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Affinity-Based Screening of Peptides Recognizing Assembly States of Self-Assembling Peptide Nanomaterials

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Abstract: Novel peptides capable of binding to self-assembled peptide nanomaterials were identified from a random heptapeptide library displayed on phages. Affinity-dependent peptide screening against helically coiled nanofibers constructed of β-sheet peptides gave phage clones displaying peptides with a variety of affinities and selectivities. An enzyme-linked immunosorbent assay using phage-displayed peptides revealed that the screened peptides specifically bind to target nanofibers, in contrast to reference nanofibers comprised of peptides with slightly different amino acid sequences. A Dot blot assay using chemically synthesized peptides demonstrated that peptide 01 (p01), with the sequence Thr-Gly-Val-Lys-Gly-Pro-Gly, showed an affinity constant ($3.7 \times 10^5\ M^{-1}$) for the target nanofibers 200 times greater than its affinity for monomeric peptides and 60 times greater than for short nanofibers. These results suggested that p01 selectively recognizes the assembly state of the target peptide. ATR/IR secondary structure analyses clearly showed that when p01 binds to target nanofibers, it undergoes a structural transition from random-coil to parallel β-sheet structures, resulting in greater affinity and high specificity for the target fiber. Surface modification of the peptide nanofibers by p01 demonstrated that the peptide specifically binds to the edge of the nanofibers. Using p01, uniform arrays of gold nanoparticles (proteins) could be generated on the peptide nanomaterials.

Introduction

A number of biomolecules such as proteins, peptides and nucleic acids exhibit properties required for nanoscale self-assembly and molecular recognition. In natural systems, supramolecular architectures are assembled through numerous weak interactions (noncovalent bonds) such as electrostatic, hydrogen bonding, π-stacking, van der Waals interactions and hydrophobic effects. The ability of "smart" biomolecules to self-assemble has been utilized for development of bioinspired and nanoscaled materials.[1] The spontaneous assembly of biomolecules has advanced a bottom-up approach for the fabrication of nanostructured materials.[2] In particular, peptide-based assemblies are superior from the viewpoint of flexibility of design and synthesis for modulating molecular assembly and introducing functionality.[3] Nanomaterials constructed by designed peptides can act as novel, smart materials such as fibers or tubes,[4a−d] filaments,[4e,f] hydrogels[4g−i] and surfactants.[4j,k] We have developed methodologies for the fabrication of peptide nanofibers with a uniform width of approximately 100 nm using *de novo* designed short peptides composed of 10 amino acid residues (sequence: $PKX_1KX_2X_2EX_1EP$, where X indicates hydrophobic Phe, Ile, Val, or Tyr) which form antiparallel β-sheet structures.[5] The peptide nanofibers, especially those self-assembled from the peptide FI (X_1: F, X_2: I), exist as a single highly regular nanofiber constructed of helical coils. Nanofibers constructed by β-sheet peptides containing Phe as the hydrophobic residue form helical coils, and those containing Ile and Val form tape-like (not helical coils) nanofibers. Additionally, the nanofiber constructed by β-sheet peptide FI (FI nanofiber) was functionalized with arbitrary molecules by peptide biotinylation,[6a] and by using functional anchors composed of binding and functional groups.[6b]

On the other hand, exquisite molecular recognition abilities of biomolecules have been produced by evolutionally process

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in natural systems. Recent studies have revealed novel peptides with selective affinity for artificial materials screened from combinatorial bioengineered peptide libraries displayed on phage and cell surfaces.[7] These peptides have regular structure, and thus might recognize 2- or 3-dimensional regular distributions of atoms or functional groups at the materials surface, giving rise to specific affinities.[8] Furthermore, such novel peptides have potential applications as adsorbents for patterning,[8a] catalysts for the preparation of inorganic particles,[9] and surface modifiers[9a,10] used for assembly. Additionally, Belcher and coworkers disclosed that peptides can discriminate crystal defects of germanium thin films on silicon (Ge-on-Si) and Ge wafers, suggesting that the recognition ability of peptides could be exploited for nondestructively probing and identifying the localization of defects in crystalline substrates.[11]

Because peptides can recognize slight differences in delicate chemical structures on material surfaces, it might be possible to functionalize self-assembled nanomaterials by peptide binding. In this study, we screened peptides that specifically bind to self-assembled coiled "FI" nanofibers that have fine surface structures using phage display combinatorial technology. High affinities of the screened heptapeptide against the target FI nanofibers were demonstrated using an enzyme-linked immunosorbent assay (ELISA). The affinity was considerably lower when structurally similar reference IF, FF, and VI nanofibers were used, due to their slight differences in amino acid sequence or composition. Binding and secondary structure analyses using chemically synthesized peptides indicated that the peptide binds specifically to FI nanofibers with a structural transition. This is in contrast to its binding to reference nanofibers, as well as the heptapeptide binding to monomeric, oligomeric, and short fiber states of FI. Using the screened peptide, TEM clearly showed a nanoarrangement of gold nanoparticles (proteins) on the fiber. To our knowledge, this is the first report demonstrating the recognition by short peptides of self-assembled nanomaterial surfaces and assembly states.

Experimental Section

Peptide Synthesis. Peptides were synthesized by conventional solid-phase method by using 9-fluorenylmethyloxycarbonyl (Fmoc) strategy.[12] The peptide chains were assembled on a 2-chlorotrityl chloride resin (*N*-and *C*-termini were free) or rink amide resin (for screened peptides, *N*-termini was free, and *C*-termini was amidated) by using Fmoc amino acid derivatives (3 equiv), *N*,*N*-diisopropylethylamine (DIEA, 6 equiv), 2-(1*H*-9- azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, 3 equiv) in *N*-methylpyrrolidone (NMP) for coupling, and piperidine (25%) (PPD)/NMP for Fmoc removal. To cleave the peptide from the resin and remove the side chain protecting groups, the peptide resin was treated with trifluoroacetic acid (TFA)/triisopropylsilane/water (95/2.5/2.5). All peptides were purified by reverse-phase HPLC on a Cosmosil 5C18-AR-300 packed column (10 × 250 mm) by using a linear gradient of acetonitrile/0.1% TFA at a flow rate of 3.0 mL min^{-1}. The peptides were identified satisfactorily by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and amino acid analyses. MALDI-TOF-MS was performed on a Shimadzu KOMPACT MALDI III mass spectrometer by using 3,5-dimethoxy-4-hydroxycinnamic acid as a matrix. Amino acid analyses were carried out by using a Wakopak WS-PTC column (4.0 × 200 mm) after hydrolysis in HCl (6 M) at 110 °C for 48 h in a sealed tube, and labeling by phenylisothiocyanate (PITC). For synthesis of biotinylated peptides, same methodologies were applied using 2-chlorotrityl chloride resin. After coupling of last amino acids, amino groups at *N*-termini were protected by *tert*-butoxycarbonyl (Boc) groups using di-*tert*-butyl dicarbonate (10 equiv) and DIEA (5 equiv). To cleave the protected peptide from the resin, the peptide resin was treated with acetic acid/trifluoroethanol/dichloromethane (1/1/3). Carboxylic groups of protected peptides at *C*-termini were reacted with amino groups of amino- and biotin-terminated pentaethylene glycol. Detail is described in the Supporting Information. To remove side chain and *N*-terminus protecting groups, the biotinylated peptides were treated as described above.

