"Threading" of β-Sheet Peptides via Radical Polymerization

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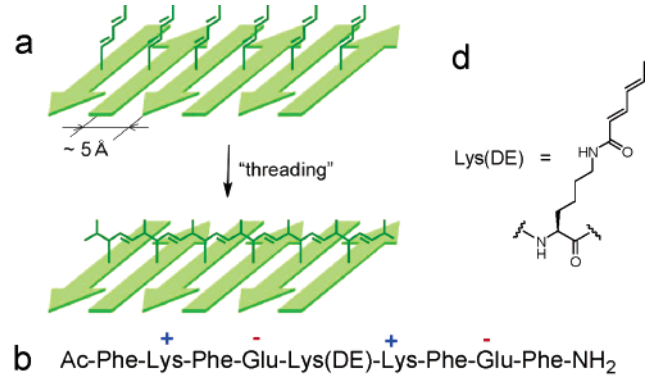
A nonamer peptide containing a diene group in the center of the sequence was synthesized. When the peptide forms an antiparallel β-sheet, the diene groups align ca. 5 Å apart on the β-sheet. The diene groups successfully photopolymerized without distorting the β-sheet structure. The obtained β-sheet showed high stability against acid denaturation and addition of 1,1,1,3,3,3-hexafluoroisopropanol.

Introduction

The importance of β-sheet peptides in materials science is increasing. For example, recent new applications for silk fibers, which are mainly composed of β-sheets, have been found in biomaterials.[1] In the field of tissue engineering, hydrogels formed from β-sheet fibers several tens of nanometers in width have been used as tissue scaffolds.[2] Furthermore, in the field of nanotechnology, β-sheet peptides are useful constituents of nanoscale materials that utilize their self-assembling properties.[3] In general, higher-order structures of peptides such as α-helix, β-sheet, collagen triple-helix, and so forth are relatively unstable against physical and chemical stimuli (for example, heat, solution pH, and solvent), so cross-linking peptides by covalent bonds is an important subject for the practical application of these peptide materials.[4] However, the higher-order structures of peptides sometimes become less stable after cross-linking due to a distortion of the peptide conformation induced by the cross-linking.[5] Thus, it is essential to design appropriate cross-linkers whose conformations match the arrangements of cross-linking points on the higher-order structures.

The association type of peptides in β-sheets differs from that of peptides in other higher-order structures; β-sheets are classified as being open association; i.e., a β-sheet is principally formed by the association of infinite numbers of peptide strands. Thus, polymerization is suitable for cross-linking the many peptide strands in a β-sheet. Here we present a "threading" method for β-sheet peptides that works by polymerization of monomeric groups attached to the peptides as shown in Figure 1a. In the β-sheet structure, peptide strands align 4.3−4.8 Å apart depending on the peptide sequence, typically 4.8 Å.[6] To cross-link peptide strands across this distance without distorting the peptide conformation, we selected a diene as the polymerizable group, since it is known that diene aligned at a distance of 4.9−5.1 Å in the crystalline state undergoes topochemical polymerization while keeping this amount of separation throughout the polymerization.[7,8] We designed a nonamer peptide containing Lys(DE) (Figure 1d) by referring to the strategy of Zhang's group.[9] The sequence and a molecular model are shown in Figures 1b and 1c, respectively. The peptide is expected to form antiparallel β-sheets at neutral pH by both hydrophobic interactions among Phe units and complementary electrostatic interaction among Lys and Glu units of the peptides. As a result, the centered diene groups should align on an antiparallel β-sheet.



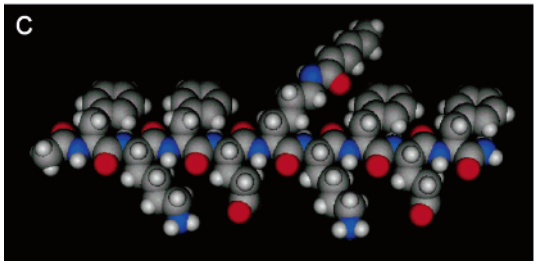


Figure 1. (a) "Threading" of β-sheet peptides utilizing polymerization of dienes attached to the peptides. (b) Sequence of the peptide used here. (c) Molecular model of the peptide with the antiparallel β-sheet conformation. (d) Chemical structure of the polymerizable amino acid Lys(DE).

Experimental Section

Materials. Sorbic acid (Wako), *N*-hydroxysuccinimide (TCI), and dicyclohexylcarbodiimide (Kanto Kagaku) were used as received. 9-Fluorenylmethyloxycarbonyl (Fmoc) amino acids and Rink-Amide MBHA resin were obtained from Watanabe Chemical.

