have shown the potential application of bionanometric material for applications ranging from molecular electronic and quantum dot orientation to antibacterial activity and drug delivery.[11−23] The controlled self-assembly of the biomolecular nanostructure, preferably from the simplest building blocks possible, is therefore of great interest.

We recently revealed the formation of well-ordered and discrete peptide nanotubes by self-assembly of the diphenylalanine core recognition motif of Alzheimer's β-amyloid polypeptide[24] (Figure 1a). These nanotubes, which are made of aromatic dipeptides as building blocks, show remarkable similarity to aromatic carbon nanotubes in their existence as discrete and stiff structures of a high persistence length.[24] We have demonstrated the ability of these tubes to serve as a degradable nanoscale mold to fabricate metallic nanostructures of high aspect ratio.[24] The ability of the simplest naturally occurring aromatic dipeptide to self-assemble into well-ordered suparmolecular structures is consistent with our suggestion for the key role of aromatic stacking interactions in many cases of ordered amyloid fibril formation.[25−28] We assume that the geometrically restricted interactions between aromatic moieties may provide both an energetic contribution as well as the order and directionality needed for the formation of very well-ordered amyloid fibrils in a process that was termed as "one-dimensional crystallization".[29] Our recent results are also consistent with the suggestion made by the late Max Perutz and co-workers suggesting that amyloid fibrils are water-filled peptide nanotubes.[30]

Here, in our search for the simplest biomolecular self-assembled system, we extended our studies to characterize the most generic form of an aromatic dipeptide, the diphenylglycine (Figure 1b). The diphenylglycine offers molecular properties similar to the diphenylalanine peptide, albeit its molecular structure is more rigid with a lower degree of freedom due to the lack of rotational freedom around the additional C−C bond and the higher steric hindrance of the

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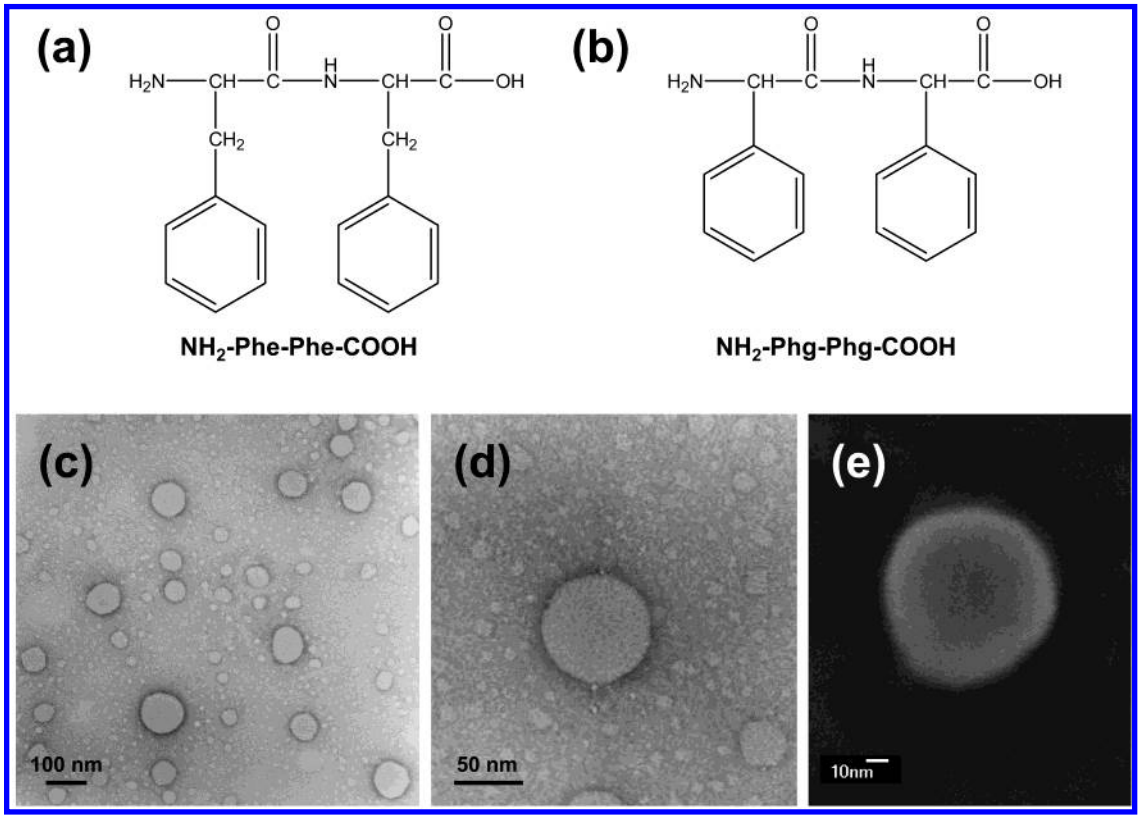

