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Supporting Information

Triplex-forming PNA modified with unnatural nucleobases: the role of protonation entropy in RNA binding

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Contents:

1) Reagents and sample preparation…………………….…………..pS3
2) PNA synthesis……………………………………………………pS3
3) Kinetic analyses…………..………………………………………pS3
4) Fluorescence titration assay..………………………………………..pS4

PNA preparation

PNA-HF488 was synthesized using solid-phase synthesis methodology and Fmoc chemistry on an Expedite 8909 DNA synthesizer as described in detail in the previous reports [1-3].

Kinetic analyses

HPLC-purified hairpin-BHQ1, purchased from Japan Bio Services, was annealed in a buffer containing 30 mM HEPES-KOH at various pH conditions, 0.01% CHAPS, and 200 mM KCl. PNA solution (0.11 nM) was diluted in the same buffer and pre-equilibrated at 25 °C to maintain the same temperature throughout the experiment. Aliquots (90 μL) of the PNA solution were injected into the wells of 384-well black plate (Corning) containing 10 μL of the hairpin-BHQ1 by using the injector module of an Infinite 200 Pro microplate reader (Tecan). Final concentration of the PNA-HF488 was 0.1 nM. Final concentrations of the hairpin-BHQ1 were varied at the range excess to the PNA-HF488 depending on the pH. Fluorescence signals of the PNA-HF488 at 530 nm were monitored (excitation at 490 nm) at 25 °C using an Infinite 200 Pro microplate reader every 8 s from 0 to 60 min. The time course of the fluorescence was corrected for the lag-time error (18 s) from the injection of the PNA solution to the first measurement.

Fluorescent titration assay

The hairpin-BHQ1 was prepared as previously described [3]. The fluorescence signals of PNA-HF488 (0.1 nM) mixed with various concentrations of the hairpin-BHQ1 (0-160 nM) in a buffer containing 30 mM HEPES-KOH, 200 mM KCl, and 0.01% CHAPS at different pH conditions were measured using Infinite 200 Pro. Excitation and emission wavelength were 490 and 530 nm, respectively. Measurements were performed in a range of temperatures from 25 to 37 °C. Before each data collection, samples were incubated at least 60 min to make the equilibrium state. The equilibrium binding constant (K_B) values were determined by fitting the titration curves obtained from at least three independent sets of experiments using following equation, which is based on a 1:1 binding equilibrium:

$$F = F_{\text{initial}} + \left(\frac{F_{\text{final}} - F_{\text{initial}}}{2 \times [\text{PNA}]}\right) \times \left\{ \left([\text{PNA}] + [\text{RNA}] + \frac{1}{K_B}\right) - \sqrt{\left([\text{PNA}] + [\text{RNA}] + \frac{1}{K_B}\right)^2 - 4 \times [\text{PNA}] \times [\text{RNA}]} \right\}$$

S1

where F is the fluorescence signal of the PNA-HF488 at each concentration of hairpin-BHQ1, F_{initial} is the fluorescence signal of the PNA-HF488 in the absence of hairpin-BHQ1, F_{final} is the fluorescence signal of the PNA-HF488 after binding to hairpin-BHQ1, [PNA] is the total concentration of the PNA-HF488 (fixed at 0.1 nM), and [RNA] is the hairpin-BHQ1 concentration. Standard differential entropy ($\Delta S°$) and enthalpy ($\Delta H°$) were obtained from the dependence of calculated K_B values (obtained after introducing the adjustment for the buffer pH changes upon temperature) on temperature according to the following Van`t Hoff equation:

$$\text{Ln}\, K_B = -\frac{\Delta H^{\circ}}{RT} + \frac{\Delta S^{\circ}}{R} \qquad \textbf{S2}$$

where R is the universal gas constant (0.00198 kcal K^{-1} mol^{-1}), and T is the absolute temperature.

References

[1] Zengeya, T., Gupta, P., Rozners, E., Triple Helical Recognition of RNA Using 2-Aminopyridine-Modified PNA at Physiologically Relevant Conditions. *Angew. Chem. Int. Ed.* 2012, *51*, 12593.
[2] Gupta, P., Zengeya, T., Rozners, E., Triple helical recognition of pyrimidine inversions in polypurine tracts of RNA by nucleobase-modified PNA. *Chem. Commun.* 2011, *47*, 11125.
[3] Endoh, T., Hnedzko, D., Rozners, E., Sugimoto, N., Nucleobase-Modified PNA Suppress Translation by Forming a Triple Helix with a Hairpin Structure in mRNA In Vitro and in Cells. *Angew. Chem. Int. Ed*. 2016, *55*, 899.

