Self-assembly of tripeptides into γ-turn nanostructures

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Self-assembly of tripeptides into γ-turn nanostructures

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Self-assembling phenylalanine-based peptides have garnered interest owing to the potential for creating new functional materials. Here, we designed four diastereomers, L-Phe-L-Phe-L-Phe (FFF), D-Phe-L-Phe-L-Phe (fFF), L-Phe-D-Phe-L-Phe (FfF) and L-Phe-L-Phe-D-Phe (FFf), to analyze the effect of D-isomer on the self-assembly. Using SEM, TG, VCD, and solid-state NMR, we found that only FFf forms a γ-turn conformation and self-assembles into a nanoplate with higher thermal stability. The supramolecular structure of FFf consists of intra- and intermolecular hydrogen bonds, and π-π stackings. From our results, we have discovered that FFf forms a new type of self-assembling γ-turn conformation, clarifying the structural role of a D-amino acid residue in supramolecular formation.

There have been special interests in research on mechanism of self-assembly for short peptides. Self-assembly is a phenomenon in which molecules spontaneously form an ordered structure. The interest stems from the possibility of new functional material inventions. For instance, self-healable materials have been developed by manipulating hydrogen bond pairs.[1] One class of substances which can self-assemble, peptides, has attracted attention for several reasons, including: the biocompatibility and high designability of certain chemical structures, such as N-terminal amino protecting groups; modification of side chains; and ease of replacement with other α-amino acids. Potential applications of self-assembled short-peptide architectures, such as in piezoelectric components,[2] multi-responsive hydrogels,[3] drug delivery,[4] and many more,[5] have been suggested since the discovery that phenylalanine dipeptide (L-Phe-L-Phe-OH) self-assembles into fiber.[6] According to the molecular structure of diphenylalanine fiber, intermolecular interactions such as electrostatic, hydrogen bonding, and π-π stacking interactions stabilize the supramolecular assembly.[7] Interestingly, the side chains are oriented on the same side against the backbone plane, enabling them to form π-π stacking. Further, Görbitz investigated how hydrophobic dipeptides are likely to adopt a packing structure with nanopores, and concluded that the structure can be roughly divided into two categories: a valine-and-alanine model, which makes hydrophobic nanopores; and a Phe-and-Phe model, which makes hydrophilic nanopores.[8] The N-terminal amino protecting group peptide, Fmoc-Phe-Phe-OH, which is known as the new building block for self-assembly to a fibrous hydrogel.[9,10] However, using the Fmoc group might be inappropriate for biological applications because of its cytotoxicity.[11]

Triphenylalanine, L-Phe-L-Phe-L-Phe-OH (**FFF**), is well known as a peptide that self-assembles into a nanoplate and assumes a β-sheet structure.[12,13] As for tripeptides with hydrophobic amino acid containing a few phenylalanine residues, many studies have been attempting to obtain a gel by using D-amino acid residue, since tripeptides using only L-amino acids are less likely to self-assemble.[14] D-Val-L-Phe-L-Phe and D-Phe-L-Phe-L-Val tripeptides self-assembled into twisted filaments at pH 7.4, resulting formation of a hydrogel.[15] It is indicated that D-form at 1st residue of hydrophobic tripeptide plays a crucial role to form the gel at near physiological pH. However, there are no reports investigating the structural influence of D-phenylalanine on its supramolecular structure for tripeptides consisting of all three Phe residue. In this study, we investigated the effect of residue-specific replacement of L-isomer with D-isomer on the supramolecular structure of tripeptides consisting of only phenylalanine. To this end, we designed and synthesized L-Phe-L-Phe-L-Phe (**FFF**) and its three diastereomeric peptides containing a D-Phe residue: D-Phe-L-Phe-L-Phe (**fFF**), L-Phe-D-Phe-L-Phe (**FfF**), and L-Phe-L-Phe-D-Phe (**FFf**) (Scheme S1). Further, those supramolecular structures of the self-assembled peptides were investigated in detail using SEM, TG-MS, solid-state VCD, and solid-state NMR. In the process, we discovered that FFf self-assembles into nanoplate which has the higher thermal stability than other peptides. It is found that there are differences in view of secondary structure, structural homogeneity and the role of D-amino acid on the formation of the supramolecular structure in the combination of solid-state VCD with

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solid-state NMR. Herein, we report a new type of self-assembled γ-turn conformation for **FFf** peptide at near pH 5.0.

