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Colloidal Nanoparticles from Poly(*N*-isopropylacrylamide)-*graft*-DNA for Single Nucleotide Discrimination Based on Salt-induced Aggregation: Extension to Long Target DNA

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The DNA-linked colloidal nanoparticle was prepared through the phase transition of the amphiphilic copolymer consisting of DNA as the hydrophilic part and thermo-responsive poly(*N*-isopropylacrylamide) as the hydrophobic part. We have succeeded in detecting the single nucleotide difference of 39-mer target DNA by using a salt-induced aggregation of the particles.

Single-base mutations are the most variations found in human genome with an estimated frequency of one polymorphic nucleotide per kilobase, commonly referred to as single nucleotide polymorphisms (SNPs).[1] These polymorphisms often acted as an important factor for estimating predispositions toward disease and drug-response.[2,3] It is an attractive challenge to develop a simple method for detection of the SNPs. Using the high binding specificity of nucleic acids in molecular recognition, various methods such as oligonucleotide microarray (DNA chip) have been proposed.[4,5] However, it is rather difficult to detect the SNPs by those assays based on the DNA hybridization, because there is only a small difference between their melting temperatures (T_ms). It is necessary to find a new approach for the highly selective detection.

Recently, we developed a simple method for detecting single nucleotide difference by using a salt-dependent aggregation of DNA-linked colloidal nanoparticles, which were composed of a hydrophobic core from poly(*N*-isopropylacrylamide) (polyNIPAAm) and a hydrophilic shell from DNA.[6] The nanoparticles were formed through the self-assembly of polyNIPAAm-*graft*-DNA copolymers above the phase transition temperature of polyNIPAAm part (ca. 35 °C). Adding the complementary DNA into the DNA-linked colloidal nanoparticles dispersion, the particles aggregated rapidly based on the salting-out phenomenon: nanoparticles carrying single-stranded DNA give a clear dispersion even in 1.5 M NaCl, while a suspension from those carrying duplex DNA becomes turbid over 0.4 M NaCl.[6] In contrast, they kept dispersing in the presence of the point-mutated DNA under the identical conditions. However, the examples of the target were limited in short oligonucleotides (9-mer).

In this study, we extended this method to single nucleotide difference of 39-mer target DNA, which is much longer than the DNA attached to the surface of the nanoparticle. For the purpose, two additional DNAs were used for forming the full-length duplex on the surface of nanoparticles (Figure 1). An anchor DNA connected with the nanoparticles is complementary with the 3′-terminal region of the target DNA. A 10-mer sequence is added as a probe. An auxiliary DNA (20-mer) is used for forming the duplex with the 5′-terminal region of the target, so that no single-stranded part should remain on the nanoparticles.

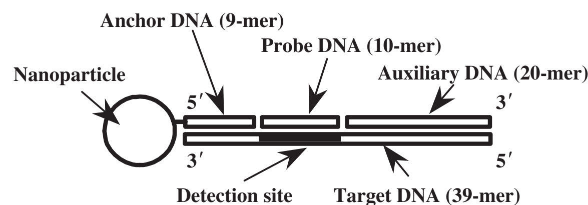

Figure 1. Schematic illustration of colloidal nanoparticle used for the detection of 39-mer target DNA.

PolyNIPAAm-*graft*-DNA copolymer was prepared by a radical copolymerization between *N*-isopropylacrylamide monomer and vinyl group-introduced DNA (DNA macromonomer; d(5′-GCCACCAGC-3′)) as reported previously.[6] The copolymer contained 0.28 mol % of the DNA macromonomer unit as determined by UV measurement. The copolymer was dissolved in 700 mM NaCl/10 mM Tris–HCl buffer (pH 7.4) to be the concentration of 0.1 mg/mL (2.3 μM in DNA strand concentration). The DNA-linked colloidal particles were formed by raising the temperature of the solution to 40 °C, which was higher than the phase transition temperature of the copolymer.[7] The averaged radius of the particles was estimated to be 80 nm under the present conditions by dynamic light scattering using cumulants method. The sequences of the target DNA are shown in Table 1. DNA **I** is a 39-mer sequence of the c-K-*ras* gene.[8] DNAs **II**–**VIII** are the point-mutated DNA. The probe DNA (d(5′-TCCAACTACC-3′)) and the auxiliary DNA (d(5′-ACAAGTTTATATTCAGTCAT-3′)) were both modified with a phosphate group at 5′-terminus. The target DNA was mixed with the probe DNA and auxiliary DNA in 700 mM NaCl/10 mM Tris–HCl buffer (pH 7.4), so that each strand concentration was 2.3 μM. After annealing, the mixture was added to the nanoparticles dispersion at 40 °C, and placed for 30 min for the single nucleotide discrimination assay.

