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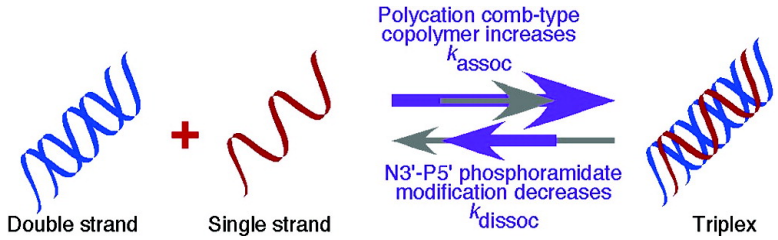

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Synergistic Stabilization of Nucleic Acid Assembly by Oligo-N3′→P5′ Phosphoramidate Modification and Additions of Comb-type Cationic Copolymers

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Abstract: Synergic stabilization of DNA triplexes by oligo-N3′→P5′ phosphoramidate (PN) modification and additions of comb-type cationic copolymers was demonstrated. The combination of the copolymer and the PN modification increased triplex K_a about 4 orders of magnitude. Kinetic analysis revealed that observed stabilization resulted from kinetic complimentarity between increased association rates by the copolymer and decreased dissociation rates by the PN modification of triplex forming oligonucleotides. No countering interference between these stabilizing effects was observed. We propose that kinetic analyses of stabilizing effects permit selection of a rational combination of stabilizing methods for successful synergy in stabilizing complex formation.

Introduction

Molecular interactions with high specificity are pivotal for chemical and biochemical processes. Base pairing in nucleic acid strands is an outstanding example of such interactions. Watson−Crick base pairing in duplexes and Hoogsteen base pairing in triplexes formed between single-stranded homopurine or homopyrimidine triplex-forming oligonucleotides (TFO) and homopurine-homopyrimidine stretches in duplexes[1−3] are involved in sequence-specific nucleic acid interactions. Recently, such assemblies of nucleic acid strands have attracted considerable interest for their wide variety of potential applications in not only life sciences[4−7] but also nanotechnology including nanomachines and opto/electronic nanodevices.[8−10] In addition to specificity, stability of nucleic acid assembly is a key factor for their practical utility. Various stabilization methods, including molecular designs of nonnatural nucleotides and hybrid stabilizers for nucleic acid assembly, have been developed.[11,12] Further complex stabilization has involved combinations of different stabilization methods.[13,14] However, combinations of two stabilizing methods have frequently resulted in unexpectedly small effects. For example, while each of the 2′,4′-bridged nucleic acid modifications[15−21] and N3′-P5′ phosphoramidate (PN) modifications[22−28] of nucleic acid strands increases the duplex and triplex thermal dissociation temperatures significantly, the combination of these two modifications results in less stabilizing activity than the 2′,4′-bridged nucleic acid modification alone.[29] In another example, while acridine

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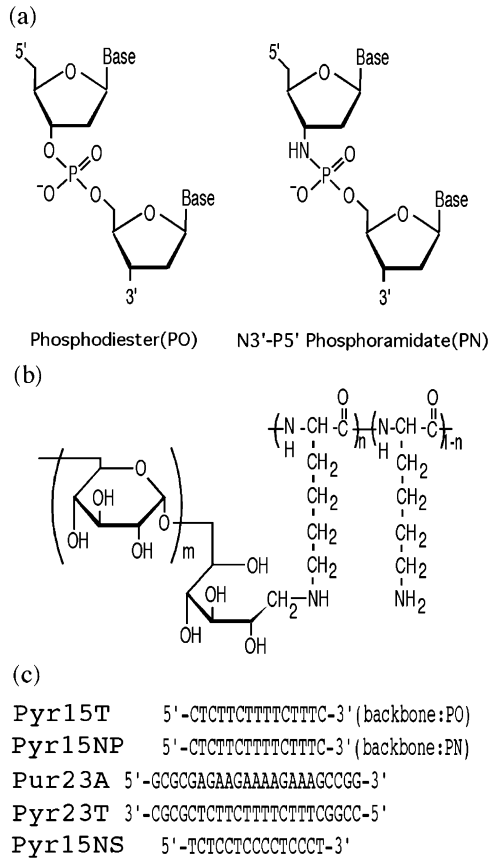

