Affinity Precipitation Separation of DNA Binding Protein Using Block Conjugate Composed of Poly(*N*-isopropylacrylamide) Grafted Double-Stranded DNA and Double-Stranded DNA Containing a Target Sequence

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In this research, we synthesized a novel DNA-polymer conjugate and evaluated its application to an affinity precipitation separation of TATA-box binding protein (TBP), which is a representative general transcription factor. The conjugate was composed of two fractions. One was a double-stranded DNA modified by the grafting of poly(*N*-isopropylacrylamide) (PNIPAAm), which is known as a thermosensitive vinyl polymer. The other fraction is a native double-stranded DNA containing a specific base sequence (5'-TATAAA-3') called a TATA-box. These two fractions, which have *Eco*RI termini, were treated with T4 DNA ligase, and the block conjugate was obtained as a precipitate after two wash processes. When the resultant block conjugate was introduced into a sample solution containing TBP (0.26 μ M) and bovine serum albumin (BSA) (0.39 μ M), a rapid and selective precipitation separation of TBP under homogeneous conditions was achieved by controlling temperature. The purity of TBP in the precipitation fraction was estimated to be above 90%.

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Introduction

DNA binding proteins play important roles in intracellular processes. For example, some transcription factors specifically recognize the base sequence on double-stranded DNA, and regulate development, differentiation, growth and malignant transformation. It is widely accepted that many unknown transcription factors exist in a cell; various transcription factors are waiting to be characterized.

To study such transcription factors, we must find a technique for separating and purifying them. Sepharose beads,¹ synthetic polymer resins² or silica gels³ have been used to separate DNA-binding proteins by immobilizing DNA on their surfaces. Though an affinity between a target protein and DNA enables the specific separation, these conventional approaches have some disadvantages. In these techniques, the affinity reaction takes place in a solid-liquid heterogeneous interface, as a result, the efficiency of the affinity reaction is reduced.⁴ Moreover, the nonspecific adsorption to the support or the denaturation due to the crash against the support can cause the loss of a target protein. Therefore the development of a novel separation technique that realizes affinity separation in a homogeneous system is important to overcome such problems.

Poly(*N*-isopropylacrylamide) (PNIPAAm) is one of the candidates for accomplishing such an ideal separation. PNIPAAm is known to have a phase-transition temperature at 31°C in an aqueous solution, and becomes insoluble above this temperature.⁵ Various approaches for separation using PNIPAAm have been performed based on the specific properties of this polymer.⁶⁻¹⁴

We have studied the synthesis and characterization of DNA-polymer conjugates¹¹⁻¹⁹ and applied those conjugates for the separation of DNA-binding molecules. The double stranded DNA-PNIPAAm conjugate achieved a one-pot affinity precipitation separation of DNA-binding dye.¹¹

We describe here a synthesis of a new-type DNA-PNIPAAm conjugate and a strategy for the separation of a DNA-binding protein that utilizes this conjugate. The conjugate, named "block conjugate", is composed of a thermosensitive-fraction and a ligand-fraction, as shown in Fig. 1. The former fraction is DNA-PNIPAAm conjugate, which gives a driving force to precipitate the block conjugate, and the latter fraction is native DNA that contains a specific base sequence that functions to recognize the target DNA-binding protein. TBP was selected as the target, given its importance in the biochemical, molecular biological and genetic engineering fields.

Experimental

Materials

N-Isopropylacrylamide (NIPAAm) was purchased from

Fig. 1 Illustration of the DNA-PNIPAAm block conjugate.

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Tokyo Kasei Kogyo Co., Ltd. and was recrystallized from a mixture of benzene and hexane. *N,N'*-Azobisisobutyronitrile (AIBN) was obtained from Kishida Chemical Co., Ltd. Trioxalen (4,5',8-trimethylpsoralen) and 3-mercaptopropionic acid (MPA) were obtained from Aldrich. Plasmid pBluescript II KS(-) was purchased from RIKEN. Single stranded DNAs (ss(TATA-A): 5'-AATTCGGAATTCGGGCTATAAAAGGGGGATCCGG-3', ss(TATA-B): 5'-AATTCGGATCCCCCTTATAGCCCGAATTCCG-3') were purchased from Amersham Pharmacia Biotech. *Eco*RI and T4 DNA ligase were obtained from New England Biolabs, Inc. T4 Polynucleotide kinase and TBP were purchased from Toyobo. Other reagents and solvents were obtained commercially and used without further purification.