The stability of the colloidal nanoparticles in the presence of various DNAs was studied by changing the concentration of NaCl from 700 to 1500 mM. The assembling behavior of the nanoparticles was monitored at 500 nm using a UV–vis spectrophotometer. The nanoparticles without adding any DNA kept dispersing in the experimental concentration range, as reported previously.[6] Similarly, the nanoparticles kept dispersing in the presence of the DNA **I**, because 30-mer single-stranded DNA protruded on the surface of the nanoparticle. The nanoparticles also kept dispersing in the presence of DNA **I** and the auxiliary DNA. On the other hand, the transmittance of the particles dispersion decreased gradually from 900 mM NaCl in the presence

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Table 1. Sequence of target DNAs

Target DNA	Sequence (3′→5′)	T_m/°C[a]
I	CGGTGGTCGAGGTTGATGGTGTTCAAATATAAGTCAGTA	47.5
II	CGGTGGTCGAGGTT**A**ATGGTGTTCAAATATAAGTCAGTA	24.5
III	CGGTGGTCGAGGTT**T**ATGGTGTTCAAATATAAGTCAGTA	36.0
IV	CGGTGGTCGAGGTT**C**ATGGTGTTCAAATATAAGTCAGTA	<20
V	CGGTGGTCGA**A**GTTGATGGTGTTCAAATATAAGTCAGTA	36.8
VI	CGGTGGTCGAGG**C**TGATGGTGTTCAAATATAAGTCAGTA	33.6
VII	CGGTGGTCGAGGTTGA**C**GGTGTTCAAATATAAGTCAGTA	<20
VIII	CGGTGGTCGAGGTTGATG**A**TGTTCAAATATAAGTCAGTA	42.7

[a]Melting temperature (T_m) of duplex between each target DNA and the probe DNA (d(5′-TCCAACTACC-3′)) in 1.5 M NaCl/10 mM Tris–HCl buffer (pH 7.4). The concentration of DNAs was 2.3 μM in strand. The temperature ramp was 0.5 °C/min.

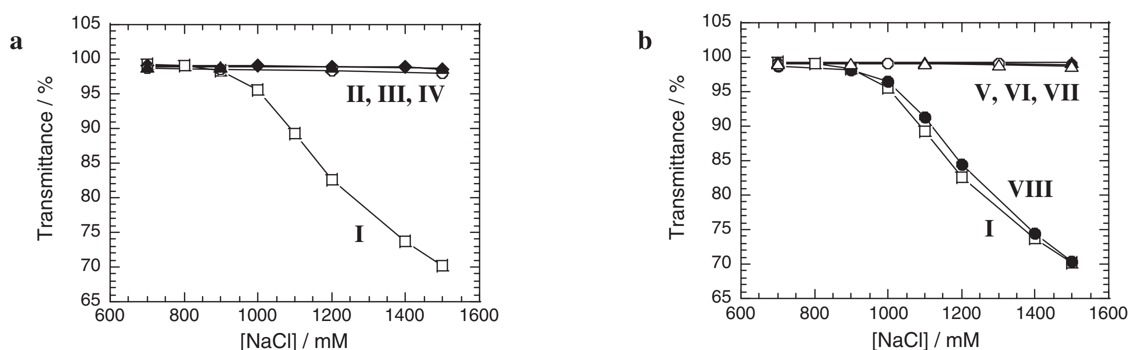

Figure 2. Plots of the transmittance at 500 nm vs the concentration of NaCl for the colloidal dispersion of the DNA-linked nanoparticles in the presence of the target DNA **I**–**IV** (a) and the target DNA **I**, **V**–**VIII** (b) with both of the probe DNA and the auxiliary DNA in 10 mM Tris–HCl buffer (pH 7.4) at 40 °C.

of DNA **I** with both of the probe DNA and the auxiliary DNA, due to the salt-dependent aggregation of the colloidal nanoparticles. As shown in Figure 2a, however, no obvious decrease of the transmittance was observed in the case of the point-mutated DNAs (**II**–**IV**) in contrast with the complementary DNA (**I**). This is explained by the fact that the duplex between DNA **II**–**IV** and the probe DNA was not stable, because all of the T_ms were under 40 °C (Table 1). The single nucleotide difference of the target DNAs (dG to dA, dT, or dC) was detected clearly by using the salt-induced aggregation of the nanoparticles.

The stability of the colloidal nanoparticles was examined for the point-mutated DNA, which has a single mismatch at different position on the detection site. As shown in Figure 2b, no change on the transmittance of the nanoparticles dispersion was observed in the presence of the target DNA **V**–**VII**. On the other hand, an obvious decrease of the transmittance was observed in the presence of the target DNA **VIII** similarly to the complementary one (DNA **I**). The T_m of the duplex between DNA **VIII** and the probe DNA is 42.7 °C, indicating that the duplex should be rather stable in the present assay conditions. These results strongly suggest that the aggregation would be strictly dependent on whether the probe DNA is bound to the target DNA. To take advantage of this behavior of the nanoparticles for single nucleotide discrimination, there are three requirements for the design of probe DNA: (i) The probe DNA should be shorter to obtain larger difference of T_ms between full match and mismatch. (ii) The detection position should be in the center region of probe DNA for the same purpose. (iii) The T_m of full-match duplex between the probe DNA and the complementary target DNA should be higher than the experimental temperature (40 °C) and the T_m of mismatch duplex between the probe DNA and the point-mutated target DNA should be lower than it, so that the probe DNA is only bound to the complementary target DNA.

In conclusion, we have succeeded in detecting a single nucleotide difference of 39-mer target DNA by using the salt-induced aggregation of the colloidal nanoparticles. Binding of short probe DNA to detection site of long target DNA triggers the drastic stability change of nanoparticle, which enables facile turbidimetric discrimination. The present extension will lead to the development of novel gene diagnostics.

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