Affinity-Dependent Peptide Screening with Nanofibers. Formation of FI nanofibers were performed as previously described.[5,6] In brief, peptides were dissolved in 1,1,1,3,3,3-Hexafluoroisopropanol in a concentration of 1.7 mM. The solution was added to microtubes, and the solvent was removed by N_2 gas. Then, sodium-phosphate buffer (100 mM, pH 7.4) was applied to the microtube, and the solution was sonicated for complete dissolution. The solution was allowed to stand at ambient temperature for 72 h for fiber formation. For immobilization, FI nanofibers in the buffer were incubated on glass substrate (high water-repellent printing glass slide, Matsunami Glass) for 1 h. The immobilization conditions of FI nanofibers on glass substrates are the same for the phage-screening. To FI peptide nanofibers was applied 1.2×10^{10} pfu/15 μL of phage library which provides 10^9 different phage clones with 7-amino acid linear peptide inserts at *N*-termini of minor coat proteins (New England Biolabs) dissolved in the buffer. The mixture was incubated for several min at ambient temperature. The incubation time was decreased with an increasing number of rounds (15 min for first round, 10 min for second and third round, and 5 min for fourth and fifth round). To remove any unbound phages, the fibers were rinsed with 15 μL of buffer. The washing time was increased with an increasing number of rounds (no wash for first round, 1 min for second round, 3 min for third round, 5 min for fourth round, and 10 min for fifth round). Then, the bound phages were eluted by mounting 15 μL of 50 mM glycine-HCl (pH 2.2) onto the fibers for 15 min at ambient temperature. The collected buffer solution containing the eluted phages was neutralized with 6 μL of 1 M Tris-HCl buffer (pH 9.1). To estimate the phage numbers collected, aliquots of elutes were used for tittering. For the next round of biopanning, the phages were amplified by infecting with *Escherichia coli* ER2738 and collected and purified with polyethylene glycol and NaCl. To remove phages binding to glass slide, phages were incubated on the glass slide, and unbound phages were applied to next round of biopanning. A total of five cycles

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FI: PKFKIIEFEP
IF: PKIKFFEIEP
FF: PKFKFFEFEP
VI: PKVKIIEVEP

Figure 1. Designed β-sheet peptides for construction of nanofibers used in this study:[5] hydrophilic amino acids are Lys (K) and Glu (E), and hydrophobic amino acids are selected from Phe (F), Ile (I), and Val (V).

or rounds of biopanning were performed. After the fifth round of biopanning, the tittering plates were used to isolate phage clones. The cloning and DNA sequencing followed conventional methodologies.

Enzyme-Linked Immunosorbent Assay (ELISA). To analyze the binding affinity of the phages, ELISA was performed. Peptide nanofibers were immobilized on each well of microtiter plate (Half area plate, Corning). Phage solution (10 μL at 100 pM) was applied to the nanofibers, and the mixture was incubated for 10 min. After washing with the buffer, horseradish peroxidase (HRP) conjugated anti-M13 phage monoclonal antibody solution containing 2% skim milk was applied for 15 min. Subsequently, the relative binding amounts of phages were estimated by measuring the fluorescence intensity of the product (QuantaBlu Fluorogenic Peroxidase Substrate Kit, Pierce, λ_{ex} and λ_{em} were 355 and 460 nm, respectively). To quantitatively investigation of the phage affinities, dependences of phage concentration against relative binding amounts were analyzed. Fluorescence intensity of each concentration was fitted to a Langmuirian equation.

Dot Blot Assay. Aliquots (1 μL) of each fiber formation state of peptide nanofiber were applied onto nitrocellulose membrane (pore size: 0.45 μm; Hybond ECL, GE Healthcare Bioscience) for 3 times. The membranes were blocked with SuperBlock Blocking Buffer in TBS (Pierce) for 10 min at ambient temperature. The membranes were washed with the sodium-phosphate buffer and incubated with each concentration of biotinylated peptide solutions. The membranes were washed again with the buffer and incubated with Cyanine5.5 (Cy5.5) conjugated antibiotin antibody (abcam) for 30 min at ambient temperature. Then, fluorescent intensity of Cy5.5 meaning the binding amounts of biotinylated peptides was analyzed by fluorescence and infrared imaging system (MS Techono Systems). To quantitatively understand about peptide affinities, peptide concentration against relative binding amounts was analyzed. Relative binding amount of each concentration was fitted to a Langmuirian equation.

IR Analysis. Attenuated total reflection (ATR)/IR spectra of peptide nanofibers (500 μM), chemically synthesized screened peptides (25−1000 μM) and those mixtures were obtained using refractive surfaces (IRPrestige-21 with DuraSampl IR-II, Shimadzu) in air at ambient temperature. Interferograms were coadded 100 times and Fourier transformed at a resolution of 4 cm^{-1}.

TEM Observation. FI nanofibers were incubated with biotinylated peptides in the sodium-phosphate buffer. Collodion-coated copper EM grid was placed coated-side down onto a droplet of the solution for 30 s, and excess solution was removed by blotting with filter paper. Then, the grid was floated onto a droplet of gold nanoparticles labeled goat antibiotin IgG antibody (diameter of gold nanoparticle: 10 nm) in Tris buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.5) and was washed by floating on buffer solution and the solution was removed by blotting. The sample was negatively stained with 2% (w/v) uranyl acetate for 1 min (in case without antibody) or 5 s (in case with antibody). The grids were blotted and allowed to dry gradually at ambient temperature. All images were taken by using a Hitachi H-7500 electron microscope operating at 100 kV.

