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Dipeptide derived from benzylcystine forms unbranched nanotubes in aqueous solution

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Abstract

The essence of modern nanotechnology is manifested in the formation of well-ordered nanostructures by a process of self-association. Peptides are among the most useful building blocks for organic bionanostructures such as nanotubes, nanospheres, nanotapes, nanofibrils, and other different ordered structures at the nanoscale. Peptides are biocompatible, chemically diverse, and much more stable and can be readily synthesized on a large scale. Also, they have diverse application in biosensors, tissue engineering, drug delivery, etc. Here, we report a short cystine-based dipeptide, which spontaneously self-associates to form straight, unbranched nanotubes. Such self-assembled nanobiomaterials provide a novel possibility of designing new functional biomaterials with potential applications in nanobiotechnology. The formation of nanotubes in solution state has been demonstrated by atomic force microscopy and scanning electron microscopy. Infrared absorption and circular dichroism demonstrated the intermolecular β-sheet-like backbone hydrogen bonding in juxtaposing and stacking of aromatic side chains.

Keywords: Nanotube, Dipeptide, π-π stacking, Cystine, Self-assembly

Background

There has been rapid advancement in the development of self-assembled nanobiomaterials, such as nanotubes, nanocrystals, and nanowires, which have potential application in electronics, biosensors, catalysis, drug delivery, and tissue engineering [1-4]. The physical and chemical properties of these nanomaterials are tunable by controlling their shapes and sizes [5]. There are various designing rules of the synthesis of these biomaterials, where secondary structure of the self-assembled fiber, the thickness of fiber, and hydrogel porosity, and different mechanical properties can all be varied predictably simply by changing the amino acid sequence, its concentration, the surrounding media, and its processing route [6-8]. Over the years, various natural self-assembling systems have served as inspiration for the design of novel building blocks on the nanoscale [9-11]. It has been already reported that cyclic peptides, amphiphilic peptides, and amyloid-inspired peptides can form ordered nanostructures with different morphologies including nanowires, nanotubes, nanovesicles, nanofibrils, and nanosheets [12-14]. The formation of these nanostructures is facilitated by the combination of different non-covalent forces that include electrostatic interactions, hydrogen bonds, hydrophobic interactions, and aromatic stacking interactions [9,13,15]. Unlike inorganic and carbon-based nanomaterials, the peptides undergo self-assembly process at ambient temperature and atmospheric pressure [16]. Besides, the peptides can be produced in large scale by simpler experimental methods. The self-assembled peptide nanostructures can further organize to form nanoscale devices [17]. Therefore, the application of self-assembling synthetic peptides as the building blocks of nanoscale devices is practical, robust, and affordable [18-20].

In this report, we present a novel peptide that, in solution, readily undergoes the formation of a linear nanotube with β-sheet-like backbone hydrogen-bonding pattern. The dipeptide constitutes two-side-chain benzyl-protected cystine moieties. This is possibly the first report for the formation of a cystine-based nanotube. First, this dipeptide has been synthesized, purified, and characterized. The β-sheet-like backbone arrangement of the peptide in the solid state has been confirmed by Fourier transform infrared (FT-IR) spectroscopy. The computational model calculation and density functional theory (DFT) study also support these data.

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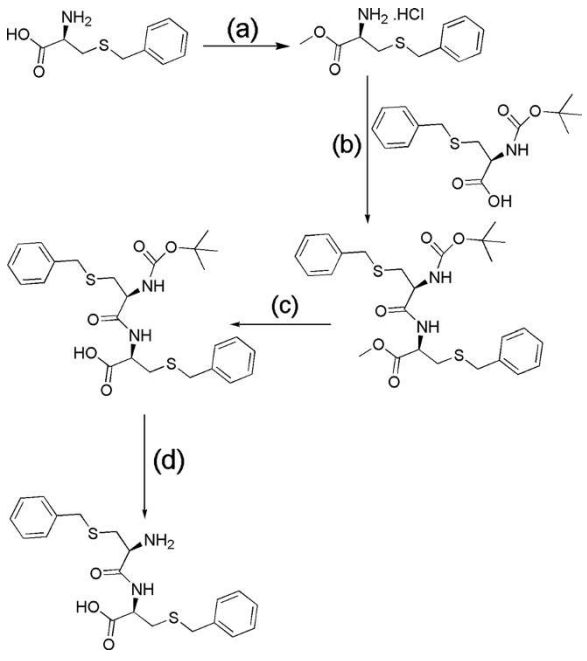
Results and discussions

Synthesis of the dipeptide

The dipeptide was synthesized by conventional solution-phase methodology (Scheme 1). The N-terminus was protected by the Bocgroup, and the C-terminus was protected as methyl ester. Couplings were mediated using 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride/hydroxybenzotriazole (EDC·HCl/HOBt). The deprotection of methyl ester leading to the acid was performed by saponification (aq. LiOH) method, and the Boc group was removed using 4 M HCl in 1,4-dioxane. The final tetrapeptide was fully characterized by ^{1}H-NMR, HRMS, and infrared (IR) spectroscopy.