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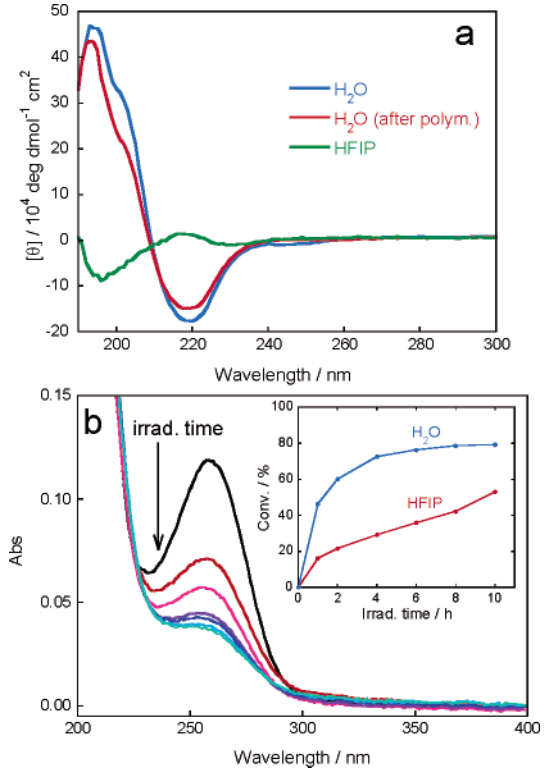

Figure 2. (a) CD spectra of the peptide in HFIP and H_2O (before and after 10 h of photopolymerization). (b) UV spectral change during photopolymerization of the peptide (0−10 h) in H_2O. The inset shows time−conversion curves of the diene group of the peptide in H_2O and HFIP. In part a, the peptide concentration is 100 μM, and the pH of the H_2O solutions is 7.4. In part b, polymerization was conducted in aqueous (pH 7.4) or HFIP media with a peptide concentration of 100 μM. After polymerization, the peptide solution was diluted with HFIP to a concentration of 50 μM, and the samples were used for UV spectra measurements.

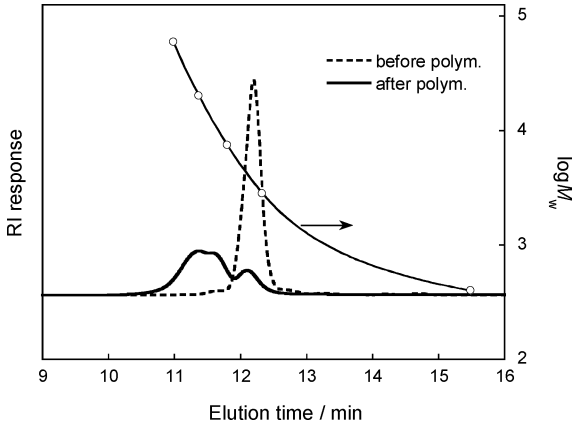

Figure 3. GPC profiles of the peptide before and after 10 h of photopolymerization. Approximately 10 mM sodium-trifluoroacetate-containing HFIP was used as an eluent to dissociate β-sheets during the analysis.

Synthesis of Fmoc-Lys(DE)-OH. Sorbyl *N*-hydroxysuccinimide ester was synthesized according to the literature.[10] Sorbyl *N*-hydroxysuccinimide ester (1.55 g, 7.46 mmol) was then dissolved in 90 mL of *N*,*N*-dimethylformamide (DMF) in a 100 mL round-bottom flask. Fmoc-Lys-OH (1.37 g, 3.73 mmol) was added to the solution, and the resulting turbid mixture was stirred for 8 h at room temperature while the flask was covered with aluminum foil to protect the contents from photopolymerization. A transparent solution resulted, and 360 mL of H_2O was slowly added to this at 0 °C. The resulting suspension (pH ≈ 3) was stirred for 1 h at 0 °C and filtered. The obtained white solid was washed with water and dried in vacuo. The resulting white solid was dissolved in MeOH/AcOEt and purified by silica gel column chromatography (AcOEt/*n*-hexane/AcOH = 2:1:0.01). The obtained compound was stored in a Pyrex bottle covered with aluminum foil at 5 °C.