Carboxy-terminated polyNIPAAm

Semitelechelic oligomer of NIPAAm was synthesized by the method described by Takei *et al.*²⁰ It was prepared by radical polymerization of NIPAAm in dimethylformamide (DMF) using AIBN as an initiator and MPA as a chain-transfer agent. The polymer was purified by repeated reprecipitation from DMF-diethyl ether. The molecular weight of the resulting polymer was determined by acid-base titration of its terminus carboxyl group.

To determine the phase transition temperature (lower critical solution temperature; LCST) of the polymer in an aqueous solution, the optical transmittance of the polymer solutions was monitored at 500 nm while the temperature was raised at the

rate of 0.4°C min⁻¹ using a spectrophotometer (Hitachi, U-3210). The temperature at 10% light transmittance of the polymer solutions was defined as the cloud point.

Psoralen-terminated polymers

The carboxyl group at the polymer terminus was esterified with *N*-hydroxysuccinimide (NHS) and

Scheme 1 Synthesis of psoralen-terminated PNIPAAm (PsoPNIPAAm).

Scheme 2 Preparation procedure of a block conjugate composed of a PNIPAAm-grafted DNA fragment and a native DNA fragment. Plasmid pBluescript II KS(-) was treated with *Eco*RI and subjected to a photoreaction with PsoPNIPAAm to obtain a thermosensitive fragment for the block conjugate. The mixture of ss(TATA-A) and ss(TATA-B) was heated up to 90°C and annealed. The resultant ds(TATA) was phosphorylated by T4 polynucleotide kinase to produce a ligand fragment for the block conjugate. The two kinds of fragments were mixed and treated with T4 DNA ligase. The ligation mixture was then heated and centrifuged two times to wash it.

Fig. 2 Gel electrophoresis (1.0% agarose) of linearized pBluescript II KS(-) (55 μ M in bp), which was irradiated with UV light in the presence or absence of PsoPNIPAAm (55 μ M). Lane 1, DNA alone (control); Lane 2, no UV irradiation; Lane 3, UV irradiation.

dicyclohexylcarbodiimide (DCC) in dry DMF at *ca.* 25°C for 24 h in a molar ratio of 1.0:1.2:1.2. The reaction mixture was concentrated *in vacuo* and was poured into diethyl ether to precipitate the product. The precipitate was dissolved again in a small portion of DMF and poured into diethyl ether. The resultant white powder was dried *in vacuo* at room temperature. The activated polymers (0.5 g, 94 μ mol) and 4'-[[N-(2-aminoethyl)amino]methyl] 4,5',8-trimethylpsoralen (0.07 g, 235 μ mol) were dissolved in dry DMF (total volume, 10 mL). After being stirred for 12 h at *ca.* 25°C, the solution was concentrated *in vacuo* and was poured into diethyl ether. The precipitate was collected and washed with 100 mL of diethyl ether. The white powder was dissolved in distilled water and dialyzed against 10 L of distilled water. The polymer thus obtained was dissolved in pure water to be a stock solution whose concentration was determined from UV absorption due to the psoralen moiety (300 nm).

Synthesis of the thermosensitive-site for block conjugate

Plasmid pBluescript II KS(-) was linearized by using *Eco*RI and purified by phenol/chloroform extraction and ethanol precipitation, then resuspended in sterile water. Then, 23.2 μ L of the linearized pBluescript II KS(-) solution (1538 μ M bp) and 40.7 μ L of the psoralen-terminated polyNIPAAm (PsoPNIPAAm) solution (878.6 μ M) and 1.1 μ L of sterile water were mixed in a micro test tube (Eppendorf, 1.5 mL) (final concentration: linearized pBluescript II KS(-); 550 μ M bp, PsoPNIPAAm; 550 μ M). The solution was cooled in an ice bath and irradiated for 15 min (*ca.* 30 mW/cm²) using a 500 W ultrahigh pressure Hg lamp equipped with a high-pass filter (Toshiba, UV-31). The mixture was loaded on a 1.0%-agarose gel, and electrophoresis was performed at 7 V/cm for 1 h in TBE (80 mM Tris-borate, 25 mM EDTA, pH 8.0) buffer. After electrophoresis, DNA in the gel was stained by ethidium bromide.

