Biomimetic organization: Octapeptide self-assembly into nanotubes of viral capsid-like dimension

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The controlled self-assembly of complex molecules into well defined hierarchical structures is a promising route for fabricating nanostructures. These nanoscale structures can be realized by naturally occurring proteins such as tobacco mosaic virus, capsid proteins, tubulin, actin, etc. Here, we report a simple alternative method based on self-assembling nanotubes formed by a synthetic therapeutic octapeptide, Lanreotide in water. We used a multidisciplinary approach involving optical and electron microscopies, vibrational spectroscopies, and small and wide angle x-ray scattering to elucidate the hierarchy of structures exhibited by this system. The results revealed the hexagonal packing of nanotubes, and high degree of monodispersity in the tube diameter (244 Å) and wall thickness (~18 Å). Moreover, the diameter is tunable by suitable modifications in the molecular structure. The self-assembly of the nanotubes occurs through the association of β-sheets driven by amphiphilicity and a systematic aromatic/aliphatic side chain segregation. This original and simple system is a unique example for the study of complex self-assembly processes generated by de novo molecules or amyloid peptides.

The ability of simple molecules to spontaneously organize into well defined nanostructures is of fundamental importance and has wide ranging applications in biotechnology and materials sciences (1). In fact, characteristic lengths <100 nm are not easily accessible at present by lithographic techniques, but can be realized with biological self-assemblies such as tobacco mosaic virus, capsid proteins (2), tubulin (3), or actin (4, 5). These proteins under appropriate conditions possess the unique capability to form long filaments with a well defined diameter. However, the fabrication cost often restricts their potential interest in practical applications. Therefore, a simple alternative route has been emerged based on de novo molecules that self-organize in a programmed way (6–11). The design of such biomimetic systems requires the understanding of the relationship between the molecular structure and the self-assembly process of the nanostructures. This inspiration from natural fibers is difficult to implement when the building blocks themselves are complex, as in the case of proteins. Up to now, no simple synthetic molecule was able to self-assemble into hollow nanotubes with well defined characteristic length in the range of 20–30 nm. Lanreotide is an octapeptide synthesized as a growth hormone inhibitor. Lanreotide forms hydrogels (Autogel), which are easily accessible at present by lithographic techniques, but can be realized with biological self-assemblies such as tobacco mosaic virus, capsid proteins, tubulin, actin, etc. Here, we report the molecular and supramolecular organization of self-assembling nanotubes formed by Lanreotide in water (10% wt/wt, acetate salt). We chose a multidisciplinary approach, by combining polarized light microscopy, electron microscopy, vibrational spectroscopies, small and wide angle x-ray scattering (SAXS and WAXS, respectively) to elucidate the hierarchical structures formed by this system. The nanotubes are remarkably monodisperse, with a diameter of 244 Å and a wall thickness of ~18 Å. The study of a Lanreotide derivative indicates the possibility to control the diameter of these tubes from the molecular structure. The self-assembly of these nanotubes occurs through the association of β-sheets driven by amphiphilicity and a systematic aromatic/aliphatic side chain segregation. This original and simple system is a unique example of molecules able to self-organize into well defined nanostructure. The resolution of the structure at the molecular scale highlights the simplicity of the interactions involved in the self-assembly process, and could find implication for β-amyloid fibers or de novo self-assemblies.

Materials and Methods

Materials. Cyclic Lanreotide of sequence NH2-(d)Naph-Cys-Tyr-(d)Trp-Lys-Val-Cys-Thr-COH2 (BIM 23014C) and its cyclic derivative of sequence NH2-(d)Naph-Cys-Tyr-(d)Phe-Lys-Val-Cys-Thr-COH2 (BIM 23A462C) were obtained from Ipsen Pharma (Barcelona) as acetate salts (molecular masses of 1,095 and 1,060 Da, respectively, purity >98%). Mixtures were made by dissolving the peptides powders at 10–14% wt/wt in pure water. Glycerol (99.9%) was purchased from Sigma.

Optical Microscopy. Very thin preparations between glass slides were observed with a Nikon microscope equipped with two crossed polarizers. A color plate was used to analyze the deformation orientations.

Electron Microscopy. Electron microscopy observations were preceded by freeze-fracture and freeze-etching of samples containing 30% wt/wt dried glycerol as cryoprotectant. Small aliquots of the samples were placed on copper grids, frozen in liquid propane, and stored in liquid nitrogen. Freeze-fracture, freeze-etching, and replication were successively performed by using a Balzers 301 apparatus equipped with an electron gun for platinum shadowing. Replicates were examined by using a Philips 301 electron microscope.

