CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200902758

Peptide Nanofibers Modified with a Protein by Using Designed Anchor Molecules Bearing Hydrophobic and Functional Moieties

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Abstract: Self-assembly of peptides and proteins is a key feature of biological functions. Short amphiphilic peptides designed with a β-sheet structure can form sophisticated nanofiber structures, and the fibers are available as nanomaterials for arranging biomolecules. Peptide FI (H-PKFKIIEFEP-OH) self-assembles into nanofibers with a coiled fine structure, as reported in our previous work. We have constructed anchor molecules that have both a binding moiety for the fiber structure and a functional unit capable of capturing target molecules, with the purpose of arranging proteins on the designed peptide nanofibers. Designed anchors containing an alkyl chain as a binding unit and biotin as a functional moiety were found to bind to peptide fibers FI and F2i (H-ALEAKFAA-FEAKLA-NH_2). The surface-exposed biotin moiety on the fibers could capture an anti-biotin antibody. Moreover, hydrophobic dipeptide anchor units composed of iminodiacetate connected to Phe–Phe or Ile–Ile and a peptide composed of six histidine residues connected to biotin could also connect FI peptide fibers to the anti-biotin antibody through the chelation of Ni^{2+} ions. This strategy of using designed anchors opens a novel approach to constructing nanoscale protein arrays on peptide nanomaterials.

Keywords: electron microscopy · nanostructures · peptides · proteins · self-assembly

Introduction

The self-assembly of peptides and proteins is an attractive method for the fabrication of nanostructured materials.[1,2] Recently, bottom-up approaches have focused on the field of nanotechnology, and self-assembling peptides show advantages in this field.[3–5] Short peptides are of interest for controlling the morphology and size of the nanostructures by using the information stored in various amino acid sequences. Several peptides with self-assembling properties have been constructed by using α-helix and β-sheet structures,[6–12] however, proteins related to misfolding diseases such as Alzheimer's and Parkinson's diseases can form nanostructured fibers in vitro, and these fibers are usually formed by β-sheet structures.[13–15] Hydrogen-bonding networks of β-sheets have the advantages of design and control over the molecules that self-assemble. Previous works by our group have shown a sophisticated design strategy of short peptides based on amphiphilic β-sheet structures and found that some peptides form tightly oriented nanofibers composed of ten amino acid resides.[16,17] Peptide FI (sequence H-PKFKIIEFEP-OH) self-assembles into straight fibers with clear edges and coiled fine structures, as observed by transmission electron microscopy (TEM) and atomic force microscopy (AFM) measurements.[16] The FI peptide spontaneously forms these nanostructured fibers in a hierarchical manner. Moreover, covalent conjugation of the FI sequence with a functional unit, such as a biotin group, incorporates the functionality into the nanoscale fibers.[17]

Protein-analyzing microarrays and labs-on-a-chip have been an emerging approaches for comprehensive analyses of protein function in living systems.[18,19] Nanomaterials that can arrange the capture molecules for protein detection are beneficial for constructing detecting nanounits for protein chips, and the peptide nanofiber could be one of the candidate materials.[20] If a variety of capture agents, such as antibodies and engineered proteins, are easily arranged on a nanofiber surface, it might be possible to design a nanoscaled protein array. Towards this aim, we have also designed an anchoring approach that involves the noncovalent binding of small molecules with both a binding moiety for

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the fiber structures and a functional unit for the protein nanoarranging system. Because the naturally occurring membranes and envelopes of viruses contain molecules on their surfaces, the hydrophobic moieties of which are buried in the membrane,[21] we have used hydrophobic interactions to design the binding moieties for the peptide fibers. The designed peptide fibers are formed by the hydrophobic interactions as a main driving force, as in the case of naturally occurring systems. Thus, hydrophobic moieties such as alkyl chains and hydrophobic dipeptide units are available as binding units for the peptide fibers. Moreover, an antibody that recognizes biotin can be arranged on the fiber surfaces through the designed functional anchor molecules. The new strategy promises to widen the usability of these peptide nanofibers.

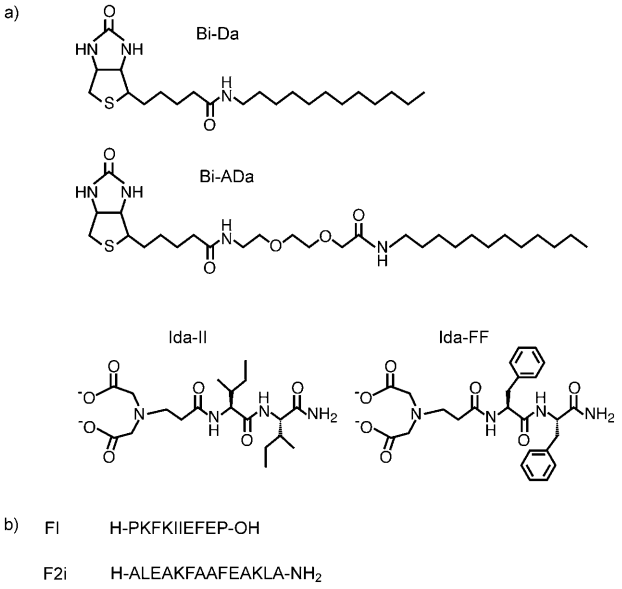

Figure 1. a) Structures of the designed anchor molecules that contain both hydrophobic and functional groups. b) Sequences of FI and F2i peptides that self-assemble into nanofibers.

