Self-assembly and hydrogel formation ability of Fmoc-dipeptides comprising α-methyl-L-phenylalanine

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Abstract
Various biofunctional hydrogel materials can be fabricated in aqueous media through the self-assembly of peptide derivatives, forming supramolecular nanostructures and their three-dimensional networks. In this study, we describe the self-assembly of new Fmoc-dipeptides comprising α-methyl-L-phenylalanine. We found that the position and number of methyl groups introduced onto the α carbons of the Fmoc-dipeptides by α-methyl-L-phenylalanine have a marked influence on the morphology of the supramolecular nanostructure as well as the hydrogel (network) formation ability.

Introduction
Supramolecular nanostructures, which consist of self-assembling peptide derivatives, have attracted rapidly increasing attention for the fabrication of various biofunctional materials [1–9]. Recent seminal works have revealed that short peptides bearing an aromatic group at their N-termini have a robust potential to self-assemble into supramolecular nanostructures under aqueous conditions with subsequent hydrogel formation, facilitating active exploration [10–17]. Rediscovery that simple dipeptides bearing the Fmoc (9-fluorenylmethyloxycarbonyl) group (one of the most typical protecting groups) give rise to hydrogels, as reported by Xu and coworkers [10], has facilitated the research activity [4]. In addition, among such self-assembling peptides, an aromatic phenylalanine–phenylalanine dipeptide [FF, (F: L-phenylalanine)] is one of the simplest and most advantageous scaffolds used to fabricate a variety of supramolecular nanostructures [18, 19]. In fact, two research groups simultaneously reported that the FF dipeptide bearing the Fmoc group (Fmoc-FF) could form stable supramolecular hydrogels due to the formation of a supramolecular nanofiber network, which were utilized as scaffold materials for cell cultures [11, 12].

As discrete structural derivatives of natural peptides can be synthesized, α,α-disubstituted α-amino acids (αAAs) can offer a distinctive opportunity to modulate the morphology of supramolecular nanostructures [20, 21]. It is well recognized that peptides consisting mainly of α-aminoisobutyric acid (Aib), which is one of the most actively investigated αAAs, adopt a 310-helix instead of an α-helix [21, 22]. Control of the helicity of the 310-helix in response to various chiral stimuli has been actively investigated [22]. On the other hand, the self-assembly of peptides containing αAAs has remained unexplored. Hammer and coworkers demonstrated that a peptide containing αAAs inhibits β-amyloid formation [23]. Moreover, Moretto and coworkers developed unique peptides containing αA, which have two azobenzene moieties as side chains that are capable of participating in the photoresponsive transformations of supramolecular nanostructures [24]. Due to limited examples [25, 26], the systematic construction of peptides

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containing ααAAs and the investigation of their self-assembly are significant in understanding the influence of subtle but precise chemical modifications of constituent peptides on the ability of these peptides to form supramolecular nanostructures.

Herein, we describe the self-assembly and supramolecular hydrogel formation ability of Fmoc-dipeptides (Fmoc-XXs, Fmoc = 9-fluorenylmethyloxycarbonyl, X = F or D F or m F) comprising α-methyl-L-phenylalanine (m F) as one of the ααAAs and/or L-phenylalanine (F) or D-phenylalanine (D F). To our knowledge, the influence of such chemical modification of the Fmoc-FF at the α carbons on the self-assembling propensity is unexplored. Due to a reliable and scalable synthetic procedure with asymmetric phase-transfer catalysts, developed by Maruoka and coworkers, a variety of ααAAs, including m F, with high purity are now commercially available [27, 28]. In this study, we found that the position and number of methyl groups introduced onto the α carbons of the Fmoc-dipeptides (Fig. 1) have an unexpectedly marked influence on the morphology of the supramolecular nanostructure as well as the hydrogel formation ability.