SAXS. X-ray diffraction experiments were performed at the High Brilliance beam line (ID2), European Synchrotron Radiation Facility in Grenoble, France (13). The undulator x-ray beam (of wavelength 0.99 Å) was selected by a channel-cut Si(111) crystal, and focused by rhodium-coated toroidal mirror. The beam size defined by the collimating slits was 0.2 mm × 0.2 mm. The detector was an image intensified charge-coupled device camera, and the sample-to-detector distance varied between 150 and 650 cm.

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Abbreviations: SAXS, small angle x-ray scattering; WAXS, wide angle x-ray scattering.

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X-Ray Fiber Diffraction and Analysis. The setup was the same as in SAXS, with a sample-detector distance of 90 cm. In addition, high-resolution X-ray patterns were collected with a 45 × 36-cm$^2$ image-plate detector at a distance of 65 cm. Theoretically, the fiber diffraction pattern (14) is horizontal layer lines localized in the reciprocal space at $q_c = l c^*$ and whose intensity $I(q_c)$ is

$$I(q_c) = |(F_c(q_c, \Psi, l c^*))|^2,$$

where $\Psi$, $q_c$, and $c$ are the angular, radial, and axial cylindrical coordinates of the reciprocal space, and with

$$F_c(q_c, \Psi, l c^*) = \sum_{n=-\infty}^{\infty} F_{nl}(q_c) \exp[i n \Psi]$$

$$F_{nl}(q_c) = \exp\left(i \frac{\pi}{2}\right) \int_0^c \rho_n(\rho) J_n(2\pi \rho) 2\pi \rho \, d\rho,$$

where $J_n$ is the Bessel function of order $n$ and

$$\rho_n(\rho) = \frac{1}{2\pi} \int_0^c \rho(\rho, \psi, z) \exp\left[-i \left(n \psi - \frac{2\pi \rho z}{c}\right)\right] \, d\rho \, dz,$$

where $\rho(\rho, \psi, z)$ is the electron density of the tube function in cylindrical coordinate $\psi$, $r$, and $z$. In the case of a thin cylinder of radius $r_0$, the diffuse scattering is reduced to horizontal lines with intensity profiles $I(q_c, r_0)$. For peaks corresponding to $q_c = h_i c^* + k_j c^*$, with $i^*$ and $j^*$ the reciprocal vectors of the $(i, j)$ lattice, the profile is a Bessel function of order $l = h_i n + k_j m$, i.e.,

$$I(q_c, r_0) = F_{nl}(q_c, r_0),$$

and the vertical position is $q_z = h_i z^* + k_j z^*$.

**Fourier-Transform Raman Spectroscopy.** Spectra were recorded at 4 cm$^{-1}$ resolution by using a Bruker IFS 66 interferometer coupled to a Bruker FRA 106 Raman module equipped with a continuous Nd:YAG laser providing excitation at 1,064 nm. All spectra to a Bruker FRA 106 Raman module equipped with a continuous Nd:YAG laser providing excitation at 1,064 nm. All spectra were recorded at room temperature with backscattering geometrically and thin nanotubes (Fig. 2b) and its derivative (c and d) observed between cross-polarizers (±45°) through thin preparations (magnification, ×2,500). A color plate is added in b. (c) Texture growing from isotropic liquid (magnification, ×1,250). The observed fan-shape textures are characteristic deformations of parallel planes in developable surfaces (arrows). These deformations are compatible with hexagonal columnar liquid-crystal phases (or lamellar phases).

**Lanreotide Conformation.** FT-Raman spectroscopy shows the presence of a disulfide bridge in the Lanreotide structure, with a gauche–gauche–gauche conformation as evidenced by the presence of the characteristic 506-cm$^{-1}$ vibration (Fig. 3a) (17). The amide I vibrations observed by Fourier transform infrared spectroscopy indicate that 35% of the hydrogen bonds implying backbone carbonyl groups are involved in antiparallel β-sheet. These data strongly support a planar β-hairpin conformation for the peptide backbone with a turn located at the D3-trypophan residue, which is stabilized by the disulfide bridge and intramolecular hydrogen bonds. This conformation enhances the amphiphilic nature of the peptide by exposing the hydrophilic disulfide bridge on one face of the β-hairpin, whereas the hydrophobic residues are exposed on the other face. Furthermore, the aromatic residues are segregated from the aliphatic ones, each being located on one β-strand. Great attention is given to this β-hairpin, three couples of hydrogen bond donors/acceptors are in the right orientation to form a β-sheet fiber.

