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Surface Modification of Peptide Nanofiber by Using Antigen-Antibody Interaction

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A biotin-terminated nanofiber-formable oligopeptide (**1**), which consists of 16-mer hydrophobic Leu and hydrophilic Lys, was newly prepared in order to modificate nanofiber surface through antigen-antibody interaction. The secondary structure and self-assembling properties of **1** in water were evaluated by means of CD and AFM measurements. As a result, the peptide **1** was found to form relatively short nanofibers, compared to those from biotin-free peptide **2**, with β-sheet conformation in aqueous solution at pH 9. On the other hand, biotin-containing β-sheet nanofibers with high-axial-ratio were successfully obtained by mixing the peptide **1** with **2**. Fluorescence, AFM and TEM measurements clearly revealed that streptavidin interacted with the **1/2**-nanofiber, and was immobilized on nanofiber surface. These results demonstrate the potential of this nanofiber as novel biomaterials with a wide range of applications, such as nano-scaffold and nano-template.

Key words: Self-assembly, Nanofiber, Amphiphilic Peptide, Biotin, Antigen-Antibody Interaction

1. INTRODUCTION.

Much attention has been focused on the self-assembly of artificial peptides and proteins into β-sheet nanofibers because of their importance as a structural model for amyloid formation, and as a powerful strategy for constructing supramolecular nanofibers. In general, such β-sheet nanofibers possess unbranched and long morphology (lengths > 1 μm) with diametric ranges of *ca.* 1-10 nm. A number of artificial peptides and peptidomimetics have been prepared and investigated their self-assembling behaviors into nanofibers in aqueous solutions.[1-6] In previous study, we have also reported that fine tuning of peptide sequence, chirality of amino acids, and solution pH enables the structural regulation of peptide nanofibers, by controlled self-assembly of amphiphilic 16-mer peptides.[7,8] Furthermore, temporal control of peptide self-assembly has recently been accomplished by using poly(ethylene glycol)-attached oligopeptide which forms β-sheet nanofibers in response to enzymatical triggering.[9] These self-assembled nanofiber architectures are expected to possess potential as novel biomaterials with a wide range of applications based on their characteristic nanostructures. In fact, more recently, Stupp's group and Mihara's group used peptide nanofibers with surface-binding motif as nano-templates to accomplish one-dimensional organization of functional molecules such as gold nano-particle and protein.[10,11]

Here, we report a surface modification of β-sheet nanofiber by using antigen-antibody interaction toward a construction of one-dimensional nano-array. For this purpose, we employed a biotin-terminated β-sheet formable triblock-type oligopeptide (**1**), which composed of hydrophobic Leu and hydrophilic Lys, as building unit for self-assembly (Fig. 1). It has been known that the biotin-avidin interaction is one of the strongest non-covalent biological interactions with a binding constant of *ca.* 10^{15} M^{-1} and a high specificity.[12] This molecular system has found widespread use in immunology and cell biology. Since the streptavidin (SAv) has four biotin-binding sites, the additional and versatile functionalization of SAv-immobilized nanofiber would be possible by using various biotin-derivaives.

A detailed analysis of conformation and morphology of the biotin-modified peptide **1** and its mixture with biotin-free **2** was performed in water. In addition, we examined the specific interaction between biotin-containing nanofiber and SAv by various microscope techniques. These studies should be useful for the design of novel peptide-based nano-scaffolds.

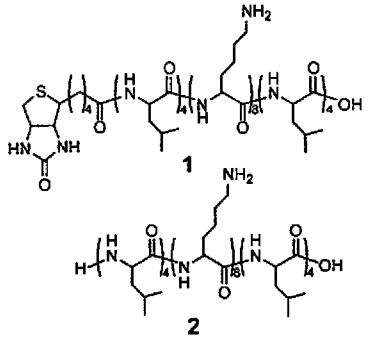

Fig. 1. Chemical structures of biotin-terminated β-sheet formable triblock oligopeptide (**1**), and biotin-free triblock peptide (**2**), used as building units for self-assembly.

