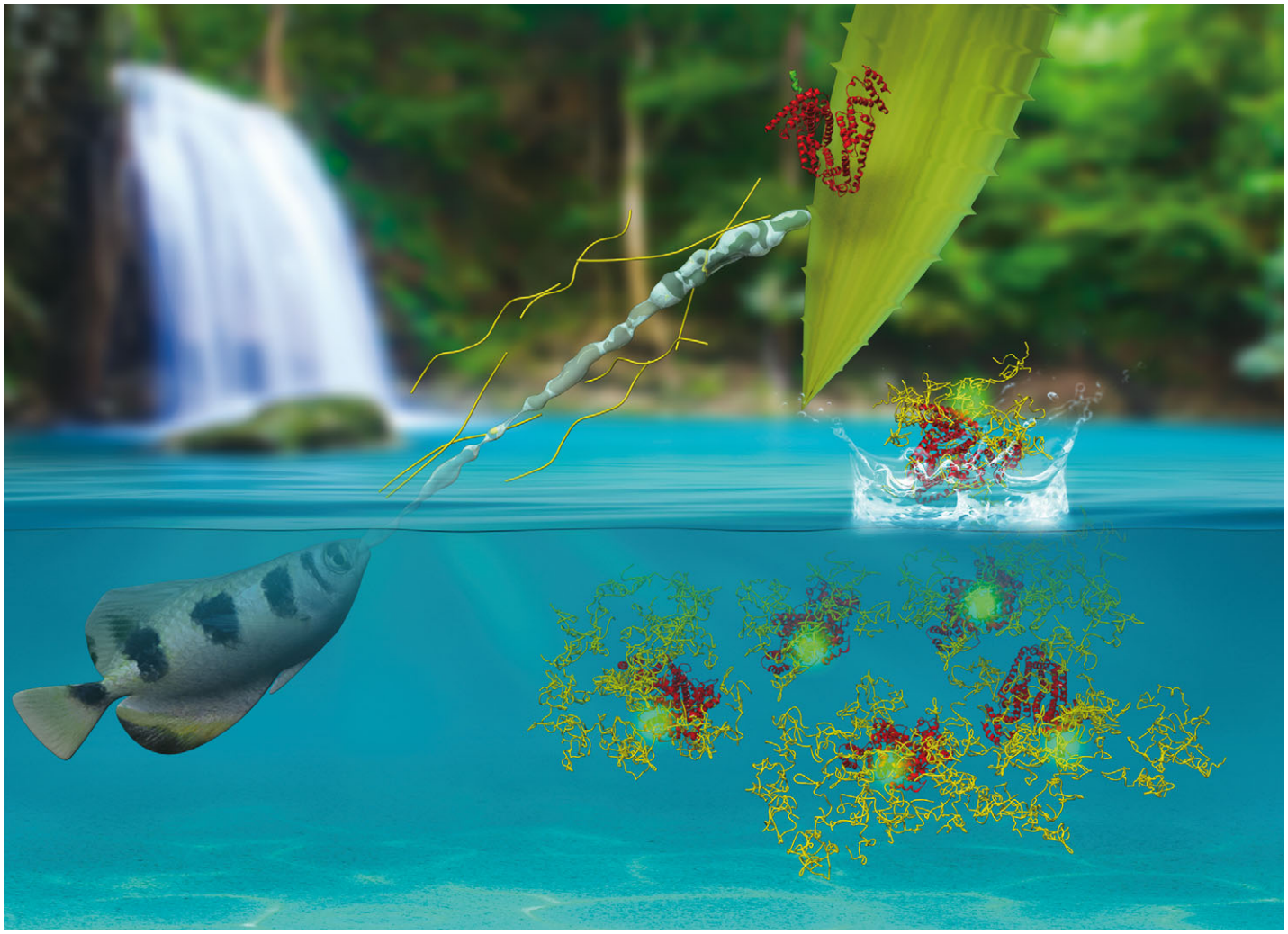

**Showcasing research from Serizawa's Laboratory, Department of Organic and Polymeric Materials, Tokyo Institute of Technology, Japan.**

Affinity-based thermoresponsive precipitation of proteins modified with polymer-binding peptides

A model protein (*i.e.*, human serum albumin) chemically modified with a 12-mer peptide with an affinity for the *meso* diad sequence of poly(*N*-isopropylacrylamide) (PNIPAM) was successfully precipitated with PNIPAM above the lower critical solution temperature of PNIPAM.