First, the reaction for the self-assembly was carried out at pH 4.9-5.2. by adding 90 µL of ultrapure water kept at 5 °C to a solution of 1.0 mg peptide per 10 µL HFIP following the previously reported procedure.[6] As shown in the SEM images of Figure 1 (a) and Figure S1, the four types of peptides considered in this study formed nanoplates with width of ca. 500 nm in the nanometer scale. It seems

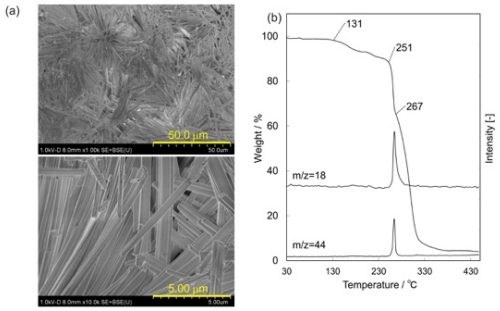

Figure 1. (a) SEM images and (b) TG curve of the **FFf** nanoplate.

like that the nano-plate of **FFF** is closely similar with the reported structure of **FFF**.[12] Moreover, all nanoplate structures resembled each other in the shape and size. The result of the TG-MS measurement is shown in Figure 1(b). The mass spectrometric analysis was performed with target molecular weights (m/z =18 and 44) on the gas generated during thermal decomposition. The thermal decomposition was defined as the temperature at which the weight (%) of the assembled peptides rapidly dropped by 1%. The major thermal decomposition usually occurs over 200°C via the reactions of deamination, decarboxylation, and decomposition of peptide bonds.[16] The less than 10% weight loss ratio observed before 150°C is due to the gradual thermal release of components of peptide.[17,18] The remaining 80% or greater of weight loss occurred via thermal decomposition. The major decomposition temperatures of the respective peptides were determined to **FFF** = 230°C, **fFF** = 229°C, **FfF** = 238°C, and **FFf** = 251°C (Figure 1(b) and Figure S2). **FFf** showed the highest thermal stability among the three tripeptides. Thus, it is implied that the self-assembly of **FFf** forms the most robust structure and its supramolecular structure is quite different from the other peptides.

Hence, in order to investigate supramolecular chirality and peptide secondary structure in the nanostructure, we performed

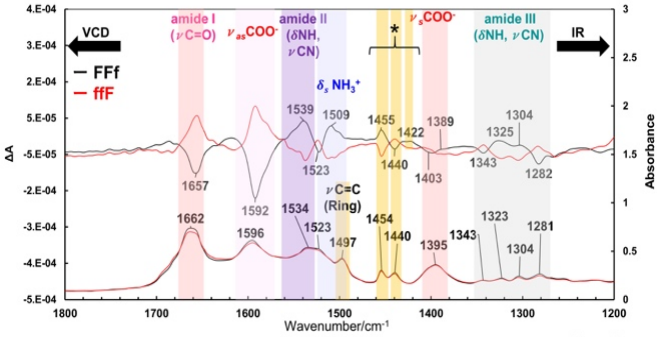

Figure 2. VCD (top) and IR (bottom) spectra of self-assembled **FFf** (black) and **ffF** (red). The signals marked with asterisk * indicate the bands of δC-H(Cα), δC-H(Cβ) and coexistence of δC-H(Cβ) and δC-H(Cβ) at 1455, 1440, 1422 cm^{-1}.