Results and Discussion

Peptide Screening against Nanofibers. Peptides with affinity to FI nanofibers (Figure 1) were screened five subsequent times. After five rounds of biopanning, phage pools that specifically bound to FI peptide nanofibers were screened and their binding

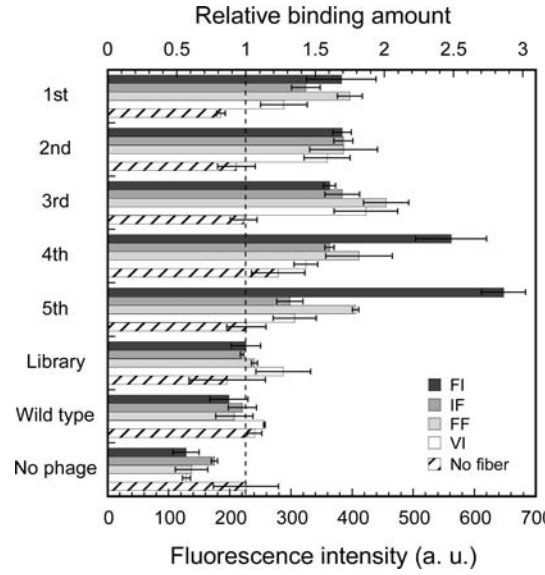

Figure 2. Binding amounts of the phage pools at each round of biopanning. The black, dark gray, grayish white, white, and shaded bars indicate the amounts against FI, IF, FF, and VI nanofibers and microtiter plate (no fiber), respectively. The Fluorescence intensity values were converted to binding amounts relative to that of a phage library against FI nanofibers. For all samples $n = 3$. Error bars represent the standard deviation.

affinities were quantitatively investigated by ELISA. Affinity to FI nanofibers tended to increase with each successive round, while affinity to IF, FF and VI nanofibers (references) did not (Figure 2). Note that the quantity of phages nonspecifically bound to microtiter plates (polystyrene substrates) was within experimental error, indicating that the measured binding to nanofibers was representative of the true binding affinities of the phages. Additionally, the amounts of peptide nanofibers adsorbing to microtiter plates, determined by the fluorescamine assay[13,14] (Figure S1, Supporting Information), showed a similar amount of bound FI nanofibers to that of IF and VI nanofibers, but less than FF nanofibers. This result also supports the greater phage amounts bound to FI nanofibers. After cloning and DNA sequencing, 26 phage clones were isolated. The diversity of the peptides was distinctly decreased. Amino acid sequences of the screened peptides are shown in Table 1, where frequencies indicate the fraction of the same clone in all the isolated clones. Since there does not seem to be a sequence similarity in peptides, these peptides may interact with FI nanofibers by individual mechanisms and binding sites. Some clones, such as clone 01 (c01) and c12, were observed at high frequency, constituting more than 6 out of the 26 clones. The percent amino acid composition of the screened peptides was compared with that of the library phage (Figure S2, Supporting Information) and showed that Lys and Gly were clearly increased. All nanofibers (FI, IF, FF and VI) were negatively charged in the same range (Figure S3, Supporting Information), and most of the clones have amine-containing amino acids (Lys, Arg, or His), indicating that positively charged amino acids are important for peptide interactions with FI nanofibers. In contrast, Gly was present in just 2 clones, suggesting that the role of Gly is unique for individual peptides. These observations suggest that the phage

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Table 1. Phage-Displayed Peptides with Its Apparent Binding Constants against Nanofibers

			K_{app}/ 10^{10} M^{-1}				ratio[a]		
clone	frequency	sequence	FI	IF	FF	VI	FI/IF	FI/FF	FI/VI
c01	12/26	**TGVKGPG**	40	5.1	4.3	2.2	7.8	9.3	18.2
c12	6/26	**SAPSSKN**	7.9	3.8	3.3	0.84	2.1	2.4	9.4
c05	2/26	**GLQNSLP**	10	6.6	5.8	0.68	1.5	1.7	14.7
c06	2/26	**DSWPANF**	16	3.2	12	0.45	5.0	1.3	35.6
c02	1/26	**KPTTSDY**	13	12	10	12	1.1	1.3	1.1
c07	1/26	**AHYYPKP**	3.4	3.8	1.7	2.0	0.89	2.0	1.7
c11	1/26	**TASPRAP**	1.9	1.5	1.2	2.1	1.3	1.6	0.90
c25	1/26	**TASPHQP**	1.7	2.8	1.8	1.6	0.61	0.94	1.1
library	–	–	0.89	1.3	1.8	0.48	0.68	0.49	1.9
wild type	–	–	0.91	1.7	1.6	0.74	0.54	0.57	1.2

[a] Ratios of phage clone K_{app} between against FI and other nanofibers.

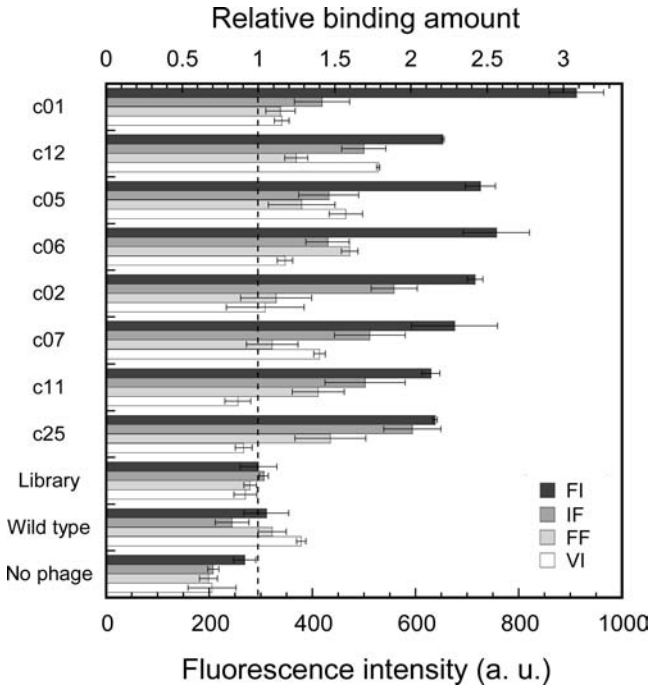

Figure 3. Binding amounts of phage clones screened from the phage displayed peptide library for FI nanofibers. The black, dark gray, grayish white, and white bars indicate amounts against FI, IF, FF, and VI nanofibers, respectively. The fluorescence intensity values were converted to amounts relative to that of phage library against FI nanofibers. For all samples $n = 3$. Error bars represent the standard deviation.

library was successfully manipulated to evolve phage clones specific for the nanofibers.