**Fiber Diffraction.** The organization within the nanotube wall is crystalline as shown by an exceptionally well aligned WAXS pattern (mosaicity <0.5°) acquired with the Lanreotide derivative (Fig. 4a). This diffraction pattern can be unambiguously interpreted in terms of a 2D curved crystal (further analysis reveals the formation of ripples along the l vector constituted by two filaments). The position of diffuse scattering maxima can be indexed by a 2D monoclinic lattice $l = 20.7 \, \AA$, $j = 20.8 \, \AA$, $\gamma = 117.2°$, with $j$ at an angle of 48.3° with respect to the direction of the cylinder axis. The line shapes of the diffuse scattering can be simulated (Fig. 4b and c) by Bessel functions corresponding to the Fourier transform of a 2D lattice (Fig. 4d). The molecular organization of both Lanreotide and its derivative are identical as indicated by the similarity of all of the cell parameters (Table 1). In both cases, the Patterson function of the nanotube walls, calculated from the main diffuse scattering, reveals a 20.8 Å alternation of low and high electron density along the $j$ vector.

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**Results**

**Nanotube Morphology and Organization.** Lanreotide acetate and its derivative spontaneously form gels in water at 10% (wt/wt) concentration. Optical textures observed between crossed polarizers (Fig. 1) are developable surfaces, which are compatible with a columnar hexagonal-crystal phase. Electron micrographs of freeze-etching replicates show tightly packed long and thin nanotubes (Fig. 2a and b). In the case of Lanreotide, SAXS experiments reveal that these nanotubes have a 2D hexagonal packing with a lattice parameter ($d_{hex}$) of 365 Å, a monodisperse diameter ($\phi$) of 244 Å and a wall thickness ($e$) of ≈18 Å (Fig. 2c and d). Moreover, the absence of order along the direction of the tubes axis demonstrates that the nanotubes freely slide in a hexagonal liquid crystalline phase (15, 16).

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i.e., a segregation between aromatic/aliphatic residues. A β-sheet stacking made by a unique peptide translation leads to a repeat distance of $9.4 \pm 2.3$ Å and is not in agreement with the experimental one of 20.8 Å. Moreover, such a stacking would not create the aromatic/aliphatic alternation. On the contrary, β-sheet fibers built from an alternated stacking of antiparallel peptides (Fig. 4e) would be in agreement with both the observed repeat distance and aromatic/aliphatic segregation.

### Structural Model of Nanotube Wall

The surface $S$ per unit cell is 380 Å² and contains two molecules. If we assume that, as for proteins, the Lanreotide density ranges from 1.2 to 1.3, then the volume $V$ of the molecule can be estimated to be 1,400–1,500 Å³. The thickness $t$ of one fiber estimated by using $t = 2V/S$ ranges between 7.4 and 8 Å, which is about half the wall thickness of the nanotubes (18 Å). Therefore, two superimposed β-sheet fibers constitute a single filament, which is visualized on the Patterson
respectively, constituting a nanotube.

identical organizations cannot be spontaneously curved, the
function (Fig. 4d). Thus, the amphiphilic nature of the peptide
induces the formation of a bilayer (Fig. 5 a and b), in which the
confined hydrophobic residues are protected from water by the
inner and the outer \( \beta \)-sheet fibers and by the hydrophilic
residues.

Four fiber organizations within the filament are in agreement
with the alternation of aliphatic and aromatic residues. All of
them are constituted by two molecules related by a 2-fold axis.
The first two fiber organizations (Fig. 7, which is published as
supporting information on the PNAS web site, www.pnas.org) would
exhibit a repeat distance equal to four times the hydrogen
bond repeat distance of a \( \beta \)-sheet (4.75 Å), i.e., 19.0 Å. These two
fiber possibilities are in contradiction with the experimental
repeat distances (20.8 Å for Lanreotide and 21.1 Å for its
derivative) and, consequently, the fiber solution is among the
two other ones. The two remaining solutions (Fig. 5b) would
exhibit a repeat distance of \( [(4 \times 4.75) + 7\times 12] = 20.2 \) Å, in which
the 7 Å represents the length of two residues along the
peptide backbone. Because a filament is constituted by two
amphiphilic \( \beta \)-sheet fibers and because a bilayer formed by two
identical organizations cannot be spontaneously curved, the
organization of the inner and the outer \( \beta \)-sheet fibers has to be
different. At the molecular level, the two remaining \( \beta \)-sheet
organizations only differ in the nature of the amino acids
involved in the intermolecular hydrogen bonds. The stacking of
these two different \( \beta \)-sheets, described in Fig. 5b, exhibit 2-fold
axes that would meet exactly when aromatic residues interact in
a Naph/Tyr/Tyr/Naph sequence. Therefore, we proposed that
the filaments are constituted by the association of these two types
of fibers.