solid-state VCD/IR experiments. To check the mirror-imaged VCD signals, a D-Phe-D-Phe-L-Phe (**ffF**) nano-plate, which is the enantiomer of **FFf**, was prepared and measured. In Figure 2, the upper spectra are obtained from the VCD signals and the lower spectra are from the IR signals. The vibrational signals and their assignments of IR bands are shown in Table S1 and S2. Typically, amide I (~1650 cm^{-1}), amide II (~1550 cm^{-1}), and amide III (1400~1200 cm^{-1}) signals of a peptide can be distinctly observed. Further, the amide I band, which is mainly from C=O stretching, and the amide III band, which is from N-H in-plane bending and C-N stretching, is used to determine the peptide secondary structure.[19-21] In the observed **FFf** spectra, a signal of 1662 cm^{-1} in the amide I region and a plurality of high frequency signals of 1343, 1323, 1304, and 1281 cm^{-1} in the amide III range were obtained. In the case of the turn structure, amide I gives a signal at 1662-1686 cm^{-1},[20] and it is theoretically predicted that amide III gives peaks at 1317, 1303, 1298, and 1291 cm^{-1} when a structure forms a γ-turn.[21] In addition, it is experimentally known that the random coil and β-sheet structures cannot give peaks at higher than 1295 cm^{-1}. Other research further defines that the amide III band obtained at 1333, 1317, 1304, and 1291 cm^{-1} in the case of γ-turn structures.[21,22] Therefore, it is revealed that **FFf** takes a stable γ-turn conformation in the self-assembly. On the other hand, the amide I bands of **FFF, fFF, FfF** appeared at 1671/1639, 1685/1653, 1685/1639 cm^{-1} (Figure S3 and Table S1). In the case of the anti-parallel β-sheet structure, the amide I band appeared at an intensity ratio of 1:5 at 1695 and 1630 cm^{-1}.[13] Therefore, it is suggested that the **FFF**, **fFF** and **FfF** form anti-parallel β-sheet structures because similar IR spectrum of self-assembled **FFF** with β-sheet conformation were observed.[12] Many self-assembling short peptides containing diphenylalanine and D-amino acids are considered to have an anti-parallel β-sheet structure as well. [9, 10, 14] Cyclic-peptides and Ala-Phe-Ala (AFA) tripeptide have not been adequately studied to understand the feature of the γ-turn structure in solution.[26-28] Further, self-assembling short peptides with γ-turn structures have not been spectroscopically reported yet. A band of carboxylic group can be distinctly observed as follows: the vibration of νCOOH typically appears in the high frequency region at 1720-1800 cm^{-1}, while asymmetric and symmetric stretching vibrations of COO^- ($\nu_{as}COO^-$) appears at ~1600 cm^{-1} and stretching vibration (ν_sCOO^-) ~1400 cm^{-1}.[29] The IR bands of the self-assembled **FFf** appeared at 1596 and 1395 cm^{-1}. This indicates that the C-terminal group in the self-assembled peptides are ionized as COO^-. In fact, in the FT-IR spectrum of AFA tripeptide, the bands of $\nu_{as}COO^-$ and ν_sCOO^- are observed at 1596 cm^{-1} and 1395 cm^{-1}, respectively.[30] Mirror imaged VCD signals of **FFf** and **ffF** were observed corresponding to the IR assignments in the mid-infrared region. Huge

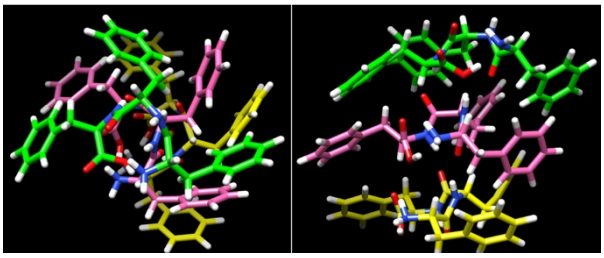

Figure 3. The model of associated three γ-turn peptides from the top (left) and side (right) views. The first (green), second (pink) and third (yellow) repeating **FFf** peptide units are counting from the front of the model in the top view.

VCD signals of amide I and $\nu_{as}COO^-$ appeared at 1657 and 1592 cm^{-1}. The VCD signals of amide II and III were also observed with opposite signs. As described below, the intensity and sign of VCD signals give structural insight for the supramolecular formation (Table S2).