**Materials and methods**

**Experimental generals**

Unless stated otherwise, all commercial reagents were used as received. All water used in the experiments was ultra-pure water obtained from a Millipore system with a specific resistance of 18 MΩ·cm. Thin-layer chromatography was performed on silica gel 60F254 (Merck). 1H and 13C NMR spectra were obtained on a JEOL JNM ECS-400 spectrometer (400 MHz for 1H, 100 MHz for 13C) with tetramethylsilane (TMS) or residual nondeuterated solvents as the internal references. Multiplicities are abbreviated as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = double doublet, and br = broad. MALDI-TOF mass spectra were recorded using a Shimadzu AXIMA-CFR plus mass spectrometer. High-resolution mass spectra were obtained on a JEOL JMS-T100LP AccuTOF LC-plus (ESI) or a Waters Xevo QTof (ESI) mass spectrometer. Fluorescence spectra were obtained using a PerkinElmer LS-55 fluorescence spectrometer.

![Diagram](image_url)

**Fig. 1** Hydrogel formation ability of Fmoc-XXs. a Chemical structures of Fmoc-dipeptides (Fmoc-XXs, X = F or D F or m F) comprising phenylalanine and α-methyl-phenylalanine. b Summary of the gelation tests (Gel transparent or translucent gel, pGel partial gel, tSol turbid solution, Sol clear solution) of Fmoc-XXs. Conditions: 100 mM HEPES-NaOH (pH 7.4) containing DMSO (2.0 vol% for 0.20 wt% of Fmoc-XXs and 4.0 vol% for 0.40 wt% of Fmoc-XXs). Fmoc 9-fluorenylmethyloxycarbonyl, F L-phenylalanine, D F D-phenylalanine, m F α-methyl-L-phenylalanine.
Solid-phase synthesis of Fmoc-dipeptides (Fmoc-XXs)

Syntheses of Fmoc-dipeptides (Fmoc-XXs) were carried out in the solid phase by using a multiple reaction device (HiPep Laboratories, PetiSzyzer) according to conventional solid-phase peptide synthesis protocols. All reactions were carried out in cartridges with polypropylene frits (HiPep Laboratories, LibraTube) using 2-chlorotrityl chloride resin (Watanabe, 100–200 mesh, 1% DVB, ~1.6 mmol/g). The condensation reaction was carried out in the presence of Fmoc-amino acid (3.0 eq.), 1-[bis(dimethylamino)methyl]imidazolium-1H-benzotriazole-3-oxide hexafluorophosphate (HBTO, 3.0 eq.), 1-hydroxybenzotriazole hydrate (HOBT·H2O, 3.0 eq.), and N,N-diisopropylethylamine (DIEA, 6.0 eq.) in N-methylpyrrolidone at room temperature (from 1 to 3 h). For the final step, a mixture of hexafluorospirane (HFIP)-dichloromethane (1:4 v/v) was added, and the resin was removed by filtration and washed with the cleavage mixture. The collected filtrate was concentrated under reduced pressure, and the peptide was precipitated by the addition of diethyl ether and hexane. Each obtained peptide was analyzed by 1H and 13C NMR and ESI-TOF-HRMS (Electronic supplementary materials).

Preparation of hydrogel or dispersion

Gelation ability was evaluated by an inverted tube test [30]. Typically, Fmoc-XX powder was dissolved in dimethyl sulfoxide (DMSO), and the resultant stock solution (10 wt %, 4.0 µL) was mixed with aqueous buffer [100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4), 196 µL] inside a glass vial (inner diameter: 9.0 mm). The suspension was heated until a homogenous solution was obtained. The solution was left to cool at room temperature without any disturbance. Gel formation was determined by whether the sample flows or not when the glass vial is inverted.

Transmission electron microscope (TEM) observation

A sample (~10 µL) was dropped on a copper TEM grid covered by an elastic carbon-support film (20–25 nm) with filter paper underneath, and the excess solution was immediately blotted with filter paper. The TEM grid was dried under a reduced pressure for at least 6 h prior to TEM observation. TEM images were acquired using a Hitachi H-7000 (accelerating voltage: 100 kV) equipped with a CCD camera and AMTV600 software.