Yield 60%; white solid. ^{1}H NMR (400 MHz, d_6-DMSO, ppm) δ: 1.3−1.4 (m, 2H), 1.4−1.5 (m, 2H), 1.78 (d, J = 6.2 Hz, 3H), 3.12−3.16 (m, 2H), 3.93−3.98 (m, 2H), 4.22−4.31 (m, 3H), 5.91 (d, J = 15.0 Hz, 1H), 6.03−6.08 (m, 1H), 6.16−6.22 (m, 1H), 7.01 (dd, J = 15.0, 11.0 Hz, 1H), 7.34 (t, J = 7.0 Hz, 2H), 7.43 (t, J = 7.5 Hz, 2H), 7.64 (d, J = 8.1 Hz, 1H), 7.74 (d, J = 7.7 Hz, 2H), 7.90 (d, J = 7.7 Hz, 2H), 7.97 (t, J = 5.7 Hz, 1H), 12.0−13.0 (br, 1H). ^{13}C NMR (100 MHz, d_6-DMSO, ppm) δ: 18.2, 23.1, 28.8, 30.5, 38.4, 46.7, 53.8, 65.6, 120.1, 123.1, 125.3, 127.0, 127.6, 129.9, 136.2, 138.9, 140.7, 143.8, 156.2, 174.0. Anal. Calcd for $C_{27}H_{30}N_2O_5$: C, 70.11; H, 6.54; N, 6.06. Found: C, 69.84; H, 6.51; N, 5.92.

Peptide Synthesis. The peptide was synthesized manually based on the Fmoc-based solid-phase method using Rink-Amide MBHA resin. Approximately 4.0 equiv of Fmoc amino acids were coupled by *N*,*N*′-diisopropylcarbodiimide (4.0 equiv) and 1-hydroxy-1H-benzotriazole (4.0 equiv). DMF was used as the coupling solvent. The completion of the coupling reactions was monitored by Kaiser's ninhydrin test. The peptide was purified by high-performance liquid chromatography (HPLC), and the purification procedure was as follows. HPLC was carried out using a Tosoh CCPM multipump, a PX-8010 controller, and a UV-8011 detector. YMC-Pack ODS-A analytical (4.6 mm × 250 mm) and preparative (20 mm × 250 mm) columns were used with flow rates of 1.0 and 5.0 mL/min, respectively. For analytical HPLC, a linear gradient of acetonitrile containing 0.05% tetrafluoroacetate in 0.05% aqueous tetrafluoroacetate, from 20% to 50% in 30 min, was used. The obtained peptide was identified by electrospray ionization mass spectrometry (ESI-MS). MS (ESI) *m*/*z*: calcd for $C_{72}H_{97}N_{13}O_{15}$ ($M + H^+$), 1384.7; found, 1383.8. After lyophilization, the obtained peptide was stored in a Pyrex bottle covered with aluminum foil at −30 °C. The peptide was dissolved in deionized water, and the pH of the peptide solution was adjusted by addition of aqueous NaOH or HCl.

Photopolymerization. Peptide solutions (3.0 mL) were bubbled with Ar for 3 min and poured into a quartz cuvette (pass length, 1.0 cm; width, 1.0 cm). Photopolymerization of the peptide was conducted in the cuvette at room temperature. UV light was irradiated using an ultrahigh-pressure Hg lamp (Ushio USH-500D, 500 W) with a filter (Toshiba, UV-27; cutoff wavelength, 270 nm). The distance between the front surface of the cuvette and the light source was 10 cm.

Transmission Electron Microscopy Observation. The transmission electron microscopy (TEM) observation was performed on a Hitachi H-800 electron microscope operated at an accelerating voltage of 75 kV. Specimens for TEM observation were prepared as follows. A drop of the aqueous peptide solution before and after polymerization was cast on a 200 mesh copper TEM grid deposited by carbon and dried in vacuo. A drop of aqueous phosphotungstic acid (2.0 wt %) was then placed on the specimen, and it was dried in vacuo.

Measurements. ESI-MS was performed on an Esquire 2000-T mass spectrometer (Bruker). Circular dichroism (CD) spectra were measured by a Jasco J-820 spectropolarimeter using a 1 mm path length quartz cuvette (resolution, 1 nm; scan speed, 50 nm/min). UV spectra were measured by a Hitachi U-3210 spectrophotometer equipped with a temperature controller (SPR-10, Shimadzu) using a 1 mm path length quartz cuvette. Gel permeation chromatographyt (GPC) analysis of the peptide was performed on a Tosoh HLC-8220GPC system with two TSKgel SuperH2500 columns at 40 °C. Approxmiately 10 mM sodium tetrafluoroacetate/1,1,1,3,3,3-hexafluoroisopropanol (HFIP) (elution rate, 0.3 mL/min) and poly(methyl methacrylate)s were used as an eluent and standards, respectively. The molecular model of the peptide was generated by using the MOE, version 2004.03, program (Chemical Computing Group).