2. EXPERIMENTAL

2.1 Materials.

The biotin-terminated amphiphilic 16-mer oligopeptide (**1**) used in this study was prepared from standard solid phase peptide synthesis using Fmoc chemistry. The peptide segment was synthesized on a CREAR (cross-linked ethoxylate acrylate resin, PEPTIDE INSTITUTE, INC.), by using Fmoc-L-amino acid derivatives (3 equiv), 1-hydroxy-7-azabenzotriazole (3 equiv) and 1,3-diisopropylcarbodiimide (3 equiv) in *N,N*-dimethylformamide (DMF) for coupling, and piperidine (25%)/DMF for Fmoc removal. To introduce antigen-group, D-biotin was coupled by the same method. To cleave the peptide with biotin group from the resin and remove the side chain protecting groups, the peptide-resin was treated with trifluoroacetic acid (TFA)/CH_2Cl_2 (9/1 v/v). The crude peptide was purified by reversed-phase HPLC (Bio-Rad, DuoFlow) on a YMC-Pack Pro C18 (20 x 150 mm) by using a linear gradient of water-acetonitrile (containing 0.1% TFA). The purified peptide was identified by matrix-assisted laser desorption ionization-time of flight MS (MALDI-TOFMS) (Shimadzu KOMPACT MALDI) and ^{1}H-NMR spectroscopy (400 MHz, JEOL JNM-AL-400). The peptide **1** was dissolved in 2,2,2-trifluoroethanol (TFE) as a stock solution before the aggregation assay. The aggregation solutions of the peptides (final peptide concentration: 40 μM, TFE content: 5 % in vol.) were prepared by diluting the stock solution with the 5 mM $NaHCO_3$/NaOH buffer or phosphate buffer. All the incubations for the aggregation of the peptides were performed at room temperature. MALDI-TOFMS: found $[M+H]^+$ (calcd. $[M+H]^+$); **1**: *m/z* 2177.6 (2176.3). ^{1}H-NMR (D_2O, DSS); δ (ppm) 0.9 (CH_3-(Leu)), 1.3-1.8 (-CH_2-CH-(Leu), -CH_2-(Lys), -CH_2-(biotin)), 2.3 (-CH_2CO-(biotin)), 2.7 (SCH_2-(biotin)), 3.0 (-CH_2-NH_2 (Lys)), 3.3 (SCH-(biotin)), 4.2-4.5 (-COCHNH-(main peptide chain)). The preparation of biotin-free peptide **2** has been described elsewhere.[8]

The streptavidin (SAv) was purchased from Wako Pure Chemical Co. The gold-nanoparticle (10 nm)-labeled SAv and FITC-labeled SAv were purchased from ICN Co. and Vector Laboratories, Inc., respectively. All regents were used without further purification.

2.2 Measurements.

Circular dichroism (CD) spectra were recorded on a J-720 spectropolarimeter (JASCO Ltd.) under a nitrogen atmosphere. Experiments were performed in a quartz cell with a 5 mm path length over the range of 190-250 nm at room temperature. Sample solutions were prepared by diluting the TFE stock solution of peptide with buffers. Final peptide concentration was 40 μM in aqueous media and TFE content was 5 %. Fluorescence spectra were measured on a FP-770 fluorescence spectrophotometer (JASCO Ltd.), at an excitation wavelength of 290 nm. Experiments were performed in a quartz cell with 10 mm path length at room temperature.

