Video Article

Formation of Ordered Biomolecular Structures by the Self-assembly of Short Peptides

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Abstract

In nature, complex functional structures are formed by the self-assembly of biomolecules under mild conditions. Understanding the forces that control self-assembly and mimicking this process *in vitro* will bring about major advances in the areas of materials science and nanotechnology. Among the available biological building blocks, peptides have several advantages as they present substantial diversity, their synthesis in large scale is straightforward, and they can easily be modified with biological and chemical entities[1,2]. Several classes of designed peptides such as cyclic peptides, amphiphile peptides and peptide-conjugates self-assemble into ordered structures in solution. Homoaromatic dipeptides, are a class of short self-assembled peptides that contain all the molecular information needed to form ordered structures such as nanotubes, spheres and fibrils[3-8]. A large variety of these peptides is commercially available.

This paper presents a procedure that leads to the formation of ordered structures by the self-assembly of homoaromatic peptides. The protocol requires only commercial reagents and basic laboratory equipment. In addition, the paper describes some of the methods available for the characterization of peptide-based assemblies. These methods include electron and atomic force microscopy and Fourier-Transform Infrared Spectroscopy (FT-IR). Moreover, the manuscript demonstrates the blending of peptides (coassembly) and the formation of a "beads on a string"-like structure by this process.[9] The protocols presented here can be adapted to other classes of peptides or biological building blocks and can potentially lead to the discovery of new peptide-based structures and to better control of their assembly.

Video Link

The video component of this article can be found at http://www.jove.com/video/50946/

Introduction

Nature forms ordered and functional structures by the process of biomolecular self-assembly. Understanding the forces that govern this spontaneous process may lead to the ability to mimic self-assembly *in vitro* and consequently to major advances in the area of material sciences[10,11]. Peptides, specifically, hold great promise as a biomolecular building block, since they present large structural diversity, ease of chemical synthesis, and can easily be functionalized with biological and chemical entities. The field of peptide self-assembly was pioneered by Ghadiri and his colleagues, who demonstrated the self-assembly of peptide nanotubes by cyclic peptides with alternating D- and L-amino acids[12]. Other successful approaches to the design of peptide assemblies include linear bolaamphiphile peptides[5,] amphiphiles (AP)[6], nonconjugated self-complementary ionic peptides[13], surfactant-like peptides[4,14], and diblock copolypeptides[15.]

A more recent approach involves the self-assembly of short aromatic peptides, termed homoaromatic dipeptides. These peptides comprise only two amino acids with aromatic nature (*e.g.* Phe-Phe, tert-butyl dicarbonate (Boc)-Phe-Phe)[7,8,16-21]. The structures formed by these homoaromatic peptides include tubular structures, spheres, sheet-like assemblies and fibers[6,8,15,21-32]. The fibers in some cases generate a fibril mesh that yields a hydrogel[33-37]. These assemblies have been exploited for applications of biosensing, drug delivery, molecular electronics, *etc.*[38-45]

This paper describes the experimental steps needed in order to start the spontaneous self-assembly of homoaromatic peptides. In addition, it presents the process of peptide coassembly. This process involves the self-assembly of more than one type of peptide monomer.

Our demonstration includes the coassembly of two commercially available peptides: the diphenylalanine peptide (NH_2-Phe-Phe-COOH) and its Boc protected analogue (Boc-Phe-Phe-OH). Each of the peptides self-assembles into a supermolecular structure: the diphenylalanine peptide forms tubular assemblies and the Boc-Phe-Phe-OH peptide self-assembles into either spheres or fibers depending on the solvent[7,17,46]. We blended the two peptides in certain ratios and characterized the resulted assemblies by electron microscopy, force microscopy and FT-IR spectroscopy. The methods demonstrated the formation of a peptide-based structure which is comprised of spherical elements with a diameter of several microns (1-4 μm) that are connected by elongated assemblies with a diameter of a few hundred nanometers (~300-800 nm). The assemblies resemble beaded strings in their morphology, as the spherical structures seem to be threaded on the elongated assemblies. We therefore termed these assemblies "biomolecular necklaces". The "biomolecular necklaces" might serve as a new biomaterial, as a drug delivery agent or as a scaffold for electronic applications. Moreover, the procedure that leads to the self-assembly of peptides may be utilized with other

classes of peptides and biomolecules. It may lead to a better understanding of the forces involved in self-assembly and the formation of new ordered structures.