Figure 1. (a) Structural formulas for phosphodiester (PO) and phosphoramidate (PN) backbones; (b) structural formula for PLL-*g*-Dex copolymer; (c) oligonucleotide sequences for the target duplex (Pur23A·Pyr23T), the specific TFOs (Pyr15T and Pyr15NP), and the nonspecific TFO (Pyr15NS).

modification of TFO and addition of a triplex-binding ligand increase the binding constant of triplex formation by 10-fold and 100-fold, respectively,[30] combination of these two methods results in only a 50-fold increase in triplex stability.[30] Hence, rational methods to design new stabilizing combinations for more effective nucleic acid complexation are lacking and should be established.

We have previously reported that PN modification of TFO (see Figure 1a) increases the binding constant for triplex formation at neutral pH by nearly 2 orders of magnitude.[28] Kinetic studies have revealed that this increased complex stabilization by the PN modification results mainly from a decreased dissociation rate constant.[28] By contrast, we have previously reported that poly(L-lysine)-*graft*-dextran (PLL-*g*-Dex) copolymer [poly(L-lysine) grafted with hydrophilic dextran side chains; see Figure 1b] significantly increases the stability of duplex[31] and triplex[32−39] formation by reducing counterion-induced condensation effects. This copolymer increases the binding constant of triplex formation at neutral pH by nearly 2 orders of magnitude.[35,36] Kinetic studies have demonstrated that the increase in binding constant by the copolymer results from a considerable increase in the association rate constant rather than a decrease in the dissociation rate constant.[35,36] The kinetic effect of adding the copolymer is in sharp contrast with that of a PN-modification in TFO. The difference in the kinetic effects between the copolymer and the PN-modification of TFO produced our hypothesis that the copolymer should further increase stability of triplexes involving PN modification.

In the present study, we demonstrate that the combination of these two stabilizing methods synergistically increase the binding constant for triplex formation at neutral pH. Kinetic analyses reveal that observed stabilization results from kinetic complimentarity between increased association rates by the copolymer and decreased dissociation rates by PN modification of TFO. No countering interference between these stabilizing effects was observed. We propose that kinetic analyses of stabilizing effects permit selection of a rational combination of stabilizing methods for successful synergy in stabilizing complex formation.

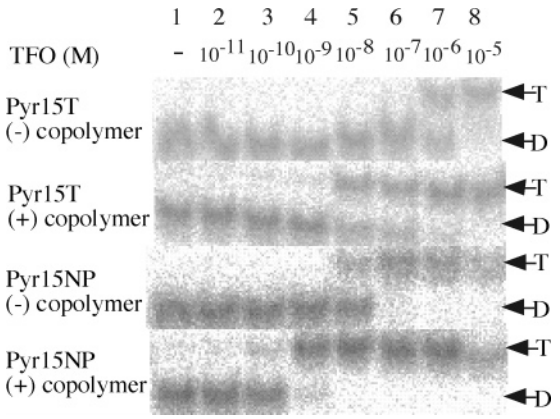

Figure 2. EMSA results for triplex formation involving Pyr15T or Pyr15NP at neutral pH with or without addition of PLL-*g*-Dex copolymer. Triplex formation was initiated by adding ^{32}P-labeled Pur23A·Pyr23T duplex (~10 pg) with the indicated final concentrations of Pyr15T or Pyr15NP. Pyr15NS was added to adjust the equimolar concentrations (10 μM) of TFO (Pyr15T + Pyr15NS or Pyr15NP + Pyr15NS) in each lane. Reaction mixtures involving Pyr15T or Pyr15NP in buffer [50 mM Tris-acetate (pH 7.0), 100 mM sodium chloride, and 10 mM magnesium chloride] with or without 4 μM PLL-*g*-Dex copolymer (charge ratio of [amino groups]$_{copolymer}$/[phosphate groups]$_{DNA}$ = 2) were incubated for 6 h at 37 °C and then electrophoretically separated on a 15% native polyacrylamide gel at 4 °C. Positions of the duplex (D) and triplex (T) are indicated.