FT-IR study

FT-IR spectroscopy studies were performed to determine the secondary structures of the peptide (Figure 1). In the case of β-sheet structure, the FT-IR spectra show two amide I transitions, one is a strong transition between 1,610 and 1,640 cm^{-1} and the other one is a weak transition between 1,680 and 1,700 cm^{-1}. Here, the FT-IR spectra of the peptide show characterized CO stretching bands at around 1,631 and 1,680 cm^{-1} and the NH stretching band at around 3,304 cm^{-1}, typical for intermolecularly hydrogen-bonded β-sheet structures in solid. Furthermore, the NH bending frequencies of this dipeptide appear at 1,512 cm^{-1}, suggesting the formation of a β-sheet structure, whereas absorption in the range 1,610 to 1630 cm^{-1}, which is a characteristic of aggregated amyloid-like peptides, was not observed. IR frequencies were calculated using DFT level of theory. NH and COstretching frequencies were found to be 1,635 and 1,681 cm^{-1}. It nicely corroborates with the experimentally obtained data.



Scheme 1 Reagent and conditions. (a) $SOCl_2$, MeOH, 0°C to reaction time (rt), 4 h; **(b)** EDC·HCl (1.5 equivalency (equiv.)), HOBt (1.2 equiv.), TEA (3 equiv.), 0°C to rt, 7.0 h; **(c)** lithium hydroxide (1.5 equiv.), MeOH/water (5:1), rt, 1.5 h; **(d)** 4 M HCl in 1,4-dioxane, 0°C to rt, 2.0 h.

SEM and AFM imaging

The scanning electron microscopy (SEM) images of the dipeptide (Figure 2A) show that it forms nanofibrils with a diameter of 80 nm and length of 30 μm. They are almost uniform in nature. The atomic force microscopy (AFM) image of the dipeptide also confirmed the formation of nanotubes with a diameter nearly 80 nm and length of 30 μm.

Model of self-assembly process

The dipeptide showed intermolecular β-sheet-like backbone hydrogen bonding in juxtaposing and stacking of aromatic benzyl side chains (Figure 3). They have a hydrophobic head with an array of overlapping benzyl groups similar to what is found in silk fibroin or spider silk assemblies [21]. The benzyl groups form packed hydrophobic interactions in water, which can be disrupted mechanically during sonication. However, these hydrophobic cohesive ends can find each other quickly in water since the exposure of hydrophobic alanine arrays to water is energetically unfavorable. Since the hydrophobic alanines' interaction is non-specific, they can slide diffuse along the nanostructure, like trains sliding along the rails [21]. Such fragments could form various assemblies readily through hydrophobic and intermolecular hydrogen-

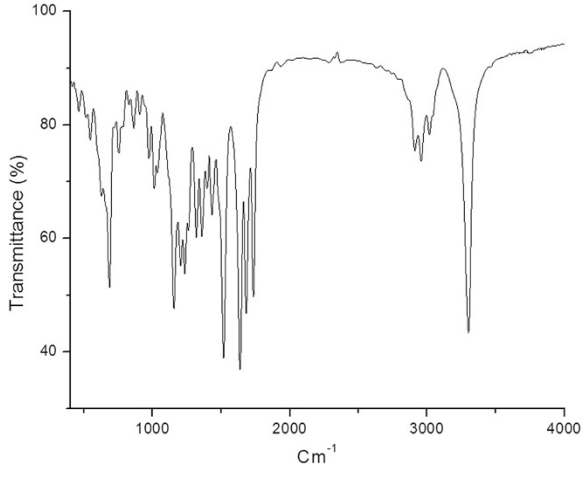


Figure 1 FT-IR spectra of the benzyl-protected cystine dipeptide.

