Supporting information

Regulation of Thrombin Activity by Ligand-Induced Topological Alteration in Thrombin-Binding Aptamer

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1. Supplemental Methods

Circular dichroism (CD) spectrometry

CD spectra were recorded on a J-720 spectropolarimeter (JASCO, Tokyo, Japan) using a quartz cell of 1 mm optional path length and an instrument scanning speed of 500 nm min^{-1} with a response time of 1 s, and over a wavelength range of 230–320 nm. Oligonucleotides (Integrated DNA Technologies, IDT) used in this protocol were dissolved as a 1.0 mM stock solution in MilliQ water to be used without further purification. Further dilution of the oligonucleotide was 50 mM Tris-HCl buffer with 100 mM KCl from 1 mM stock solutions to give a concentration of 10 μM. The solutions were annealed by heating at 95 °C for 5 min, and then slowly cooled to room temperature. After that, each concentration of 6LCO soliton was titrated into the oligonucleotide samples, with a total volume of 50 μL (final concentration= DNA: 2.0 μM, 6LCO: 0-20 μM), and incubated overnight. Finally, CD spectra are representative of five averaged scans taken at 25 °C.

Thermal Difference spectra (TDS)

In the UV experiment, oligonucleotides were dissolved in 50 mM Tris-HCl buffer with 100 mM KCl (pH 7.4) to give a final concentration of 10 μM. The samples were annealed at 95 °C for 5 min followed by slow cooling to 20 °C. After that, the 6LCO solutions were added into the oligonucleotide samples, with a total volume of 100 μL (final concentration= DNA: 5.0 μM, 6LCO: 0-50 μM), and incubated overnight. UV spectra were recorded with a V-730 spectropolarimeter (JASCO) using a quartz cell with an optical path length of 10 mm (scanning speed: 1000 nm min^{-1}, wavelength range: 230-320 nm) at 20 °C and 95 °C.

FRET melting assay

The FRET melting assay was performed with an excitation wavelength of 470–505 nm and a detection wavelength of 523–543 nm using the Thermal Cycler Dice Real Time System III (Takara). The dual fluorescently labeled oligonucleotides (FAM-TBA; Table. S1) was used in this protocol. The donor fluorophore was 6-carboxyfluorescein (FAM) and the acceptor fluorophore was 6-carboxytetramethyl- rhodamine (TAMRA). A oligonucleotide (Sigma Genosys) was dissolved as stock solutions (100 μM) in MilliQ water to be used without further purification. Further dilutions of the oligonucleotides were performed with 60 mM potassium cacodylate buffer (pH 7.4), and FRET

experiments were carried out with a 0.4 μM oligonucleotide solution. Dual-labeled DNA was annealed by heating at 95 °C for 5 min, and then slowly cooled to room temperature. A ligand was prepared as DMSO stock solutions (10 mM) and diluted to 1 mM using DMSO, and then diluted to 100 μM using 60 mM potassium cacodylate buffer (pH 7.4). Next, the annealed DNA (20 μL) and the compound solution (20 μL) were distributed across 96-well plates (Takara), with a total volume of 40 μL, with the labeled oligonucleotide (0.2 μM) and the compound (1.0 μM). The plates were incubated at 25 °C for 12 h. Subsequent experiments used the following temperature procedure in RT-PCR, finishing as follows: 25 °C for 20 min, and then a stepwise increase of 1 °C every minute from 25 °C until 99 °C. During the procedures, we measured the FAM fluorescence after each step. The change in the melting temperature at 1.0 μM compound concentration—ΔT_m (1.0 μM)—was calculated from at least three experiments by subtraction of the blank from the averaged melting temperature of each compound.