Results

Electrophoretic Mobility Shift Assay of Triplex Formation at Neutral pH. Triplex formation of the target duplex, Pur23A·Pyr23T (Figure 1c), with TFO, Pyr15T, or Pyr15NP (Figure 1c), was examined either with or without added PLL-*g*-Dex copolymer (Figure 1b) at pH 7.0 by EMSA (Figure 2). Total oligonucleotide concentration ([specific TFO (Pyr15T or Pyr15NP; Figure 1c)] + [nonspecific oligonucleotide (Pyr15NS; Figure 1c)]) was kept constant at 10 μM to achieve equal charge ratios of [amino groups]$_{copolymer}$/[phosphate groups]$_{DNA}$ and to assess

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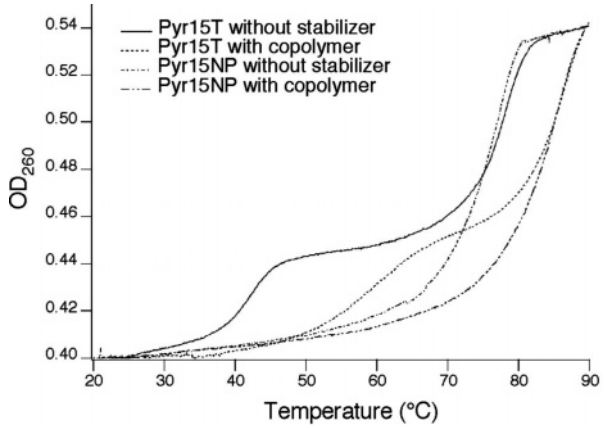

Figure 3. UV melting profiles for the triplex involving Pyr15T or Pyr15NP at neutral pH with or without the PLL-*g*-Dex copolymer. Triplexes involving Pyr15T or Pyr15NP in buffer A (See Materials and Methods in the Supporting Information) with or without 1.7 μM PLL-*g*-Dex copolymer (the charge ratio of [amino groups]$_{copolymer}$/[phosphate groups]$_{DNA}$ was 2) were melted at a scan rate of 0.5 °C/min with detection at 260 nm.

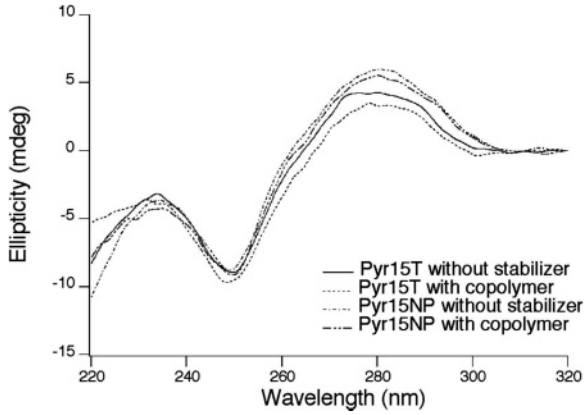

Figure 4. CD spectra for triplex involving Pyr15T or Pyr15NP at neutral pH with or without the PLL-*g*-Dex copolymer. The triplexes involving Pyr15T or Pyr15NP at 15 °C and pH 6.8 in buffer A (see Materials and Methods in the Supporting Information) with or without 3.3 μM PLL-*g*-Dex copolymer (the charge ratio of [amino groups]$_{copolymer}$/[phosphate groups]$_{DNA}$ was 2) were measured in the wavelength range 220−320 nm.