Considering this structural model of the filaments, the nano-
tubes would be formed by the self-assembly of 26 identical
filaments of Lanreotide (18 identical filaments for the deriva-
tive) resulting in the high monodispersity of the nanotube
diameter (Fig. 5c and Table 1). Furthermore, fiber diffraction
pattern indicates that these filaments coil up around the tube at
an angle of 48.5° with respect to the direction of the cylinder axis.
Considering this filament orientation, the model gives a hydro-
gen bond orientation of 29° with respect to the direction of the
cylinder axis.

Discussion

Interactions Driving Nanotube Formation. The structure of Lan-
reotide nanotubes in water reveals the interactions responsible
for the self-assembly. Along the three directions of the nanotube
wall crystal, the driving forces are the hydrophobic effect generating a bilayer of fibers forming the filament (Fig. 5 a and b), the hydrogen-bond network maintaining the filament structure along the \( j \) vector (Fig. 5b), and the hydrophobic effect again stabilizing the lateral packing of 26 filaments along the \( i \) vector (Fig. 5c). In addition, the whole structure highlights a systematic

![Fig. 5. Schematic view of the different hierarchical levels in the self-assembly of Lanreotide-acetate nanotubes in water.](image)

segregation of aromatic from aliphatic residues. Indeed, fila-
ments exhibit a rigorous and systematic alternation of aromatic
and aliphatic regions along the hydrogen bonds direction (\( j \)) and
within the bilayers of fibers (Fig. 5b). Moreover, the Patterson
electron density map (Fig. 4e) shows that interactions between
filaments lead to the formation of continuous regions of either
aliphatic or aromatic residues along the i vector. This feature essentially results from the constraints exerted by the disulfide bridge and intramolecular interactions on the Lanreotide backbone. Indeed, the resulting β-hairpin conformation enhances the initial segregation present in the peptide sequence.

**Implication for β-Sheet Fibrils.** E. Gazit (19) recently pointed out the importance of aromatic π-stacking in the self-assembly process leading to amyloid fibrils. This analysis was essentially based on the statistics of aromatic residues occurring in amyloid-related sequences. Similarly, in the two β-strands of the native PrP human prion protein, responsible for the Creutzfeld–Jakob disease (20), three residues among the eight hydrophobic ones are aromatic (tyrosine), suggesting the involvement of aromatic segregation plays a significant role in the β-sheet fibrils.

The importance of the peptide charge in the formation of fibrils has been reported (21, 22). Fibrils formation does not occur (i) if the peptide charge is vanishing because of precipitation or (ii) when the effective charge is too high and inhibits the fiber formation by electrostatic repulsion. In the case of Lanreotide, the effective charge is of +2. A filament in a nanotube is under two electrostatic repulsion forces, one coming from the neighbor filaments in the same nanotube and the other coming from the neighbor nanotubes. The former repulsion tends to increase the size of the nanotube, whereas the latter tends to make it decrease. The electrostatic field generated by the neighbor filaments can be approximated by $\sigma/2\varepsilon$ with $\sigma$ the surface charge of the wall and $\varepsilon$ the dielectric permittivity. For the neighbor nanotubes, the electrostatic field can be estimated in first approximation by $\sigma/2\varepsilon d^2$, with $\phi$ being the nanotube diameter and $d$ being the distance between the considered filament and the center of the neighbor nanotube. The filament would be at mechanical equilibrium when both electric fields are balanced, i.e., when $d$ is $\approx \phi$. This means that the mechanical equilibrium would be reached when the distance between the nanotubes is about their radius (122 Å for Lanreotide and 83 Å for its derivative). The experimental data, for both Lanreotide and derivative, are in agreement with this simple model (Table 1) and suggest that electrostatic forces play a major role in the formation of nanotubes in the hexagonal lattice.

The supramolecular organization of Lanreotide reported here demonstrates that this system is able to investigate the minimal interactions required for generating large self-assembling nanotubes already observed with proteins (23–26) or lipids (27, 28). Furthermore, the potential applications of this type of nanotubes include nanofiltration of biological molecules (29) and templates to fabricate ordered mesoporous materials (1). Currently, a Lanreotide acetate hydrogel of higher concentration than the one studied here is used as a therapeutic in the treatment of acromegaly in the form of a long-acting s.c. implant (Autogel) (12). The exceptional self-assembling properties of Lanreotide acetate in water suggest a correlation between Lanreotide nanotube organization and the controlled release properties of this pharmaceutical product.

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