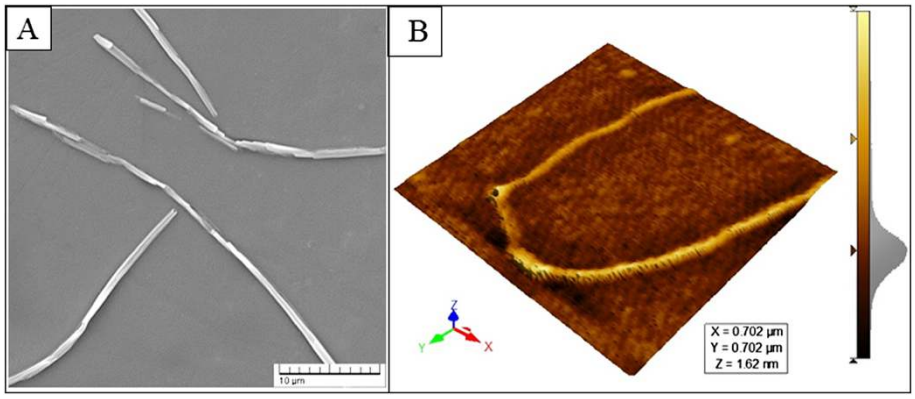


Figure 2 SEM image (A) and AFM image (B) of the dipeptide.

bonding interactions. The model of self-assembly process is shown in Figure 4.

Here, we have shown a model for plausible intermolecular interactions where the benzene ring of the benzyl groups shows π-π stacking interaction with the benzene ring of the benzyl group of another dipeptide. The hydrogen bonding occurs between the N-H of the amide group and the oxygen atom of the backbone carbonyl. The importance of hydrogen bonds and π-π stacking interaction in the formation of nanotubes has been recognized and emphasized in the literature. It was pointed out that the formation of the hydrogen bonds is the necessary controlling factor in the formation of cylindrical shape. In this present system, the assembled phenyl rings could generate the possibilities of π-π stacking interaction between the nearby dipeptide, which leads to the circular ring of the self-assemblies, though no β-sheet structure formed in solution phase. These two forces help form the nanotubes.

CD spectroscopy

The solution-state conformation of the peptide was also defined by circular dichroism (CD) spectroscopy. Circular dichroism revealed an extended conformation. The CD spectra of the dipeptide in water are shown in Figure 5. The peptide in dilute conditions showed a negative CD band at 201 nm. However, with an increase in peptide concentration, the CD absorption band red-shifted (approximately 3 nm) to longer wavelengths, which indicates that at a higher concentration of the peptide due to the formation of oligomer, the amide geometry may be changed.

Ultraviolet spectroscopy

Two distinct absorption peaks in Figure 6 were observed due to the benzyl groups present in the dipeptide molecule: one at 260 nm and the other at 267 nm. Scattering was also observed which indicates self-aggregation and higher oligomer formation. With the increasing concentration, scattering was also found to be increased.

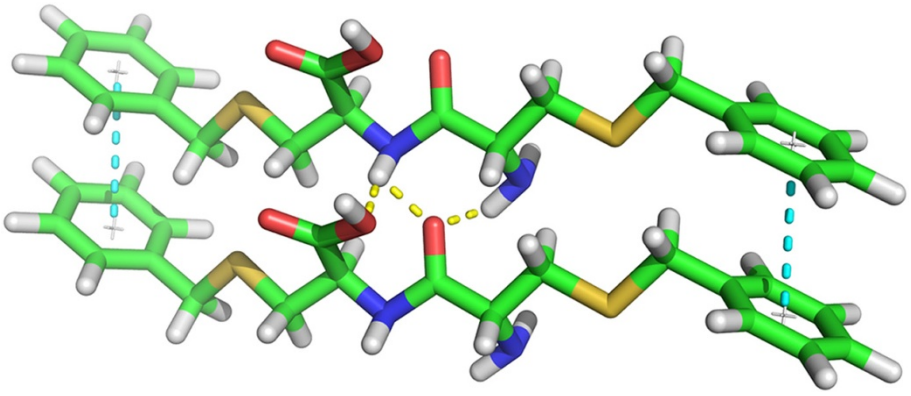


Figure 3 Plausible intermolecular interaction pattern. Illustrating the role of intermolecular β-sheet-like backbone hydrogen bonding in juxtaposing and stacking of phenyl rings in the benzyl group side chains. Pi stacking is shown with cyan dotted lines, and the hydrogen bonding is shown in yellow dotted lines.