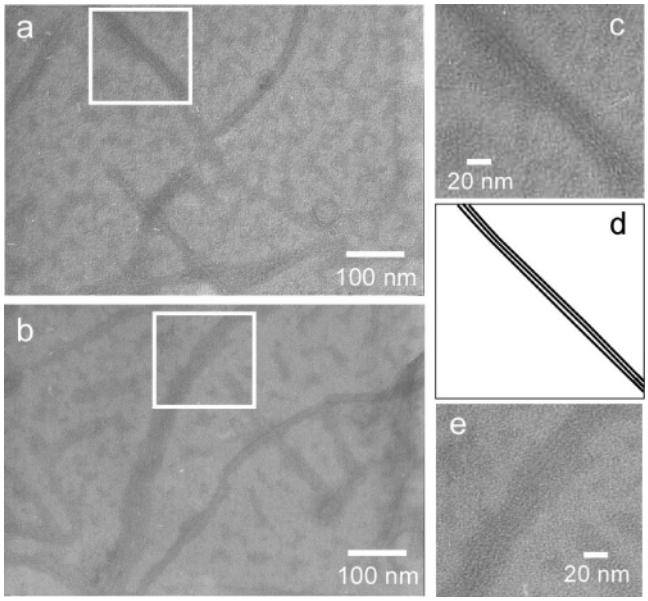

Figure 4. TEM images of the peptide (a and c) before and (b and e) after photopolymerization. Parts c and e are magnified images of the regions indicated as white squares in parts a and b, respectively. Part d is a trace of the fine fibers constituting the thick fibers of part c.

Results and Discussion

CD spectra of the aqueous (pH 7.4) and HFIP solutions of the peptide are shown in Figure 2a. The spectral pattern of the aqueous solution was typical of a β-sheet structure as we expected, whereas the peptide adopts an unstructured conformation in HFIP solvent. UV absorption of the peptide due to the diene group showed not only a blue shift but also hypochromism when the solvent was changed from HFIP to neutral water (Figure S2 in the Supporting Information). This spectral change reflects the hydrophobic environment around the diene group when the peptide forms the β-sheet in aqueous media, because the diene group locates on the hydrophobic side of the β-sheet.

Next, photopolymerization of the diene groups attached to the peptide strands was carried out in the aqueous and HFIP media, in which the peptide adopts β-sheets and unstructured conformations, respectively. During polymerization in the aqueous media, the peptide solution became slightly turbid. The turbid solution was diluted by HFIP before measuring UV spectra because the solution became clear by dilution. As shown in Figure 2b, the absorption due to the diene (λ_{max} = 257 nm) gradually decreased with irradiation time. The consumption of diene groups as estimated from the absorption intensity was faster in aqueous solvent than that in HFIP solvent and was almost saturated at 4 h (Figure 2b, inset). Thus, it was concluded that β-sheet formation accelerates the polymerization of the diene groups of the peptides. The increase of molecular weight of the peptide after photopolymerization was followed by GPC using HFIP as the elution solvent (Figure 3). After 10 h of polymerization, a peak resulting from the monomer peptide (elution time ca. 12 min) decreased considerably, and new peaks corresponding to higher molecular weights appeared. The size increase of the peptide after photopolymerization was revealed by a dynamic light scattering method (Figure S3). In the HFIP solvent, the polymerized peptide showed an average hydrodynamic diameter of approximately 170 nm, whereas the diameter of the unpolymerized peptide was difficult to measure with our instrument because of the small scattering intensity. The CD spectrum of the peptide after 10 h of photopolymerization is shown in Figure 2a. Although a slight decrease in the CD intensity was observed at 195 and 220 nm, the peptide kept its β-sheet structure even after polymerization. This result indicates that the polymerization of diene side groups hardly distorts the secondary structure of the peptide main chain.