Figure 1. Self-assembly of spherical nanometric structures by the simplest aromatic peptide, diphenylglycine. (a) The diphenylalanine motif, the central core of the β-amyloid polypeptide, which forms discrete well-ordered peptide nanotubes. (b) Schematic presentation of the simplest aromatic dipeptide, the diphenylglycine peptide. (c) Low magnification transmission electron microscopy (TEM) image of negatively stained nanospheres formed by the diphenyglycine peptide. (d) High magnification TEM image of the negatively stained nanosphere. (e) High magnification (400,000×) cold field emission gun (CFEG) high-resolution scanning electron microscope (HR−SEM) image of the nanospheres formed by the diphenyglycine peptide.

molecule. Structural analysis using TEM (transmission electron microscopy) revealed that under the same conditions in which peptide nanotubes were formed by the diphenylalanine, spherical nanometric structures self-assembled by the diphenylglycine peptide (Figure 1c,d). These nanometric particles exist as individual entities and have a uniform spherical appearance as seen by TEM visualization (Figure 1d). The assembly of the spherical particles was very efficient and regular, as could be seen using low magnification TEM analysis (Figure 1c). The efficiency and regularity are similar to those observed with the peptide nanotubes.[24]

To further examine the three-dimensional characteristics of the novel nanoparticles, they were subjected to analysis by SEM (scanning electron microscopy). Cold field emission gun (CFEG) high-resolution scanning electron microscope (HR−SEM) confirmed the three-dimensional spherical shape and the regularity of the self-assembled nanostructures (Figure 1e). In addition, we used AFM (atomic force microscopy) analysis in order to get an independent indication about the topography of nanostrucutres. The AFM analysis clearly confirmed the three-dimensional spherical configuration of the nanostructures (Figure 2a, b). While AFM is a less suitable tool to determine the exact dimensions of the structures at the horizontal and vertical axes due to tip convolution, it is an excellent method to determine the height of nanostructures at the Z-range. Indeed, AFM analysis clearly indicated that the spheres are about 90 nm in height (Figure 2b), which is consistent with both TEM and SEM analysis.

During our studies on the ability of the diphenylalanine peptide nanotubes to serve as a mold for the fabrication of metal nanowires, we observed their complete stability toward extensive boiling.[24] This property is highly useful for future industrial nanotechnological utilization of the nanostructures and is consistent with a well-ordered molecular arrangement. Here, we extended our studies to determine the stability of the nanoparticles also under extreme chemical conditions (Figure 3). The nanospheres were found to be stable under acidic conditions after incubation for 5 h at 10% TFA as they maintained their configuration and uniform structure (Figure 3a). Their stability under alkaline conditions was also tested when the nanospheres were subjected to 1 M NaOH for 5 h (Figure 3b). In the presence of NaOH the nanosphere structure appears to be more uniform while having a smaller diameter. This remarkable stability of the nanoparticles is very intriguing both from the scientific point of view as well as the technological one. The significant stability of the peptide nanostructures is rare but consistent with the structural stability of amyloid fibrils, as was recently reported.[22] This is in line with our motivation for the initiation of our studies that stemmed from the apparent role of peptide motifs in the molecular recognition and self-assembly of amyloid fibrils. Moreover, the unusual stability of the peptide nanostructures is extremely useful for their use as part of a