Synthesis of block conjugate

Single stranded DNAs, ss(TATA-A) and ss(TATA-B) (10.3 μ L, 3400 μ M base, each), were mixed and heated at 90°C for 3

Fig. 3 The amount of ligand DNA in the supernatants. The values are estimated from the absorbance at 260 nm of supernatants I and II.

min in a micro test tube. After annealing, the resultant double stranded DNA (ds(TATA)) was phosphorylated by treatment with T4 polynucleotide kinase. The phosphorylated ds(TATA) (30 μ L, 1000 μ M bp) was then mixed with the PNIPAAm-grafted DNA prepared above (54.5 μ L, 550 μ M bp); then the mixture was treated with T4 DNA ligase at 16°C for 24 h. The final solution conditions were as follows: ds(TATA), 300 μ M bp; PNIPAAm-grafted DNA, 300 μ M bp.

The ligation mixture was examined from the viewpoint of thermally-induced precipitation. To the ligation mixture (100 μ L), an aqueous solution of PNIPAAm (10 w/v%) was added until it reached 1.5 w/v%. The mixture was then centrifuged (15000 rpm, 3 min) at 40°C. After the supernatant (defined as supernatant I) was removed, the precipitate was re-dissolved in 100 μ L of sterile water; then excess ligase was heat-inactivated. After the sample was centrifuged (15000 rpm, 3 min) at 40°C, the supernatant (defined as supernatant II) was extracted. The block conjugate was obtained as a precipitate. The absorbance at 260 nm for both supernatants was measured to evaluate the composition of the block conjugate.

Selective precipitation separation of TBP

The block conjugate was introduced into the aqueous solution, which contained TBP (0.26 μ M) and BSA (0.39 μ M) (buffer: 12 mM Tris-HCl, 20 mM HEPES, 10 mM MgCl₂, 60 mM KCl, 0.5 mM dithiothreitol, 12% glycerol). The sample solution (total volume, 100 μ L) was incubated at 20°C for 1 h, then heated to 40°C for 3 min in a water bath and centrifuged for 5 min at 40°C (15000 rpm). An 80 μ L volume of the supernatant was collected as a supernatant component and the residual supernatant was removed from the sample. The precipitate was re-dissolved in 100 μ L of sterile water, and 80 μ L of the sample was then taken as a precipitate component. Each component was respectively mixed with the buffer (80 μ L, 0.05 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue). After the heat treatment, each sample was subjected to sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE). The conditions of PAGE were as follows: 8% polyacrilamide, 25 mM Tris base, 192 mM glycine, 0.1% SDS. After electrophoresis, proteins in the gel were stained by Coomassie Brilliant Blue G-250.

Scheme 3 A schematic illustration of affinity precipitation separation of TBP (a), and a flow chart of the procedure (b).

Results and Discussion

Psoralen-terminated PNIPAAm

Scheme 1 shows the synthesis of psoralen-terminated PNIPAAm (PsoPNIPAAm). The number-averaged molecular weight (M_n) of PNIPAAm was determined by NaOH titration of the terminal carboxyl group. The calculated value for the carboxy-terminated PNIPAAm was about 14000 (*ca.* 120 mer).

PNIPAAm is a well-known polymer that exhibits a phase transition: when an aqueous solution of PNIPAAm is gradually heated, it turns insoluble abruptly at 31°C and its precipitate appears. The transition temperature of the polymer prepared in this study was found to be about 31°C, and the transition was fully reversible.

PNIPAAm grafting on plasmid pBluescript II KS(-)

Psoralens are known to intercalate into DNA double strands in the dark and to form covalent bonds at their 3,4 and 4',5' double bonds with pyrimidines upon near-UV (~365 nm) irradiation.²¹ If the psoralen moiety introduced to the polymers reacts with the double-stranded DNA, it should induce the covalent grafting of the polymers on DNA.