CD melting and annealing assay

The oligonucleotide was diluted to 100 μM with 50 mM Tris-HCl buffer in 100 mM KCl, and heated at 95 ºC for 5 min, then slowly cooled to room temperature. A solution of 6LCO (100 μM) was added into the oligonucleotide sample, and incubated at least 12 h (the final concentration = DNA: 2 nM, 6LCO: 20 μM). Melting and cooling curves were obtained by monitoring the CD intensity at 260 or 295 nm on a J-720 spectropolarimeter (JASCO, Tokyo, JAPAN) by using a quartz cell of 1 mm optical path length during heating from 20 ºC to 90 ℃ and then cooling from 90 ºC to 20 ºC (each rate: 1 ºC /min).

Native Polyacrylamide Gel assay

The oligonucleotide was diluted to 100 μM with 50 mM Tris-HCl buffer in 100 mM KCl, and heated at 95 ºC for 5 min, then slowly cooled to room temperature. Each solution of 6LCO (0-50 μM) was added into the oligonucleotide samples, and incubated at least 12 h. After incubation, the oligonucleotide samples were mixed 2 μL of ficoll 400 (100 mg/mL) to give a concentration of 5 μM. 5 μL of each mixture was run on 20 % non-denaturing polyacrylamide gel, (gel contained 50 mM Tris-HCl with 100 mM KCl buffer (pH 7.4)) at 250 V for 10 min. Gel was stained with SYBR® Gold and scanned with phosphor imager (Typhoon 8600, GE Healthcare) using 580-640 band pass filter.

Dot-blot assay

The oligonucleotide (TBA) was diluted with 50 mM Tris-HCl buffer in 100 mM KCl to give a concentration of 0.2 μM, and thrombin was diluted with MilliQ water. The solution of the oligonucleotide was annealed by heating at 95 °C for 5 min, and then slowly cooled to room temperature. Each concentration of 6LCO solution was titrated into the oligonucleotide samples, and incubated overnight. The solution of thrombin was immobilized on a nitrocellulose membrane (8.9 nM/spot). The thrombin-spotted membrane was blocked by 2% (w/v) skim milk solution in 50 mM Tris-HCl buffer with 100 mM KCl containing 0.05% Tween-20 (Tris-T buffer). After washing with Tris-T buffer, the membrane was incubated for 1h with each solution of oligonucleotide samples. After incubation, the membrane was washed with Tris-T buffer. Finally, the membrane was scanned with phosphorimager (Typhoon 8600, GE Healthcare) using 580-640 band pass filter.

Thrombin activity assay

Fibrin clotting was measured at 550 nm (increase in optical density) using a Jasco V-650 spectrophotometer equipped using a quartz cell of 10 mm optional path length at 25 °C for 2100 s. The oligonucleotide was diluted by 50 mM Tris-HCl buffer with 100 mM KCl from 1 mM stock solutions to give a concentration of 10 μM. The solution was annealed by heating at 95 °C for 5 min, and then slowly cooled to room temperature. Each concentration of 6LCO solution was titrated into the oligonucleotide samples, and incubated overnight. Human thrombin (89 nM, purchased from Funacoshi, HCT-0020) was mixed with the solution of DNA. This mixture was incubated for 1 hour at room temperature. The reaction was started by addition of human fibrinogen (purchased from Sigma-Aldrich, F3879) in a total volume of 500 μL (the final concentration = DNA: 890 nM, 6LCO: 4.5 or 8.9 μM, thrombin: 8.9 nM, fibrinogen: 0.8 mg/mL), and the absorbance at 550 nm was measured each second. The "$t_{1/2}$" was defined as the time when the normalized absorbance at 550 nm reached 0.5.