The γ-turn structure is usually generated via intramolecular hydrogen bonding between CO(*i*) and NH(*i*+2).[31] There are two types of γ-turn structures: the classic γ-turn (ϕ = 70° to 85°, Ψ = -60° to -70°) and the inverse γ-turn (ϕ = -70° to -85°, Ψ = 60° to 70°). [31] It seems that inverse γ-turn is difficult to form intermolecular hydrogen bonds which is necessary for forming supramolecular structure. Firstly, considerable classic γ-turn **FFf** was constructed. Then three **FFf** peptides were aligned and optimized by DFT calculation. As a result, the model of associated three peptides was obtained, as shown in Figure 3. The calculated spectra of VCD and IR given by the optimized structure are shown in Figure S5 (a) and estimated dihedral angles are shown in Table S3. However, calculated VCD from the optimized structure didn't correspond with obtained VCD completely because an unavoidable event of proton transfer from NH_3^+ to COO^- for the optimized structure occurred at the step of DFT calculation. Accordingly, we carried out preliminary VCD calculation for an initial model of trimer having similar γ-turn conformation with ionized N- and C-terminal groups before optimization of a structure. As a result, calculated VCD spectrum of the manual model showed the intense signals of amide I and amide II due to having a harmonic vibration among three γ-turn peptides (Figure S5 (b)). Besides, the signal patterns were quite similar with observed signals. The VCD intensity was stronger in comparison with that of optimized structure (Figure S5). It would be more interesting if the present VCD results are simulated more precise theoretically in terms of large scale of molecular packing. Furthermore, we used solid-state NMR to ascertain whether it has a γ-turn structure, in accordance with the model structure. Although self-assembled **FFf** has a natural abundance ratio of ^{13}C and ^{15}N, well-resolved signals were observed enough to clearly identify all carbon and nitrogen atoms of the peptide in both ^{13}C and ^{15}N CP-MAS spectra, as shown in Figure 4. On the other hand, the ^{13}C NMR signals of **FfF** and **fFF** were slightly lower resolved than that of **FFf** (Figure S6). The estimated chemical shifts were evaluated by converting the shielding constants calculated from the model structure. The observed and estimated chemical shift values of **FFf** are summarized in Table S4. A value of the Cα carbon is related with conformation of peptide backbone based on the dihedral angles (ϕ, φ) of the secondary structure.[32] Furthermore,

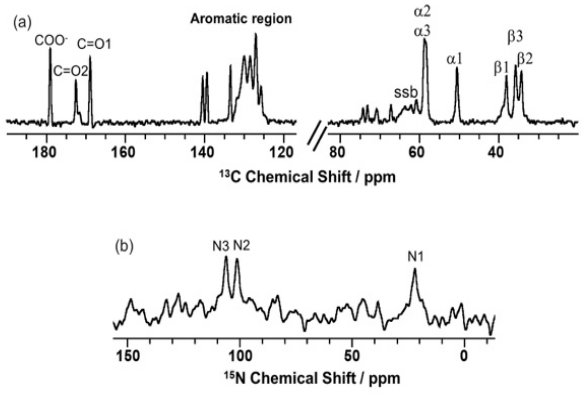

Figure 4. Solid-state (a) ^{13}C and (b) ^{15}N CP-MAS NMR spectra of the **FFf.** nanoplate.

