Asymmetric cooperativity in tandem hybridization of enantiomeric metal complex-tethered short fluorescent DNA probes

Yusuke Kitamura,[a] Toshihiro Ihara,*[ab] Kenji Okada,[a] Yusuke Tsujimura,[a] Yoshinori Shirasaka,[a] Masato Tazaki[c] and Akinori Jyo[a]

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The complex $[Ru(phen)_2(dppz)]^{2+}$ (phen = 1,10-phenanthroline, dppz = dipyrido[3,2-*a*:2′,3′-*c*]phenazine) was attached to the 5′ end of a short oligonucleotide to form conjugates, the Δ-isomer of which showed a high cooperativity during the recognition of the repetitive sequence, while the Λ-isomer did not.

In chromosomal DNA there are a large number of repetitive sequences such as tandem repeats and palindromes. These sequences play important roles in genetic events. Telomeres, for example, which are the tandem repeat sequences located at the ends of linear chromosomes, would protect them from degradation and are related to apoptosis and tumour formation.[1]

For several years, we have been engaged in the specific recognition of such sequences in a smart manner through the cooperative action between the short oligodeoxyribonucleic acid (ODN) conjugates that are complementary to one unit of the repeating sequence.[2,3] The cooperative recognition of full-length targets should result in a higher sequence specificity as well as a greater sensitivity to concentration changes. This would form a general concept for designing probes for certain sequences.[4]

In this study, we produced a probe candidate for a repetitive sequence by anchoring the $[Ru(phen)_2(dppz)]^{2+}$ complex onto the 5′ end of the short ODN (cTL (**1**): d(CCCTAA)). The sequence is complementary to one unit of the repetitive sequence of the human telomere. The structures and the sequences of the conjugates and the targets used in this study are shown in Fig. 1. The $[Ru(phen)_2(dppz)]^{2+}$ complex has a high binding affinity for the double-stranded DNA.[5] The complex expands the planar aromatic ring of dppz for intercalation, with the $Ru(phen)_2$ moiety remaining in a groove. The $Ru(phen)_2$ moiety of **3** has to thread through the double helix so that the dppz group effectively stacks with the base pairs, because the complex is connected to the terminus of the ODN through the dppz ligand.[6] Once formed, however, the threading-type complex is kinetically stable.[7] The preference of the tethered $[Ru(phen)_2(dppz)]^{2+}$ complex for the duplex structure should promote the binding of another **3** to an adjacent site. That is, a cooperative behavior is expected for **3** when it binds with repetitive sequences. There is no precedence in the study of probes that spontaneously gather onto the repetitive sequences to the best of our knowledge.

[a]*Department of Applied Chemistry and Biochemistry, Kumamoto University, 2-39-1 Kurokami, Kumamoto, 860-8555, Japan. E-mail: toshi@chem.kumamoto-u.ac.jp; Tel: +81-96-342-3873*
[b]*PRESTO, JST, 4-1-8 Honcho Kawaguchi, Saitama, 332-0012, Japan*
[c]*General Education, Faculty of Engineering, Sojo University, 4-22-1 Ikeda, Kumamoto, 860-0082, Japan*

The tethering of the dppz, $[Ru(phen)_2(dppz)]^{2+}$ or $[Ru(phen)_3]^{2+}$ complex to the 5′-end of **1** was carried out by the mixing of the 5′-aminohexyl-linked **1** with the corresponding NHS (*N*-hydroxysuccinimide) ester. The conjugates were purified by RP-HPLC. The conjugates **3** and **4** were obtained as equimolar mixtures of each diastereomer (**3Δ/3Λ** and **4Δ/4Λ**, respectively). The diastereomeric mixtures of the conjugates were also resolved by HPLC using a chiral column and then all the isolated conjugates were identified by MALDI-TOF MS and CD[6a] spectroscopy.

The thermal stability of the duplexes was studied by UV melting experiments. All of the duplexes studied here showed plain monophasic transitions. The apparent thermodynamic parameters for each duplex formation were obtained by the usual melting curve analysis, assuming a two-state model.[8] The apparent melting temperatures (T_m) and the binding constants at 25 °C (K_{app25}) of the tandem duplexes of **1**, **2**, and diastereomeric mixtures **3** ($\mathbf{3_{dm}}$) and **4** ($\mathbf{4_{dm}}$) are summarized in Table 1. The duplexes of **2** and $\mathbf{3_{dm}}$ were very stable compared to the corresponding duplexes of **1**. For example, the T_m of the tandem duplex **2/telo-d/2** was 35.0 °C, while that of **1/telo-d/1** was 13.8 °C. A more remarkable stabilization was observed for $\mathbf{3_{dm}}$. The T_m of the tandem duplex $\mathbf{3_{dm}}$**/telo-d/**$\mathbf{3_{dm}}$ was 42.2 °C; the $[Ru(phen)_2(dppz)]^{2+}$ group in **3** stabilized the duplex by 28.4 °C. The apparent binding constant of $\mathbf{3_{dm}}$ to **telo-d** was greater than that for **1** by more than three orders of magnitude. On the other hand, the $[Ru(phen)_3]^{2+}$ structure did not significantly affect the duplex stability, as observed in the T_m of $\mathbf{4_{dm}}$**/telo-d/**$\mathbf{4_{dm}}$.