Affinities of Phage Clones. The binding amount of each phage clone, identified after five rounds of screening against the nanofibers, was analyzed by ELISA, as shown in Figure 3. Note that the bound amounts of all phage clones to polystyrene substrates were within the experimental error (data not shown), indicating that the fluorescence data accurately reflected the bound amount of phage clones described above. The binding amount of all clones was 2 or 3 times greater than that of the library and wild type (WT) phages, indicating that the phage clones were successfully screened using their affinity to FI nanofibers. Furthermore, the amount of bound phage clones to the target FI nanofibers was significantly greater than the amount bound to the reference IF, FF or VI nanofibers. Some clones bound with 2 to 3 times more affinity to FI nanofibers than to IF, FF or VI nanofibers. The binding amounts of the library and WT phages were similarly very small, and independent of the type of nanofiber. These observations indicated that heptapeptides displayed on screened phage clones bind specifically to the target FI nanofibers. In other words, screened peptides can discriminate structural differences in FI, IF, FF and VI nanofiber surfaces.

To quantitatively analyze both affinity and specificity, the relative amounts of bound phages (ELISA fluorescence intensity) as a function of the phage clone concentration were obtained for the target FI nanofiber and the reference IF, FF and VI nanofibers (Figure S4, Supporting Information). The amounts saturated at a certain level for all clones. The curves were fitted to a Langmuirian equation to obtain apparent binding constants (K_{app}). The K_{app} values and ratios between FI and the other nanofibers, which correspond to the selectivity, are summarized in Table 1. All clones showed larger K_{app} values to the target FI nanofibers compared with the library and WT phages, indicating that the clones were successfully screened by their affinity to FI nanofibers. One clone, c01, showed the greatest affinity, with a K_{app} for the target FI nanofibers about 40 times greater than that of library and WT phages. Some of the clones had moderately selective affinities for the target FI nanofibers. Due to differences in amino acid sequences and compositions of the screened peptides, the affinity and selectivity of each peptide were unique, and there was no correlation between the strength of binding and selectivity. It appears that each amino acid comprising a screened peptide contributes to the interaction with the nanofibers, implying that different peptides have different recognition sites. To understand these differences, additional experimental evidence is required. Comparing K_{app} values of the phage clone for FI nanofibers, the selectivities of the clones were more than 7, 9 (c01), and 30 times (c06) greater than to the reference IF, FF and VI nanofibers, respectively. Selectivities tended to be much higher for VI than for IF and FF. FI, IF and FF nanofibers form helical coils, and only VI nanofibers form tape-like structures,[5] suggesting that all the screened peptides can discriminate not only the amino acids arrangement of the fibers, but also its macro morphology. In those clones, c01 has the greatest affinity to FI nanofibers and the highest selectivity of all the reference nanofibers out of for VI. C06 has the highest selectivity for FI nanofibers compared to VI, indicating that the Phe and Trp contained in c06 result in specific interactions with Phe contained in FI, IF and FF nanofibers.

Affinities of Chemically-Synthesized Peptides. In the previous section, affinities (and selectivities) of peptides displayed on phages toward nanofibers were analyzed. The K_{app} values, however, did not provide direct evidence for peptide affinity due to several features of the phage display system employed for these interaction analyses:[9b,15] (i) phages with high molecular weight (16300000)[16] adsorb nonspecifically, (ii) displaying plural peptide copies might enhance their affinities through the multivalent effect, and (iii) displayed peptides fused to the surface proteins of phages might have a conformation suitable for affinity to targets due to intramolecular interactions. Accordingly, to better understand the interactions between the screened peptides and peptide nanofibers, analyses using chemically synthesized peptides (nontethered peptides) were required. We selected the sequence of c01 (which displayed the best

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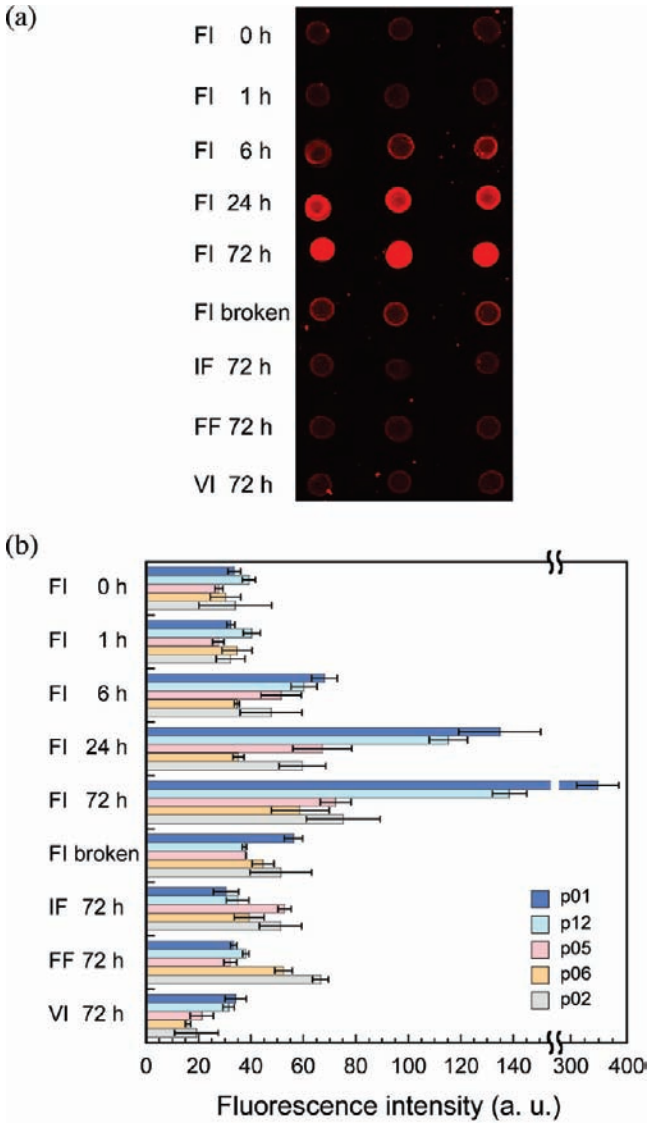