Dynamic light scattering (DLS) measurement

DLS measurements were performed on a Malvern Zetasizer Nano equipped with a He-Ne laser (633 nm). Aqueous solutions were filtered using a Millipore membrane filter (0.45 µm pore size) before the measurements. The particle size distribution was derived from a deconvolution of the measured intensity autocorrelation function by the “general purpose mode” (nonnegative linear least-squares) algorithm included in the software of the instrument.

Confocal laser scanning microscope (CLSM) observation

A freshly prepared homogeneous solution (0.20 or 0.40 wt %, 100 mM HEPES (pH 7.4), 20 µL) of Fmoc-XXs obtained by heating in a glass vial was transferred to a microtub e and left to cool at room temperature without any disturbance and was subsequently mixed with a DMSO solution of Nile red (0.3 mM (final concentration is 14 µM), 1.0 µL). The gel (~20 µL) was spotted on a glass coverslip (diameter: 25 mm, thickness: 0.13–0.17 mm, Fisher Scientific) and placed in an Attolfuor cell chamber (Thermo Fisher Scientific) with water drops (50 µL) around the sample drop to avoid dryness. The sample observed using an inverted CLSM (FV1000-D, Olympus) equipped with an Ar laser (488 nm) and LED laser (559 nm). A 60 × (numerical aperture (NA) = 1.49) oil objective was employed to obtain images. The obtained images analyzed by the acquisition software FV10-ASW4.2 supplied with a microscope. The images were also quantified and analyzed by Fiji (freely available at https://fiji.sc/) [31, 32].

Critical aggregation concentration (CAC) determinations

CACs of Fmoc-XXs were evaluated by a thioflavin T (ThT) assay [33]. The solutions of Fmoc-XXs [100 mM HEPES-NaOH (pH 7.4) containing DMSO (less than 2.0 vol%)] at various concentrations containing ThT (15 µM) were prepared. Fluorescence spectra (ex/em = 440 nm) of the solutions were obtained using a PerkinElmer LS-55 fluorescence spectrometer with a quartz cell (1.0 cm × 0.2 cm). Fluorescence intensities at λem = 494 nm were plotted against the concentration of Fmoc-XXs to evaluate the CACs.

Rheological measurement

Dynamic frequency and strain sweep experiments were carried out on a TA Instruments AR-G2 rheometer using a...
20-mm stainless steel parallel plate (the temperature of the plate was controlled at 25 °C by a Peltier system) at a gap of 1.0 mm. Hydrogel samples were placed on the plate. All the gels showed an almost linear viscoelastic regime up to 1.0% strain (frequency: 1.0 rad/s). Therefore, a frequency sweep (0.1–100 rad/s) was performed under 0.2% strain.

Results and discussion

Five distinct Fmoc-dipeptides, Fmoc-FmF, Fmoc-mFmF, Fmoc-mFF, Fmoc-DFmF, and Fmoc-mFD (Fig. 1a), were newly synthesized following standard Fmoc-based solid-phase peptide synthesis using a 2-chlorotryptyl chloride resin.
Note that, since coupling with Fmoc-mF was slow and inefficient, most likely due to the steric hindrance of the α-methyl group, the amount of Fmoc-mF used was increased, and HATU was used as the coupling reagent instead of HBTU. All Fmoc-XXs showed reasonable solubility toward DMSO and HFIP. A conventional tube-inversion method [30] was applied to evaluate the hydrogel formation ability. As summarized in Fig. 1b, Fmoc-FF, which is employed as a typical hydrogelator for control experiments, produced hydrogels at concentrations above 0.20 wt%. Gratifyingly, we found that both Fmoc-mFF and Fmoc-DFmF showed the ability to form supramolecular hydrogels at 0.20 wt%, suggesting the formation of networked supramolecular nanostructures. In contrast, Fmoc-mFmF, Fmoc-FmF, and Fmoc-mFmF showed no hydrogel formation ability; instead, rather stable suspensions were obtained, suggesting the formation of nonnetworked but self-assembled nanostructures. These differences in hydrogel formation ability, which can be attributed to the introduction of the α-methyl group and/or the chirality of the phenylalanine group in the Fmoc-FF dipeptide scaffold, were further investigated in the following study.