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# Affinity-based thermoresponsive precipitation of proteins modified with polymer-binding peptides†

Seigo Suzuki, Toshiki Sawada, Takashi Ishizone and Takeshi Serizawa*

**A 12-mer peptide with an affinity for the *meso* diad sequence of poly(*N*-isopropylacrylamide) (PNIPAM) was identified through affinity-based peptide screening. A model protein (*i.e.*, human serum albumin (HSA)) chemically modified with the peptide was successfully precipitated with PNIPAM above the lower critical solution temperature (LCST) of PNIPAM.**

Thermoresponsive polymers have received considerable attention due to their great potential for the development of well-designed functional materials.[1–3] PNIPAM is a representative classic thermoresponsive polymer and shows a reversible coil-to-globule transition at the LCST.[4–8] Control of the LCST broadens the applicability of PNIPAM; therefore, the effects of additives such as salts[9] and surfactants,[10] copolymerizations,[11] and stereoregularities[12–14] on the LCST have been investigated. Because the globular PNIPAM chains above the LCST readily aggregate to form precipitates through gentle centrifugation, PNIPAMs chemically modified with biomolecular ligands have been utilized to collect target biomolecules for diagnostics and bioseparations.[15–18] However, introducing chemical modifications into PNIPAM with biomolecular ligands is sometimes complicated and time-consuming. Therefore, more straightforward strategies are required for combining PNIPAM with target biomolecules.

In the past few decades, peptides with affinities for inorganic and organic materials have been identified by affinity-based peptide screening from random peptide libraries, which are displayed on the surfaces of genetically engineered phages or cells.[19–23] We have also identified peptides with affinities for a wide variety of synthetic polymers with different stereoregularities,[24,25] linear/branched structures,[26] crystallinity,[27] and porous structures.[28] The resulting polymer-binding peptides have been successfully utilized for modification of polymer surfaces,[29] water-dispersion of hydrophobic polymers,[30] and release of small drugs from polymer hydrogels.[31] Despite the fact that polymer-binding peptides have great potential to discriminate structural differences of synthetic polymers, previous representative targets have been water-insoluble polymers, except in the case of certain water-soluble conjugated polymers.[32] To further broaden the applicability of polymer-binding peptides, it is important to focus on water-soluble and functional polymers as peptide targets.

In this study, affinity-based peptide screening against films of PNIPAM with high *meso* diad content was demonstrated using phage-displayed random peptide libraries, and the effect of the identified peptide on the LCSTs of PNIPAMs with different *meso* diad contents was evaluated. Then, a model protein (*i.e.*, HSA) was chemically modified with the peptide for thermoresponsive protein precipitation above the LCST of PNIPAM (Fig. 1). The identified peptide showed a greater binding constant ($K_a$) for the target PNIPAM compared with reference polymers. It is known that a decrease in the *meso* diad content of PNIPAM increases water-solubility, which is followed by an increase in the LCST.[12,13] Therefore, thermoresponsive precipitation experiments were conducted using PNIPAMs with reduced *meso* diad content. The peptide modulated the LCST of PNIPAM, and the peptide-modified HSA was efficiently

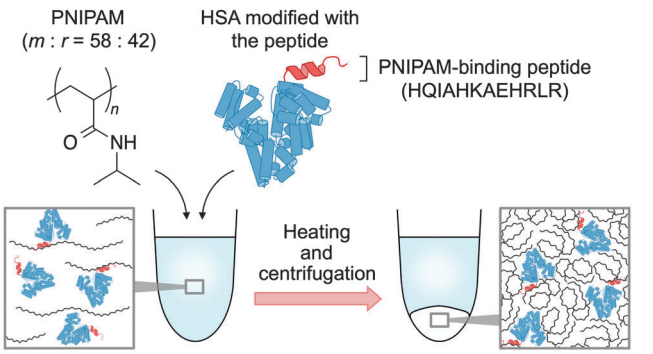


Fig. 1 Schematic illustration of the affinity-based thermoresponsive precipitation of HSA modified with the PNIPAM-binding peptide.

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precipitated with PNIPAM above the LCST. We demonstrate that the affinities between thermoresponsive polymers and their binding peptides may be potentially useful for the thermoresponsive precipitation of the desired peptide-modified proteins.

