DNA-templated Cooperative Formation of the Luminous Lanthanide Complex and Its Analytical Application to Gene Detection

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Ethylenediamine tetraacetic acid (EDTA) and 1,10-phenanthrorine (phen) were covalently attached to the 5′ and 3′ ends of two different oligonucleotides (ODNs), respectively. The sequences of the ODN conjugates were designed to contiguously hybridize on the target DNA with their auxiliary units facing each other, providing a microenvironment to accommodate the lanthanide ions. The characteristic emissions of the Tb^{3+} and Eu^{3+} ions were clearly observed in the presence of the full-matched target DNA. On the other hand, the emissions from the mixed solutions with the mutant containing one base displacement were marginal.

For the effective screening of a large number of single nucleotide polymorphisms (SNPs), split recognitions of the targets by the two conjugates have been proposed, in which the solution-based homogeneous assays were carried out using distance-sensitive fluorescence techniques such as the monomer/excimer color change[1a] and fluorescence resonance energy transfer (FRET) between the modified groups.[1b–1d]

We have also been engaged in the development of gene detection by the split recognition. In a series of studies, we showed that the two short ODN conjugates bearing a metal chelating group on their terminus cooperatively hybridize to the adjacent sequences in the presence of a half mole of Cu^{2+} as an allosteric effector.[2] The tandem hybridization of the conjugates was promoted by their dimerization on the target through their convergent coordination to a metal ion. Considering the stability constant, however, the complex is supposed to scarcely form under the stated experimental conditions. The target DNA gathered the conjugates and increased the effective concentration of the ligands to provide the appropriate microenvironment for the complexation. This work inspired us that the general concept obtained from the study, "metal ion-directed cooperative recognition," could be applied to highly selective DNA detection in a homogeneous solution by the appropriate choice of the combination of the ligands and the metal ions.

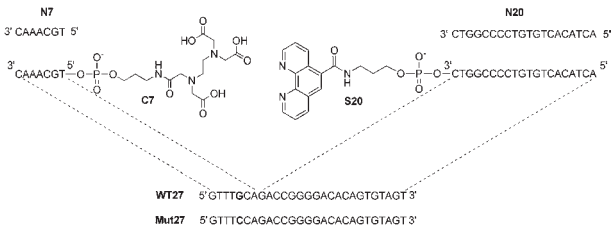

Figure 1. The ODN conjugates and the targets. The target DNAs are the part of the TPMT gene, which is related in a metabolism of anticancer agent, containing a hot spot (in boldface) in the complementary site of the capture probe.

We now present the techniques of split probing through the complexation between a luminous lanthanide ion (Ln^{3+}: Tb^{3+} or Eu^{3+}) and the two ODN conjugates carrying a metal chelator. EDTA and phen were covalently attached to the ODNs to form conjugate probes. When the ternary duplex consisting of the target and the two conjugates forms, as shown in Figure 1, the EDTA and phen moieties of the conjugates face each other and are expected to function as dehydration/capturing and sensitizer units for Ln^{3+}, respectively.[3] The stability constants of phen-Ln^{3+} are not high. Therefore, without the guide by the template (target), phen could not coordinate to Ln^{3+} under the experimental conditions.

We demonstrated the benefits of this approach for the analysis of the thiopurine *S*-methyltransferase (TPMT) gene. **WT27** and **Mut27** are the 27 mer sequences of the gene containing one of the hot spots.[4] The capture probe, **C7**, is a 7 mer ODN tethered with EDTA at the 5′ terminus, which is complementary to the part of **WT27** containing the SNP site. The sensitizer probe, **S20**, is a 20 mer ODN conjugate bearing phen at the 3′ terminus, which is complementary to the rest of **WT27**. The phen-active ester and EDTA dianhydride were coupled with the primary amino groups tethered to the ends of the corresponding ODNs. The conjugates were then purified by RP-HPLC and identified by MALDI-TOF/MS.

The thermal stability of the ternary duplexes was studied by UV melting experiments taking note of the melting behavior of **C7**. **C7** (shorter ODN) has lower melting temperatures (T_m) than those of **S20** (longer ODN). Therefore, only the T_m of **C7** would be affected by the Ln^{3+}-mediated interaction between the conjugates. T_m values for the melting of **C7** from the ternary duplexes were estimated by the usual melting curve analysis assuming a two-state model, because all of the ternary duplexes studied here showed completely separated biphasic transitions. Distinct melting behavior of **C7** was not observed for the corresponding duplexes containing **Mut27** in a temperature range of the measurement (0–90 °C). The obtained T_m values are summarized in Table 1. The addition of the Tb^{3+} or Eu^{3+} ion to the ternary duplex consisting of **C7**, **S20**, and **WT** moderately enhanced the binding affinity of **C7** to **WT** ($\Delta T_m = 2.6$ or 2.7 °C, respectively). However, Ln^{3+} scarcely affected the stability of the ternary duplex lacking in the phen (**C7**/**WT27**/**N20**). This means that the phen moiety on terminus of **S20** coordinates to Ln^{3+} under the experimental conditions. That is, although the stability constants of phen–Ln^{3+} are too low to form the complexes, the template effect of **WT27** seems to dramatically increase the effective concentrations of Ln^{3+} with the assistance of the EDTA moiety of the neighboring **C7** and makes it possible to form the complexes.