Figure 4. Binding amounts of chemically synthesized peptides against various states of target FI and references, IF, FF, and VI nanofibers. (a) A fluorescence scanner image of bio-p01 binding to the nanofibers and (b) quantified data of the biotinylated peptides. The blue, light blue, pink, orange and gray bars indicate amounts of bio-p01, 12, 05, 06 and 02, respectively. For all samples $n = 3$. Error bars represent the standard deviation.

binding strength and selectivity for FI nanofibers), and then the chemically synthesized peptides were used in dot blot assays (the chemically synthesized peptide with the c01 sequence was named p01; the other synthesized peptides were named similarly). In order to detect peptide binding, biotin was conjugated to the *C*-termini of the screened peptides via pentaethylen glycol as a spacer. A fluorescence scanner image and the resulting quantification of bound biotinylated p01 (bio-p01) at 1 mM were detected by Cy5.5 conjugated antibiotin antibodies, as shown in Figure 4. The target, FI nanofiber, was spotted after various incubation time intervals. FI at 0 h incubation was mostly monomeric FI peptide, while incubation for 1 or 6 h showed coexisting states of monomeric and oligomeric peptide. Likewise, after 24 or 72 h incubation, short fibers and long, coiled fiber states, respectively, were observed. Broken FI nanofibers prepared by rapid stirring after 72 h incubation were aggregates containing shorter fibers. IF, FF and VI nanofibers, used as references, were spotted onto membranes after 72 h incubation. The binding amount of bio-p01 to the long and coiled FI nanofiber target (FI at 72 h) was 10 times greater than that to the reference IF, FF and VI nanofibers. Furthermore, compared with amounts bound to other states of the FI nanofibers (peptides), the amount of bio-p01 bound to FI (72 h) was significantly greater than that bound to the monomeric, oligomeric, aggregated, and short fiber states. These results indicate that the screened peptides were constructed appropriately for interaction with FI (72 h) surfaces, and therefore bound with lower selectivity to other states of FI and to the reference IF, FF and VI nanofibers.

Table 2. Binding Constants of Chemically-Synthesized Peptides against Nanofibers with Various States

	K_a/ 10^3 M^{-1} (% ratio[a])								
	FI								
peptide	0 h	1 h	6 h	24 h	72 h	broken	IF	FF	VI
bio-p01	01.7 (0.46)	3.6 (0.97)	5.4 (1.5)	5.9 (1.6)	370 (100)	2.1 (0.57)	8.8 (2.4)	5.5 (1.5)	3.1 (0.84)
bio-p12	6.8 (31)	4.3 (20)	6.0 (27)	10 (45)	22 (100)	1.2 (5.5)	9.8 (45)	8.8 (40)	6.6 (30)
bio-p05	6.9 (38)	7.0 (39)	8.5 (47)	11 (61)	18 (100)	11 (61)	8.2 (46)	7.5 (42)	9.1 (51)
bio-p06	8.1 (8.1)	10 (10)	7.3 (7.3)	7.5 (7.5)	100 (100)	7.7 (7.7)	8.7 (8.7)	21.0 (21)	4.1 (4.1)
bio-p02	12.00 (16)	10 (13)	32 (42)	55 (71)	77 (100)	27 (35)	32.0 (42)	62.0 (81)	12.0 (16)

[a] Percent ratios of peptide K_a between against FI (incubated for 72 h) and other states of nanofibers.

To further investigate peptide recognition, four biotinylated peptides (p12, p05, p06 and p02, which have significant affinities for FI nanofibers when displayed on phages) were also synthesized and analyzed, and the relative amount as a function of the biotinylated peptide concentration was obtained. The saturation curves were fitted to a Langmuirian equation (Figure S5, Supporting Information) to obtain the binding constants (K_a). The K_a values and ratios between K_a toward FI (incubated for 72 h) and toward the other targets correspond to the selectivity, and are summarized in Table 2. The K_a values toward FI (72 h) ranged from 10^4 to 10^5 M^{-1}, whereas those toward other states of FI and other fibers were approximately 10^3 M^{-1}, except for the K_a values of bio-p12, which ranged in 10^4 M^{-1} toward all states of FI and other nanofibers. Thus, the peptides bound more strongly to long and coiled FI nanofibers than to reference nanofibers with slight differences in amino acid sequence and composition, and also more strongly than to identical peptides but in a different assembly state. The peptide with the strongest affinity for FI (72 h), bio-p01, has a K_a value of 3.7×10^5 M^{-1}, which is sufficiently large for a heptapeptide to interact with self-assembled nanomaterials constructed of peptides. This value is nearly the same as that of short peptides binding to materials such as synthetic polymers[15] (heptapeptide) and titanium[9b] (dodecapeptide) which interact through hydrogen bonding, van der Waals, and/or electrostatic interactions, suggesting that p01 also binds to FI nanofibers through those interactions.

In order to estimate peptide specificity, the ratios of K_a between FI (72 h) and FI (other incubation times) were determined (Table 2). These ratios show a rather wide range, and revealed that the peptides evidently discriminate FI (72 h) from not only other nanofibers, but also from other assembly states of FI. In particular, p01 has the greatest discrimination ratios against monomeric, oligomeric, aggregated, and short fiber states, suggesting excellent selectivities: p01 recognizes FI (72 h) surfaces strictly. With regards to the interaction between the peptides and nanofibers, p01 and peptide FI should communicate