Results and Discussion

Design of alkyl anchors and peptide nanofibers: In our previous report, designed peptide FI (H-PKFKIIEFEP-OH) forms well-regulated nanostructured fibers (80–130 nm in width and ≈10 μm in length).[16] The fine structure of the FI peptide fibers is comprised of striations at regular intervals. Moreover, we also previously reported that designed peptide β16 (H-GGALEAKLAALEAKLA-NH_2) forms amyloid-like fibrils, in which the peptide undergoes a self-initiated structural transition from a random coil to a β-sheet structure.[22] These peptides are designed to self-assemble through hydrophobic interactions between side-chains such as Ile, Leu, and Phe and electrostatic interactions between Lys and Glu residues. To arrange the proteins on their peptide fibers, FI and β16 peptides modified with biotin at their N-terminals were also synthesized.[17,22] A combination of biotin-modified peptides with free peptides generated a biotinylated surface on the nanofibers; streptavidin or anti-biotin antibody could then successfully bind to the fibers at regulated intervals. In these studies, we found that the periodic structures of peptide nanofibers can be good recognition points for protein nano-arrangements. To expand the availability of the peptide nanofibers for constructing protein nanoarrays, we have designed anchor molecules that contain a binding moiety for the peptide fibers and a functional group, such as biotin (Figure 1). The Bi–Da anchor is composed of the biotin and dodecyl groups, whereas the Bi–ADa anchor contains aminoethoxyethoxyacetate as a spacer between the biotin and dodecyl moieties of Bi–Da (Figure 1). It was expected that the dodecyl unit would interact with the hydrophobic region of the peptide fibers that might appear at regular intervals. We also redesigned the β16 peptide by replacing two Leu residues of the sequence with Phe to give peptide F2i (H-ALEAKFAAFEAKLA-NH_2) with enhanced hydrophobic interactions during self-assembly. Although the β16 peptide tends to form bent fibers, F2i forms nanofibers that are straight. Moreover, the width of the F2i fibers is 40 to 80 nm, which is about two times thinner than that of the FI peptide fibers (Figure 2).

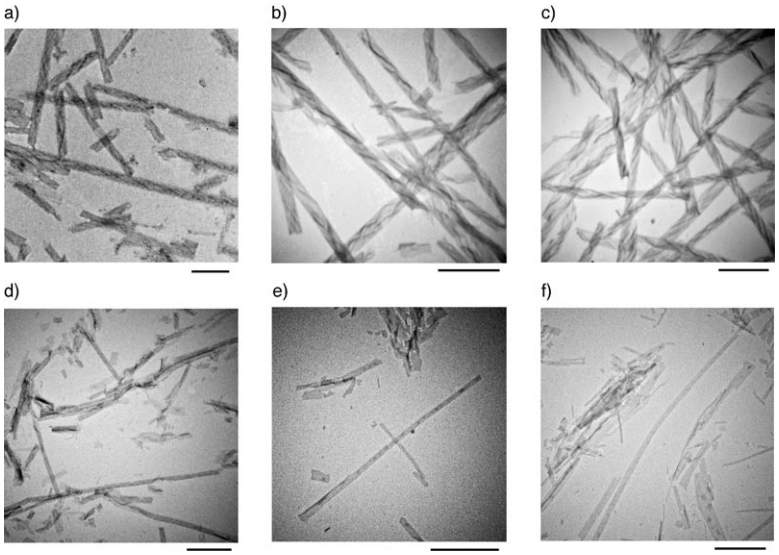

Figure 2. TEM images of FI (top) and F2i (bottom) peptide nanofibers in the absence (a, d) or presence of Bi–Da (b, e) or Bi–ADa (c, f) anchor molecules. Peptide fibers are stained with uranyl acetate (2% w/v) in H_2O. Scale bars = 500 nm. [FI] = 100 μM, [F2i] = 100 μM, [Bi–Da] or [Bi–ADa] = 100 (b, c) and 250 μM (e, f).