Protocol

1. Self-assembly of Homoaromatic Dipeptides

1. Weigh the desired peptide in its lyophilized form (*e.g.* NH_2-Phe-Phe-OH, Boc-Phe-Phe-COOH) and prepare a stock solution by dissolving the peptide in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) to the appropriate concentration (*e.g.* 100 mg/ml for NH_2-Phe-Phe-OH and Boc-Phe-Phe-COOH)[7,17,46].
2. Mix the solution using vortex and place on the bench until the peptide is completely dissolved and the solution seems clear (a few minutes).
3. Dilute the peptide stock solution, with a suitable solvent, to the appropriate concentration (*e.g.* 2 mg/ml of NH_2-Phe-Phe-OH in triple distilled water (TDW) for the formation of nanotubes; by adding 2 µl of the peptide stock solution to 98 µl TDW, 5 mg/ml of Boc-Phe-Phe-COOH in ethanol for the formation of spherical structures).
4. Keep the solution at RT for 24 hr.
5. In order to avoid any preaggregation, prepare fresh stock solutions for each experiment.

2. Coassembly of Two Homoaromatic Dipeptides

1. Prepare a solution of 50% ethanol by mixing equal volumes of TDW and absolute ethanol. Use vortex to mix the two solutions.
2. Weigh 2 mg of the NH_2-Phe-Phe-OH peptide and 1 mg of the Boc-Phe-Phe-OH peptide. Dissolve each peptide in HFP to a concentration of 100 mg/ml.
3. Mix the peptides stock solutions using vortex and place them on the bench until the peptides are completely dissolved and the solutions seem clear.
4. Blend the peptides stock solutions to the desired ratio. In this specific experiment blend 10 µl of the NH_2-Phe-Phe-OH peptide with 6 µl of the Boc-Phe-Phe-OH peptide (to a final ratio of 5:3 respectively). Due to the high volatility of the HFP solvent, it is recommended to prepare a large amount of this stock solution (at least 10 µl).
5. Use vortex to mix the blended peptides stock solution.
6. Dilute the blended peptides stock solution with 50% ethanol to the desired final concentration. In this specific experiment, in order to obtain a final concentration of 5 mg/ml for NH_2-Phe-Phe-OH and 3 mg/ml for Boc-Phe-Phe-OH respectively, add 8 µl of the blended peptides stock solution to 92 µl of the 50% ethanol solution. Use a pipette to gently mix the solution.
7. Keep the solution at RT for 24 hr.
8. It should be noted that due to the highly volatile nature of the solvent, the experiments are sensitive to small changes in the concentration of the peptides. Therefore, fresh stock solutions should be prepared for each experiment.

3. Characterization of the Self Assembled Structures Using Scanning Electron Microscopy (SEM)

1. After 24 hr of incubation, apply a 10 µl drop of the peptides solution on a glass cover slip and dry at RT.
2. Coat the sample on the glass with a thin layer of gold (a few nanometers) using a sputter coater for 90 sec.
3. Image the assemblies using SEM operating at 10-20 kV.

4. Characterization of the Self Assembled Structures Using Transmission Electron Microscopy (TEM)

1. Place a 10 µl drop of the peptides solution on a 200-mesh copper grid covered with carbon and stabilized by a polymer film support.
2. After 1 min remove the excess fluid using filter paper.
3. Prepare a solution of 2% uranyl acetate in TDW. Filter the solution using 0.22 µm filter unit.
4. To stain the sample (negative staining), place a drop of 10 µl uranyl acetate solution on the grid.
5. After 30 sec remove excess fluid using filter paper. It should be noted that although negative staining improves the contrast of the images, it is not essential in all cases.
6. Image the sample on the grid by TEM operating at 120 kV.

