Temperature-Induced Precipitation of Specific DNA Fragments Using DNA-Poly(*N*-isopropylacrylamide) Conjugate

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Soluble conjugates between double stranded DNA and poly(*N*-isopropylacrylamide) (polyNIPAAm) was synthesized and applied to the selective separation of DNA fragments: the target fragments were connected to the DNA-polyNIPAAm conjugate with the aid of T4 DNA ligase, followed by temperature-induced precipitation. Target DNA was collected from the resultant precipitate in high purity.

Single-stranded (ss) DNAs are specifically separated or analyzed on the basis of their sequence information,[1-6] while double-stranded (ds) DNAs are separated generally based on the difference in molecular weight.[7,8] Gel electrophoresis is one of the most popular methods for the preparation of DNA fragments, but it includes cumbersome procedures such as slicing and melting of gels, phenol and chloroform extraction, and ethanol precipitation.[9] In addition, it does not always guarantee sufficient yield and intactness, especially not for the DNAs with high molecular weight.

In many cases, DNAs to be separated are the digestion products by restriction endonucleases and have their characteristic sticky or blunt ends, which will work as 'tags' providing a chance to be distinguished from each other: they can be accurately connected to DNAs having complementary ends when treated with DNA ligase. By taking advantage of this, DNA-immobilized solid supports would be applicable for the separation of DNA restriction fragments. However, the ligation efficiency of DNAs immobilized was unfortunately low,[10] probably because of the heterogeneous conditions for the ligation reaction.

We have reported water-soluble conjugates[11] between dsDNA and poly(*N*-isopropylacrylamide) (polyNIPAAm) which is known to undergo temperature-directed phase transition between soluble (<31 ℃) and insoluble (>31 ℃) forms.[12] The conjugates were shown to capture DNA-binding molecules in homogeneous conditions, and to separate them from aqueous solution (*i.e.*, to concentrate them into precipitate) when heated.[13,14] In this paper, we describe a simple method for the purification of specific DNA fragments using dsDNA-polyNIPAAm conjugates with specific cohesive ends (Figure 1).

Circular pBR 322 DNA was digested with *Eco*RI (5'-G ↓ AATTC-3') to give a linear dsDNA having 5'-protruding single-stranded region (5'-AATT-3') on its ends. According to the previous report,[13] vinyl groups were introduced onto the DNA through a photoreaction between the DNA (500 μM in base pair, bp) and vinyl-derivative of psoralen (10 μM). A portion of the resultant DNA (300 μM bp: macromonomer) was then copolymerized with NIPAAm (150 mM) to give a water-soluble conjugate between DNA and polyNIPAAm. Gel electrophoresis (0.5% agarose) of the polymerization mixture did not show any trace of the unreacted macromonomer: the yield of the modified DNA was *ca.* 100%. The reaction

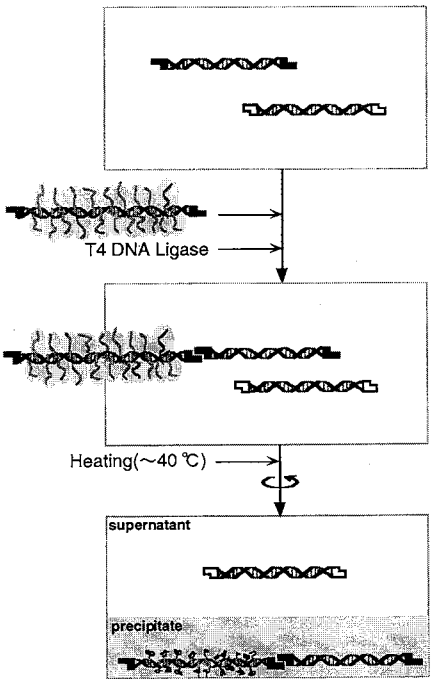

Figure 1. Schematic representation of the temperature-directed precipitation of specific DNA fragments using DNA-polyNIPAAm conjugate. The conjugate is added to the mixture of DNA fragments, and the ligation reaction (with target DNAs) is performed in homogeneous conditions. The ligation may take place at the both ends of the conjugate as well as those of the target fragment. Then the system is heated to 40 ℃ for the precipitation of the product. Target fragments are disconnected from the conjugate by treatment with restriction endonucleases and obtained from supernatant after the second 'heating & centrifugation' procedure, while the conjugate is recovered from the precipitate.

mixture was then heated to 40 ℃ and centrifuged to give a precipitate. The precipitate was washed twice with hot water (40 ℃) in order to remove the unreacted components, and was dissolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 7,4) buffer to be a stock. A pBR322-polyNIPAAm conjugate with *Hin*d III termini (5'-protruding ends: 5'-AGCT-3') was also synthesized by the same way.

Three types of target DNA fragments, namely, linear pBR 322 with *Eco*RI, *Hin*d III, and *Sty* I termini, were prepared by the digestion of the plasmid DNA with *Eco*RI, *Hin*d III, and *Sty* I, respectively. To a test tube containing one of the three types of linearized pBR322 (0.46 nM in terminus concentration) was added the conjugate between polyNIPAAm and pBR 322 having *Eco*RI termini (4.6 nM). After incubation in the presence of T4 DNA ligase (800 units) for 12h at 13 ℃, the reaction mixture (100 μL) was heated to 40 ℃ and centrifuged at the temperature. The supernatant was extracted with chloroform to

Table 1. Precipitation of DNA using DNA-polyNIPAAm conjugates.

