Peptide Recognition

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Peptides Recognize Photoresponsive Targets**

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Research towards the control of reactions by external stimuli is increasing for the fabrication of stimuli-responsive material systems. Stimuli such as light,[1] temperature,[2] pH,[3] electric fields,[4] and specific molecules[5] have been used to control material responses. Regarding the control of biological reactions, photostimuli are particularly advantageous, because targeted photoirradiation with adequate power and time conserves biomolecular structure and function. Among potential candidates for photoresponsive molecular tools, azobenzene groups, which predominantly isomerize to *trans* and *cis* forms under visible and ultraviolet light, respectively, have been widely investigated.[1c–e]

Short peptides that specifically bind to surfaces of inorganic[6] and organic[7] materials have recently been isolated. The isolation of peptides is based on affinity selection from peptide-displayed phage and cell libraries using the so-called phage display and cell-surface display methods, respectively. Those peptides have been utilized as non-covalently bound linkers for the modification of material surfaces.[8] Our previous studies have successfully demonstrated that film surfaces composed of polymers with extremely simple chemical structures can be satisfactory targets for peptides.[9] These observations suggest that a surface with a certain regular structure can be recognized by specific peptides. This recognition is due to the dimension of the structured surface that can accommodate a (nano)structure formed by the peptide itself. These observations have led to our interest in stimuli-responsive peptides, which recognize the surface structures of materials.

Herein we report the application of phage libraries displaying 7-mer random peptides on film surfaces composed of azobenzene-containing synthetic copolymers (Figure 1),

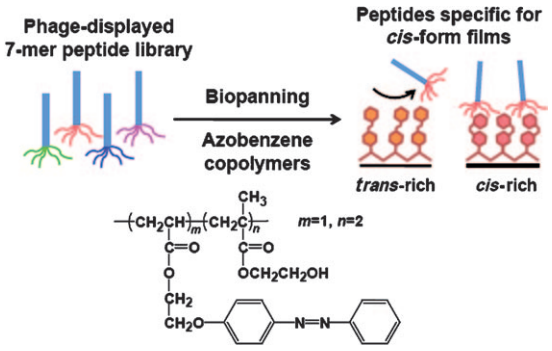

Figure 1. Schematic representation of the phage display screening (biopanning) of peptides that show affinities for the *cis* form of azobenzene groups on the polymer-film surface.

and verify for the first time that the presence of peptides can recognize photoresponsive polymer-film surfaces. Even when the selection process was conducted under visible light, peptides that specifically bind to the *cis* forms of azobenzene groups in a smaller quantity than *trans* forms were unexpectedly isolated. A possible mechanism for the origin of such unique selection is also discussed.

The target copolymer was synthesized by free radical copolymerization of azobenzene-containing acrylate monomers and 4-hydroxyethylmethacrylate (HEMA) in a 1:2 (mol/mol) ratio, as determined by ^{1}H NMR measurements. The number-average molecular weight (M_n) of the target copolymer was 36000, and M_w/M_n was 1.69, where M_w indicates the weight-average molecular weight. Static contact angles of water droplets for films under visible and ultraviolet light in air were estimated to be $(86.7\pm0.8)°$ and $(77.7\pm1.2)°$, respectively. As the *trans* form of azobenzene groups is more hydrophobic than the *cis* form,[10] the difference in angles is reasonable. The angles seldom changed after immersion of films in water (typically, for 1 h). The angle of polyHEMA films is $(34.1\pm1.0)°$.[11] These observations suggest that azobenzene groups will be sufficiently exposed to film surfaces during the biopanning and assays. The angle of the glass surface used for the biopanning was estimated to be $(46.9\pm0.2)°$, indicating that the substrate will be covered by target polymers. Atomic force microscopic (AFM) observations showed that films under visible or ultraviolet light have a similar surface roughness of less than 1 nm (Supporting Information, Figure S1).