PNIPAM with a high *meso* diad content (PNIPAM, $M_n$ = 11 000, $M_w/M_n$ = 1.20, $m:r$ = 85 : 15, where $m$ and $r$ represent *meso* and *racemo* diads, respectively) synthesized by living anionic polymerization[12] was used for the affinity-based screening of 12-mer peptides. The experimental details of the peptide screening followed those reported in our previously published protocols,[24,31] as described in the ESI.† In brief, aqueous droplet-containing linear 12-mer peptide libraries with a sequence diversity of approximately $10^9$ were mounted on PNIPAM films for 10 min at 30 °C. The unbound and weakly bound phages were washed from the films, and the strongly bound phages were subsequently eluted from the films. The eluted phages were amplified by infecting with *Escherichia coli*, and the amplified phages were utilized for the next round of screening. Five screening rounds were repeated to determine the peptide sequences based on phage cloning and DNA sequencing. In fact, the percent yields, which were estimated by the ratios between the input and output phages, tended to increase with increases in the screening rounds (Fig. S1 in the ESI†).

The affinity-based screening revealed two peptides with highly homologous amino acid sequences (Table 1, see the underlined amino acids). Importantly, basic amino acids, such as Arg, His, and Lys, were enriched in the peptides, and their isoelectric points were 10.84. Considering the chemical structure of PNIPAM, these peptides may have interacted with PNIPAM through hydrogen bonds. The bound amounts of the phage clones and wild type (WT) phages against the target PNIPAM films at 20 °C and 30 °C were analyzed by titer counting assays according to our previously published protocol[26] (Fig. S2 in the ESI†). Regardless of temperature, greater amounts of the phage clones obviously bound to the films compared with WT phages, suggesting that the peptides displayed on the phage clones had the potential to bind the target PNIPAM. In particular, c2 showed greater amounts of binding compared with c1. Therefore, the c2 peptide with an amino acid sequence consisting of His-Gln-Ile-Ala-His-Lys-Ala-Glu-His-Arg-Leu-Arg was synthesized chemically, and its $K_a$ values for the target PNIPAM and reference polymer films were estimated by surface plasmon resonance (SPR) measurements according to our previously published protocol.[33]

SPR measurements detected association (0–180 s) and dissociation (180–1000 s) of the c2 peptide against the target PNIPAM films at different peptide concentrations at 20 °C (Fig. 2). The bound

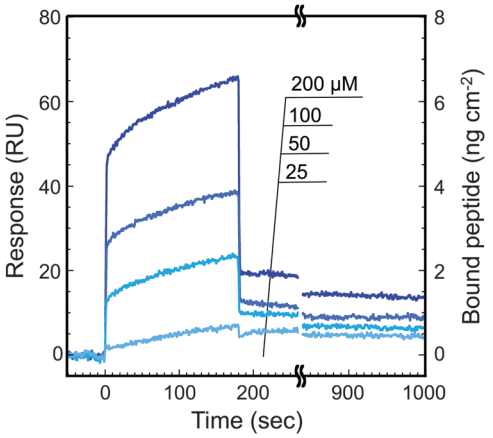


Fig. 2 SPR sensorgrams for binding of the c2 peptide to the target PNIPAM films at different peptide concentrations.

amounts tended to increase with increases in peptide concentrations. The resulting sensorgrams were fit using a Langmuir adsorption model, and the association ($k_1$) and dissociation ($k_{-1}$) rate constants were determined to be 63 $M^{-1}$ $s^{-1}$ and 3.1 × $10^{-4}$ $s^{-1}$, respectively. Subsequently, the $K_a$ value ($k_1/k_{-1}$) was estimated to be 2.0 × $10^5$ $M^{-1}$ (Table 2), which was comparable with those of previously identified polymer-binding peptides.[26,27,31–33] However, the $K_a$ value of the c2 peptide for a reference polymer, *isotactic* poly(methyl methacrylate) (*it*-PMMA, $M_n$ = 35 500, $M_w/M_n$ = 1.12, $mm:mr:rr$ = 98 : 2 : 0), was estimated to be 1.7 × $10^4$ $M^{-1}$ (see Fig. S3a for sensorgrams in the ESI†), which was 12 times smaller than that of PNIPAM, indicating that the c2 peptide discriminated between the PNIPAM and *it*-PMMA polymer structures. Furthermore, the $K_a$ value of the peptide with an inverted sequence of the c2 peptide for the PNIPAM was estimated to be 2.2 × $10^4$ $M^{-1}$ (see Fig. S3b for sensorgrams in the ESI†), which was 9 times smaller than that of the original peptide, indicating that the primary sequence of the c2 peptide was essential for its binding with PNIPAM. All of these observations suggested that the c2 peptide had the potential to specifically bind to the target PNIPAM.