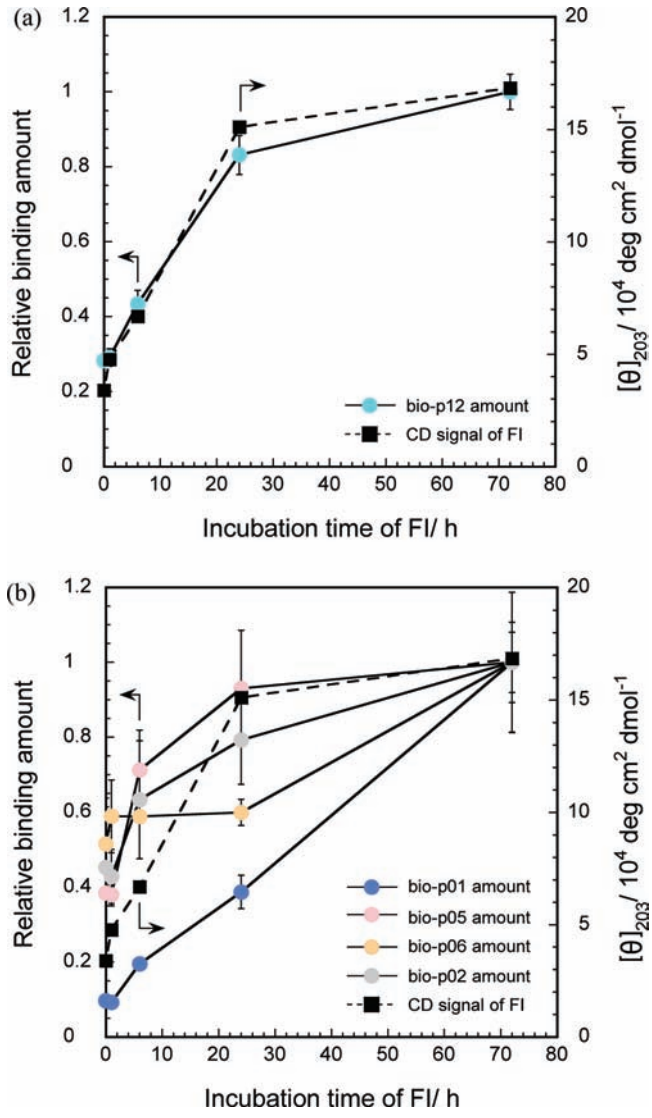

Figure 5. Comparisons of relative binding amounts of screened peptides and CD signals at 203 nm against incubation time of peptide FI. (a) Relative binding amounts converted from fluorescent intensity values at 1 mM of bio-p12 and (b) other biotinylated peptides are shown. Light blue (a), blue, pink, orange and gray circle (b) and black square (both) represent relative binding amounts of bio-p12, 01, 05, 06, 02 and CD signals, respectively. For all samples $n = 3$. Error bars represent the standard deviation.

at the surface of the nanofiber. Therefore, p01 may recognize particular amino acids arranged in a pattern caused by self-assembly of the FI peptide. Furthermore, FI nanofibers are constructed from several helically coiled ribbons, which wind up to form helical coils with fine structures.[5] Since coils are first observed after 72 h incubation, it appears that p01 might specifically recognize the helically coiled ribbon. In contrast, p06 showed medium specificity, and p12, p05 and p02 showed even less specificity, implying that these peptides recognize both FI (72 h) and other states of FI nanofibers with lower selectivity than does p01.

Analyses of the relative binding amounts of the biotinylated peptides at 1 mM (completely saturated) as a function of incubation time of peptide FI were obtained, and compared with the circular dichroism (CD) signals (molar ellipticity) at 203 nm of peptide FI. The fluorescence intensity data were converted to relative binding amounts in comparison to those at 1 mM for FI (72 h) (see Figure S5, Supporting Information). In our previous study, it was shown that the CD signal at 203 nm is

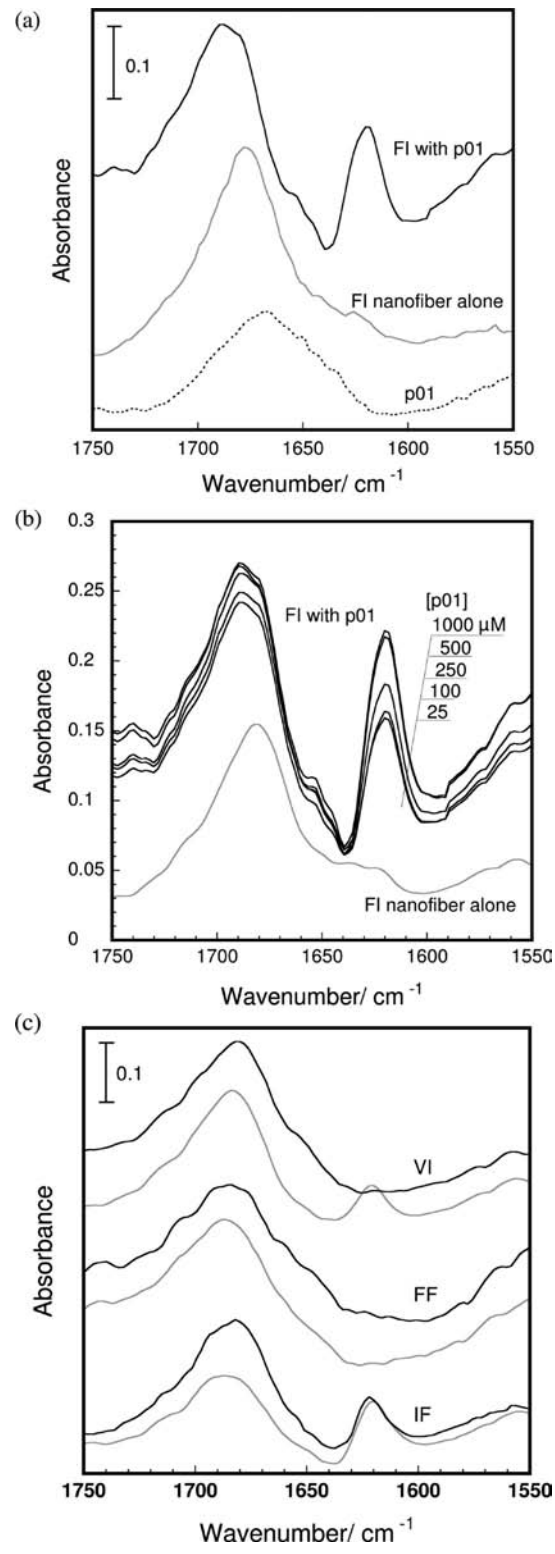

Figure 6. Secondary structure analyses of the peptides. (a) IR spectra of FI nanofiber (solid gray line), p01 (dashed black line), and mixture of p01 and FI nanofiber (solid black line) are shown. All samples were measured at 500 μM, and the scale of absorbance is shown in figure. (b) IR spectra of FI nanofiber (solid gray line) and mixture of p01 and FI nanofiber (solid black line) are shown. FI nanofibers were measured at 500 μM, and measured concentrations of p01 are 25−1000 μM as shown in figure. (c) IR spectra of IF, FF, and VI nanofibers alone (solid gray line) and mixtures with those nanofibers (solid black line) are shown. All measurement concentrations were 500 μM, and the scale of absorbance is shown in the figure.