Modification of peptide nanofibers by using alkyl anchors: To modify the FI and F2i fibers with proteins, the Bi–Da and Bi–ADa anchors were treated with fiber peptides. The anchor molecules were incubated with FI or F2i to form fibers on which biotin units were appended. TEM images of the FI peptide fibers with and without anchors were recorded (Figure 2). When FI (100 μM) in the absence and presence of Bi–Da and Bi–ADa anchors (100 μM each) was incubated in phosphate buffer (100 mM, pH 7.4) at room temperature, straight fibers with coiled fine structures were observed on the TEM grid after being stained with uranyl acetate. Moreover, FI with excess amounts of Bi–Da and Bi–ADa anchors (100 to ≈500 μM) appeared to form fibers with similar structures to FI only (Figure 2). The observed TEM images indicated that the anchor molecules did not disturb the formation of the FI fibers. TEM images of the F2i peptide fibers with Bi–Da and Bi–ADa anchors exhibited similar structures to F2i fibers alone (Figure 2). F2i formed flat fibers without any coiled fine structures. In the absence of peptides, the Bi–Da and Bi–ADa anchors did not form any aggregated structures, as observed by TEM. Thus the F2i fibers were fabricated by self-assembly and the anchor molecules did not significantly perturb the fiber elongation. Circular dichroism (CD) spectroscopy measurements were also performed to evaluate the fiber formation of F2i with and without anchors at various incubation periods at 50 °C to accelerate the fiber formation. The CD signals at $\lambda = 198$ and 218 nm assigned to the β-sheet of the F2i peptide gradually increased as the fibers matured (Figure 3). The signals at $\lambda = 198$ nm were plotted and are shown in Figure 3d. The CD intensities of F2i only were significantly increased by incubation, whereas slight increases in the signals were observed for F2i with Bi–Da and Bi–ADa anchors. These results indicated that the designed alkyl anchors could interact with F2i peptide and affect the arrangement of β-sheet structures, although the macromorphology was not largely changed.

Protein modification on FI and F2i peptide fibers with designed alkyl anchors: The FI and F2i peptides that were incubated with Bi–Da and Bi–ADa anchors could form characteristic nanofibers, which indicates that the biotin moiety of the anchor may be arranged along the fiber structure. Based on this hypothesis, the peptide nanofibers were modified with an antibody that recognizes the biotin moiety. To avoid destruction of the peptide fibers during protein modification procedures, such as washing, peptide fibers formed with anchor molecules were stabilized through the amino group in the lysine side-chain by a crosslinking reaction by using 2.5 % (v/v) glutaraldehyde in water. We applied an anti-biotin antibody conjugated with a colloidal gold nanoparticle (Au–AB; diameter 10 nm) for the detection of the antibody on the FI and F2i peptide fibers (Figure 4a). Modification of Au–AB was performed by absorption on a carbon-coated TEM grid. Non-specific interactions between fibers and Au–AB were diminished by using Tris–HCl buffer (20 mM, pH 7.4) containing bovine serum albumin (BSA; 0.1 % w/v) and Tween 20 (0.01 % v/v) during incubation and washing of the grid (see the Experimental Section).

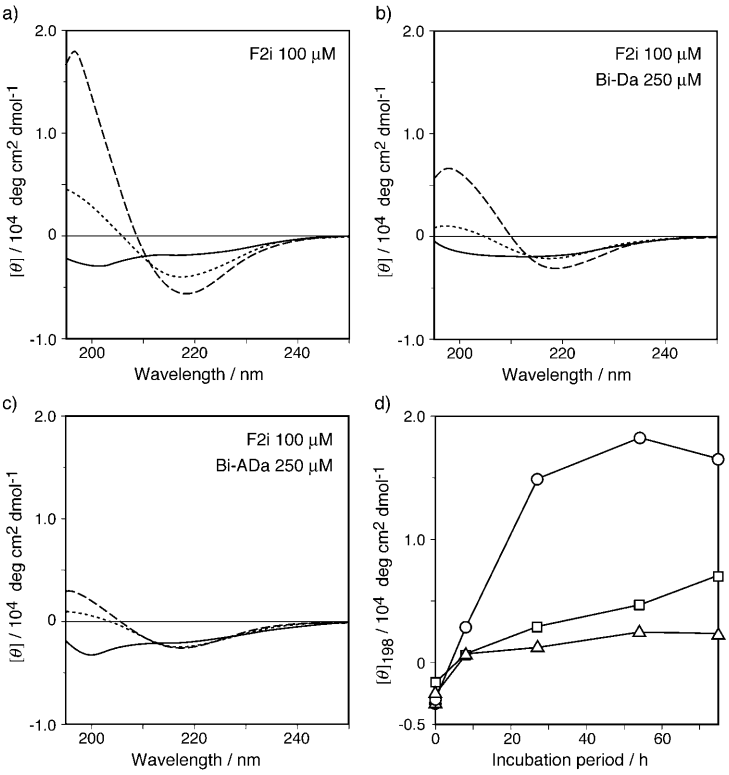

Figure 3. CD spectra of F2i fibers in the absence (a) and presence of Bi–Da (b) and Bi–ADa (c) after incubation for 0 (——), 8 (•••••), and 75 h (– – –) at 50 °C. [F2i] = 100 μM, [anchor] = 250 μM in Tris–HCl buffer (20 mM, pH 7.4). d) CD intensities of F2i peptide fibers at $\lambda = 198$ nm in the absence (○) and presence of Bi–Da (□) and Bi–ADa (△) as a function of incubation time.

