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Transformation of peptide nanotubes into a vesicle *via* fusion driven by stereo-complex formation†

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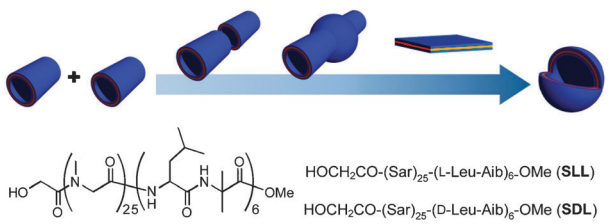
Two types of peptide nanotubes, one is prepared from an amphiphilic peptide having a right-handed helix segment and the other from that having a left-handed helix segment, are shown to transform the morphology into a vesicle by membrane fusion due to stereo-complex formation between these helical segments.

Helical peptides are easily designed to take either helicity, right-handed or left-handed, by using L- or D-residues in the sequence. When the right-handed helix is mixed with the left-handed helix, they are expected to form a stereo-complex probably due to the convexo-concave fitness between their surfaces. By using stereo-complex formation of these helical peptides, we demonstrate here vesicle formation from two types of peptide nanotubes by membrane fusion.



Several morphologies in the shapes of micelle, rod-shaped micelle, sheet, tube and vesicle have been prepared in solution by the current self-assembling techniques.[1–4] We have reported on molecular assemblies of amphiphilic peptide molecules especially by using hydrophobic helical peptides at the hydrophobic core of the molecular assemblies.[5–9] Helical peptides have a good ability to be packed regularly in the molecular assembly as shown by frequent observation of helix bundles in nature.[10,11] Indeed, several hydrophobic helical peptides with attachment of suitable hydrophilic groups formed vesicular assemblies with a diameter of *ca.* 100 nm in water, which are named "peptosome".[8] Further, a peptide nanotube with a diameter of *ca.* 60 nm and a length of *ca.* 200 nm was obtained from an amphiphilic block polypeptide with a hydrophobic helix, $(Sar)_{27}$-*b*-(L-Leu-Aib)$_6$.[12] In the latter case, the block polypeptide initially formed a curved square sheet assembly, which was transformed quantitatively into a nanotube morphology upon heating at 90 °C for 10 min. The transformation mechanism from the curved sheet to the nanotube is just to stick two opposing hydrophobic sides of the square sheet, which is unique from other reports on nanotube formation, where a twisted long sheet or helix ribbon generally fused the edges together to grow into nanotubes.[13–17] On the other hand, in our nanotubes, the size of the nanotube is determined by the initial size of the curved square sheet, which has an advantage of a very narrow size distribution of the produced nanotubes. The reason for the sheet curving is considered to be due to the regular packing of the right-handed helices in the hydrophobic core, similarly to the recent studies on molecular assemblies with chiral molecules.[18–31]

Poly(sarcosine) is used here as a hydrophilic segment, because it is as hydrophilic as poly(ethylene glycol), and sarcosine is biodegradable by endogenous sarcosine dehydrogenase. We have applied the amphiphilic peptide micelles made of $(Sar)_n$-*b*-$(Glu\text{-}OMe)_m$[5] or $(Sar)_n$-*b*-$(lactide)_m$[6] for *in vivo* tumor imaging by using a near-infrared fluorescence labelling probe. These poly(sarcosine) conjugates are shown to be highly biocompatible. The $(Sar)_{25}$ block was attached to the N-terminal of the hydrophobic helical block, (L- or D-Leu-Aib)$_6$, *via* polymerization of sarcosine *N*-carboxy anhydride (NCA) to obtain **SLL** or **SDL**.

Morphologies of molecular assemblies are studied by a transmission electron microscope (TEM) with negative staining or cryogenic freezing. As previously reported, **SLL** as injected in buffer takes a homogeneous nano curved-sheet morphology.[12] The same nano curved-sheet assembly is also obtained from **SDL**. These nano curved-sheet assemblies are transformed into nanotubes by heating the molecular assembly solution at 90 °C for 10 min. Circular dichroism (CD) measurements show that **SLL** and **SDL** in the molecular assemblies take the right-handed and left-handed α-helix, respectively. Further, the Cotton effect at 222 nm is slightly stronger than that at 208 nm, indicating that the helices form a tightly packed bundle structure (Fig. S2 in ESI†).