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Transmission-mode UV/Vis absorption spectra of copolymer films under visible light in air showed a λ_{max} at around 340 nm, and the isomer composition (*trans*:*cis*) was estimated to be 95:5, corresponding to the spectra of the copolymer and the azobenzene monomer in chloroform (Supporting Information, Figure S2). However, when the azobenzene monomer was dissolved in acetonitrile/water solvent mixture, the *cis* form slightly increased (Supporting Information, Figure S3). Considering that the *cis* form is more hydrophilic than the *trans* form,[10] the local content of the *cis* form on the film surface, which remained in contact with the aqueous phage solutions, might be greater than the aforementioned content (contact angles proved that *trans* and *cis* forms were still predominant under visible and ultraviolet light, respectively). The isomer composition under ultraviolet light could not be determined, because copolymer film spectra differed from those of the copolymer and the azobenzene monomer. However, the clear disappearance and appearance of λ_{max} at around 340 nm and 440 nm, respectively, suggested sufficient isomerization from *trans* to *cis* forms under ultraviolet light (Supporting Information, Figure S2).

After four rounds of biopanning under visible light, amino acid sequences of 7-mer peptides displayed on twenty-seven phage clones were determined (Table 1). Multiple appearances of the same clones, such as c05, c03, c02, and c04, suggested that the phage library was enriched to the objective phage pool after the biopanning rounds. However, frequencies in Table 1 did not simply determine affinities, possibly owing to the unfavorable amplification of genetically engineered phages during the biopanning process.[9a–c] Regarding positional enrichment of amino acids, a possible 7-mer motif for the polymer film was proposed to be Trp-His-Thr-(hydrophobic)-Pro-Asn-Ala, which was homologous to the c16 peptide. Aromatic Trp might bind to phenyl rings of the azobenzene group using π–π interactions, and His and Asn might form hydrogen bonds with azo, hydroxy, and/or other groups of the polymer. The possibility of hydrophobic interactions by Ala and other hydrophobic amino acids is reasonable when the relatively hydrophobic film surface is considered. Pro, which can induce a kinked and rigid structure in peptides, was similarly observed in other polymer-binding 7-mer peptides.[9a,b,d] These observations in amino acid appearance suggest that resulting peptides with a regular conformation interact with the copolymer using intermolecular interactions at film surfaces.

Table 1: Characterization of selected phage clones.

Clone	Frequency[a]	Sequence[b]	$K_{app,Vis}/K_{app,UV}$[c]
c05	12/27	**WPTPPYA**	1.2
c03	3/27	**WHAVPKP**	1.9
c02	2/27	**SQSIMRL**	1.7
c04	2/25	**MHQGSNT**	1.9
c06	1/27	**TTPNGVH**	1.8
c07	1/27	**SPSWLIQ**	2.2
c11	1/27	**HLHYALP**	1.7
c12	1/27	**WPTPPNP**	2.8
c16	1/27	**WHTLPNA**	2.0
c19	1/27	**ASTLPLA**	1.3
c21	1/27	**MHQGPNT**	2.4
c23	1/27	**WHTAPYA**	1.8
Library	–	–	3.2

[a] Frequencies for the same clone in 27 isolated clones. [b] The most abundant amino acids at each position are highlighted in gray. [c] K_{app} values under visible light ($K_{app,Vis}$) are divided by K_{app} values under ultraviolet light ($K_{app,UV}$).

Bound amounts of phage clones onto polymer films under visible or ultraviolet light were analyzed by enzyme-linked immunosorbent assays (ELISAs). When relative affinities were plotted against phage concentrations, saturation curves were obtained (Supporting Information, Figure S4). Therefore, assuming Langmuir-type adsorption, the curves obtained were fitted to acquire apparent affinity constants (K_{app}) of the clones. Figure 2 shows K_{app} values of all the clones and the library for polymer films under visible or ultraviolet light. The values obtained were fairly large, because those were derived from the sum of the affinities of multiple peptide copies displayed on phage pIII coated protein, and the non-specific adsorption of huge phage bodies.[9a–c,e] To normalize data with respect to the difference in the hydrophobicity of films owing to photoinduced *cis*–*trans* isomerization, ratios of K_{app} values ($K_{app,Vis}/K_{app,UV}$) were taken (Table 1; note that the $K_{app,Vis}$ and $K_{app,UV}$ values of the library differ from one another). Interestingly, ratios of clones (1.2–2.8) were clearly smaller than those of the library (3.2), indicating that clones tended to show relatively strong binding to the film under ultraviolet light compared to binding under visible light. These observations do not directly agree with the fact that the present target was the copolymer film under visible light.