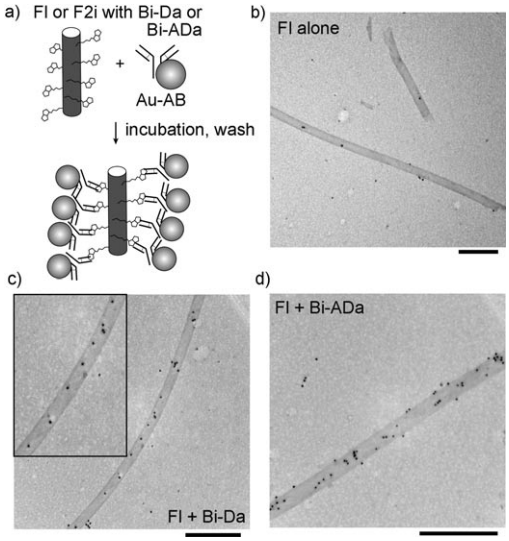

Figure 4. a) Schematic representation of the protein modification of FI and F2i fibers by using the alkyl anchors and Au–AB. b–d) TEM images of the FI in the absence (b) and presence of Bi–Da (c) and Bi–ADa (d) anchors. Scale bars = 500 nm. [FI] = 400 μM, [Bi–Da] and [Bi–ADa] = 1 mM, [Au–AB] = 3.1 μg mL^{-1}.

Figure 4 shows TEM images of the protein modifications of FI peptide fibers in the absence and presence of Bi–Da and Bi–ADa anchors. Gold dots are clearly observed on FI fibers formed with anchors bearing the biotin moiety, but almost no gold dots were present on the TEM grid with FI alone. The gold dots on the FI fibers in the presence of Bi–Da and Bi–ADa anchors appeared three and seven times, respectively, which is more frequently than those in the absence of the anchor molecule (Table 1). Interestingly, the gold dots appear to be arranged along the edges of the coiled fine structures of FI fibers, especially with the Bi–ADa anchor (Figure 4; discussed in the section below). These results indicated that the FI peptide can form a fiber structure with the anchor molecule and the Bi–ADa anchor can be arranged on patterned structures in the nanofibers.

Table 1. Quantitative analysis of the number of gold dots observed on the fibers by using Au–AB.

Fiber peptide	Anchor	Number[a] [μm^{-1}]
FI	–[b]	2.8 ± 1.3
FI	Bi–Da	9.0 ± 3.7
FI	Bi–ADa	20.3 ± 6.5
F2i	–[b]	1.4 ± 0.9
F2i	Bi–Da	9.1 ± 2.3
F2i	Bi–ADa	5.1 ± 1.7
FI	–[c]	2.6 ± 0.8
FI	Ida-II	17.3 ± 6.1
FI	Ida-FF	13.5 ± 4.7

[a] The number of gold dots on the peptide fibers is calculated by determining the average number on a 1 μm section of fiber. [b] The protein modification was performed by using peptide fibers in the absence of any anchor molecule. [c] The protein modification was performed by using peptide fibers in the absence of dipeptide anchors but in the presence of Bi–His6 and Ni^{2+} ions.

F2i peptide fibers were also modified with Au–AB through Bi–Da and Bi–ADa anchors (Figure 5). In the presence of anchor molecules, gold dots are clearly observed on the F2i fibers (Table 1), which indicates that the F2i fibers contain the anchor molecules. Interestingly, when we focused on the regions that contain Au–AB in high density, we observed that Au–AB binds to F2i fibers with a constant periodicity of 110–130 nm (Figure 5b, inset). These findings suggest that the biotin moieties of the anchor molecules, which act as a recognition point for the antibody, are distributed with constant periodicity on the surface of the fiber. On comparing the F2i and FI peptide fibers, it was found that the amounts of appended Au–AB is apparently not different.

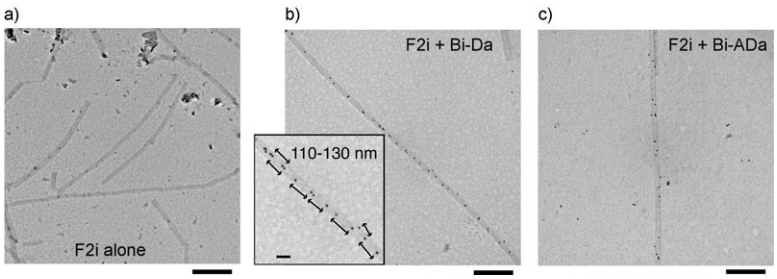

Figure 5. TEM images of F2i peptide fibers in the absence (a) and presence of Bi–Da (b) and Bi–ADa (c) anchors. Scale bars = 500 nm (inset scale bar = 100 nm). [F2i] = 20 μM, [Bi–Da] and [Bi–ADa] = 50 μM, and [Au–AB] = 3.1 $\mu g\,mL^{-1}$.

Design of dipeptide anchors bearing an iminodiacetate moiety: Bi–Da and Bi–ADa anchors can bind to the FI and F2i peptides and are available for protein modification through the biotin moiety. To refine the anchoring methodology, we designed dipeptide anchors bearing an iminodiacetate (Ida) moiety capable of chelating metal ions, such as Ni^{2+} and Zn^{2+}. Ida-II and Ida-FF anchors (Figure 1a) were composed of two Ile or two Phe residues, respectively, with Ida at the N-terminal and a β-alanine residue positioned as a spacer between the dipeptide unit and Ida. It was expected that the hydrophobic side-chains of Ile and Phe in the dipeptide anchors would form hydrophobic interactions with the periodic hydrophobic sites of the FI and F2i peptide fibers.