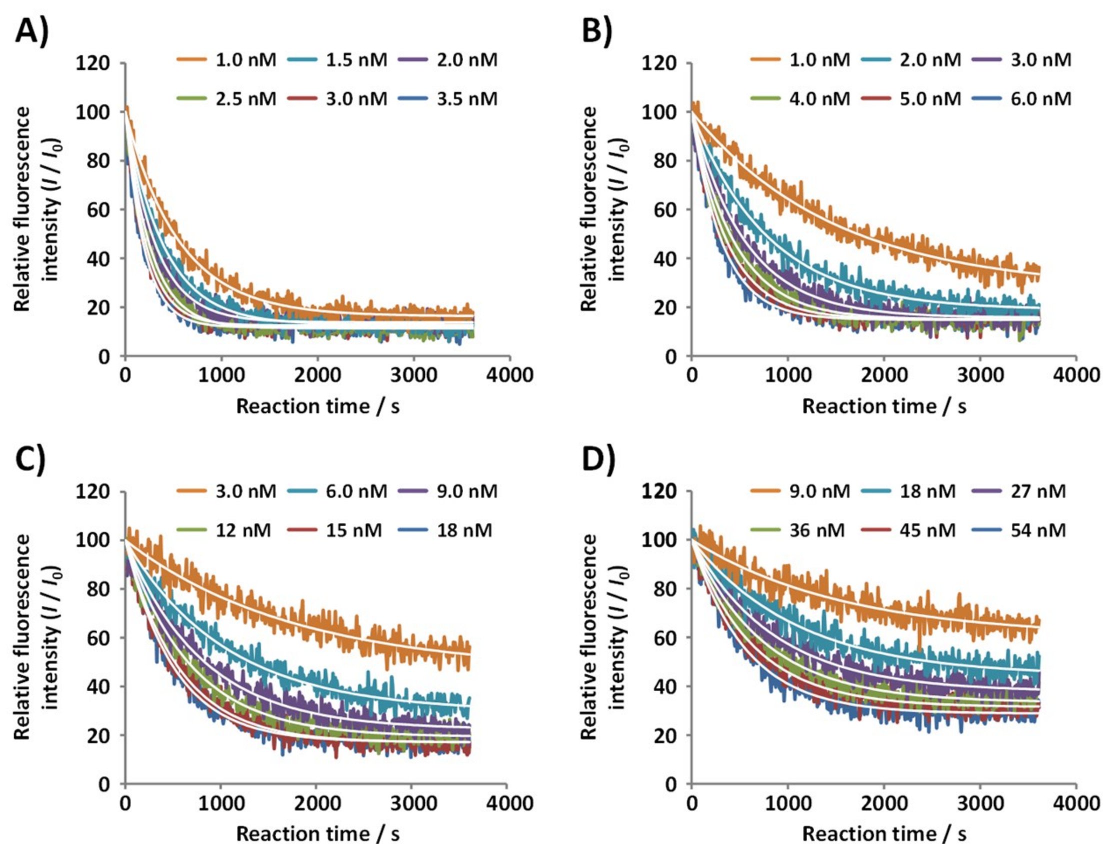

Figure S1. Kinetic profiles of binding reactions between PNA-HF488 (0.1 nM) and hairpin-BHQ1 at (A) pH 6.8, (B) pH 7.0, (C) pH 7.2, and (D) pH 7.4. The samples were prepared in 30 mM HEPES-KOH, 0.01% CHAPS, 200 mM KCl. Concentrations of hairpin-BHQ1 mixed with PNA-HF488 are indicated in each graph. I_0, the PNA fluorescence intensity in the absence of hairpin-BHQ1 RNA, was set as 100%. The calculated relative fluorescence intensity (I/I_0) corresponds to the percentange of fluorescence decrement compared to I_0. White lines show theoretical signal changes fitted into pseudo-first order equation.