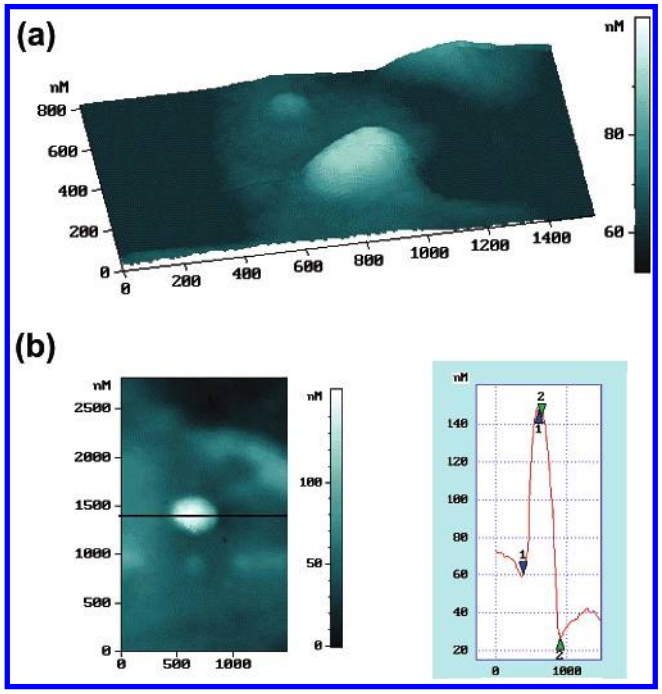

Figure 2. Structural analysis of the self-assembled nanospheres. (a) Three-dimensional AFM topography image of the nanospheres. (b) The nanospheres height was analyzed from atomic force microscopy (AFM) topography image.

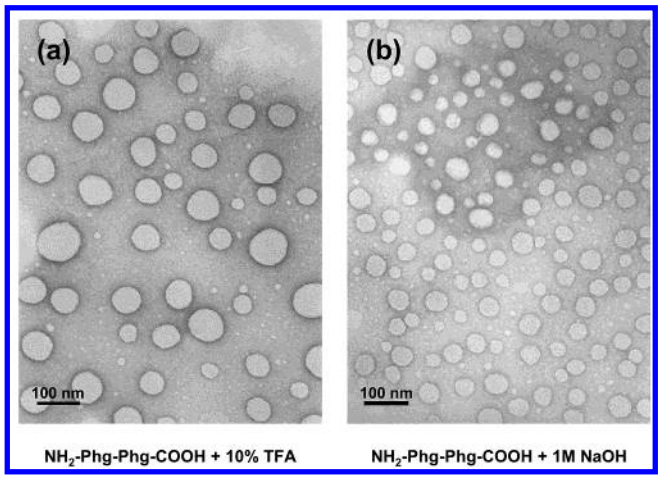

Figure 3. The nanostructures are stable at extreme chemical conditions, as seen by TEM. Self-assembled nanospheres were incubated in the presence of strong acid or base. (a) The nanospheres after 5 h incubation at 10% TFA. (b) The nanospheres after 5 h incubation at 1 M NaOH.

combined (bio)organic and/or inorganic nanoscale fabrication process, including optic and electron-beam lithographic protocols. Although biologically based scaffolds offer many advantages to nanotechnology, their relative instability in general questions their ability to serve in robust and long-lasting nanodevices. The newly described peptide nanostructures offer both molecular recognition and chemical flexibility of biological nanoobjects, together with stability that is compatible with industrial procedures and the requirements for robust and stable devices.

In parallel experiments of a different path, we studied the ability of the cysteine-diphenylalanine tripeptide (CFF, Figure 4a) to form peptide nanotubes. The rationale behind these studies was to introduce a thiol group into the nanotubes that would allow their covalent attachment to fabricated gold electrodes in nanodevices. However, we discovered that the CFF peptide does not self-assemble into nanotubes but rather into nanospheres that are extremely similar to those formed by the diphenylglycine peptide (Figures 4b, c). To study whether the spherical structures that were formed by the CFF peptide were the result of the peptide length or rather the presence of the thiol group, we chemically modified an amine to a thiol in the context of the diphenylalanine peptide (Figure 4d). For that purpose, we used 2-iminothiolane (Traut's reagent), which reacts with the single primary amine in the diphenylglycine and introduces a sulfhydryl group. We reacted the peptide with the reagent in organic solvent mixture that was then followed by dilution into an aqueous solution that allowed the self-assembly process. Clearly, the addition of a thiol group to the diphenylalnine peptide transformed the geometry of the assembled structures from nanotubular into spherical ones (Figure 4d). As a control, we used the same reaction mixture but without the addition of the *N*,*N*-diisopropylethylamine base that is required for the reaction. Under these conditions only nanotubular structures were observed.