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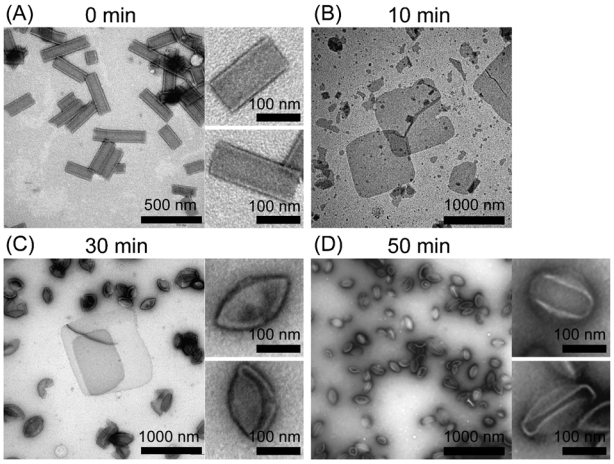

Fig. 1 TEM images of a mixture of two types of nanotubes prepared from **SLL** or **SDL** upon heating at 90 °C for specified times. The nanotubes were prepared in 10 mM Tris-HCl buffer (pH 7.4) (3 mg/1 mL) by the ethanol injection method at 90 °C for 10 min.

The important point of the nano curved-sheet and nanotube is that they are metastable, where upon heating transformation from the nanosheet to the nanotube occurs quantitatively, and upon further heating elongation of the nanotube to double and triple was observed.[12] Even though the hydrophobic edges of the nanosheet and the nanotube are stabilized by a shielding effect of poly(sarcosine) chains nearby, the edges can be fused with other hydrophobic surfaces upon heating.

Nanotubes are prepared separately from **SLL** and **SDL**, and two types of the nanotubes are mixed at an equimolar ratio. The mixture is heated at 90 °C for 50 min, and the time course of the morphology change is analyzed by TEM (Fig. 1A–D). Before heat treatment, nanotubes with a diameter of *ca.* 70 nm and a length of *ca.* 200 nm are homogeneously formed (Fig. 1A). Upon heating for 10 min, planar square sheets with *ca.* 1 μm side appear in the TEM image (Fig. 1B). Then, the sheets are gradually transformed into vesicles (Fig. 1C and D). Transformation of the sheet into the vesicle is completed within 50 min at 90 °C. With **SLL** or **SDL** alone, no such change was observed but just elongation of the nanotubes partially (Fig. S3 in ESI†). It is thus speculated that the **SLL** nanotube and the **SDL** nanotube should fuse together to transform the nanotube morphology into the planar square sheet. Indeed, the observation of a planar sheet can be reasonably explained by mixing of enantiomeric polypeptides **SLL** and **SDL** with stereo-complex formation accordingly to generate the achiral planar sheet. Whereas, **SLL** or **SDL** alone forms the curved sheet due to the one-handed helix association with a regular tilt angle between the helices. To investigate the morphology transformation mechanism in detail, the same fusion system was examined at lower temperature.

Upon heating at 50 °C, the peptide nanotube fusion process proceeds slowly compared with that at 90 °C. TEM observations reveal that upon heating at 50 °C for the initial 1–2 h, longer nanotube assemblies connecting two or more tubes are observed (Fig. 2B and C). Hydrophobic edges at the open mouths of the nanotubes therefore trigger the association of the nanotubes. Interestingly, some bunchy connecting regions of two nanotubes are observed (Fig. 2C and Fig. S4 in ESI†).

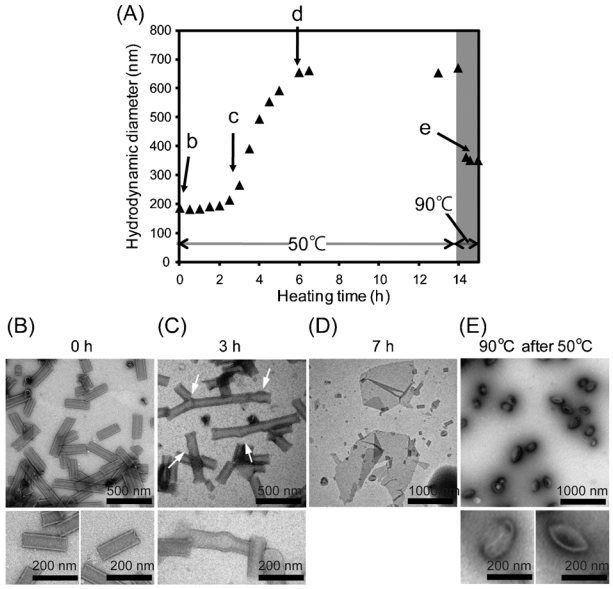

Fig. 2 DLS data (A) and TEM images (B–D) of a mixture of two types of nanotubes prepared from **SLL** or **SDL** upon heating at 50 °C. (E) TEM images of the same sample upon heating at 90 °C for 1 h after heating at 50 °C for 14 h.