Figure 2 shows the electrophoretic migration profiles that confirm the UV crosslinking between a plasmid DNA and PsoPNIPAAm. The degree of migration of the linearized pBluescript II KS(-) plasmid was almost the same as that of its mixture with the PsoPNIPAAm. On the other hand, after photoreaction with PsoPNIPAAm, the linearized plasmid did not migrate at all because of the increase of the molecular weight of the plasmid according to the crosslinking reaction with the polymers. This result indicates that PNIPAAm-grafted

Fig. 4 SDS-PAGE (8% polyacrilamide) of a supernatant and a precipitate component of the sample solution after the affinity separation of TBP using the block conjugate. Lane 1, control (mixture of TBP and BSA); Lane 2, supernatant; Lane 3, precipitate.

DNA was formed as a thermosensitive-fragment in block conjugate.

Conjugation between the thermosensitive-fragment and ligand-fragment

A ligation reaction between the ds(TATA) fragment and the PNIPAAm-grafted pBluescript II KS(-) fragment was performed. Each fragment has an *Eco*RI terminus whose 5' end was phosphorylated. After the ligation reaction, the wash process was performed twice to purify the block conjugate, and the block conjugate was obtained as a precipitate (Scheme 2). Figure 3 shows the amount of ds(TATA) fragment existing in supernatants I and II as estimated from their absorbance at 260 nm. It was found that 64% of the ds(TATA) fragment remained in the supernatant. This finding indicates that 36% of the ds(TATA) fragment was bound to the PNIPAAm-grafted DNA fragment. Considering that 300 μ M bp of the ds(TATA) fragment was used in the ligation reaction, the produced block conjugate was calculated to contain 110 μ M bp of the ds(TATA) fragment, which is equivalent to 0.32 nmol of the TBP-binding site (ds(TATA); 34 bp). The block conjugate gave its precipitate efficiently, similarly to PNIPAAm itself, when its aqueous solution was centrifuged at 40°C. On the other hand, block conjugates modified with shorter-lengthed PNIPAAms could not give their precipitates efficiently when centrifuged at 40°C. Therefore, the block conjugate with 120 mer of PNIPAAm prepared here is considered to be an adequate one for the affinity separation of TBP.

Affinity precipitation separation of TBP by using block conjugate

The block conjugate was applied to the precipitation separation of TBP. TBP recognizes the TATA-box sequence (5'-TATAAA-3') in ds(TATA) fragment specifically.²² Scheme 3(a) shows an illustration of the selective affinity separation of TBP using the block conjugate.

The protocol is summarized in Scheme 3(b). The block conjugate was added to a buffer solution containing TBP and BSA, which are the target protein and the control, respectively. The mixture was incubated at 20°C for 1 h, and then centrifuged (15000 rpm) at 40°C to give a precipitate. The supernatant was carefully collected, and the precipitate was re-dissolved in

sterile water. The results of SDS-PAGE for each sample are shown in Fig. 4. As seen in Lane 2, the supernatant component contained BSA, which had no interaction with the block conjugate. In contrast, the precipitate component contained TBP, as shown in Lane 3. These results strongly suggest that the ds(TATA) fragments in the block conjugate recognize and capture TBP selectively. The purity of TBP in the precipitate component was estimated to be above 90% in homogeneity. The results indicate that the block conjugate acts as an affinity ligand for TBP and is exclusively available for the separation of the protein.

In this paper, we have demonstrated the affinity precipitation separation of TBP using the block conjugate composed of polymer-grafted DNA and native DNA containing the recognition base sequence for TBP. In this system, the complexation between the block conjugate and the target protein occurs under homogeneous conditions, and the separation is achieved in a single reaction vessel. Therefore, the block conjugate has the potential to provide a rapid and widely applicable separation technique.

Conclusion

We have synthesized a block conjugate constructed by PNIPAAm-grafted DNA and native DNA having a TATA-box sequence for the affinity precipitation separation of TBP. Since PNIPAAm is known as a thermoresponsive polymer, the resulting conjugate showed the temperature-responsive precipitation due to the aggregation of PNIPAAm chains. The conjugate was added to a solution containing TBP and BSA. When the solution was heated above the phase transition temperature, the conjugate selectively precipitated TBP from the mixture. The purity of TBP in the precipitate fraction was very high. It was clearly indicated that TBP was captured by the conjugate precisely on its recognition site, was precipitated from the aqueous solution, and was concentrated in the precipitation phase. The conjugate can provide a rapid and widely-applicable separation technique since the complexation with the target occurs in homogeneous conditions and the separation is achieved in one pot.

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