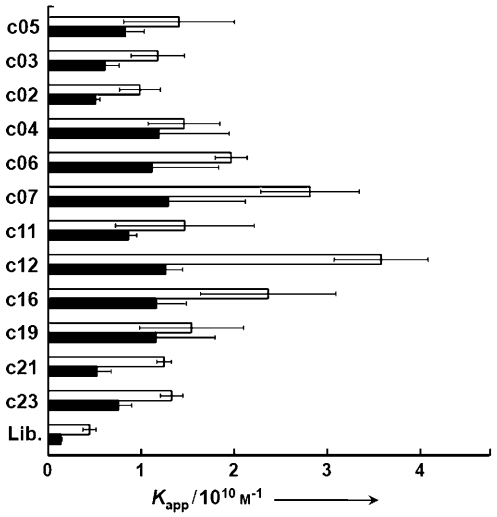

Figure 2. Apparent affinity constants (K_{app}) of clones for polymer films under visible (unfilled bars) or ultraviolet (filled bars) light. Ratios of values are given in Table 1. Lib. = libraray values.

We therefore proposed that the biopanning process preceded the *cis* form of azobenzene groups on the film surface, although the film applied under visible light contained a smaller content of the *cis* form than of the *trans* form. Phage particles seemed to more readily contact and interact

with the *cis* form on the film surface. To verify our hypothesis, the c16 peptide, which is thoroughly consistent with the proposed motif, was selected, and its C terminus was biotinylated by an additional Lys residue. After the binding of the c16 derivative peptide (1 μM for 1 h) was accomplished, amounts of the c16 derivative peptide were further amplified by the binding of streptavidin.[12] Binding of streptavidin was analyzed in situ by a 27 MHz quartz-crystal microbalance (QCM) apparatus.

Figure 3 shows the binding curves of streptavidin at 50 pM for treated and untreated QCM substrates. For films not treated with a c16 derivative peptide, detectable changes in

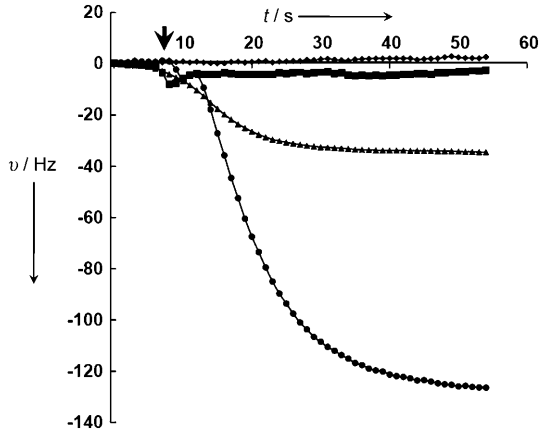

Figure 3. Binding curves of streptavidin at 50 nM for polymer films under visible light (◆), irradiated with ultraviolet light (■), and pre-bound with the c16 derivative peptide at 1 μM for 1 h under visible (▲) or ultraviolet (●) light. Further details are given in the Supporting Information. The arrow indicates the point at which streptavidin was injected.

frequencies were not observed. Streptavidin did not adsorb nonspecifically onto films under visible and ultraviolet light at the present low concentration. On the contrary, when the peptide was pre-bound onto films, frequencies clearly decreased with time, indicating that streptavidin was specifically bound to pre-bound peptides. Accordingly, bound amounts of the peptide were successfully quantified by streptavidin. The observed lag time for the decrease in frequency (approximately 7 s) is possibly due to the time required to mix the streptavidin solution in a batch cell. It is noted that QCM and surface plasmon resonance[9d–f] measurements failed to directly quantify bound amounts of the c16 peptide owing to unexpected baseline instability.