Atomic force microscopy (AFM) images were collected at ambient temperature on a Nanoscope IIIa (Digital Instrument, Inc.) operated in a tapping-mode and a phase-mode using silicon cantilevers (125 μm, tip radius *ca.* 10 nm). An aliquot of peptide **1** in water was placed on freshly cleaved mica. After adsorption for 3-5 min, the excess solution was removed by absorption onto filter paper and the samples were stored in a covered container to protect them from contamination until they were imaged (within 1 h). A 10 μm x 10 μm scanner was used for imaging. The scanning speed was at a line frequency of 1 Hz, and the original images were sampled at a resolution of 512 x 512 points. Transmission electron microscopy (TEM) images were collected on a H8100 (Hitachi Co.) at 200 kV accelerating voltage. A small volume of nanofiber solutions with gold-labeled SAv was applied to carbon-coated copper grid, and the excess solution was blotted with filter paper. The TEM images were obtained from non-stained samples. Fluorescence images (x 1000) were obtained on a BH2-RFC (Olympus Co.) equipped with an excitation filter (BP-490) and a barrier filter (O-515).

3. RESULTS AND DISCUSSION

3.1 Specific Interaction between Streptavidin and Biotin-functionalized Amphiphilic Peptide in Water.

Complexation of the biotin-functionalized peptide **1** with SAv was first of all confirmed by the fluorescence spectroscopy of trypophan (Trp) existing in the binding site of SAv. It has been known for the emission maximum (λ_{em}) of Trp to be considerably affected by its microenvironment. Maste et al. have proposed a classification for the microenvironment of Trp in protein: (1) Trp residues are placed at a hydrophobic site (λ_{em}=330 nm), (2) Trp residues are placed at a site in which they partly contact water (λ_{em} =340 nm), and (3) Trp residues are placed at a site in which they are completely exposed to water (λ_{em}=354 nm).[13] We can therefore evaluate the biotin-SAv interaction from λ_{em},

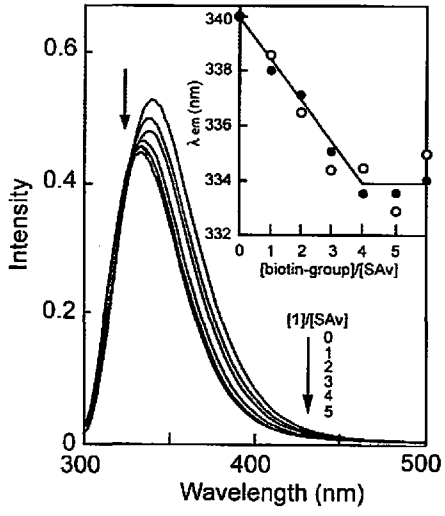

Fig. 2. Fluorescence spectra of SAv-**1** mixtures in phosphate buffer at pH 7.0. λ_{ex}=290 nm. The molar ratios ([1]/[SAv]) are indicated. The inset displays relationship between λ_{em} and [1]/[SAv] values as black circles. The data of pure biotin is also included for comparison as white circles. [SAv]=2.7x10^{-6} M.

since the complexation of biotin causes a change in microenvironment of the binding site of SAv. Fig. 2 shows the fluorescence spectra of SAv in the presence of different amount of peptide **1** in phosphate buffer at pH 7.0. The emission of Trp residue was clearly observed at 340 nm without peptide **1**. On the other hand, the λ_{em} shifted to a shorter wavelength (334 nm) and the intensity decreased upon addition of **1**. The inset in Fig. 2 illustrates the relationship between λ_{em} and the molar ratio of **1**/SAv. In this figure, the data for pure biotin/SAv system was also included for comparison. As a result, the shift in λ_{em} values was found to stop when the molar ratio of **1**/SAv reached to be 4.0. Similar spectral change of SAv was also observed by the addition of pure biotin. Thus, the peptide **1** is capable of forming complexes with the tetrameric SAv effectively in water, as well as the pure biotin.