When the FI peptide was incubated with the Ida-II and Ida-FF anchors, straight fibers with a similar morphology to FI only were observed by TEM. Moreover, no fibrous material was observed for Ida-II and Ida-FF in the absence of the FI peptide. These results indicated that the designed dipeptide anchors did not disturb the fiber formation of the FI peptide. CD spectra of the FI peptide in the presence and absence of Ida-II and Ida-FF anchors at various incubation periods were measured and are shown in Figure 6. The CD signal intensities at $\lambda = 203$ nm (indicative of β-sheet assembly and fiber maturation)[16] for the FI peptide in the presence and absence of Ida-II increased with time in a similar manner. Interestingly, the time-dependent increase in the CD intensity of FI with Ida-FF at $\lambda = 203$ nm was larger than that without the anchor (Figure 6d). This finding suggested that the CD signal at $\lambda = 203$ nm may be related to the arrangement of Phe residues in the fibers.

On the other hand, CD spectra of the F2i peptide were measured during fiber formation in the presence and absence of the dipeptide anchors at various incubation periods at 25 °C. In the spectral change at $\lambda = 198$ nm, the increases in the CD intensities during incubation of F2i with Ida-II and Ida-FF were smaller than that without the anchor molecule (data not shown). This result was similar to the case of the F2i peptide with the Bi–Da and Bi–ADa anchors. The interactions between F2i and the hydrophobic regions of the designed alkyl and dipeptide anchors may affect the β-sheet arrangement in the formation of F2i peptide fibers.

Protein modification of the peptide fibers by using the dipeptide anchors: To modify the

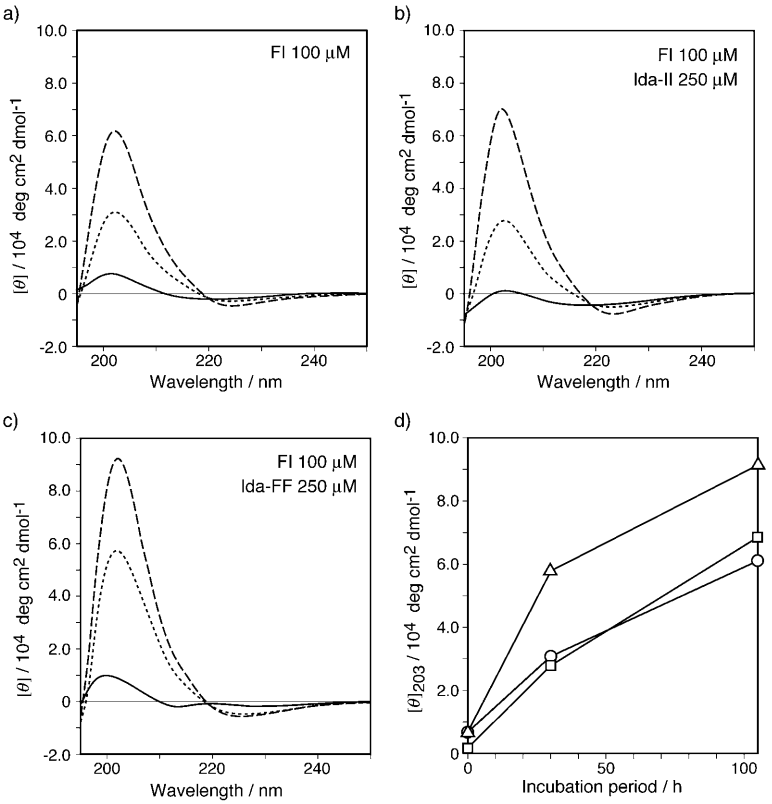

Figure 6. CD spectra of FI fibers in the absence (a) and presence of Ida-II (b) and Ida-FF (c) anchor molecules after incubation for 0 (——), 30 (•••••), and 105 h (– – –) at RT. [FI] = 100 μM, [anchor] = 250 μM in 100 mM phosphate buffer (pH 7.4). d) CD intensities of FI peptide fibers at $\lambda = 203$ nm in the absence (○) and presence of Ida-II (□) and Ida-FF (△) as a function of incubation time.

F2i and FI peptide fibers by using the dipeptide anchors, we have designed a peptide with six histidine residues that bears the biotin moiety (Bi–His6). Figure 7 shows the protein modification of peptide fibers by using the designed dipeptide anchors. Ni^{2+} ions were used to conjugate the Ida-II and Ida-FF anchors with Bi–His6. After Ni^{2+} coordination by addition of $NiSO_4$ (1 mM) to the FI fibers containing Ida-II and Ida-FF, a mixture of Bi–His6 and Au–AB was added on the TEM grid, and the observed TEM images are shown in Figure 8. Unfortunately, almost no gold dots corresponding to Au–AB were observed in either F2i only or in F2i with Ida-II or Ida-FF. This result indicates that Au–AB with Bi–His6 either was not captured in the F2i fibers or was removed during modification procedures such as washing. In addition, the Ida group of the dipeptide anchors might not be exposed on the surface of the F2i fibers. In contrast to the F2i fibers, the FI peptide fibers with dipeptide anchors successfully captured Au–AB through the Bi–His6 peptide, whereas the gold dots were rarely observed in the FI fibers in the absence of Ida-II and Ida-FF. The numbers of the gold dots on the FI fibers increased when Ida-II and Ida-FF anchors were used (Table 1). These results indicated that the iminodiacetate group of the anchor was present on the fiber surfaces. Interestingly, the gold dots were clearly arranged along the striations of the fiber fine structure (Figure 8d). This finding indicates that the dipeptide anchors that interact with the hydrophobic sites of the FI peptide are exposed on the apertures between the fiber edges formed by the fine striation structures. In other words, the positions of the striations might provide a hydrophobic environment for trapping functional anchors, including the designed dipeptide and alkyl anchors.

