Simultaneous Occurrence of Nanospheres and Nanofibers Self-Assembled from Achiral Tripeptides

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The achiral tripeptide Boc-Aib-MABA-Aib-OMe has the ability to co-exist as nanospheres and as a network of nanofibers in methanol. Furthermore, AFM and TEM images show the presence of bulges in the network of nanofibers. Interestingly, the formation of nanofibers is seen to emerge from the outer boundary of the spherical structures. Some of the nanofibers curl up at the tip and later result in the formation of hollow nanospheres with thick boundaries. The presence of β-turn-like structures with hydrogen bonding is observed using FT-IR studies. The presence of hydrogen bonding is also demonstrated by using NMR studies.

The biomolecular self-assembly process uses molecular fragments to construct well-ordered complex systems. This process is usually mediated by non-covalent interactions including van der Waals interactions, II–II interaction, electrostatic interactions and hydrogen bonding. These interactions help the self-assembled peptides to be in lower energy stable states.

Sometimes, the self-assembled peptide nanostructures demonstrate flexibility in their structures depending upon its sequence and experimental conditions. The structural transition involving covalent interaction is generally irreversible while reversibility phenomenon is generally accompanied in non-covalent interactions due to energy minimization. The irreversible structural transition of nanostructures can occur thermally while reversible transition can occur in different solvents and also with the increase in temperature in the solvents. Furthermore, different pH values, peptide concentrations, surface and enzymatic effects influence the structural transition of nanostructures. Interestingly, in phosphopeptide inspired from phospholipid membrane, a structural transition from nanospheres to half-elliptical nano sheets have been observed in the process of self-assembly of D-phosphopeptide while curved nano tapes are observed in the case of L-phosphopeptide by changing the solvent from HFIP to water. The irreversible structural transition in peptide nanostructures causes changes in physical properties including optical, electronic and molecular properties.

However, understanding about how the sequence of amino acids in peptides causes structural transition between supramolecular assemblies is still in early stage. It has been observed that positional isomeric dipeptides Boc-Aib-MABA-OMe and Boc-MABA-Aib-OMe formed by sequence reversal lead to the formation of nanostructures of different morphology with difference in self-assembly and cell viability under the same conditions. Inspired by the formation of structurally diverse nanostructures by dipeptides Boc-Aib-MABA-OMe and Boc-MABA-Aib-OMe, we designed and synthesized tripeptide sequence Boc-Aib-MABA-Aib-OMe to explore the effect of achiral aromatic amino acid surrounded by protected achiral aliphatic amino acids on the self-assembling ability to form nanostructures. It can be expected that there might be simultaneous presence of more than one nanostructure in this peptide Boc-Aib-MABA-Aib-OMe. The schematic representation of the structure of the peptide is shown in Figure 1. We demonstrated this achiral tripeptide sequence Boc-Aib-MABA-Aib-OMe containing achiral aliphatic and aromatic amino acids shows co-existence of two nanostructures.

To examine the three dimensional topology of peptide nanostructures, atomic force microscopy (AFM) was used. The AFM images at 1 mM peptide concentration confirm the co-existence of both nanospheres (Figure 2A) and network of nanofibers (Figure 2B), atomic force microscopy images confirm the formation of nanostructures.

![Figure 1. Schematic representation of the peptide Boc-Aib-MABA-Aib-OMe.](image-url)
The nanofibers (Figure 2B). The nanofibers form networks (Figure 2B) or look like they are branch roots with presence of bulges with tapering end. Figure 2B also shows the presence of nanospheres. Since, AFM is excellent tool to measure along Z-direction, it was found that the height of some of nanospheres varies from 20 to 75 nm with distances varying between 250–500 nm (Figures 2Ai-2Aiii) while the height of some nanofibers is about 14 nm (Figure 2Bi).

The self-assembly of the peptide Boc-Aib-MABA-Aib-OMe was further examined under transmission electron microscopy (TEM). The TEM image (Figure 3) shows the presence of network of nanofibers. These nanofibers are inter-tangled in such a way that it looks as if there is presence of branched root like structure. The diameter of one of the individual strand is approximately 16 nm. The network of nanofibers also suggests the presence of bulges. Some of bulges have diameter varying between approximately 67 to 100 nm as shown in Figure 3.

To understand the self-assembly pathway for the formation of nanospheres and nanofibers we monitored TEM images at two different concentrations and at three time points. At 5 mM peptide concentration in 14 days, nanospheres of various sizes are formed (Figure S1(a) in the Supporting Information). On close observation at different magnification some spherical structures have become distorted with the generation of nanofibers formed from the boundary of spherical structures (Figure S1(b) in the Supporting Information) which become more prominent in 19 days (Figure S1(d) in the Supporting Information). Interestingly, at 1.25 mM peptide concentration in 14 days, there was generation of nanofibers from spherical structures (Figure 4(A); Figure 4(B)) that curls up at their tip (Figure 4(C)). In 16 days, we see there is presence of hollow...
nanospheres with thick boundary and also presence of irregular nano closed structures [Figure 4(D)] and [Figure 4(E)]. In 19 days, the dominance of hollow nanospherical structures with thick boundary was observed [Figure 4(F)].