Computational analysis

Molecular docking simulations were performed to evaluate the binding affinity of two type TBA G4 (anti-parallel and parallel) to 6LCO. The three-dimensional structure of anti-parallel TBA G4 was obtained using the solution structure of an anti-parallel forming G4 DNA (PDB-ID: 1C32).[1] Since the 3D structure of parallel TBA G4 with the same nucleotide sequence of anti-parallel TBA G4 has not been determined, molecular modeling was performed using human telomeric DNA (PDB-ID: 1kf1),[2]

which has a parallel forming structure, as the template structure. The target parallel TBA G4 is assumed to have a two-layer G-quartet, but the template structure has a three-layer G-quartet, so two consecutive two-layer models (bottom-center and center-top layers) of G-quartet structure cores were utilized. Loop structures between the G-quartet were predicted by the Conformation/Low-mode sampling tool in MOE ver. 2020.04 (Chemical Computing Group, LLC). MMFF94x was used for the force field parameters, and default values were set for all other parameters. The structure with the lowest potential energy from the two possible layer models was chosen as the final parallel TBA G4 structure for docking simulations.

For initial coordinate of 6LCO, ionization and energy minimization were performed with the OPLS3e force field in the LigPrep script in the Maestro (Schrödinger, LLC). This minimized structure was employed as input structures for docking simulations. Molecular docking simulation of 6LCO on the TBA G4 structures with two types of topologies, anti-parallel and parallel, were performed using the Glide SP docking program[3,4] (Schrödinger, LLC). Because the 6LCO is very flexible molecule, we extracted up to the energetically lowest 100 candidate docking poses and scores for each TBA G4 models.

2. References

1. V. M. Marathias, P. H. Bolton, *Nucl. Acids Res.,* 2000 **28**, 1969-77.
2. G. N. Parkinson, M. P. Lee, S. Neidle, *Nature*, 2002, **417**, 876-80.
3. R. A. Friesner, J. L. Banks, R. B. Murphy, T. A. Halgren, J. J. Klicic, D. T. Mainz, M. P. Repasky, E. H. Knoll, M. Shelley, J. K. Perry, D. E. Shaw, P. Francis, P. S. Shenkin, *J. Med. Chem.,* 2004, **47**, 1739-49.
4. T. A. Halgren, R. B. Murphy, R. A. Friesner, H. S. Beard, L. L. Frye, W. T. Pollard, J. L. Banks, *J. Med. Chem.,* 2004, **47**, 1750-9.

3. Oligonucleotides Sequences

Table S1. Sequences of oligonucleotides used in this paper

Oligonucleotides	Sequence
TBA *1	d[GGT TGG TGT GGT TGG]
Flu-TBA *2	FAM-d[GGT TGG TGT GGT TGG]-TAMRA
15 mer DNA *1	d[GGC TGG CAA TGG CGG]
30 mer DNA *1	d[GGA TCA CCG GTG GAA TTG CTA TCG CAA TGG]

*1 Oligonucleotides were purchased from Integrated DNA Technology (IDT) as desalting purification grade and used without further purification.

*2 "Flu-TBA" was purchased from Sigma Genosys as HPLC purification grade and used without further purification.

4. Thermal difference spectra (TDS)

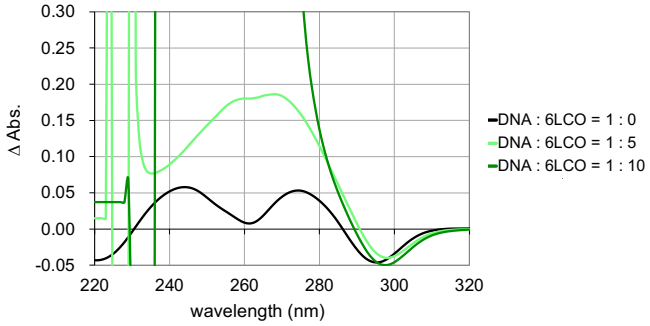
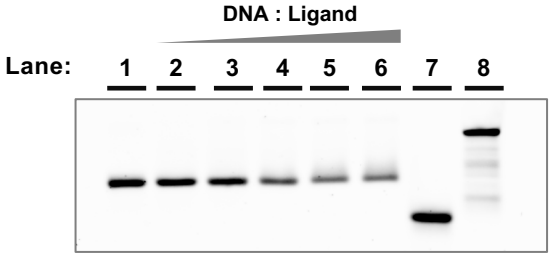


Fig. S1 The thermal difference spectra (TDS) of TBA (5 μM) in 50 mM Tris-HCl with 100 mM KCl in the presence of 6LCO (0-50 μM).