Saturated amounts of streptavidin bound to both films are shown using the left two histograms in Figure 4 (Vis, UV). A fivefold increase in bound streptavidin to the film under ultraviolet light is observed compared to that of visible light, confirming the c16 peptide interacts favorably with *cis* forms of azobenzene groups. Bound streptavidin on films under visible light might suggest that the peptide selectively bound to the surface corresponding to *cis* forms of azobenzene groups. As aforementioned, the film under visible light was more hydrophobic than that under ultraviolet light. If the c16 peptide simply behaved as surfactants for polymer films in water, the peptide may preferentially bind to the former film. Therefore, the peptide selection specific for *cis* forms is

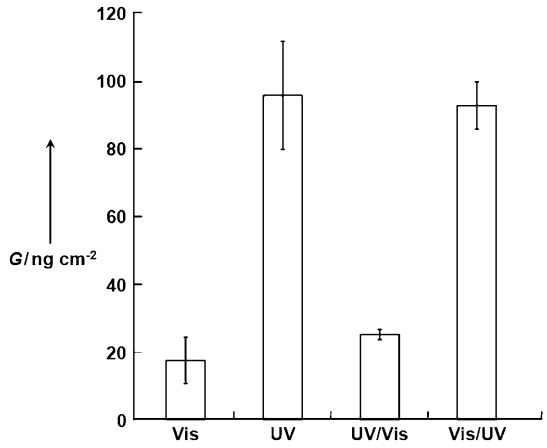

Figure 4. Saturated binding amounts of streptavidin at 50 nM for polymer films pre-bound with the c16 derivative peptide at 1 μM for 1 h under visible (Vis) and ultraviolet (UV) light, initially under ultraviolet and subsequently visible light (UV/Vis), and the reversed combination (Vis/UV). Further details are given in the Supporting Information.

suggested. Considering the molecular dimension of streptavidin (ca. $4.2 \times 4.2 \times 5.6\ nm^3$),[13] the surface coverage of streptavidin under ultraviolet light was estimated to be approximately 17–23% under aforementioned conditions. This estimation confirmed that streptavidin adequately quantified pre-bound peptides without steric hindrance.

To test the photoresponse of the c16 peptide, pre-bound films under visible and ultraviolet light were irradiated with ultraviolet and visible light, respectively, and were subsequently incubated in the same peptide solutions for 1 h before detection by streptavidin. The amounts of streptavidin for photoirradiated films are represented by the two right histograms Figure 4 (UV/Vis, Vis/UV). More significantly, amounts of streptavidin returned to the appropriate amount for each irradiated film within experimental error. This observation indicates that the amounts of the c16 peptide reversibly changed by the isomerization of azobenzene groups, reaching a new equilibrium under the corresponding photoirradiation. These observations indicate for the first time that the c16 peptide successfully responded to external photostimuli, changing its bound amount.

To further characterize the peptides, unsubstituted azobenzene molecules were placed in water (a poor solvent) in the presence or absence of peptides under visible or ultraviolet light[14] (Supporting Information, Figure S5). Larger amounts of azobenzene were dissolved in water under ultraviolet light than under visible light. Amounts dissolved under visible light did not change even in the presence of peptides, whereas those dissolved under ultraviolet light in the presence of the c16 and c11 peptides were, respectively 1.7 and 1.4 times greater than those in the absence of peptides. These observations strongly suggest that the biopanning process preceded the *cis* form of azobenzene groups on the film surface, and the identified peptides have potentials to recognize the molecular-level difference in azobenzene isomers. It is, however, difficult to discuss if the peptides interact with single or multiple azobenzene groups. Consid-

ering the size of 7-mer peptides, we expect one-to-one interactions; however, detailed structural analyses are necessary to adequately illustrate the nature of the interaction.