To analyze the affinity of the c2 peptide for PNIPAMs dissolved in aqueous solutions, their LCSTs were analyzed with or without peptides. Two PNIPAMs with different *meso* diad contents (*meso*-rich PNIPAM: $M_n$ = 7600, $M_w/M_n$ = 1.12, $m:r$ = 58 : 42; *meso*-poor PNIPAM: $M_n$ = 13 000, $M_w/M_n$ = 1.15, $m:r$ = 17 : 83) were used, and their LCSTs were measured at a heating rate of 0.1 °C $min^{-1}$ at polymer and peptide concentrations of 0.5 mg $mL^{-1}$ (4.4 mM per monomer unit) and 880 μM, respectively. The LCST (defined as the temperature of 50% transmittance at 500 nm) of *meso*-rich PNIPAM

Table 1 Amino acid sequences of screened phage clones

| Clone | Frequency[a] | Sequence | pI[b] |
|---|---|---|---|
| C1 | 5/6 | HSFKWLDSPRLR | 10.84 |
| C2 | 1/6 | HQIAHKAEHRLR | 10.84 |

[a] DNA sequencing was conducted to thirteen clones totally; however, seven clones did not display peptides. [b] The isoelectric point (pI) of the peptides with a free C-terminus was calculated using the pI/mass program (http://www.expasy.org).

Table 2 Kinetic parameters for the binding of peptides to polymer films

| Peptide | Polymer | $k_1$ ($M^{-1}$ $s^{-1}$) | $k_{-1}$ ($10^{-4}$ $s^{-1}$) | $K_a$ ($10^4$ $M^{-1}$) |
|---|---|---|---|---|
| C2 | PNIPAM | 63 | 3.1 | 20 |
| C2 | *it*-PMMA | 3.3 | 1.9 | 1.7 |
| Inverted c2 | PNIPAM | 5.0 | 2.3 | 2.2 |

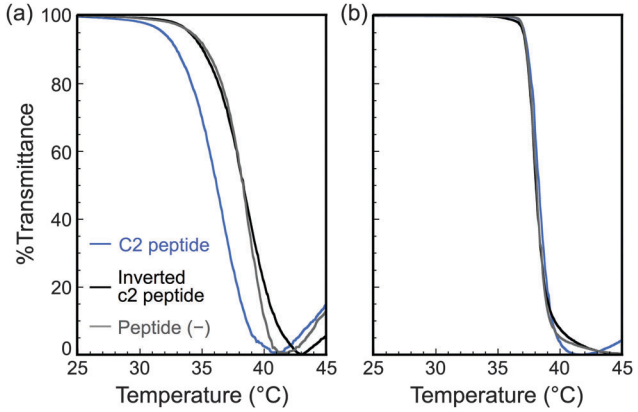


Fig. 3 Temperature-dependent changes in the transmittance of visible light (500 nm) for (a) *meso*-rich PNIPAM and (b) *meso*-poor PNIPAM solutions with and without peptides.

with the c2 peptide was remarkably lower than those without the peptide or with the inverted c2 peptide (Fig. 3 and Table S1 in the ESI†). However, the LCSTs of *meso*-poor PNIPAM with or without the two peptides were almost the same (*i.e.*, within experimental error). These observations strongly suggested that the c2 peptide had the potential to show the affinity for the *meso* diad sequence of PNIPAM even when it was dissolved in an aqueous solution and that there was a threshold level of peptide binding to modulate the LCST of PNIPAM. It is hard to explain why the c2 peptide lowered the LCST of *meso*-rich PNIPAM. It is generally known that dehydration of dissolved PNIPAM chains lowers LCSTs.[34] Therefore, *meso*-rich PNIPAM was likely dehydrated through peptide binding below the LCST, which was followed by lowering of the LCST. It is noted that the LCSTs of PNIPAMs without peptides were lower than those published in a previous paper.[12] This is possibly due to the differences in the experimental conditions, such as PNIPAM concentration, heating rate, cell thickness, and stirring procedure.

We hypothesized from the aforementioned observations that the c2 peptide with an affinity for *meso*-rich PNIPAM could be utilized for a functional molecular tool to precipitate the desired peptide-modified proteins with PNIPAM above the LCST. Therefore, a model protein (*i.e.*, HSA) was chemically modified with the c2 peptide through a Cys34 residue using a bismaleimide linker (see the ESI† for synthetic details). Ellman's reagent was used to quantify the amounts of Cys residues,[35] which revealed that 97% of HSA was modified with the linkers. Matrix-assisted laser desorption/ionization time-of-flight mass (MALDI-TOF-MS) spectra supported the modification of HSA with the c2 peptide (Fig. S4 in the ESI†). Circular dichroism (CD) spectra revealed that the secondary structure of HSA hardly changed before and after peptide modification (Fig. S5 in the ESI†), suggesting that the secondary structure of HSA was not influenced by peptide modification. These observations indicated the successful preparation of HSA modified with the c2 peptide. Importantly, SPR measurements revealed the $K_a$ value of the peptide-modified HSA for the target PNIPAM to be $4.4 \times 10^7$ $M^{-1}$, which was 17 times greater than that of native HSA ($2.6 \times 10^6$ $M^{-1}$) (see Fig. S6 for sensorgrams and Table S2 for kinetic parameters in the ESI†). This observation suggested that the c2 peptide displayed on HSA still showed an affinity for the *meso* diad sequence of PNIPAM.