Conclusion

We have successfully designed anchor molecules that have both hydrophobic and functional moieties that bind to sophisticated peptide nanofibers. The Bi–Da and Bi–ADa anchors,

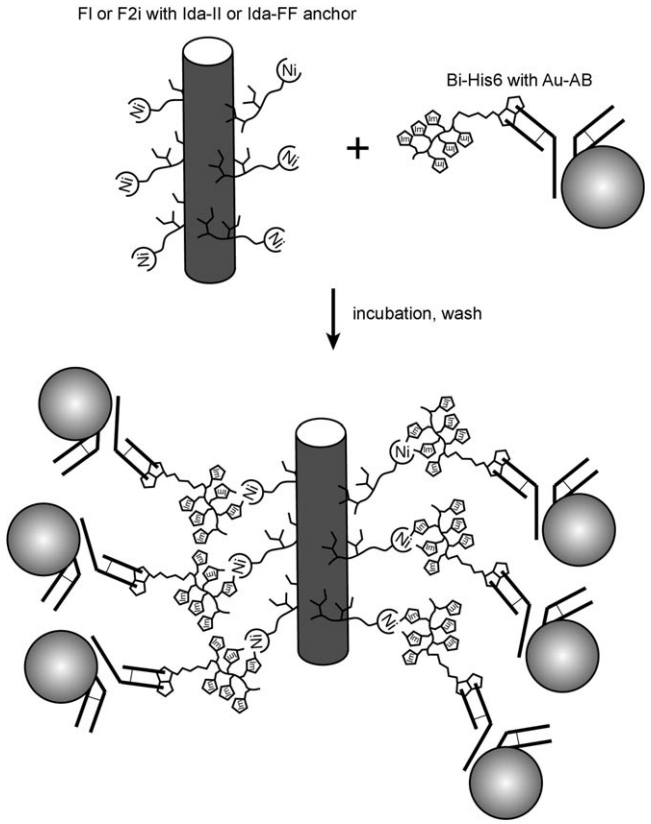

Figure 7. Schematic representation of the protein modification of FI and F2i fibers by using dipeptide anchors and Bi–His6.

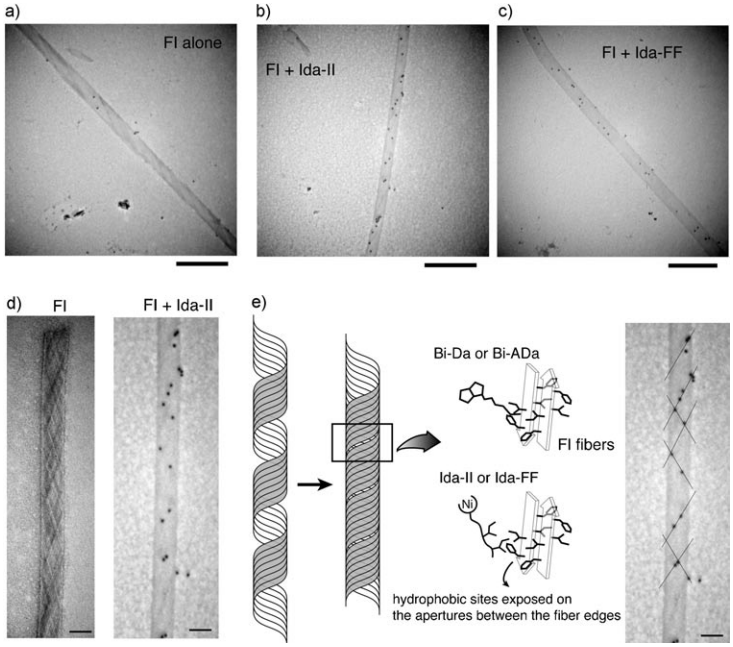

Figure 8. TEM images of FI fibers in the absence (a) and presence of Ida-II (b) and Ida-FF (c) anchors. The fibers were incubated with Ni^{2+} and washed, then further incubated with Bi–His6 and Au–AB. Scale bars = 500 nm. [FI] = 400 μM, [Ida-II] and [Ida-FF] = 2.0 mM, and [Au–AB] = 3.1 $\mu g\,mL^{-1}$. d) TEM images of FI only without any treatment (left) and FI fibers formed with Ida-II (right); scale bars = 100 nm. e) Schematic representation of FI fiber formation in the presence of hydrophobic anchors. Functional groups (biotin and Ida) of anchors that can interact with recognition molecules (Au–AB and Bi–His6) may be arranged on the apertures between the edges of the fibers.