To probe the distribution of size of the nanostructures dynamic light scattering (DLS) experiment,\(^{[23]}\) was carried out with methanolic solution of the peptide Boc-Aib-MABA-Aib-OMe. The DLS study suggests that this peptide self-assembles into nanoparticles of mean diameter of 36.9 nm at 1.25 mM peptide concentration (Figure S2 in the Supporting Information). The FT-IR study was used to study the secondary structure of the peptide. In the amide I region, two major peaks at 1686 cm\(^{-1}\) and 1642 cm\(^{-1}\) (Figure 5) was observed that indicates \(\beta\)-turn like conformation.\(^{[24–26]}\) Furthermore, a major band at 3320 cm\(^{-1}\) corresponds to presence of hydrogen bonded NH group\(^{[27,26,28,29, 30]}\) in the FT-IR spectrum of the peptide. The tripeptide was also characterized by NMR. The NMR studies was carried out in CDCl\(_3\) + 1.1 % [D\(_6\)] DMSO mixture as this results in better resonance of backbone NH protons without significant change in chemical shifts of the protons. The presence of hydrogen bonding in the peptide is examined by the change in the chemical shift of amide protons as shown in Figure 6. The different concentrations of [D\(_6\)] DMSO when added to the peptide solution in CDCl\(_3\) + 1.1 % [D\(_6\)] DMSO causes large downfield shift of the amide protons exposed to the solvent [D\(_6\)] DMSO by interacting with it. The observed \(\Delta\delta\) valves for Aib (1) NH and MABA (2) NH are large (1.13 and 1.01 ppm) suggestive of non-involvement in intramolecular hydrogen bonding while the \(\Delta\delta\) valve for Aib (3) NH is 0.26 which is appreciably large indicative of increase of solvent exposure of Aib (3) amide proton\(^{[31]}\) in the case of tripeptide.

It is certainly desirable to capture multiple nanostructures together under same roof as found in our studies carried out in single solvent methanol. Our studies suggest that the achiral tripeptide Boc-Aib-MABA-Aib-OMe co-exists as nanospheres and network of nanofibers in methanol. In 1.25 mM peptide concentration, we saw nanofibers generating from spherical structures. Some of the nanofibers curls up at its tips and later with time there is formation of hollow nanospheres with thick boundary. The FT-IR study shows the formation of \(\beta\)-turn

\[\text{Figure 4. TEM images of the peptide Boc-Aib-MABA-Aib-OMe at 1.25 mM concentration, showing (A) The presence of both spherical and fibrous structures in 14 days. (B) and (C) are magnified versions of image (A). (D) The image in 16 days. (E) Image is the magnified version of image (D). (F) The image in 19 days.}\]

\[\text{Figure 5. FT-IR of the peptide Boc-Aib-MABA-Aib-OMe.}\]
structure with hydrogen bonding. The NMR study supports the formation of hydrogen bonded C-term. This interesting structural co-existence of nanospheres and nanofibers is possible due to the structural design of the peptide containing protected Aib residues at both N- and C-terminal of MABA amino acid.. We hope that this designed structural property of the peptide Boc-Aib-MABA-Aib-OMe may be harnessed in application involving optical, and drug delivery areas.

Experimental Section

Peptide Synthesis: The peptide Boc-Aib-MABA-Aib-OMe (MABA = meta-amino benzoic acid) was synthesized by solution phase method using 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo [4,5-b]pyridinium 3-oxid hexafluorophosphate/1-hydroxybenzotriazole (HATU/HOBt), and N-Ethylisopropylamine as base. The N-terminus was protected using tert-butylxycarbonyl (Boc) group while methyl ester was used for C-terminus protection. The de-protection at C-terminal was carried out by saponification of methyl ester. The synthesis of the final peptide Boc-Aib-MABA-Aib-OMe was made by fragment condensation of Boc-Aib-MABA-OH with NH₂-Aib-OMe. The peptide was purified by column chromatography using silica gel (60-120-mesh size) as stationary phase and methanol mixture as eluent. The homogeneous nature of the purified peptide was confirmed using analytical HPLC. The mass of the purified peptide was analyzed by using positive ion electrospray ionization mass spectrometry, 

$$[M_{obs} = 421 \text{Da}], \quad [M_{obs} (M + H^+ = 422.23 \text{Da}, M + Na^- = 444.21 \text{Da}, 2 M + Na^- = 865.43 \text{Da})].$$

Preparation of Peptide Particles: Peptide was dissolved in methanol at 1.0 mM, 1.25 mM and 5 mM concentration and was allowed to age at room temperature.

NMR: Experiments were carried out on a Bruker Advance III HD 500 MHz spectrometer. The solvent titration experiment illustrating the delineation of exposed NH protons was achieved by titrating CDCl₃ + 1.1% [D₆] DMSO mixture containing the peptide with low concentrations of DMSO-d₆. The processing was done using BRUKER Software TOPSPIN 3.5.

Transmission Electron Microscopy (TEM): The peptide dissolved in methanol was loaded onto the carbon/formvar coated 200 mesh copper grids for 3 minutes. The peptides loaded grids were negatively stained with 2% Uranyl acetate for 45 seconds, excess Uranyl acetate wiped off using Whatman filter paper and allowed to air dry. The grids were viewed under the transmission electron microscopy at 120KV (JOEL, Japan). For 1.25 mM and 5 mM peptide concentrations, TEM images were recorded after aging for 14, 16 and 19 days at room temperature.

Atomic Force Microscope (AFM): The peptide sample in methanol was deposited on a freshly cleaved mica surface (mica is a layered mineral with an atomically flat surface several microns thick) and allowed to dry for about 5–10 minutes before imaging. The images were acquired on Hydra SPM Multiview 4000 AFM system (Nanonics Imaging Ltd, Manhat Technology Park, Malcha, Jerusalem, Israel) using non contact tapping mode.

Fourier Transform Infrared Spectroscopy (FT-IR): FT-IR spectra of the peptide were recorded on Thermo Nicolet Nexus 670 Spectrometer. The peptide was freeze-dried and then pressed with powder of potassium bromide (KBr) thereby forming pellets.

Dynamic light scattering (DLS): DLS experiment was carried out with 1.25 mM solution of peptide in methanol using Horiba Scientific nano partica, nano particle analyser SZ-100.

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Conflict of Interest

The authors declare no conflict of interest.

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