5. Three-dimensional Characterization of the Assemblies by Atomic Force Microscopy (AFM)

1. Prepare a sample for the AFM analysis using the procedure described in paragraph 3.1.
2. Analyze the sample on the glass using an AFM instrument working in AC mode. Use silicon cantilevers with a spring constant of 3 N/m and a resonant frequency of 75 kHz.
3. Start by scanning a large area of the grid, in order to find the desired structure. Then focus on a specific smaller area and scan it (the scan size was 2.5 µm x 2.5 µm for the image included in this manuscript).

6. Characterization of the Secondary Structure by FT-IR

1. Apply a 30 µl drop of the peptides solution to a CaF_2 window.
2. Allow the solution to dry at RT.
3. The adsorption of water in the IR spectrum is at 1,650 cm^{-1}. This peak is at the center of the amide I band of the peptide bond. It is also a typical peak for α-helical structures of peptides and proteins[47]. In order to overcome this problem and avoid the signal of water, a hydrogen-to-deuterium exchange must be performed. Place a drop of deuterium oxide (D_2O) on the dried peptide sample. The drop should be large enough to completely cover the peptide deposit on the window.
4. Allow the sample to dry under vacuum.
5. Repeat steps 6.3 and 6.4 2x to ensure maximal hydrogen-to-deuterium exchange. Save the sample under vacuum until its analysis.
6. Record the FT-IR spectra using a deuterated triglycine sulfate (DTGS) detector. The FT-IR system includes a purge gas generator, in order to prevent humidity in the surroundings of the sample. For samples of short peptides, it is best to scan the sample 2,000x at a resolution of 4 cm^{-1}. The transmittance minimal values can be determined by the software supplied with the instrument.

Representative Results

This paper describes a method for the formation of ordered structures at the nano-and micrometer scale by the self-assembly of peptides. In order to demonstrate this simple process we present and characterize the coassembly of two simple aromatic peptides (**Figure 1**). One of the peptides is the NH_2-Phe-Phe-OH (diphenylalanine) peptide, which can self-assemble in an aqueous solution into hollow tubular structures with nanometric dimensions[7]. The other peptide is its Boc protected analogue, Boc-Phe-Phe-OH. This peptide can form fibrillar structures in aqueous solutions and spherical assemblies in ethanol[17,46]. We assumed that these peptides would coassemble into a structure that combines the two elements mentioned. Using SEM analysis, we revealed that the blended peptides formed an architecture of spherical assemblies with a diameter of several microns connected by elongated structures with a diameter of a few hundred nanometers (**Figure 2**). Due to the high resemblance in morphology to beaded strings, we termed these structures "molecular necklaces". AFM analysis of these structures clearly demonstrated their three-dimensional arrangement (**Figure 3**). In addition, SEM analysis of different regions of various samples indicated that this process occurred with high yield (**Figure 2b**).

FT-IR analysis provided information on the secondary structure of the peptides assemblies. The absorbance spectrum of the amide I band of the spherical assemblies formed by the peptide Boc-Phe-Phe-OH (5 mg/ml, 50% ethanol) showed a single amide I peak at 1,657 cm^{-1} indicating an α helix conformation. The tubular structures formed by the NH_2-Phe-Phe-OH peptide (2 mg/ml, 50% ethanol) showed two distinctive peaks, one at 1,613 cm^{-1} and the other at 1,682 cm^{-1}. These peaks correlated with a β-sheet secondary structure. The FT-IR spectrum of the biomolecular necklaces, formed by the coassembly of the two peptides, differed from the assignment for each individual peptide as it comprised two peaks: one peak at 1,653 cm^{-1} which corresponds with an α helix structure and another peak at 1,684 cm^{-1} which relates to a β-turn conformation (**Figure 4**)[48]. The difference between the various spectra indicates a unique structure for biomolecular necklaces.