In conclusion, the phage-display method was applied to film surfaces composed of azobenzene-containing copolymers under visible light. Peptides with the characteristic amino acid sequence were enriched after four rounds of biopanning. ELISAs using phage clones and QCM measurements using typical synthetic peptides suggested that peptides preferentially bound to *cis* forms of azobenzene groups on film surfaces. Importantly, bound amounts of peptides were controlled by the photoisomerization of azobenzene groups. This is the first instance in the literature demonstrating that material-binding peptides respond to external stimuli based on their potential specificities. *Cis* forms of unsubstituted azobenzene molecules were effectively dissolved in water in the presence of peptides, thereby suggesting that identified peptides precisely recognize the azobenzene isomer. Detailed characterization and possible application of peptides, and the utilization of other stimuli-responsive materials, are now in progress.

Experimental Section

The azobenzene-containing acrylate monomer (3.6 mmol), synthesized according to previous studies,[15] and HEMA (3.6 mmol) were copolymerized in the presence of a free radical initiator 2,2′-azobisisobutyronitrile (0.071 mmol) in DMF (14.7 mL) at 60 °C for 24 h. The resulting copolymer was purified by reprecipitation in diethyl ether. ^{1}H NMR spectra (Bruker 500 MHz) revealed the copolymer composition. The molecular weight was measured by gel permeation chromatography using DMF containing LiBr at 10 mM.

Films of the copolymer with approximately 20 nm thickness were prepared by spin-coating methods on glass or quartz plates using a chloroform solution. For the preparation of *trans* and *cis* rich films, films were handled at ambient temperature in a bright room and in a darkroom after the irradiation of ultraviolet light using a hand-held UV lamp (SLUV-4, K.K. Iuchi Seieido) at 365 nm for 4 min, respectively. Static contact angles were measured by a contact-angle meter CA-X (Kyowa Interface Science) in air at ambient temperature using 2 μL water droplets. UV/Vis spectra (JASCO V-530) were measured in air at ambient temperature.

Biopanning: 1.0×10^{10} pfu/30 μL of phage solution was placed onto copolymer films and incubated for 1 hr at ambient temperature. To remove unbound phages, films were rinsed five times with 150 μL of Tris-buffered saline (pH 7.5) containing 0.1 wt % Tween-20. The bound phages were then eluted by mounting 20 μL of 0.5 M glycine-HCl (pH 2.2) onto films for 15 min at ambient temperature. For the next round of biopanning, phages were amplified by infecting with *Escherichia coli* strain ER2738. After four rounds of biopanning, eluted phages were cloned. DNA extracted from clones was sequenced.

ELISAs: Triplicate ELISAs were performed. Copolymer films were prepared on glass multiplates. A 100 μL aliquot of the phage solution at adequate concentrations dissolved in phosphate-buffered saline (PBS, pH 7.4) was applied onto films for 1 h at ambient temperature. Films were rinsed five times with PBS containing 0.05 wt % Tween-20. Horseradish peroxidase conjugated anti-M13 bacteriophage antibodies (Amersham Biosciences) were applied for 1 h. Relative amounts of bound phages were estimated by measuring the absorbance of products from substrates (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) at 405 nm.

Peptide Affinity: The c16 peptide was biotinylated by conjugating biotinylated Lys (N^{α}-Fmoc-N^{ε}-(d-biotin)-L-lysine) to the peptide C terminus, which was amidated. Copolymer films were spin-coated on the 27 MHz QCM sensor tip (Affinix Q, Initiam). Peptides bound onto films at 1 μM in PBS for 1 h at room temperature. After gently rinsing film surfaces with pure water, streptavidin was bound to films at 50 nM in PBS using a batch cell, and the amounts of bound streptavidin were analyzed over time. Frequency decreases of the QCM tip were converted to amounts of bound streptavidin using Sauerbrey's equation.[16]

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