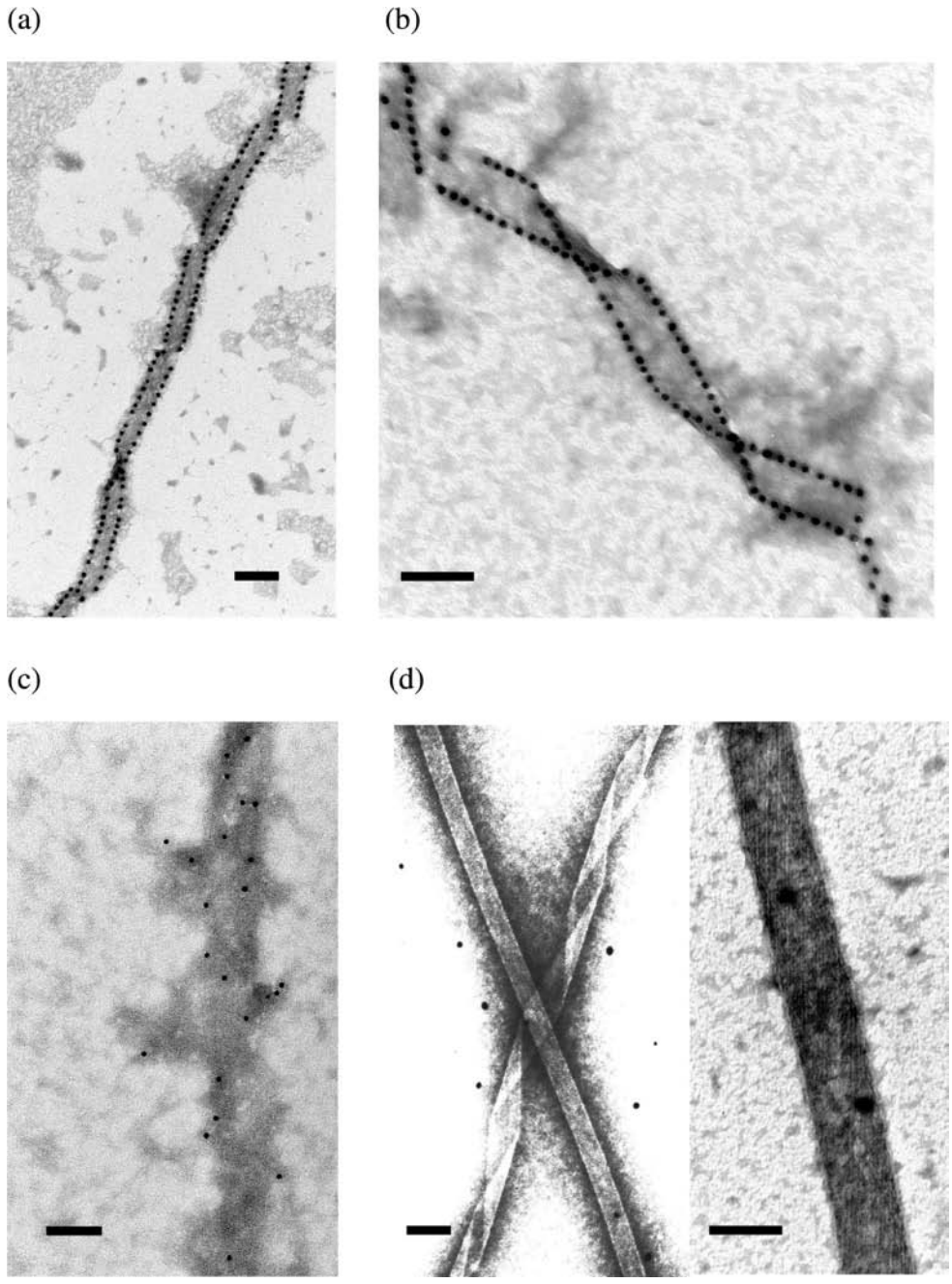

Figure 7. Surface modification of the peptide nanofibers by gold nanoparticles labeled antibiotin antibody (Au-Ab) via (a and b) bio-p01 and (c) bio-p12. (d) FI nanofibers alone are also shown as a reference.[5] All samples were negatively stained by 2% uranyl acetate (5 s and 1 min for with and without Au-Ab, respectively). All scale bars represents 100 nm.

positively correlated with fiber formation and organization of peptide FI.[5] It is interesting that the increasing profile of the CD signals is the same as the profile of the binding amount of p12 (Figure 5a), suggesting that p12 might recognize the nanofiber formation process. The profiles of other peptides are each different from each other, and independent of the CD signals (Figure 5b). The slope of the binding amounts of p01 was much shallower than that of the other peptides, which further supports the predominant recognition of the mature assembly state at 72 h. Comparisons of binding amounts of the peptides and the CD signals also revealed that the recognition mechanism of each peptide for FI was unique, since each recognition pattern was different. In fact, the binding profiles of the peptides toward various fibers and states were independent (Figure S5, Supporting Information), suggesting that the peptides, and especially p01, can discriminate the assembly states of FI nanofibers, and recognize slight differences in the arrangement of the amino acids on nanofibers formed by peptide self-assembly.

Secondary Structure Analyses of Peptides. In order to further investigate the strong binding and high selectivity of p01, the secondary structures of the peptides were analyzed by ATR/IR. IR spectra of the amide I region (1600−1700 cm^{-1}), which is primarily assigned to C═O stretching, was attributed to parallel β-sheet (1620−1640 cm^{-1}), α-helix (1648−1655 cm^{-1}) and antiparallel β-sheet (1670−1690 cm^{-1}).[17] The spectra of FI nanofibers with and without p01 are shown in Figure 6a. The spectrum of FI nanofiber shows a sharp band at 1690 cm^{-1} and a small shoulder band at 1625 cm^{-1}, corresponding to antiparallel β-sheet and parallel β-sheet structures, respectively.