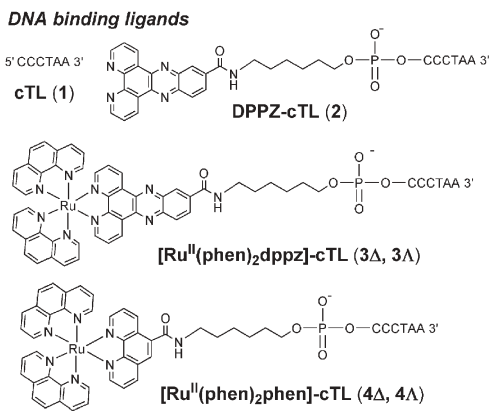


Fig. 1 DNA conjugates and ODNs used in this study.

Table 1 The apparent T_m values and binding constants for the tandem duplexes

Duplex	T_m[a]/°C	ΔT_m/°C	K_{app25}[b]/M^{-1}
1/telo-d/1	13.8 ± 0.2	—	(1.8 ± 0.1) × 10^5
2/telo-d/2	35.0 ± 0.1	21.2	(2.3 ± 0.0) × 10^7
3_{dm}/telo-d/3_{dm}	42.2 ± 0.2	28.4	(1.8 ± 0.2) × 10^8
4_{dm}/telo-d/4_{dm}	19.9 ± 0.0	6.1	(3.3 ± 0.2) × 10^5

[a] The apparent melting temperatures for the tandem duplexes (1.0 μM conjugates and 0.5 μM **telo-d**) were measured in phosphate buffer solution (1.0 mM, pH 6.5) containing 1.0 M LiCl and 1.0 mM EDTA. [b] The apparent binding constants were calculated at 25 °C.

Therefore, the large increases in the duplex stability would be due to the effective intercalation of the dppz moiety. Especially for **3**, the electrostatic interaction and hopefully the effect of the kinetic trap of the shackled structure by the threading intercalation of $[Ru(phen)_2(dppz)]^{2+}$ should also account for this. The $[Ru(phen)_3]^{2+}$ moiety would weakly bind on the surface in a groove of the duplex through electrostatic and hydrophobic interactions because the small phen aromatic ring could not fully intercalate due to steric hindrance.[9] These results apparently show that the structure of the large aromatic ring of dppz plays a significant role in stabilizing the duplex.

The following studies were carried out for each diastereomer of **3**s and **4**s, after isolation. Fig. 2(a) shows the absorption spectra of **3Δ** with and without **hp**. Here, the **hp** forms a partial hairpin structure with a protruding 6-mer (TTAGGG), under the experimental conditions. A hypochromic effect was observed for both of the transition bands, *i.e.*, the intraligand (IL) π–π* transition of the dppz chromophore (λ_{max} = 380 nm) and the metal-to-ligand charge transfer (MLCT) transition (λ_{max} = 440 nm), by the addition of an equimolar amount of **hp**. The IL band exhibited a red shift of *ca.* 5 nm, while the MLCT band did not.[5] **3Λ** showed essentially the same change in its spectra. These results indicate that the dppz ligand in **3** intercalates into the stem moiety of **hp** or the duplex consisting of **hp** and **3** itself. This only becomes possible by the threading of the bulky $Ru(phen)_2$ moiety of **3**s, and coincides with the observed significant increase in the thermal stability of the **3_{dm}/telo-d/3_{dm}** tandem duplexes compared to **1/telo-d/1**.