3.2 Fabrication of Biotin-containing Nanofibers through Peptide Self-assembly.

The conformational and self-assembling properties of the peptide **1** were investigated by means of CD and AFM measurements. We demonstrated previously that biotin-free peptide **2** could be self-assembled into β-sheet nanofibers upon partial neutralization of the charge of Lys residues at pH 9.0.[8] Fig. 3(a) shows the time dependence of CD spectra for **1** at pH 9.0 (peptide concentration 40 μM). When the sample solution was freshly prepared, the CD spectra gave mixed pattern of α-helix and random coil structure with two negative maxima at 220 and 201 nm. In contrast, the CD spectra showed a β-sheet-like pattern with single negative maxima at 217 nm after incubation for 3 h, although the $[\theta]_{217}$ value was small (-8000 deg cm^2 dmol^{-1}) as compared to the typical β-sheet structure. In parallel with such conformational change, it was found from tapping-mode AFM analysis that the peptide **1** formed relatively short nanofibers (lengths: 500-800 nm, heights: *ca.* 5 nm) in comparison with those from biotin-free peptide **2** (lengths > 1 μm) (Fig. 3(c)). This difference in nanofiber lengths is probably due to strong hydrogen-bonding property of biotin-group, which prevents hydrogen bonding between peptides for β-sheet formation. It should be noted that at pH 7.0, the peptide **1** was predominantly in the monomeric random coil conformation even after incubation for 3 h, as was previously observed in peptide **2** system.

In order to fabricate biotin-containing nanofiber with high-axial-ratio, **1/2**-mixed peptide system was then employed. The molar fraction of **1** was adjusted to be 0.1, taking into account the molecular dimensions both of SAv and β-sheet structure. Fig. 3(b) and (d) showed the CD spectral change and AFM image (3 h) of **1/2** (1/9) mixture at pH 9.0, respectively. In contrast to the peptide **1**, the CD spectra of **1/2**-mixture showed a gradual change typical for a β-sheet ($[\theta]_{217}$=-13200 deg cm^2 dmol^{-1})) through an isodichroic point at 208 nm. Moreover, AFM image demonstrated the formation of matured nanofibers. From these results, it can be concluded that well-developed biotin-functionalized β-sheet nanofiber, which has a height of *ca.* 3-5 nm and lengths in excess of 1 μm, can be successfully obtained from the **1/2**-mixed peptides by self-assembly.

3.3 Antigen-Antibody Interaction on Self-assembled Peptide Nanofiber.

Specific biotin-SAv interaction on the **1/2**-peptide nanofiber was subsequently examined by using fluorescence, AFM, and TEM techniques. All experiments were carried out by adding guest streptavidins into the matured nanofiber solutions, that were obtained by incubation at pH 9.0. Fluorescence spectra indicated that the biotin-SAv complex was formed even on the nanofiber surface at pH 9.0, namely

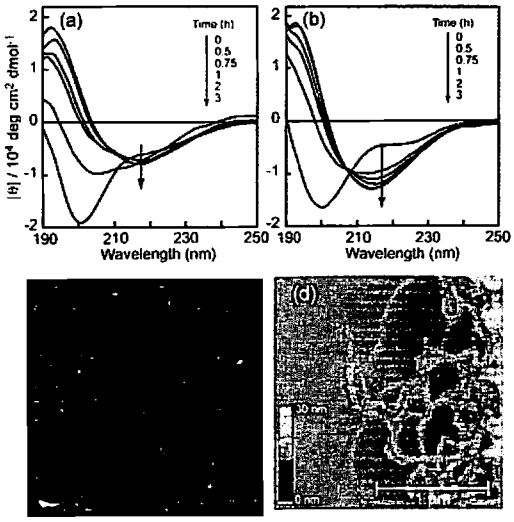

Fig. 3. CD spectral changes of 1 (a) and 1/2 (1/9) mixture (b) in 5 mM $NaHCO_3$/NaOH buffer (containing 5 % TFE in vol.) at pH 9.0, room temperature. [Total peptide]=40 μM. Tapping-mode AFM images (2 μm x 2 μm) of 1 (c) and 1/2 (1/9) mixture (d) at 3 h, pH 9.0. z-scale: 30 nm.