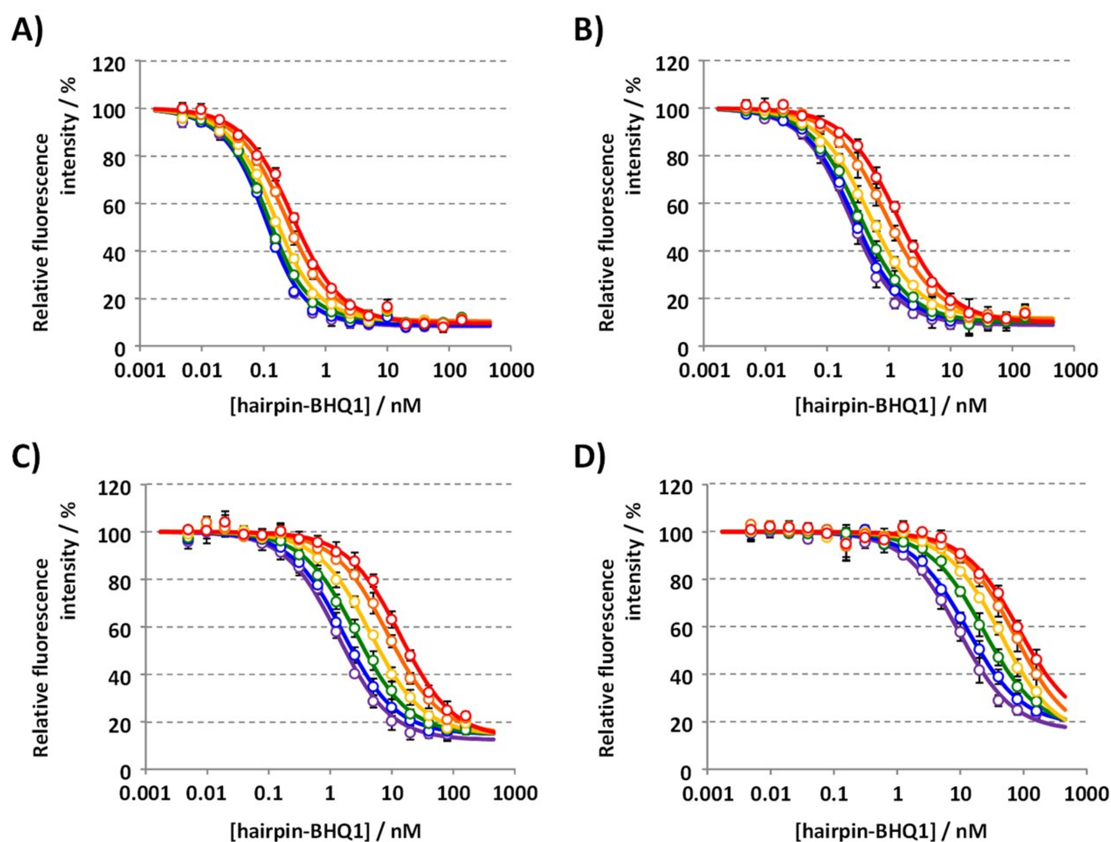

Figure S2. Relative fluorescence intensity of PNA-HF488 mixed with various concentrations of hairpin-BHQ1 in a buffer containing 30 mM HEPES-KOH, 200 mM KCl, and 0.01% CHAPS at progressively increasing temperature and at (A) pH 6.8, (B) pH 7.0, (C) pH 7.2, and (D) pH 7.4. For each data set, measurements were carried out at 25 °C (purple), 27.5 °C (blue), 30 °C (green), 32.5 °C (yellow), 35 °C (orange), and 37 °C (red). Fluorescence intensitites of PNA-HF488 were normalized relative to that in the absnce of hairpin-BHQ1. Values are means ± standard deviations from three independent measurements. Lines show theoretical signal changes fitted into Equation S1.

Table S1. $K_{B\ (thermodynamics)}$ values between PNA-HF488 and hairpin-BHQ1 obtained by fluorescence titration at different pH and temperature conditions[a]

Hairpin RNA	$K_{B\ (thermodynamics)}$ (× 10^8 M^{-1})			
	pH 6.8[b]	pH 7.0[b]	pH 7.2[b]	pH 7.4[b]
25 °C	182 ± 8.4	55.0 ± 5.2	7.78 ± 0.63	1.07 ± 0.18
27.5 °C	192 ± 6.8	48.3 ± 4.3	6.41 ± 0.88	0.799 ± 0.11
30 °C	158 ± 9.3	35.7 ± 3.0	3.92 ± 0.62	0.430 ± 0.050
32.5 °C	105 ± 2.4	24.1 ± 1.7	2.27 ± 0.24	0.230 ± 0.020
35 °C	60.3 ± 1.8	12.3 ± 1.6	1.19 ± 0.11	0.139 ± 0.019
37 °C	42.3 ± 2.6	7.78 ± 0.53	0.741 ± 0.11	0.120 ± 0.011

[a] The values correspond to means ± standard deviations from three independent measurements.

[b] Theoretical values were not normalized for the temperature effect on buffer pH.

Table S2. Temperature dependent HEPES-KOH pH shift[a]

Buffer pH	25 °C	27.5 °C	30 °C	32.5 °C	35 °C	37 °C	40 °C
6.8	6.853	6.818	6.798	6.773	6.745	6.720	6.703
7.0	7.020	6.988	6.963	6.935	6.910	6.890	6.868
7.2	7.248	7.210	7.193	7.163	7.140	7.118	7.103
7.4	7.440	7.405	7.380	7.358	7.338	7.320	7.290

[a] Buffer composition: 30 mM HEPES-KOH, 200 mM KCl, and 0.01% CHAPS. Values are averaged from four times measurements.