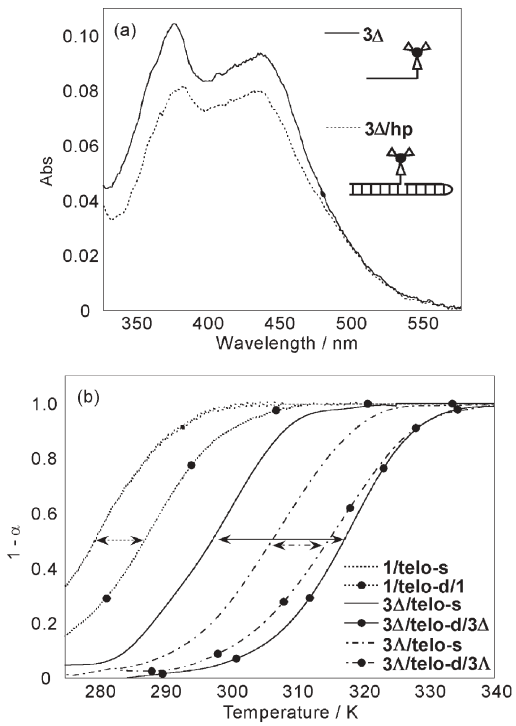


Fig. 2 (a) Visible absorption spectra of **3Δ** with (dotted line) and without (solid line) **hp**. (b) Normalized UV melting curves recorded at 260 nm for the single and tandem (with circles) duplexes. Melting experiments were carried out in phosphate buffer solution (1.0 mM, pH 6.5) containing 1.0 M LiCl. The concentrations of the conjugates, **telo-s** and **telo-d** were 1.0, 1.0 and 0.5 μM, respectively. The solutions were heated at a rate of 0.5° min^{-1} after equilibration for 1 h at 0 °C followed the annealing procedure. α is the fraction of single strands in the duplex state.

The cooperativities during the tandem hybridization of the ODN conjugates to **telo-d** were studied using UV melting curves (Table 2). The thermal stabilities of all the tandem duplexes with **telo-d** were higher than those of the corresponding single duplexes with **telo-s**. Even for the unmodified ODN **1**, the stacking between the terminal bases of **1**s at the joint obviously contributed to the formation of the tandem duplex (**1/telo-d/1**).[10] The difference in the T_m value on the basis of the corresponding single duplex (*vs.* **1/telo-s**, $\Delta T_{m,t-s}$) was 7.7 °C. The $\Delta T_{m,t-s}$ values for the tandem duplexes of **4Δ** (*vs.* **4Δ/telo-s**) and **4Λ** (*vs.* **4Λ/telo-s**) with **telo-d** were 7.8 and 7.6 °C, respectively. Although the tandem duplexes were apparently more stable than the corresponding single duplexes, the effect was not greater than that of **1**. That is, the $[Ru(phen)_3]^{2+}$ group does not interfere with the successive binding of **4** to a neighbouring site, nor does it promote it. It was very interesting that an asymmetric cooperativity was observed for **3Δ** and **3Λ** in their tandem duplex formation with **telo-d**. Fig. 2(b) shows the normalized melting curves for the tandem and corresponding single duplexes of **1** and **3**s. The $\Delta T_{m,t-s}$ values of the tandem duplexes of **3Δ** (*vs.* **3Δ/telo-s**) and **3Λ** (*vs.* **3Λ/telo-s**) were 20.0 and 8.6 °C, respectively. The Δ and Λ isomers of $[Ru(phen)_2(dppz)]^{2+}$, tethered at the end of the ODN, seem to work differently during successive hybridizations, probably due to the sequence selectivity or diastereomeric interaction with the duplex moiety. The disparity in the cooperative effect observed for **3Δ** and **3Λ** decreased when one nucleotide gap was inserted between the two binding sites (**gap**). While the thermal stability of the tandem duplex of **3Δ** decreased by 7.6 °C due to the gap insertion, the difference in the stability for the corresponding tandem duplexes of **3Λ** was trivial. This means that the tandem hybridizations of **3Δ** and **3Λ** proceed in a cooperative and independent manner, respectively.

To assess the cooperativity, we carried out a quantitative treatment of the results according to Weber's method.[11] The

Table 2 Cooperative effects in the tandem duplex formation of **3**s[a]

Duplex	T_m/°C	$\Delta T_{m,t-s}$[b]/°C	ω
3Δ/telo-s	24.1 ± 0.2	—	—
3Δ/telo-d/3Δ	44.1 ± 0.1	20.0	53.5
3Δ/gap/3Δ	36.5 ± 0.0	12.4	4.0
3Λ/telo-s	33.1 ± 0.1	—	—
3Λ/telo-d/3Λ	41.7 ± 0.1	8.6	1.6
3Λ/gap/3Λ	39.5 ± 0.2	6.4	0.8

[a] The melting studies were carried out under the same conditions as described in **Table 1**. [b] The T_m difference between the duplex with **telo-s** and that with **telo-d** or **gap**.