because it is considered that peptide folding is largely controlled by orientation of the hydrophobic side chain, an interpretation of the chemical shift at D-Phe residue is important to understand the role of D-isomer in the structure. Specifically, a dihedral angle of D-isomer for one residue in a peptide has a symmetrical relationship of L-isomer on a Ramachandran plot because the allowed area is determined by the orientation of the side chain.[33] Therefore, the dihedral angle of the D-amino acid residue having a symmetrical relationship with the L-amino acid residue on the Ramachandran plot means that the NMR signal appears at the same chemical shift. Interestingly, in the chemical shifts of Cα carbons obtained from the model structure, the values of the 2nd L-isomer and the 3rd D-isomer are very similar to each other. The relationship on the plot is almost symmetrical in terms of position as the (ϕ, ψ) angles of the 2nd residue = (71.99°, -85.44°) and those of the 3rd residue (ϕ, ψ) = (-75.19°, 76.96°) as shown in Figure S7 (a) and (b). These angles are very close to those of typical classic γ-turns. [31] Experimentally, a peak intensity ratio of 2:1 was obtained for 58.73 ppm and 50.54 ppm peaks in the region of the resonance of Cα carbons. Based on the aforementioned consideration, 58.73 ppm peak can be assigned to the 2nd and 3rd residues and 50.54 ppm as that for the 1st residue. In addition, the calculated chemical shifts of Cβ and amide nitrogen atoms were also in good agreement with the experimental data (Table S4). Since the Cα and Cβ chemical shift values of 2nd and 3rd Phe indicate that both residues have α-helical-like torsion angles,[32] our model structure can be also supported by the NMR data. In addition, in the results of the VCD measurement, distinct mirror spectra between **FFf** and the antipodes **ffF** were obtained. This result strongly suggests that the molecular packings in the **FFf** and **ffF** nanoplates have a mirror image relationship. It means that self-assembled **FFf** and **ffF** form left- and right-handed helical-like structure toward the extension direction, respectively. The intensity of VCD generally tends to significantly increase with the hydrogen bonding between molecules against one extension direction.[34,35] In the VCD spectra of **FFf** and **ffF**, stronger peaks of $\nu_{as}COO^-$ appeared at 1592 cm^{-1} as opposite signs (Figure 2). The reason for observed huge VCD signal could be that the C-terminal group of **FFf** in the model structure can participate in not only inter- but also intramolecular hydrogen bonds (Figure S8). In the NMR spectra, the ^{13}C and ^{15}N signals of the C- and N-terminal groups in **FFf** appeared at 178.98 and 22.01 ppm which may be corresponding to the increase of strength of hydrogen bonds.[32,36] In the IR results, the fact that $\nu_{as}COO^-$ appeared at a lower wavenumber compared to other peptides also suggests strongly hydrogen bonding states.[19] Thus, **FFf** is a γ-turn structure having intramolecular hydrogen bonds between the N- and C-terminal groups. Additionally, it is not yet clear where in the γ-turn the amide I band appears; the amide I appearing at 1690-1647 cm^{-1} shows weak intra-hydrogen bonding between CO(*i*) and NH(*i*+2).[31] Therefore, it is considered that the C=O (L-Phe1) and N-H (D-Phe3) of **FFf** are gently intramolecular-hydrogen bonded. It is rather reasonable that the C=O (L-Phe1) and N-H (D-Phe3) is used to form complementary hydrogen bonds with the N-H (L-Phe2) and the C=O (L-Phe2) like the model structure (Figure S8). Moreover, the NMR signals in the aromatic ring region were well separated (Figure 4 and Figure S6). Thus, it can be inferred that the aromatic rings form very ordered π-π stacking. In general, Cγ peaks appear at the lower field among the aromatic region; thus, we were able to assign three peaks,

at 133.40, 140.45, and 139.39 ppm, as the Cγ carbons.[37] According to the Lindeman-Adams empirical rule,[38] the sums of three carbons consecutive correspond; therefore, we assigned the peak at 133.40 ppm as the 1st residue because the signal of Cβ appeared at the lowest field, 40.77 ppm. Similarly, we assigned the Cγ peaks at 140.45 and 139.39 ppm to the 2nd and 3rd residues. It was emphasized that the **FFf** forms a homogeneous γ-turn structure via the inter-molecular interactions of complementary hydrogen bonds and ordered π-π stackings. Generally, L-residue has a negative ϕ value owing to the orientation of the sidechain and the allowed region of the positive ϕ value in the plot, called the left-handed helical region, for Phe is very small owing to the large sidechain. In the case of the γ-turn conformation, however, the 2nd and 3rd residues limited the allowing torsional angles based on the narrow range of the angles for typical classic γ-turn (Figure S7(b)). Thus, it is necessary for the γ-turn to take extremely restricted conformation. Namely, the **FFf** self-assembles into very homogeneous and ordered structure. However, a relation between the γ-turn and the morphology of **FFf** cannot be completely illustrated through the SEM images and our spectroscopic results. In future work, we will determine the detailed left-handed supramolecular structure of **FFf** by combining powder XRD and an advanced solid-state NMR technique that measures the interatomic distance.

Conclusions

In this study, we have shown that the characterization of a series of diastereomeric Phe-Phe-Phe tripeptides using our spectroscopic approaches can reveal structural features. Here, it is clearly demonstrated, as results of solid-state VCD and solid-state NMR, that only **FFf** forms γ-turn structure in the nanostructure and **FFF**, **fFF**, **FfF** form anti-parallel β-sheet structures. We found that γ-turn conformations with the introduction of D-Phe into the 3rd residue enables the formation of stably assembled peptides. The complementary inter- and intramolecular hydrogen bonds between peptides in self-assembled **FFf** with γ-turn conformation are crucial in the development of a supramolecular structure with high thermal stability. Our results could serve as templates for the rational design of functional self-assembled short peptide systems.

Conflicts of interest

There are no conflicts to declare.

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