To compare the self-assembling properties of Fmoc-XXs, we investigated their CACs by using a widely used environment-responsive and preferentially β-sheet binding fluorescence dye, thioflavin T (ThT) [33]. Fig. 2b summarizes the CACs evaluated from the concentration-dependent fluorescence intensity plot (Fig. 2a). Here, Fmoc-DFmF showed the lowest CAC and the strongest propensity to self-assemble, while Fmoc-mFmF showed the highest CAC and a significantly lower fluorescence intensity, suggesting its weaker propensity to self-assemble and adopt β-sheet structures. Additionally, this result indicates that the methyl groups introduced onto the α carbons have a significant influence on the self-assembling propensity and suggests that there would be a tendency for the methyl group at the N-terminus to enhance self-assembly and a tendency for that at the C-terminus to attenuate it.

TEM observations were carried out to gain insight into the morphology of the supramolecular nanostructures of Fmoc-XXs. Consistent with previous reports [10, 11], the network structures of the nanofibers were clearly visualized for Fmoc-FF (Fig. 3a). On the other hand, nonnetworked nanorod structures were found for Fmoc-mFF, a derivative containing two α-methyl groups (Fig. 3b). When one F of Fmoc-FF was replaced with mF, highly spherical nanostructures were observed for Fmoc-FmF, as shown in Fig. 3c-i, ii. The average diameter was 174 ± 50 nm (the histogram analysis is shown in Fig. 3c-iii). In good agreement with the size estimated from TEM images, DLS experiments showed an intensity distribution with a peak corresponding to an average hydrodynamic diameter of 209 ± 48 nm with a relatively narrow distribution (Fig. 4 and Table 1). In sharp contrast, the network structures of fibrous architectures with diameters of several tens of nm were observed for Fmoc-mFF (Fig. 3e), which should be responsible for the formation of a supramolecular hydrogel. Clearly, the position and the number of methyl groups introduced onto the α carbons of the original Fmoc-FF have a profound influence on the morphology of supramolecular nanostructures and the hydrogel formation ability.

We also surveyed the influence of the chirality of phenylalanine on the morphology of supramolecular nanostructures [34–36] for both Fmoc-mFF and Fmoc-FmF. Marchesan and coworkers systematically investigated the supramolecular hydrogel formation ability of peptides that contain D-amino acid residues [34, 35]. For example, although the tripeptides VFF (V, valine) and FFV failed to self-assemble at physiological pH, PFFV (P, D-valine) and PFFV successfully self-assembled to form hydrogels [35]. In addition, Banerjee and coworkers reported that the incorporation of D-amino acid into Boc-FFF (Boc, tert-butoxycarbonyl) enables modulation of the physiological stability as well as the stiffness of the hydrogels [36]. Herein, we found that Fmoc-mFmF, as a result of chirality inversion at the C-terminus of Fmoc-mFF, does not show well-defined nanostructures (Fig. 3f). In contrast, clusters of spherical nanostructures were observed for Fmoc-mFmF (Fig. 3d-i, ii). The average diameter of the spherical nanostructures was 96 ± 31 nm (the histogram analysis is shown in Fig. 3d-iii). This result suggests (colloidal) hydrogel formation [37], which has rarely been reported for short self-assembling peptides [38, 39].