In contrast, a broadband at around 1665 cm^{-1} was observed in the spectrum of p01, indicating a random-coil structure or mixed structure. Upon interaction, an additional band at 1625 cm^{-1} was obviously appeared, which corresponds to parallel β-sheet. Since the area of this additional band is dependent on the concentration of p01 (Figure 6b), the band is clearly due to bound p01. Thus, p01 binds to FI nanofibers with a manner of parallel β-sheet. Considering the secondary structure of the nonbound form, p01 should transit from random-coil to parallel β-sheet upon binding to FI nanofiber, suggesting an induced fit mechanism for interaction with the FI nanofibers. Judging from the formation of the specific secondary structure, the p01 amino acids responsible for affinity were suitably positioned to form interactions with FI nanofibers. Interaction of p01 with the reference IF, FF and VI nanofibers did not result in any additional bands (Figure 6c). Each spectrum of IF and VI nanofiber alone showed a smaller band at 1625 cm^{-1}, indicating that the nanofibers contain parallel β-sheet as minor secondary structures. In case of interactions of p01 with VI nanofibers, the band was clearly decreased, whereas the band of IF nanofibers was not. The decrease might be due to disturbance of the fiber formation at surfaces through interactions with p01. Moreover, other screened peptides also did not show additional bands arising from interaction with FI nanofibers, although the peptides might have random-coil structure when they bind to FI nanofibers (data not shown). These results strongly suggest that p01 undergoes a structural change with it binds to FI nanofibers, which might result in high affinities and selectivities. Thus, parallel β-sheet formation might be suitable for interaction with nanomaterials constructed of β-sheet peptides.

Surface Modification of Nanofibers. TEM were used to investigate the binding sites of peptides screened against peptide nanofibers. FI nanofibers and biotinylated peptides were incubated in the buffer, and applied to the grid. Then, gold nanoparticle (10 nm)-labeled antibiotin antibody (Au-Ab) was applied. Binding of Au-Ab to FI nanofibers via bio-p01 clearly demonstrated uniform arrays of Au-Abs (Figure 7a), indicating that bio-p01 binds to the edges of nanofibers. Without using bio-p01, no Au-Ab was observed on the nanofiber, indicating that Au-Ab clearly binds to a biotin moiety conjugated with the peptides (Figure S6a, Supporting Information). High magnification images showed that p01 clearly binds to the edges of helically coiled ribbons (Figure 7b). FI nanofibers have a helically coiled morphology (Figure 7d), resulting in the specific features of the Au-Ab arrays. Even though the size of the Au-Ab must be taken into account when discussing the recognition site, the distance between Au-Ab was uniform, approximately 20 nm (Au particle: 10 nm), indicating that the recognition site of p01 is spaced at regular intervals of less than 20 nm. In contrast, other peptides did not show defined site specificities (for example bio-p12, Figure 7c), and appeared to bind randomly. In cases of using reference nanofibers (IF and VI) in the same experimental conditions, amounts of Au-Ab via bio-p01 or bio-p12 were obviously decreased (Figure S6b and c, Supporting Information), suggesting that the site specific binding of bio-p01 is very unique to the FI nanofiber, and bio-p12 also has specific affinity for FI nanofibers. Given the variation of the binding selectivity of p01 against various states of the FI nanofibers, the arrangement of amino acids at the edge of the ribbons could arise from the self-assembly process of the peptide FI. These results show that the screened peptides, and especially p01, clearly discriminate the arrangement of amino acids in the self-assembled nanomaterials, and could act as a tool for the construction of molecular nanoarrays. Thus, similar to our conjugation of biotin to p01, various other molecules can be conjugated to the peptide and arrayed onto the nanofiber.

(17) (a) Harris, P. I.; Chapman, D. *Biopolymers* **1995**, *37*, 251–263. (b) Srisailam, S.; Wang, H.-M.; Kumar, T. K. S.; Rajalingam, D.; Sivaraja, V.; Sheu, H.-S.; Chang, Y.-C.; Yu, C. *J. Biol. Chem.* **2002**, *277*, 19027–19036. (c) Moses, J. P.; Satheeshkumar, K. S.; Murali, J.; Alli, D.; Jayakumar, R. *Langmuir* **2003**, *19*, 3413–3418.

Conclusions

We demonstrated that short peptides recognizing the assembly state of self-assembling nanomaterials with fine-structures constructed by β-sheet peptides, FI nanofibers were screened using a phage-displayed peptide library. Phage clones displaying each peptide showed not only greater affinities to FI nanofibers, but also higher selectivities compared with binding to IF, FF and VI nanofibers used as references. Quantitative analyses of phage clone binding revealed that the best clone displaying peptides with the sequence Thr-Gly-Val-Lys-Gly-Pro-Gly (c01) bound FI nanofibers with over 40 times greater affinity than the library and WT phages, and 8−18 times greater affinity than reference nanofibers. Binding analyses using chemically synthesized peptides demonstrated that the peptide with the sequence (p01) bound to long and coiled FI nanofibers with a binding constant of 3.7×10^5 M^{-1}, which is approximately 200 and 60 times greater than its affinity to monomeric peptide FI and short FI nanofiber, respectively. These results revealed that the peptide can discriminate the assembly state of self-assembled nanomaterials. Comparing the relative binding amounts of each peptide and the CD signals of FI nanofibers supports the above recognition mechanism. The peptide recognizes the arrangement of individual amino acids in the self-assembled peptide FI. IR studies showed that p01 binds to FI nanofibers by undergoing a structural transition from a random-coil to parallel β-sheet, meaning that the short peptide p01 exhibits its affinity via an induced fit mechanism against the target, FI nanofiber. TEM of Au-Ab on FI nanofibers via biotinylated p01 clearly demonstrated that p01 specifically binds to the edge of FI coiled nanofiber with a uniform distance. The specificity of screened peptides can be used for molecular arrays with uniform nanosized arrangements on the nanofibers. This is the first report regarding the recognition of assembly states of self-assembled nanomaterials using short peptides. These peptides can have different recognition sites and be used as probes for investigating molecular assembly, and surface modifiers for achieving multimolecular functionalization at the nanolevel.

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Supporting Information Available: Synthesis of biotinylated peptides, adsorption amounts of peptide nanofibers, an amino acid appearance used in screened peptides, zeta-potentials of peptide nanofibers, and phages and peptides concentration dependences against relative amounts, TEM observation of Au-Ab on the nanofibers via the peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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