For thermoresponsive precipitation experiments, mixed solutions of *meso*-rich PNIPAM and HSA with or without peptide modifications at respective concentrations of 2.0 mg $mL^{-1}$ and 100 nM were heated to 50 °C at a heating rate of 0.1 °C $min^{-1}$, followed by centrifugation to precipitate the globular PNIPAM chains with HSA. To quantify the amount of HSA in the supernatants, HSA was previously labeled with fluorescein through chemical coupling with the primary amino groups of Lys residues before peptide modification. The experimental details are provided in the ESI.† Importantly, 98% of the peptide-modified HSA was precipitated with *meso*-rich PNIPAM, while approximately 20% of HSA was precipitated without peptide modification (Fig. 4a, left). Precipitation of the peptide-modified HSA was suppressed to 30% in the presence of 50 μM free peptides (Fig. S7 in the ESI†), suggesting the contribution of the peptide affinity to precipitation. Photos of the mixed solutions after centrifugation under ultraviolet light (365 nm) successfully visualized the presence of greater amounts of fluorescent HSA in the precipitates of the former solution (Fig. 4b). However, precipitation of the two proteins was hardly observed through the same thermal treatment without *meso*-rich PNIPAM (Fig. 4a, right), indicating that the proteins did not precipitate only through thermal treatment. It is noted that more than 90% of the peptide-modified HSA was precipitated with *meso*-rich PNIPAM even at a HSA concentration of 10 nM (Fig. S8 in the ESI†). Therefore, it was found that HSA modified with the c2 peptide was efficiently precipitated with *meso*-rich PNIPAM above the LCST through non-covalent peptide affinity.

In conclusion, affinity-based peptide screening against films of PNIPAM with high *meso* diad content using phage-displayed random peptide libraries successfully identified the peptide with an amino acid sequence consisting of His-Gln-Ile-Ala-His-Lys-Ala-Glu-His-Arg-Leu-Arg. SPR measurements revealed that

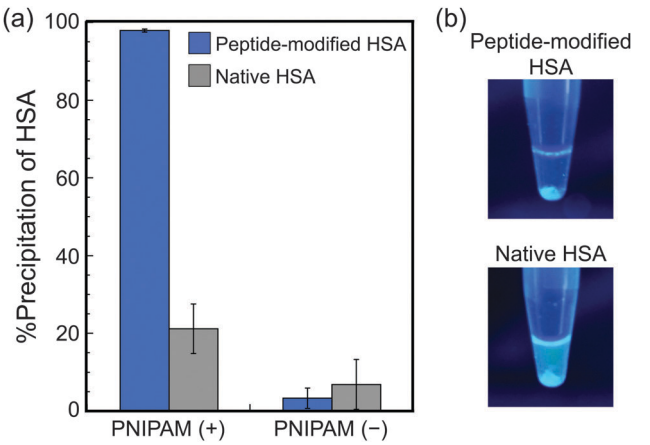


Fig. 4 (a) Percent precipitation of peptide-modified HSA with or without *meso*-rich PNIPAM above the LCST and (b) photos of the solutions containing HSA (top) with or (bottom) without peptide modification under ultraviolet light (365 nm) after centrifugation. Percent precipitation of HSA and their standard deviations was obtained from triplicate experiments.

 

the $K_a$ value of the peptide for the target PNIPAM was one order of magnitude greater than that of the reference *it*-PMMA. In addition, the $K_a$ value of the inverted peptide for PNIPAM was one order of magnitude smaller than that of the original peptide. The peptide specifically lowered the LCST of *meso*-rich PNIPAM rather than *meso*-poor PNIPAM, suggesting that the peptide potentially had affinity for the *meso* diad sequence of PNIPAM even in an aqueous phase. A model protein, HSA chemically modified with the peptide, was efficiently and specifically precipitated with *meso*-rich PNIPAM above the LCST. Our findings revealed that the peptides with affinities for thermoresponsive polymers were potentially useful as a molecular tool for collecting the desired proteins through simple temperature changes, thereby opening new possibilities for the application of polymer-binding peptides. Further investigations of peptides with affinities for thermoresponsive polymers are now in progress.

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