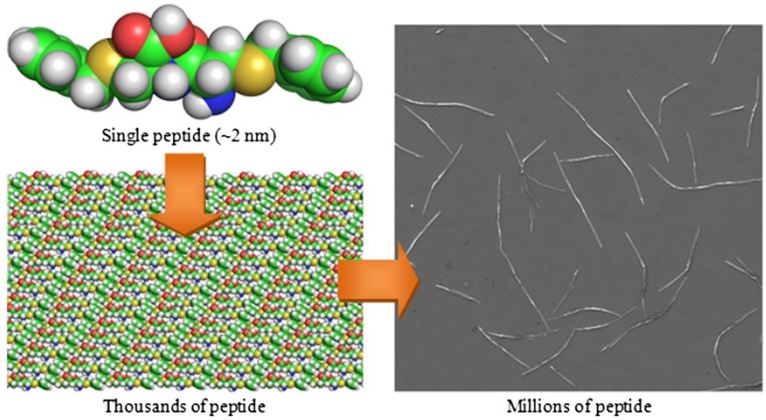


Figure 4 Schematic presentation of nanotube formation using cystine dipeptide as template. The peptide is about 2 nm long; tens and hundreds of thousands of individual peptides self-assemble into nanotubes.

Conclusion

This study clearly demonstrates that short water-soluble benzylcystine dipeptide self-assembles to form a supramolecular extended structure with β-sheet-like intermolecular hydrogen-bonding pattern in solid state, and this dipeptide also forms an ordered nanostructure from an aqueous solution under the proper conditions. The molecular arrangement of the self-association of this fibril-forming peptide is explained using molecular modelling studies. This work may help guide the design of biomaterial scaffoldings in thefuture by inscribing biological signals in the self-assemblies. Moreover, biocompatibility of peptide nanotubes makes them valuable for applications as biosensors and in tissue engineering. The field of nanoscale device development based on peptide nanotubes is still very nascent. Our efforts had continually been in developing such exotic nanomaterials for further advancement of this field.

Methods

Synthesis of the dipeptide

The starting materials, Boc-S-benzyl-L-cysteine, were purchased from Across Organics, and S-benzyl-L-cysteine was purchased from Alfa Aesar (Ward Hill, MA, USA) and were used without further purification. Solvents were freshly distilled by standard procedures prior to use. The dipeptide was purified by column chromatography. All ^{1}H and ^{13}C-NMR spectra were recorded on a Bruker 600-MHz spectrometer (Bruker AXS, Inc., Madison, MI, USA). For ^{1}H NMR, tetramethylsilane served as internal standard ($\delta = 0$), and data are reported as follows: chemical shift, integration, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), and coupling constant(s) in hertz. High-resolution mass spectra were obtained on a Jeol MS station 700 (JEOL Ltd., Akishima-shi, Japan).

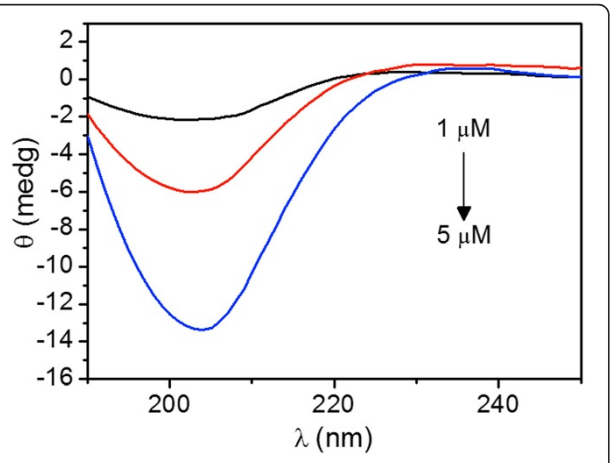


Figure 5 Circular dichroism spectra of the dipeptide at different dilutions. Approximately 3-nm shift in the CD band was observed.

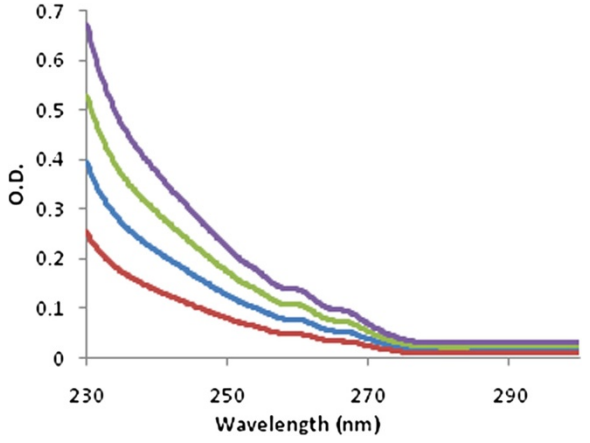


Figure 6 Absorption spectra of dipeptide at four different concentrations. Band for the benzyl groups appeared at 260 and 267 nm. Scattering was observed due to aggregation of the peptide. OD, optical density.