The study of inorganic nanotubes and fullerene-like structures, as described above,[3−6] indicated that the formation of fullerenes is not unique to carbon and is attributed to a genuine property of two-dimensional (layered) compounds.[3−6] Based on the correlation between the peptide nanotubes and carbon nanotubes, we previously suggested that the novel type of peptide nanotubes is being formed by a closure of a two-dimensional layer.[24] Our current results provide further experimental support to this notion. It appears that the energetic contribution provided by the disulfide bridge formation may allow closure of the two-dimensional layer into more closely packed spherical structures. The difference in the geometrical constraints between the short peptide may direct either mode of assembly (Figure 5).

Taken together, our recent studies clearly suggest that aromatic peptide assemblies represent a novel class of nanostructures that are mechanistically closely related to aromatic carbon nanotubes and fullerenes and to their related inorganic nanotubes and fullerene-like structures. Applications, methodologies, and theories that were applied to the study of carbon and inorganic nanostructures should be of great importance for future exploration and utilization of the peptide nanostructures. These properties of the peptide nanostructures, taken together with their biological compatibility and remarkable thermal and chemical stability, may provide very important tools for future nanotechnology applications.

Materials and Methods. *Materials.* The diphenylalanine and diphenylglycine peptides were purchase from Bachem (Bubendorf, Switzerland). The CFF peptide was purchase from SynPep (Dublin, CA). Fresh stock solutions of the diphenylalanine and the diphenylglycine were prepared by dissolving lyophilized form of the peptides in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP, Sigma) at a concentration of

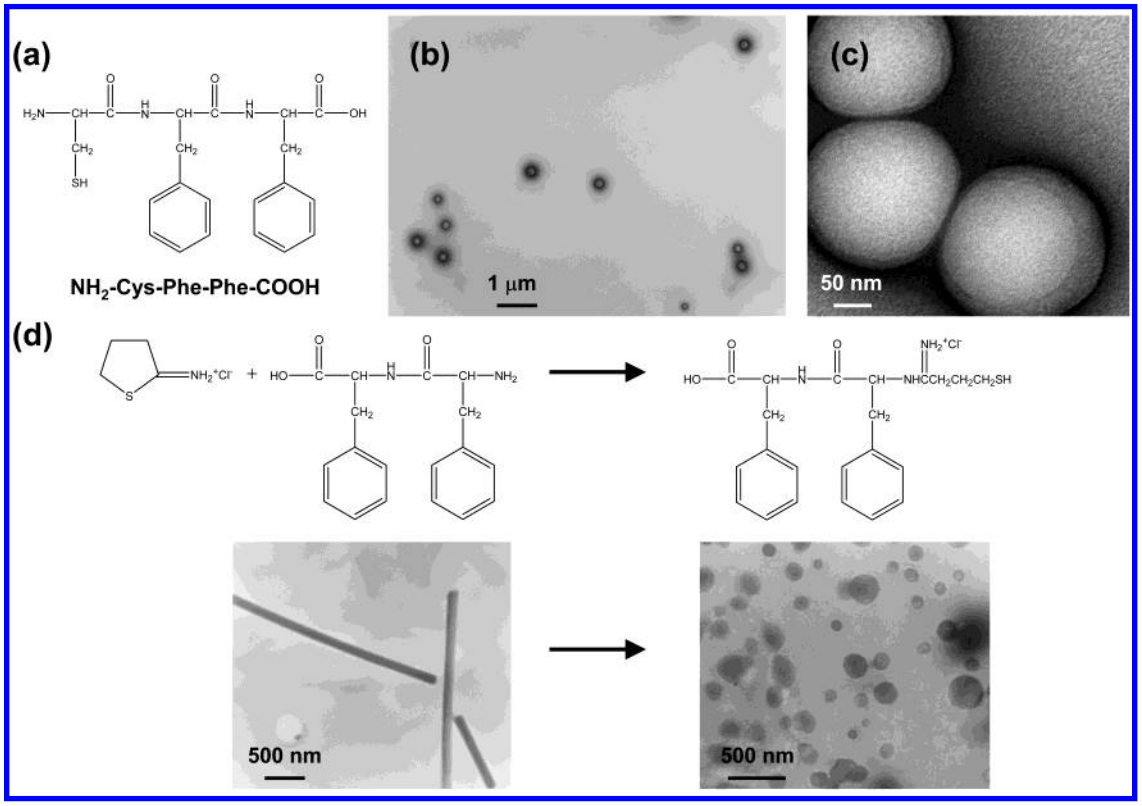

Figure 4. Similar nanosphere structures are formed in the presence of a thiol group. (a) Schematic presentation of the Cys−Phe−Phe (CFF) tripeptide. (b) Low magnification TEM microphage of the nanospheres formed by the CFF peptide. (c) High magnification HR-TEM microphage of the nanospheres formed by the CFF peptide. (d) Schematic presentation of the chemical reaction that modifies an amine to a thiol in the context of the diphenylalanine peptide. On the left, low magnification TEM microphage of the nanotubes formed by the FF peptide. On the right, low magnification TEM microphage of the nanospheres formed by FF peptide that self-assembled in the presence of 2-iminothiolane.