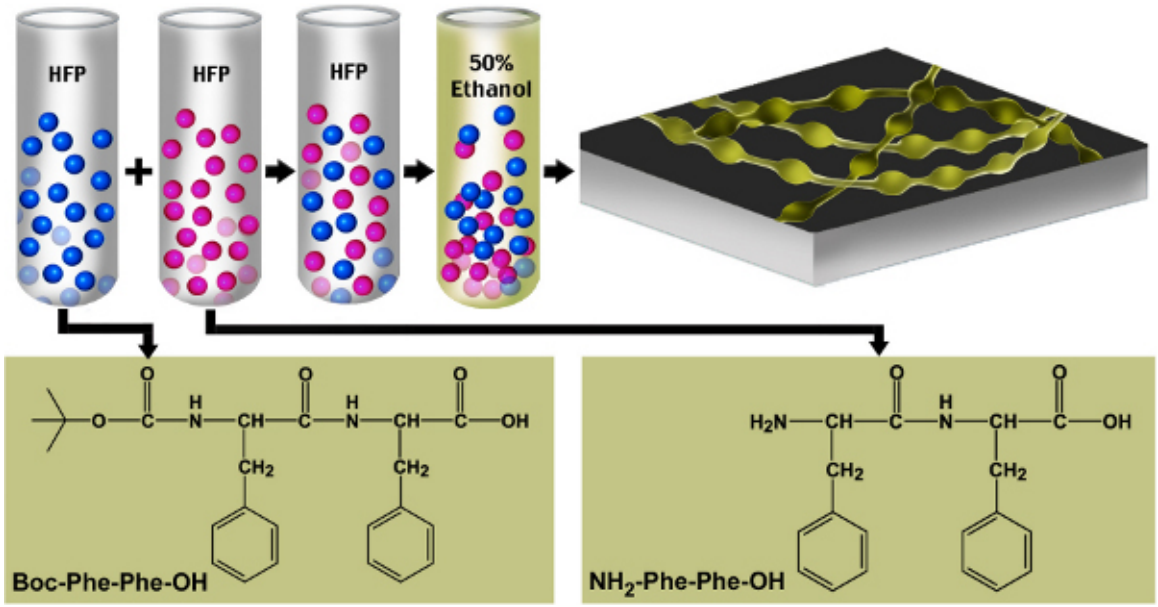

Figure 1. Coassembly of the peptides NH_2-Phe-Phe-OH and Boc-Phe-Phe-OH. Schematic illustration of the coassembly process.

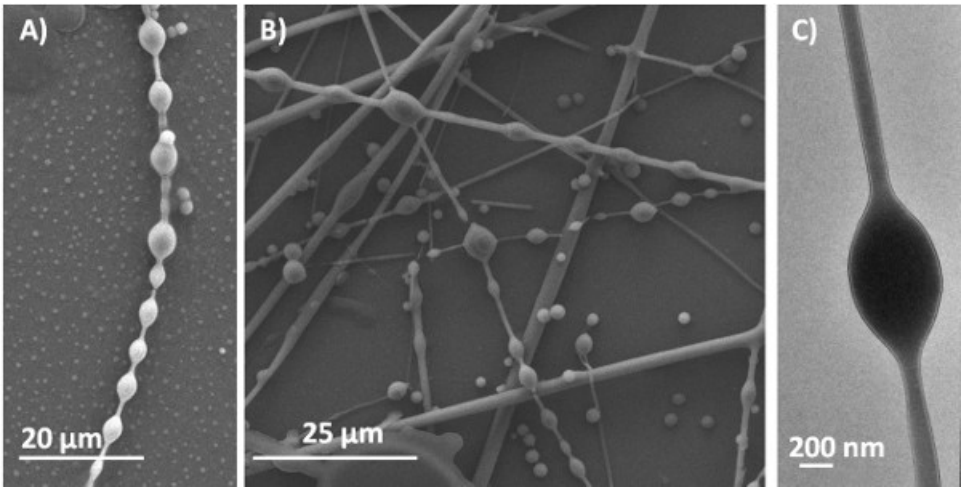

Figure 2. Electron microscopy analysis of the molecular necklaces; A) and **B**) SEM micrographs; **C**) A TEM micrograph.