This observation strongly indicates that the mixing of **SLL** and **SDL** takes place to generate a vesicle-like morphology at this connecting region. After heating for 7 h at 50 °C, the fused nanotubes are broken up and transformed into a planar square sheet with *ca.* 1.5 μm side (Fig. 2D). These results indicate that the polypeptide nanotubes are connected randomly at the initial stage (association). When two types of the peptide nanotubes are connected, the right- and the left-handed helices diffuse through the connecting moieties to cause membrane fusion, because the stereo-complex is thermodynamically stable.[14] However, the diffusion of the helical peptides in the membrane should be slow due to the tight packing of helices, which should be the reason for the long heating period for complete transformation from the nanotube to the sheet. The mixing proceeds with time, and finally the tubular structure breaks up to take the planar square sheet. It takes more than 7 h to form the planar sheet (Fig. 2D). Upon heating at 90 °C, transformation into a vesicle is attained (Fig. 2E). The morphology changes are followed by dynamic light scattering (DLS) similar to changes in hydrodynamic diameters which are calculated from the translational diffusion coefficient by using the Stokes–Einstein equation (Fig. 2A). Importantly no vesicular assembly transformation from the planar sheet was observed at 50 °C, suggesting that the transformation from the planar sheet into the vesicle requires higher energy. On the basis of analysis of many TEM images, we think that the planar sheet is flexible to bend. Upon heating at 90 °C the sheet is torn off to small sheets, which then immediately close themselves to vesicles.

We confirm the vesicle formation due to the stereo-complex formation by using another preparative method. An equimolar mixture of **SLL** and **SDL** in ethanol is injected into a buffer solution. The morphology of the self-assembly is a planar-sheet (Fig. 3A), which is different from the curved sheet prepared from the single component. The planar sheets

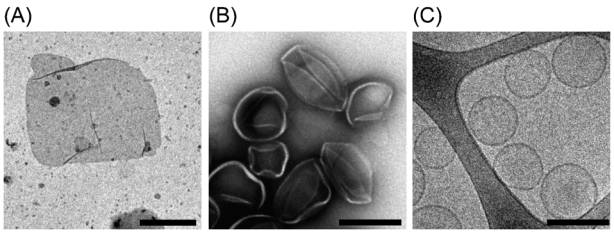

Fig. 3 TEM images (negative staining with uranyl acetate, A, B; cryogenic TEM, C) of molecular assemblies prepared from a mixture of helical polypeptides **SLL** and **SDL**, 50/50. The assemblies were prepared in 10 mM Tris-HCl buffer (pH 7.4) (3 mg/1 mL) by the ethanol injection method. Before heat treatment (A), and after heat treatment (B and C). The scale bars are 500 nm for (A), and 200 nm for (B and C).

shown in Fig. 1B, 2D, and 3A resemble each other in shape and size, supporting that these planar sheets are made of a mixture of **SLL** and **SDL**. Further, upon heating at 90 °C for 1 h, vesicles are quantitatively formed (Fig. 3B and C, cryo-TEM). The size distribution of the vesicles is very narrow around 200 nm diameter. Since this size is smaller than the expected size by closing the planar sheet with 1.5 μm side, the planar sheet should be torn off to generate the most stable size of the vesicle. This should be the reason for the narrow distribution of the vesicle diameter.

We have shown two different preparation methods for the same vesicle morphology, which confirms our interpretation of vesicle formation due to the mixing of the right- and the left-handed helices in the molecular assembly, which is thermodynamically induced by the stereo-complex formation. Indeed, the electron diffraction pattern and FTIR spectrum of the planar sheet of the mixture of **SLL** and **SDL** are different from those of the nanotube, indicating a tight molecular packing of **SLL** and **SDL** in the planar sheet and therefore vesicle due to the stereo-complex formation (Fig. 4, Fig. S5 and S6 (ESI†)).

We demonstrate a new method for vesicle preparation by a novel fusion mechanism, which is driven by the stereo-complex formation of the helical amphiphiles. The morphology transformation process from the nanotube to the vesicle is composed of four steps; (i) nanotube association, (ii) mixing of right- and left-handed helices, (iii) break-up of the tubular structure to the planar sheet structure, and (iv) closing to the vesicular structure. This fusion mechanism will pave the way to construct more complex morphologies which is now under investigation.

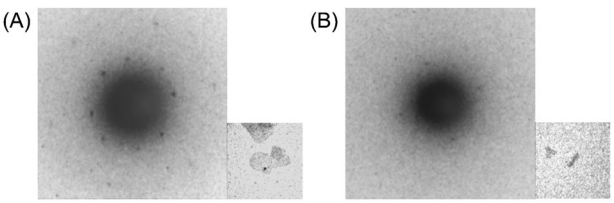

Fig. 4 TEM images and electron diffraction patterns of (A) the planar sheet assembly from an equimolar mixture of **SLL** and **SDL** and (B) the nanotube assembly from a single component of **SDL** in 10 mM Tris-HCl buffer (pH 7.4).

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