5. Native Polyacrylamide Gel assay



Lane	1	2	3	4	5	6	7	8
DNA (5 μM)	TBA	TBA	TBA	TBA	TBA	TBA	ODN 15 mer	ODN 30 mer
6LCO (equiv.)	—	1	2.5	5	7.5	10	—	—

Fig. S2 Native Polyacrylamide Gel assay (20% non-denaturing gel in TBE buffer) of TBA (5 μM) with 6LCO (0-50 μM; lane: 1-6) and single-stranded DNA, 15 mer and 30 mer, (lane: 7-8) in 50 mM Tris-HCl with 100 mM KCl.

6. FRET (Förster resonance energy transfer) melting assay

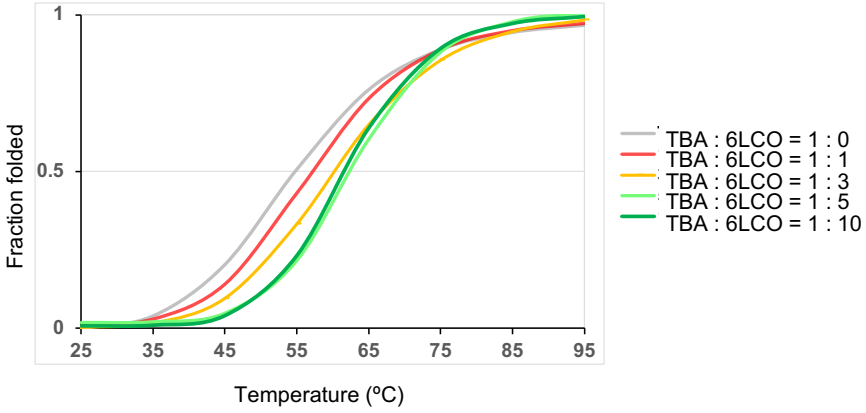


Fig. S3 FRET melting curves of Flu-TBA (0.2 μM, Table S1) with each concentration of 6LCO (0-2 μM), in 60 mM potassium cacodylate buffer (pH 7.2).

7. CD spectra assay

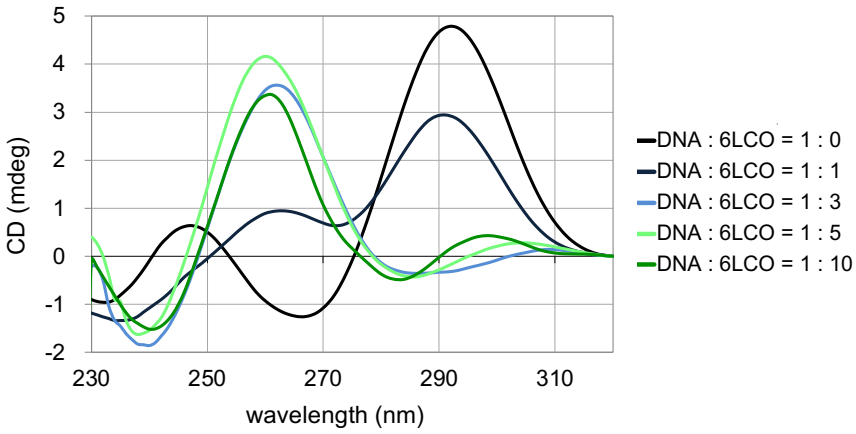


Fig. S4 The CD spectra of Flu-TBA (2 μM) in 50 mM Tris-HCl with 100 mM KCl in the presence of 6LCO (2-20 μM).