To further investigate the self-assembled morphology under wet conditions and to gain insight into the mechanism...
of hydrogel formation, CLSM observations of the hydrogels of Fmoc-FF, Fmoc-mFF, and Fmoc-DFmF were carried out. To stain the self-assembled structures, Nile red was added to the hydrogels [17]. As shown in Fig. 5a, Fmoc-FF showed networks composed of clearly discernible fibrous structures. On the other hand, Fmoc-mFF showed less clearly discernible fibrous structures (most likely owing to less bundled fibrous structures), as shown in Fig. 5b. These fiber network morphologies are consistent with those observed under TEM. In contrast, bright spots and their clusters were found almost uniformly for Fmoc-DFmF (Fig. 5c), suggesting that clustered spherical nanostructures might be responsible for the hydrogel formation, as suggested by TEM.

The viscoelastic properties of Fmoc-FF, Fmoc-mFF, and Fmoc-DFmF hydrogels were also evaluated by oscillatory rheology measurements. As shown in Fig. 6a, the strain-sweep oscillatory rheology of the three hydrogels displays a linear viscoelastic region. Figure 6b shows the frequency-sweep oscillatory rheology in the linear viscoelastic region. The storage modulus ($G'$) of Fmoc-FF (Fig. 6b-i) was significantly larger than the loss modulus ($G''$), which is almost consistent with those reported previously [12, 13]. In contrast, Fmoc-mFF (Fig. 6b-ii) and Fmoc-DFmF (Fig. 6b-iii) showed smaller $G'$ and $G''$ values than with Fmoc-FF. Nevertheless, the $G'$ values were larger than the $G''$ values, indicating the formation of weak physical hydrogels. On the other hand, Fmoc-mFF and Fmoc-DFmF hydrogels displayed relatively strong frequency dependences compared with the Fmoc-FF hydrogel (Fig. 6b). This difference could be attributed to the difference in their network structures (e.g., permanency), as suggested by TEM and CLSM observations. Theory for semiflexible polymers [40] predicts that the rigidity of a cross-linked gel is dependent on both the mesh size and the fibril bending modulus. According to the TEM and CLSM images, the fibril bending modulus of Fmoc-mFF appears to be smaller than that of Fmoc-FF, which might be consistent with the smaller $G'$ of Fmoc-mFF. On the other hand, Fmoc-DFmF exhibited apparently different morphologies of supramolecular nanostructures,
that is, clustered spherical nanostructures. Thus, the theoretical background to ascribe the rheological property should be applied after specialized experiments, which is beyond the scope of this study.

The self-assembling mode at the molecular level remains to be understood. According to the crystal structure of Fmoc-FF reported previously by Adams and coworkers [13], the π-stacking interactions of Fmoc groups and side chain phenyl groups, as well as the hydrogen bonding interactions among carbamate groups and amide groups, are crucial for giving rise to the one-dimensional self-assembled structure, reasonably corresponding to nanofiber formation. Preliminary molecular mechanics calculations (Fig. S11, electronic supplementary materials) suggest that the newly introduced methyl group of Fmoc-mFF at the N-terminus might be accommodated in a one-dimensional self-assembled structure that is somewhat expanded compared to that of Fmoc-FF, whereas introducing the methyl group at the C-terminus in Fmoc-FmF could disturb the one-dimensional self-assembled structure by weakening the π-stacking interactions as well as the hydrogen bonding interactions. Further experimental and more detailed molecular modeling studies are required to elucidate the differences from the viewpoint of self-assembling structures at the molecular level.

Conclusion

In conclusion, this study revealed that the introduction of one or two methyl groups onto the α carbons of an Fmoc-FF scaffold has a vital influence on the morphology of the supramolecular architecture at the nanometer scale as well as on the macroscopic properties (hydrogel or dispersion). Further research to elucidate the self-assembling modes of peptides comprising α-methyl-amino acids leading to distinctive supramolecular nanostructures with biofunctional properties is in progress in our group.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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