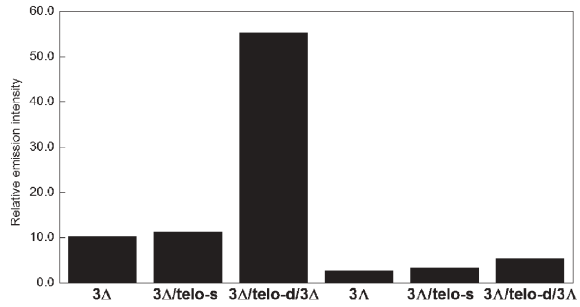
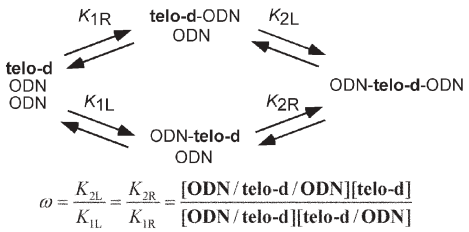


Fig. 3 Fluorescence intensities of **3**s at 615 nm (λ_{ex} = 380 nm) in the presence and the absence of **telo-s** or **telo-d** in a buffered solution (10 mM tris-HCl (pH 6.7), 100 mM NaCl). The concentrations of the conjugates, **telo-s** and **telo-d** were 5.0, 5.0 and 2.5 μM, respectively. The measurements were carried out at 10 °C after 30 min equilibration.

cooperativity, ω, which is also regarded as the equilibrium constant for a type of disproportionation reaction, is defined as follows:



$$\omega = \frac{K_{2L}}{K_{1L}} = \frac{K_{2R}}{K_{1R}} = \frac{[\mathbf{ODN/telo\text{-}d/ODN}][\mathbf{telo\text{-}d}]}{[\mathbf{ODN/telo\text{-}d}][\mathbf{telo\text{-}d/ODN}]}$$

where K_{1R} and K_{1L} are the equilibrium constants for the binding of the first ODN on the right and left binding sites of **telo-d**, respectively. K_{2R} and K_{2L} are similar constants for the second ODNs on the residual half sites. According to a previously proposed procedure,[2b] the ω value was estimated to be *ca.* 54 for **3Δ/telo-d/3Δ** at 25 °C. This means that the first binding of **3Δ** on **telo-d** magnifies the binding constant of the second one by 54 times, and the difference in the free energy of the first and second bindings is *ca.* 2.4 kcal mol^{-1}. On the other hand, the ω value of **3Λ** was 1.6.

The fluorescence of **3Δ** and **3Λ** were measured in the presence and the absence of **telo-s** and **telo-d** at 10 °C, when all of the possible duplexes should be stable (Fig. 3). The fluorescence intensities of both conjugates slightly increased with the duplex formation with **telo-s**.[6b,9b,12] The intensities for the duplexes with **telo-d** were even higher than those with **telo-s** for both conjugates. The **3Δ/telo-d/3Δ** duplex exhibited a much higher fluorescence than **3Δ/telo-s** by *ca.* 5 times, while it was *ca.* 1.5 times for **3Λ/telo-d/3Λ**. For $[Ru(phen)_2(dppz)]^{2+}$ in the duplexes with **telo-s**, it has no choice other than to intercalate between the GG/CC base pairs at the end of its own duplexes. Therefore, the effect of a "light switch" would be partially cancelled *via* oxidative quenching by the triplet G to give a fluorescence of moderate intensity.[13] On the other hand, half of the $[Ru(phen)_2(dppz)]^{2+}$complexes located at the centre of the tandem **3/telo-d/3** duplexes have two options of sites to intercalate; to extend forward to thread into the TT/AA base pairs at the end of the neighbouring duplex, or to bend backwards to thread into their own GG/CC base pairs. Considering the oxidation potentials of the nucleobases,[14] the results obtained from the fluorescence study suggest that the complex in **3Δ** mainly threads into the TT/AA base pair, and that in **3Λ** threads into the GG/CC. In fact a NMR study showed that the Δ isomer of $[Ru(phen)_2(dppz)]^{2+}$ has a higher selectivity for the A/T base pair.[15] This could well account for the observed disparity in the cooperativities between **3Δ** and **3Λ**. That is, Δ$[Ru(phen)_2(dppz)]^{2+}$ in **3Δ** promotes the successive duplex formation because it forms a stable threading complex with the neighbouring duplex. On the other hand, although the Λ$[Ru(phen)_2(dppz)]^{2+}$ in **3Λ** substantially stabilizes its own duplex (**3Λ/telo-s**) by a backward interaction, it hardly affects the stability of the next duplex.

The two ODN conjugates bearing metal complexes of opposite chiralities exhibited contrasting characteristics regarding the cooperativity of the tandem hybridization for a repetitive sequence. This is the first example of a pair of diastereomeric fluorescent DNA probes that have been studied concerning their cooperativity during hybridization to adjacent sites.

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