8. CD melting and annealing assay

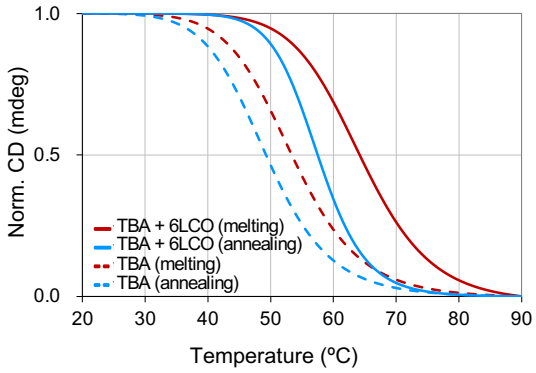


Fig. S5 The CD melting and annealing assay of TBA (2 μM) along at 295 nm (the dotted lines) and TBA with 6LCO (20 μM) at 260 nm (the solid lines) in 50 mM Tris-HCl with 100 mM KCl; Red lines: the melting curve from 20 ºC to 90 ºC (heating rate: 1 ºC/min), Blue lines: the annealing curve from 90 ºC to 20 ºC (annealing rate: 1 ºC/min).

9. Docking models

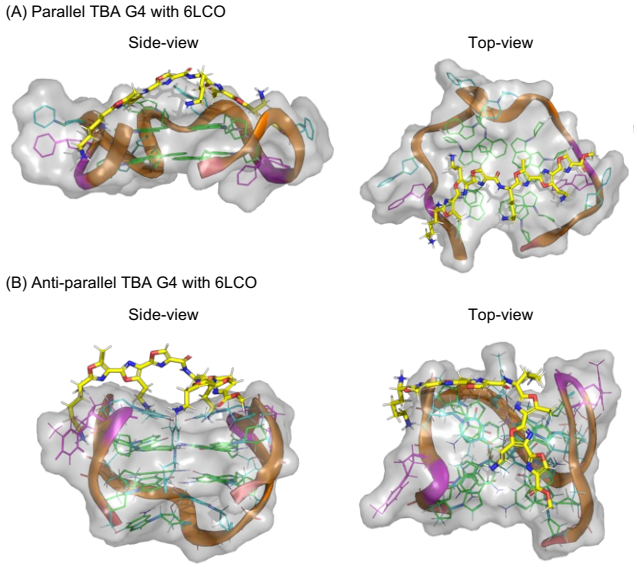
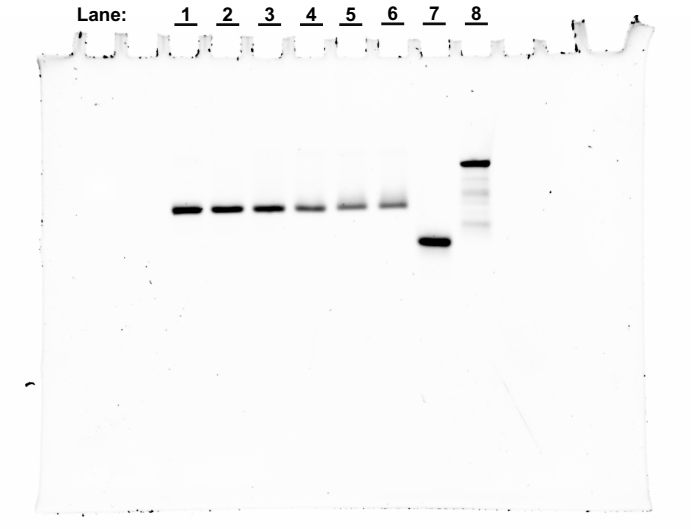


Fig. S6 The side and top views of the docking models of TBA G4 with 6LCO; (A) parallel TBA G4 with 6LCO, (B) anti-parallel TBA G4 with 6LCO (G-quartet plane: green, T3 and T12: magenta, ligand: yellow).

Whole gel image of Figure S2



Lane	1	2	3	4	5	6	7	8
DNA (5 μM)	TBA	TBA	TBA	TBA	TBA	TBA	ODN 15 mer	ODN 30 mer
Ligand **1** (equiv.)	—	1	2.5	5	7.5	10	—	—