which contain biotin and an alkyl chain, could bind to FI and F2i nanofibers, and the surface-exposed biotin moiety on the fibers could recognize the anti-biotin antibody. Therefore, the alkyl group was available for the modification of the peptide nanofibers through hydrophobic interactions. Moreover, hydrophobic amino acids such as Ile and Phe were also used to modify the peptide fibers. By combining the iminodiacetate moiety with hydrophobic dipeptide units, the anti-biotin antibody was incorporated into the FI peptide fibers by using the Bi–His6 peptide and Ni^{2+} ions. These designed anchors tend to be arranged along the striations of the fiber fine structures, in which the hydrophobic sites might be exposed. This strategy may open a novel methodology for constructing nano-scale protein arrays by using the self-assembly of designed peptides and small anchor molecules.

Experimental Section

Chemicals and reagents: All chemicals and solvents were of reagent or HPLC grade. Amino acid derivatives and reagents for peptide synthesis were purchased from Watanabe Chemical (Hiroshima, Japan). Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was measured by using a MALDI III mass spectrometer (Shimadzu, Kyoto, Japan) by using 3,5-dimethoxy-4-hydroxycinnamic acid as a matrix. Liquid-chromatography mass spectrometry (LC-MS) was measured by using a LC-MS-2100EV instrument (Shimadzu, Kyoto, Japan). HPLC was carried out by using a Hitachi L-7000 HPLC system equipped with a COSMOSIL 5C18-AR II packed column (4.6 × 150 mm; Nakalai Tesque, Kyoto, Japan), or a COSMOSIL 5C18-AR II packed column (10 × 250 mm; Nakalai Tesque, Kyoto, Japan). Amino acid analyses were performed by using the phenyl isothiocyanate (PITC) method on a Wakopak WS-PTC column (Wako chemical, Osaka, Japan). Colloidal gold-labeled anti-biotin antibody was purchased from British Biocell International (Cardiff, UK). Electron microscopy was performed by using a H-7500 electron microscope at 80 or 100 kV (Hitachi High-technologies, Tokyo, Japan).

Peptide synthesis: Peptides FI, F2i, and Bi–His6 were synthesized by using the solid-phase method and a Fmoc strategy with *O*-(7-azabezotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) or 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole hydrate ($HOBt{\cdot}H_2O$) as the coupling reagents.[23] To remove the resin and the protecting groups, the peptide–resin was treated with trifluoroacetic acid (TFA) that contained triisopropylsilane and H_2O or thioanisle, *m*-cresol, and ethandithiol as scavengers for 1 h at RT. The product was solidified with Et_2O in an ice-bath. All peptides were purified by using reversed-phase HPLC on the COSMOSIL 5C18-AR II-packed column by using a linear gradient of 0.1% TFA in MeCN at a flow rate of 3.0 $mL\,min^{-1}$. Peptides were identified by their molecular ion peaks by using MALDI-TOF MS: *m*/*z* calcd for FI: 1248.5 $[M+H]^+$; found: 1249.6; *m*/*z* calcd for F2i: 1501.6 $[M+H]^+$; found: 1501.5; *m*/*z* calcd for Bi–His6: 1138.3 $[M+H]^+$; found: 1140.0.

Synthesis of the Bi–Da anchor: Biotin (0.96 mmol) and dodecylamine (0.80 mmol) were dissolved in DMF (10 mL), then dicylcohexylcarbodiimide (DCC; 0.80 mmol) was added and the mixture was stirred at RT. Thin-layer chromatography was used to monitor the progress of the reaction. After completion of the reaction, dicyclohexylurea was removed by filtration and the filtrate was evaporated. The residue was dissolved in EtOAc and washed with 4% $NaHCO_3$, 10% citrate, and brine. The aqueous layer was extracted with $CHCl_3$. Evaporation of $CHCl_3$ and recrystallization with hexane gave Bi–Da (0.53 mmol, 67%).

Synthesis of the Bi–ADa anchor: *tert*-Butyl 2-aminoethoxyethoxyacetate (1.2 mmol) was dissolved in DMF (3.5 mL), then biotin (1.0 mmol) was added and stirred until it had dissolved. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride salt (EDC·HCl; 2.0 mmol) was added and the mixture was stirred for 7 h at RT. After evaporation of DMF, the residue was extracted with $CHCl_3$ and dried over $MgSO_4$. Evaporation of the organic layer in vacuo gave *tert*-butyl 2-biotinylaminoethoxyethoxyacetate (0.64 mmol, 64%), which was dissolved in TFA (5 mL) and stirred for 1 h at RT. After evaporation of the $CHCl_3$, diethyl ether was added to precipitate the product. The resulting solid was collected by filtration and dried in vacuo. Purification of the product by silica gel chromatography (petroleum ether/EtOAc 5:1) gave 2-biotinylaminoethoxyethoxyacetic acid (0.51 mmol, 81%). The product (0.51 mmol) was dissolved in DMF (3.5 mL), then dodecylamine (0.24 mL, 1.03 mmol) and EDC·HCl (1.03 mmol) were added and the mixture was stirred at RT for overnight. After evaporation, the residue was extracted with EtOAc and H_2O. $CHCl_3$ was added to the aqueous layer and the product was extracted by adding 4% citrate. The organic layer was dried over Na_2SO_4, filtered, and evaporated. Purification by RP-HPLC gave the Bi–ADa anchor (23 μmol, 0.4%). Bi–ADa anchor was identified by its molecular ion peak by using MALDI-TOF MS: *m*/*z* calcd for Bi–ADa: 557.8 $[M+H]^+$; found: 557.8.