Table 1. T_m values for the hybridization of 7 mers (**C7** or **N7**) in the presence or absence of the lanthanides ions[a]

Duplex	Ln^{3+}	T_m /°C	ΔT_m /°C
C7/**WT27**/**S20**	—	19.3	—
C7/**WT27**/**S20**	Tb^{3+}	21.9	2.6
C7/**WT27**/**S20**	Eu^{3+}	22.0	2.7
N7/**WT27**/**N20**	—	25.3	—
N7/**WT27**/**N20**	Tb^{3+}	25.6	0.3
N7/**WT27**/**N20**	Eu^{3+}	25.5	0.2
C7/**WT27**/**N20**	—	22.4	—
C7/**WT27**/**N20**	Tb^{3+}	22.8	0.4
C7/**WT27**/**N20**	Eu^{3+}	22.6	0.2

[a]Melting experiments were carried out in phosphate buffer solution (10 mM, pH 7.0) containing 1.0 M NaCl. Concentrations of each component for the ternary duplex and Ln^{3+} ion were all 1 μM. The solutions were heated at a rate of 0.5 deg min^{-1} after equilibration for 30 min at 0 °C.

Figure 2 shows the time-resolved emission spectra of the solution containing the two ODN conjugates with or without the targets in the presence of Tb^{3+} or Eu^{3+}. The typical emission from Tb^{3+} and Eu^{3+} were clearly observed in the presence of **WT27**. On the other hand, the emissions in the absence of the targets and the presence of **Mut27** were ca. 1/20 of that with **WT27**. The relative emission intensity for various ternary complexes was as follows: Tb^{3+}/**C7**/**WT27**/**S20**: 100 (standard, emission intensity at 545 nm), Tb^{3+}/**C7**/**Mut27**/**S20**: 6.15, Tb^{3+}/**C7**/**WT27**/**N20**: 1.88, Tb^{3+}/**N7**/**WT27**/**S20**: 0.37, Tb^{3+}/**C7**/**S20**: 5.57, Eu^{3+}/**C7**/**WT27**/**S20**: 63.3 (emission intensity at 616 nm), Eu^{3+}/**C7**/**Mut27**/**S20**: 2.80, Eu^{3+}/**C7**/**WT27**/**N20**: 0.40, Eu^{3+}/**N7**/**WT27**/**S20**: 0.33, Eu^{3+}/**C7**/**S20**: 1.01. These results indicate that **WT27** gathers the two conjugates and Ln^{3+} as the template, making it possible that the excited energy transfers from the lowest triplet state of phen to the emission levels of Ln^{3+}.[3,5] However, **Mut27** does not serve as an effective template for forming luminous complexes. The contrast in the emission intensities observed for the solution with

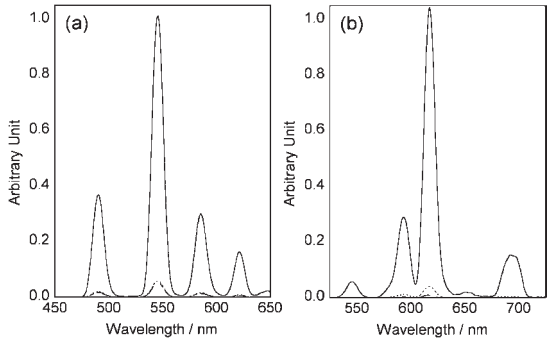

Figure 2. Time-resolved emission spectra of the solutions containing two ODN conjugates with or without the targets in the presence of (a) Tb^{3+} or (b) Eu^{3+} ion at 0 °C. The excitation wavelength was 280 nm. Concentrations of the conjugates, targets and Ln^{3+} ions added in the buffered solution (10 mM HEPES containing 1.0 M NaCl, pH 7.0) were all 1 μM. Solid curve: with **WT27**, dotted curve: with **Mut27**, dashed curve: no target. Delay time: 50 μs, Gate time: 2 ms.

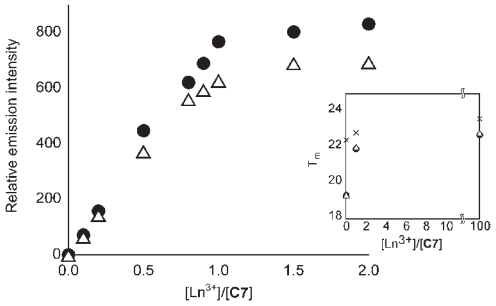

Figure 3. Job's plots for the complexation between ternary duplex and Tb^{3+} (filled circle) or Eu^{3+} (open triangle). The emission intensity was monitored at 545 and 616 nm at 0 °C for the Tb^{3+} and Eu^{3+} additions, respectively. Before each of the measurements, the sample mixtures were equilibrated at 0 °C for 30 min in the same buffer solution as Figure 2. The inset shows [metal ion]/[**C7**] vs T_m plots for the ternary duplexes (cross: **C7**/**WT27**/**N20** + Tb^{3+}, filled circle: **C7**/**WT27**/**S20** + Tb^{3+}, open triangle: **C7**/**WT27**/**S20** + Eu^{3+}. Concentrations of each component for the ternary duplex were all 1 μM.

and without **WT27** is extremely high compared with the previous studies of split probing.[6] It shows the importance of being conscious of the cooperativity when the system is designed.

The changes in the emission intensity and the T_m were also studied versus the increasing amount of Ln^{3+}. As shown in Figure 3, both values increased with the addition of Ln^{3+} and reached an inflection point around the Ln^{3+}/**C7** ratio of 1. These results indicated that the formation of the luminous complex between the ternary duplex and Ln^{3+} on the template DNA occurred at a 1:1 molar ratio.

In conclusion, the split recognition of the targets by the two ODN conjugates, **C7** and **S20**, showed its potential as a detection method for SNPs with highly selective signals. The molecular design of the conjugates that provides the cooperativity in hybridization would be critical to obtain the signals with a high contrast. The system presented here could be used for the SNP analysis in homogeneous solutions.

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