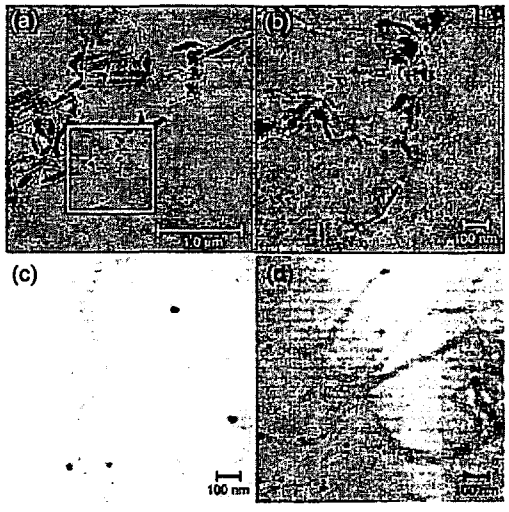

Fig. 4. Phase-mode AFM images (a and b) for the mixture of 1/2-nanofiber and gold-SAv at pH 9.0. The image (b) corresponds to the area marked with a white square in image (a). TEM images for the mixture of **2**-nanofiber/gold-SAv (c) and the mixture of **1/2**-nanofiber /gold-SAv (d) at pH 9.0.

λ_{em} of SAv shifted from 340 nm to 335 nm in **1/2**-nanofiber solutions (data not shown). To visualize such antigen-antibody complexations under microscope observation, we employed gold-nanoparticle (10 nm in diameter)-modified SAv (gold-SAv) and FITC-labeled SAv (FITC-SAv). Fig. 4(a) and (b) showed phase-mode AFM images for the mixture of **1/2**-nanofiber and gold-SAv at pH 9.0. Gold nanoparticle, which was located on nanofiber, can be clearly confirmed in this image, demonstrating the immobilization of the gold-SAv on nanofiber surface. TEM analyses also support the result of AFM study. No gold-SAv was observed on the nanofiber made from pure peptide **2** (Fig. 4(c)), whereas gold-SAvs existed only on the nanofiber in the case of **1/2**–mixed peptide system (Fig. 4(d)). In addition, visible aggregates were clearly observed in the fluorescence image when the FITC-SAv was used instead of gold-SAv, as shown in Fig. 5. These fluorescent and fibrous aggregates are attributed to the localization of FITC groups by immobilizing the FITC-SAv onto nanofiber. This result agrees well with the results of AFM and TEM studies.

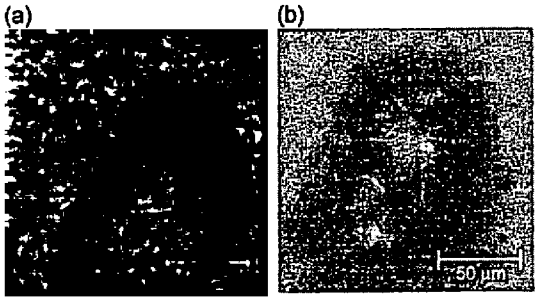

Fig. 5. Fluorescence images of the FITC-SAv in the absence (a) and the presence (b) of **1/2**-nanofibers in $NaHCO_3$/NaOH buffer at pH 9.0, room temperature.

4. CONCLUSION

In the present study we successfully synthesized biotin-functionalized self-assembling peptide (**1**), and described their conformational and self-assembling properties in water. The peptide **1** was found to form relatively short nanofibers by itself. The strong hydrogen-bonding property of biotin-group seems to interrupt the β-sheet formation. On the other hand, biotin-containing nanofiber with high-axial-ratio was successfully obtained by mixing the peptide **1** with biotin-free **2**. We have also demonstrated the biotin-SAv interaction on the surface of **1/2**-nanofiber by using various microscope techniques. These studies undoubtedly will make an important contribution to design novel peptide-based biomaterial with shape-specific nanostructure.

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