FT-IR study

The FT-IR spectra of the samples were recorded on a JASCO FT-IR 4200 spectrometer (Jasco, Easton, MD, USA) by KBr disc technique. The peptide was mixed with KBr in a clean glass pestle and compressed to obtain a pellet. The spectra were recorded from 400 to 4,000 cm^{-1}. Background spectra were obtained with KBr pellet for each sample. JASCO software was used for data processing.

SEM imaging

The dipeptide sample slides were dried completely under a critical point dryer (Quorum Technologies Inc., Guelph, Canada), and after drying, the samples were kept on the sample holder using carbon tape. Gold coating was done with the current of 10 mA at 10–6 to 10–8 mbar/Pa (model no. SC7620, Quorum Technologies Inc.). SEM imaging was performed using TESCAN Vega II LSU (TESCAN Digital Microscopy Imaging, TESCAN, Brno, Czech Republic). Imaging and measurements were done using Vega TC software (TC Software, Hampton, VA, USA).

AFM imaging

Twenty microliters of the dipeptide sample (5 mg dissolved in 1 mL water) was deposited onto freshly cleaved muscovite Ruby mica sheet (ASTM V1 Grade Ruby Mica, Micafab India Pvt. Ltd., Chennai, India) for 5 to 10 min. Mica sheets are basically negatively charged, so the dipeptide molecule binds strongly on the mica surface. After 10 min, the sample was dried using a vacuum dryer. AAC-mode atomic force microscopy was performed using a Pico Plus 5500 AFM (Agilent Technologies, Inc., Santa Clara, CA, USA) with a piezo scanner maximum range of 9 μm. Microfabricated silicon cantilevers of 225 μm in length with a nominal spring force constant of 21 to 98 N/m were used from nanosensors. Cantilever oscillation frequency was tuned into resonance frequency. The cantilever resonance frequency was 150 to 300 kHz. The images (512 × 512 pixels) were captured with a scan size between 0.5 and 5 μm at the scan speed rate of 0.5 rpm. The images were processed by flattening using Pico view software (Molecular Imaging Inc., Ann Arbor, MI, USA). The image presented in this paper was derived from the original data. Length, height, and width were measured manually using Pico view software.

CD spectroscopy

CD spectra (190 to 400 nm) were acquired at 200-nm/min scan speed, with 1-nm bandwidth on a Jasco J-810 spectrometer using a 1-mm-path-length quartz cell. Peptide stock solutions (2 mg/mL) were prepared in water and appropriately diluted during CD measurements. Five spectra were averaged to improve the signal-to-noise ratio and smoothed using the noise-reducing option. The results were represented as machine unit (θ, in units of mdeg).

UV spectroscopy

Absorption spectra were acquired using a Shimadzu UV-2401PC spectrophotometer (Shimadzu Corporation, Kyoto, Japan) with 10-mm-path-length quartz cuvette. A very dilute concentration of the peptide was used (5 to 20 μM).

DFT calculations

Structure of the dipeptides was drawn on Schrodinger Maestro molecular modeling environment (http://www.schrodinger.com/). Geometry was optimized using simulated annealing algorithm with the help of Desmond molecular dynamics simulations software tool. Simulation was run for 5.2 ns in SPC water environment in an orthorhombic periodic boundary condition. The model system was relaxed before simulation. Number of particle (N), and volume of system (V) in the ensemble were constant and the system had a well defined temperature (T). The molecule was cooked at a high temperature and then slowly cooled down to 300 K. The process was repeated several times [22]. The lowest energy conformation at 300 K was chosen for further analysis using DFT implemented in Gaussian 09 software (Gaussian Inc., Wallingford, CT, USA). In Gaussian 09, geometry optimizations and frequency calculations were performed *in vacuo* with B3LYP density functional using 6-311G + (2d,p) basis set [23,24].

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

BB, SKP, and UP conceived and designed the experiments. SKP and UP performed the experiments. All the authors analyzed the data. SKP and UP drafted the manuscript. All authors read and approved the final manuscript.

Authors' information

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