Self-assembled structures of the peptide β-sheet before and after polymerization were observed by TEM. In both cases, fibrils 10−40 nm in width and several hundreds of nanometers in length were observed (Figures 4a and 4b). The fibrils seemed to be bundles of fine fibers several nanometers in width (Figures 4c−e), and the width of the fine fibers (3.2 ± 0.4 nm) roughly agrees with the length of the peptide with β-sheet conformation (ca. 3 nm). The consistency in the size of the fibrils (width and length) between the unpolymerized and the polymerized peptides again indicates that the distortion of the peptide main chain

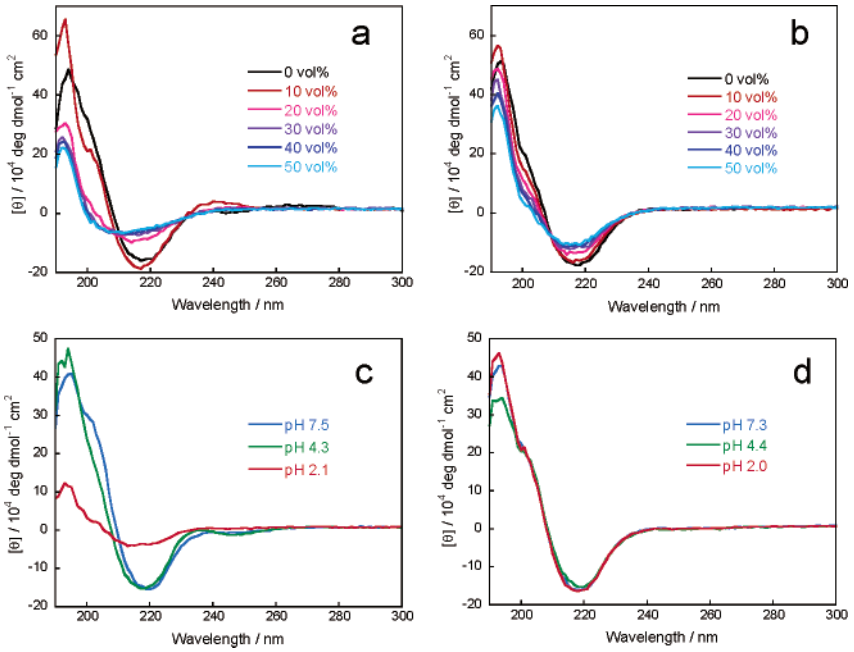

Figure 5. HFIP-induced CD spectral change of the peptide (a) before and (b) after 10 h of polymerization. Effect of acidification on the CD spectra of the aqueous peptide solutions (c) before and (d) after 10 h of polymerization. The concentrations of the peptide were (a and b) 50 and (c and d) 100 μM.

induced by polymerization of the diene side groups is very small. Thus, both high polymerizability and preservation of the β-sheet structure after polymerization were realized by matching the polymerization distance with the distances among β-strands.

The effect of the polymerization on the stability of the β-sheet was checked by two denaturation tests (HFIP addition and acidification). The unpolymerized peptide denatured when the HFIP content reached 20 vol % (Figure 5a), whereas the polymerized peptide gradually denatured with increasing HFIP content, passing through an isodichroic point at 209 nm, and retained a high β-sheet content until 50 vol % of HFIP content (Figure 5b). Under the acidic conditions, Glu units are protonated, and only the cationic charges of Lys units remain in the peptide. Consequently, the peptide will no longer form β-sheets, because of the electrostatic repulsion among the peptide strands. Indeed, the unpolymerized peptide denatured when the pH value of the solution became approximately 2 (Figure 5c). However, surprisingly, the photopolymerized peptide did not denature at all at pH 2.[11] Thus, it was shown that the polymerization of the diene side groups provides the β-sheet with high stability against HFIP addition and acidification.

Conclusion

We succeeded in "threading" β-sheet peptides by using radical polymerization of diene side groups, which effectively stabilized the β-sheet structure. To the best of our knowledge, this is the first example of utilizing a polymerization reaction for cross-linking higher-order structures of peptides. Our cross-linking method will help practical applications of β-sheet materials, for example, by strengthening silklike fibers formed from β-sheets and by reinforcing hydrogels composed of β-sheet fibers. Because the side group of Lys(DE) is relatively long and hydrophobic, Lys(DE) would essentially disturb the β-sheet formation of peptides. Thus, now we are trying to synthesize diene-modified amino acids with shorter side groups as more "inert" cross-linkers for β-sheet formation.

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Supporting Information Available. ESI mass spectra, UV spectra of peptide solutions, and results of dynamic light scattering measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) This extreme improvement in the stability of the polymerized β-sheet against acidification is probably because of (a) a decrease in the pK_a of the Glu residues due to a strong interaction between Glu residues and their neighboring Lys residues or (b) limited mobility of the β-strands due to forced linking among the strands.

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