DNA conjugate	% of precipitated DNA[a]		
	pBR322/*Eco*RI	pBR322/*Hin*d III	pBR322/*Sty* I
with *Eco*RI termini	79	1.5	6.1
with *Hin*d III termini	0.9	77	2.8

[a] pBR 322/ *Eco*RI, pBR 322/ *Hin*d III, and pBR 322/ *Sty* I were prepared by the digestion of circular pBR 322 with a restriction enzymes, *Eco*RI, *Hin*d III, and *Sty* I, respectively.

remove the ligase, then treated with *Eco*RI, and subjected to gel electrophoresis. The amount of precipitated DNAs was evaluated by band intensity of the DNA fragment remaining in the supernatant. The same experiment was conducted for each of the other two types. A pBR 322-polyNIPAAm conjugate having *Hin*d III termini was also employed for the same set of the experiments.

Precipitation (%) of DNA in each experiment was shown in Table 1. When polyNIPAAm-pBR 322 conjugate with *Eco*RI termini was used in the procedure, about 80% of *Eco*RI digest of pBR322 was separated from aqueous solution, while more than 90% of the other two digests were found in the supernatant. Similarly, when the conjugate having *Hin*d III termini was applied to this experiment, 77% of *Hin*d III digest was precipitated, while less than 3% of the other two fragments were found in the precipitate. Thus the three DNA fragments which cannot be separated by gel electrophoresis were easily distinguished by the conjugates. However, in these experimental conditions, collection efficiency did not exceed 80%. This should be ascribed to the ligation reactions among the target DNAs, especially to the intra-molecular ligation which results in circularization of target DNAs. The collection efficiency would be much improved if the target molecules were treated with alkaline phosphatases prior to the ligation reaction.

The applicability of this method was further verified by the following experiment. In this case, target DNAs were prepared by the digestion of circular pBR 322 using the mixture of three restriction enzymes, *Eco*RI (5'-G ↓ AATTC-3'), *Sty* I (5'-C ↓ CTTGG-3'), and *Pst* I (5'-CTGCA ↓ G-3'). To the mixture of the resulting three DNA fragments (1.4 μg or 29.8 nM in terminus concentration: see Figure 2), was added the pBR 322-polyNIPAAm conjugate with *Eco*RI termini (91.7 nM). After treatment with T4 DNA ligase, the system (100 μL) was shortly heated (90 ℃) to denature the ligase, and was treated with *Sty* I and *Pst* I to disconnect the non-target fragments indirectly connected to the conjugate. The solution was then heated to 40 ℃ and centrifuged. The precipitate was dissolved in TE buffer and then treated with *Eco*RI to release the fragments from the conjugate. Then the system was subjected to the second 'heating and centrifugation' procedure, and the supernatant was subjected to gel electrophoresis.

As seen in lane 2 of Figure 2, about 95% of the fragment-I was found to be present in the supernatant, while very small portions of fragment-II and –III were observed. On the other hand, the precipitate fraction (lane 3) seems to contain only fragment-II and -III. Thus the DNA fragments were clearly

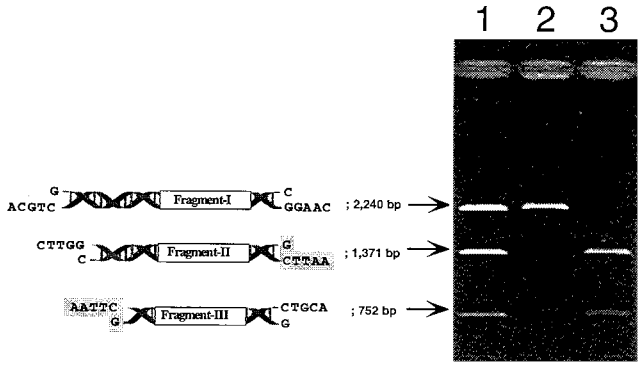

Figure 2. Selective separation of DNA fragments having *Eco*RI-terminus (5'-protruding end, 5'-AATT-3') using soluble conjugate between polyNIPAAm and pBR 322 with *Eco*RI termini. Lane 1, the starting mixture of three DNA fragments; lane 2, DNA found in the supernatant fraction after the separation procedure; lane 3, DNAs collected from the precipitation fraction.

fractionated into two groups; ones having 5'-AATT-3' terminus (Fragment-II and -III) and the other (fragment-I) without it.

In conclusion, soluble conjugate between polyNIPAAm and dsDNA with specific terminus will provide a facile method for purification of DNA fragments. As shown in Table 1, this method is applicable even to the fractionation of DNAs having the same molecular size. In addition, this method would be attractive as a technique for the collection of large DNA molecules which is very sensitive to the mechanical stresses. At present, we have confirmed that DNA fragments as large as 20 kbps can be collected by this method.

From another point of view, the connection of native dsDNA to the polyNIPAAm-coated dsDNA described in this paper may be regarded as a construction of new class of multi-functional materials: the former provides a ligand function for DNA-binding proteins, while the latter gives an ability to undergo temperature-directed precipitation. In addition, the treatment of the native DNA region with Exonuclease III should result in the ssDNA attached to the DNA conjugate. This single-stranded region may act as probe or affinity ligands. Further works in these directions are currently under way.

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