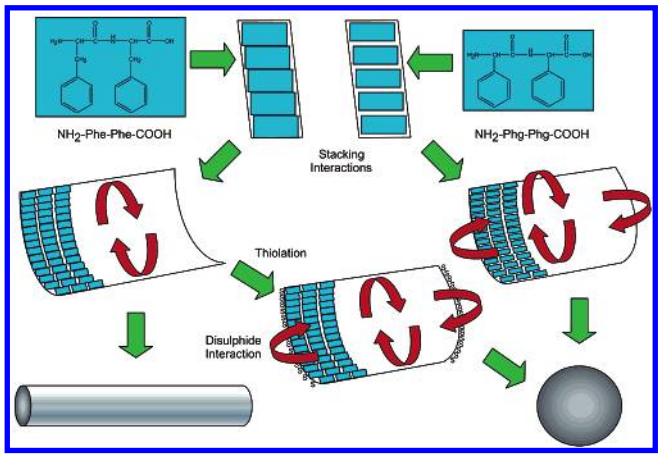

Figure 5. A model for alternative assembly of tubular and spherical peptide nanostructures. A stacking interaction between aromatic moieties of the peptides is suggested to provide energetic contribution as well as order and directionality for the initial interaction to form an extended pleated sheet 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 structures 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.

100 mg/mL. The CFF peptide was prepared by dissolving the lyophilized form of the peptide in HFP and 25% dithiothreitol, 1 M in ddH_2O to a final concentration of 25 mg\mL. To avoid any preaggregation, fresh stock solutions were prepared for each experiment. The peptides stock solutions were diluted into a final concentration of 2 mg/mL in ddH_2O.

Chemical Modification of an Amine to a Thiol. The diphenylalnine peptide was dissolved in HFP to a concentration of 100 mg/mL. This was followed by the addition of 2 μL of the solution to 8 μL of 100 mg/mL 2-iminothiolane (Sigma) dissolved in dimethyl sulfoxide (DMSO) with 2% *N*,*N*−diisopropylethylamine (DIAE). Double-distilled water was added to give a final peptide concentration of 2 mg/mL. Two control reactions were carried out to exclude the role of the reaction mixture on the assembly of the peptides; in the first control experiment the reaction mixture was prepared without the addition of DIAE. In the second control experiment, the reaction mixture was prepared without the addition of DIAE and 2-iminothiolane.

Transmission Electron Microscopy. After 24 h incubation at room temperature, a 10 μL aliquot of the peptide solution was placed on a 200 mesh copper grid. After 1 min, excess fluid was removed. In negative staining experiments, the grid was stained with 2% uranyl acetate in water and after two minutes excess fluid was removed from the grid. Samples from the chemical reaction that modifies amines to thiols were not negatively stained with uranyl acetate. Samples were viewed using a JEOL 1200EX electron microscope

operating at 80 kV. In the case of HR-TEM, a Philips Tecnai F20 field emission gun microscope was used.

Atomic Force Microscopy. AFM samples were prepared by drying the peptide solutions on TEM grids, without the staining procedure. Semicontact mode imaging was performed on a P47 solver−NT-MDT (Moscow, Russia), by using OTESP integrated cantilever probes with resonance frequency 390 kHz.

High-Resolution Scanning Electron Microscopy. TEM grids that were used for AFM analysis were viewed using a JSM-6700 field emission scanning electron microscope equipped with a cold field emission gun operating at 1 kV.

Stability in Alkaline and Acidic Conditions. In the case of stability to alkaline conditions, NaOH was added into the peptide nanosphere solution to a final concentration of 1 M NaOH. In the case of stability in acidic conditions, TFA was added to the nanostructure solution to a final concentration of 10% TFA. After 5 h peptide solutions were placed on TEM grids and analyzed by TEM.

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