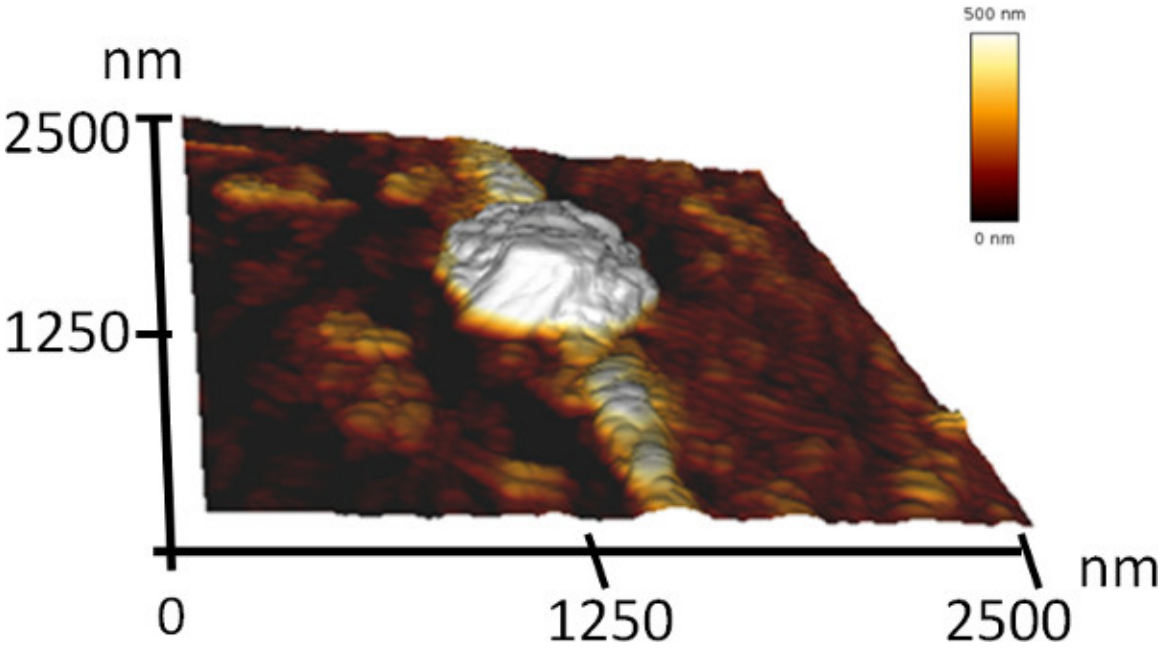

Figure 3. Three-dimensional AFM topography image of the molecular necklaces.