Synthesis of dipeptide anchors: The dipeptide anchors bearing Ida were synthesized on Rink amide resin by using the Fmoc solid-phase method, as described above. H-βAla-Ile-Ile-NH_2 and H-βAla-Phe-Phe-NH_2 were synthesized on the resin and obtained by TFA cleavage. H-βAla-Ile-Ile-NH_2 (0.38 mmol) or H-βAla-Phe-Phe-NH_2 (0.36 mmol) was dissolved in anhydrous DMF (8 mL), then iPr_2EtN (3.0 equiv) and *tert*-butyl 2-bromoacetate (3.0 equiv) were added and the mixture was stirred for 5 h at RT. The reaction progress was monitored by analytical HPLC. After evaporation of the reaction mixture, diethyl ether was added to precipi-

tate the product. The solid was obtained by filtration and dried in vacuo. Purification by RP-HPLC gave Ida-II (0.039 mmol, 13%) and Ida-FF (0.017 mmol, 6%). These anchors were identified by the molecular ion peaks by using LC-MS: *m*/*z* calcd for Ida-II: 431.5 $[M+H]^+$; found: 431.0; *m*/*z* calcd for Ida-FF: 499.5 $[M+H]^+$, found; 499.0.

Sample preparation: The peptide and functional anchor stock solutions were prepared in trifluoroethanol (TFE) or 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) at concentrations of 2 mM and 5 mM, respectively. To fabricate the fibers, the peptide stock solution was evaporated with a stream of N_2 and the residues were diluted in 0.1 M sodium phosphate buffer (pH 7.4) or 20 mM Tris–HCl buffer (pH 7.4) to initiate the fiber formation, then allowed to stand at RT for more than 24 h. To attach the designed anchor molecules to the peptide fibers, the peptide and anchor stock solutions were mixed. After removal of the solvent by N_2 gas, the residues were diluted in the buffer described above.

CD spectroscopy measurements: CD spectra were measured by using a Jasco J-720WI spectropolarimeter equipped with a thermo-regulator and a quartz cell with 1.0 mm pathlength. Spectra were recorded in terms of mean residue ellipticity ($[\theta]$). The peptide solution was prepared by dilution with sodium phosphate buffer (0.1 M, pH 7.4) or Tris–HCl buffer (20 mM, pH 7.4).

Transmission electron microscopy: The sample solution was absorbed onto a collodion-coated copper EM grid (200 mesh) by floating the grid on a drop (5–10 μL) of the peptide solution for 1 min. The excess solution was removed by blotting with filter paper, and the grid was washed by floating on H_2O, then the H_2O was removed by blotting. The sample on the grid was negatively stained with aqueous uranyl acetate (2% w/v) for 1 min. After removing the excess solution and drying, the samples were visualized by using a Hitachi H-7500 electron microscope operating at 80 or 100 kV.

Au–AB was used to modify the peptide nanofibers with a protein through the designed hydrophobic anchors. To stabilize the peptide fibers by crosslinking, the stock solution of peptide nanofibers mixed with functional anchors (2 μL) was incubated with aqueous glutaraldehyde (2.5% v/v; 2 μL) for 60 min. After addition of H_2O (6 μL), the solution was absorbed onto a grid for 20 min. The grid was washed with H_2O, then floated on a drop of 0.3 M NH_4Cl in TBS buffer (20 mM Tris–HCl buffer containing 154 mM NaCl, pH 7.4) for 30 min to diminish the reactivity of the excess aldehyde groups in glutaraldehyde. In the case of the alkyl anchors, Bi–Da and Bi–ADa, the nanofibers on the grid were incubated with TBS (10 μL) that contained Tween 20 (TBST; 0.01%; v/v) and bovine serum albumin (BSA; 0.1% w/v) for 10 min to prevent the nonspecific adsorption of Au–AB. The grid was incubated with Au–AB (10 μL; 10× dilution) in TBST that contained BSA (0.1%) for 30 min, then washed with H_2O and stained with uranyl acetate (2% w/v) for 1 min. In the case of the dipeptide anchors, Ida-II and Ida-FF, the grid was incubated with nickel sulfate (1.0 mM) in TBS (10 μL) for 30 min, then treated with TBST (10 μL) that contained BSA (0.1%) for 10 min. The grid was incubated with a mixture (10 μL) of Au–AB (10× dilution) and Bi–His6 peptide (20 μM) in TBST (10 μL) with BSA (0.1%) for 30 min, then washed with H_2O and stained with uranyl acetate (2%) for 1 min. After drying, the samples were visualized by using a Hitachi H-7500 electron microscope operating at 80 kV.

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Received: October 6, 2009
Published online: April 23, 2010