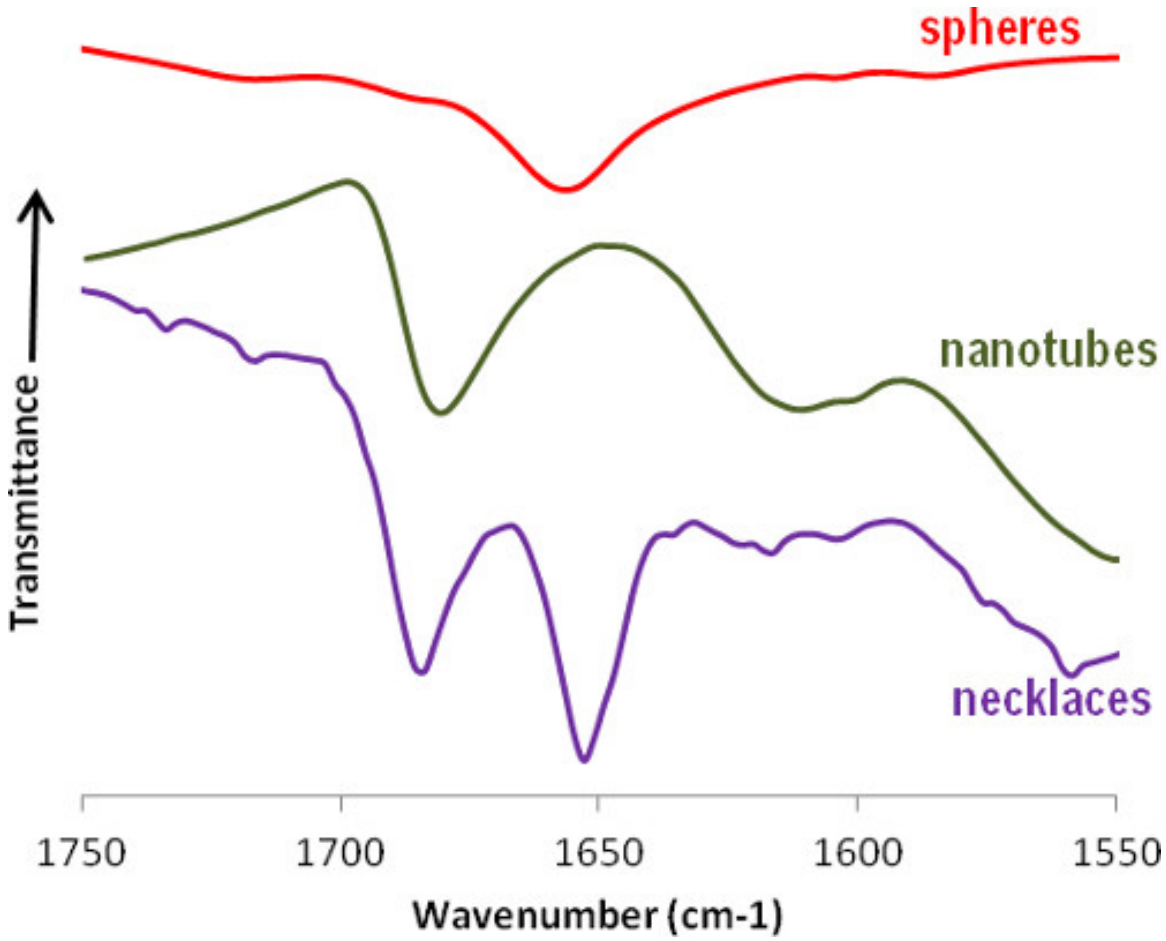

Figure 4. FT-IR analysis of the different self-assembled structures. FT-IR spectrum obtained from the sample of the spheres formed by Boc-Phe-Phe-OH (red), the tubular structures formed by NH_2-Phe-Phe-OH (green) and the molecular necklaces formed by the coassembly of these two peptides (purple).

Discussion

In summary, this paper demonstrates the ease in which peptide-based assemblies can be formed *in vitro*. The process involves commercially available peptides and solvents, and it occurs spontaneously under ambient conditions, upon the addition of a polar solvent to the test tube. It is crucial to use HFP as a solvent of the peptides, due to the low solubility of the peptides in other organic solvents. In addition, due to the high volatility of HFP it is necessary to prepare fresh stock solution for each experiment. Moreover, the volume of the stock solution should be higher than 10 µl and the transfer of the dissolved peptide into the polar solvent (water) should be done quickly.

It should be noted that this method for the solvation and self-assembly of the peptide is one possible approach, typically used for these aromatic peptides. Other approaches, however, are possible. In addition, the concentration of the stock solution of the peptide in HFP is high in these experiments in order to minimize the concentration of HFP in the final solution.

This manuscript also presents some of the major techniques for the characterization of peptide-based structures, such as AFM, TEM, SEM, and FT-IR. Using microscopy techniques it is possible to obtain information on the morphology of the assemblies. Since the dimensions of these assemblies range from hundreds of nanometers to several microns, it is sufficient to use standard electron microscopy for their characterization. Ultra-high resolution microscopes would be useful for structures that are less than 100 nm in diameter and when imaging without a conductive coating (*e.g.* gold) is desired. In some cases, the charging of the structures by the electron beam of the electron microscope may occur due to the organic nature of the structure. This can be solved by lowering the voltage of the operating system.

Additional analysis, FT-IR spectroscopy, is a medium resolution method that provides information on the secondary structure of the assemblies. In this manuscript, the measurements were performed on dry samples, however it is possible to study the structure of the assemblies in the solution phase using a fluid cell.

Taken together, the approach presented here for the self-assembly of peptides can be adapted to other classes of peptides and might lead to a better understanding of the forces and interactions during the process. In addition, it can also lead to the formation of new biomolecular assemblies.

Disclosures

The authors declare that they have no competing financial interests.

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