sequence specificity. Incubation with Pyr15T or Pyr15NP at specific concentrations caused retardation of duplex migration due to triplex formation.[33−35] The dissociation constant, K_d, of triplex formation was determined from the concentration of the TFO, producing 50% conversion of duplex to triplex.[33−35] The K_d for the triplex involving Pyr15T without the copolymer (*Pyr15T, (−) copolymer*) was estimated to be $\sim 10^{-6}$M. In contrast, K_d for the triplex involving either Pyr15T with the copolymer (*Pyr15T, (+) copolymer*) or Pyr15NP without the copolymer (*Pyr15NP, (−) copolymer*) was $\sim 10^{-8}$ M, indicating that either the copolymer or the PN modification increases triplex stability by nearly 2 orders of magnitude. Incubation with 10 μM Pyr15NS (nonspecific oligonucleotide) alone in the presence of the copolymer did not shift electrophoretic migration of the target duplex (*see lane 1, (+) copolymer*), indicating that the copolymer preserved sequence specificity of triplex formation. We then assessed cooperativity between the copolymer and the PN modification (*Pyr15NP, (+) copolymer*). Note that triplex formation, even at TFO concentration of 10^{-10}M, was observed, demonstrating nearly a 10^4-fold increase in the triplex formation binding constant by combining these stabilizing methods.

Spectroscopic Characterization of Triplex Formation at Neutral pH. Thermal stability of the triplex involving Pyr15T or Pyr15NP was investigated either with or without the PLL-*g*-Dex copolymer at pH 6.8 by UV melting measurements (Figure 3). The triplex involving Pyr15T without the copolymer exhibited a biphasic thermal dissociation profile. The first transition at lower temperature, T_{m1} (42 °C), was attributed to the thermal dissociation of triplex to duplex and a TFO. The second transition at higher temperature, T_{m2} (77 °C), was that for the duplex. Addition of the copolymer to the triplex involving Pyr15T increased both T_{m1} and T_{m2} up to 60 °C and 86 °C, respectively, indicating that the copolymer increased the stability of both the triplex and duplex. By contrast, the triplex involving Pyr15NP without the copolymer exhibited only one transition at higher temperature, T_m = 77 °C. As the UV absorbance change at T_m under this condition was nearly equal to the sum of those at T_{m1} and T_{m2} (Figure 3), the transition was identified as a direct thermal dissociation of the triplex to its constituent single strands. The PN modification, therefore, increased the triplex T_m by 35 °C, confirming, like previous work,[23,26] that PN modification increases triplex stability. Next, we evaluated the effect of the copolymer on the stability of the triplex involving Pyr15NP. The triplex involving Pyr15NP with the copolymer also exhibited only one transition at higher temperature, T_m = 86 °C. The copolymer further increased the T_m of the triplex involving Pyr15NP by ~10 °C without affecting the hyperchromicity, indicating that the copolymer and the PN modification cooperatively increased the thermal stability of the triplex.

Triplexes involving Pyr15T or Pyr15NP either with or without the PLL-*g*-Dex copolymer were further characterized by CD spectra (Figure 4) and CD melting (data not shown) measurements. The CD profiles at 15 °C and pH 6.8 were similar for the four different conditions (Figure 4), confirming triplex formation involving Pyr15T or Pyr15NP either with or without the copolymer.[40] No significant change in highly ordered triplex structure by either the copolymer or the PN modification was observed. The T_m values for the triplexes at pH 6.8 determined by CD melting measurements (data not shown) were consistent with those obtained from UV melting experiments described above.

Kinetic Analyses of Triplex Formation at Neutral pH. To understand the stabilization cooperativity observed between PLL-*g*-Dex copolymer and PN modification (Figures 2 and 3), the kinetic parameters for the association and dissociation of TFO (Pyr15T or Pyr15NP) with Pur23A·Pyr23T were assessed either with or without the PLL-*g*-Dex copolymer at 25 °C and pH 6.8 by IAsys surface affinity measurements (Figure 5). Figure 5a compares the sensorgrams representing triplex formation and dissociation involving 2.0 μM of the specific TFO with or without the copolymer. Pyr15T over the immobilized Bt-Pyr23T·Pur23A caused an increase in sensor response. However, the response was more substantially increased when the same measurement was performed in the presence of the copolymer, indicating that the copolymer significantly increased the association rate constant for the triplex. In contrast, although the change in the association curve was moderately enhanced by Pyr15NP, the dissociation curve change over time for Pyr15NP was much smaller than that for Pyr15T. These results indicate that PN modification remarkably decreases the dissociation rate

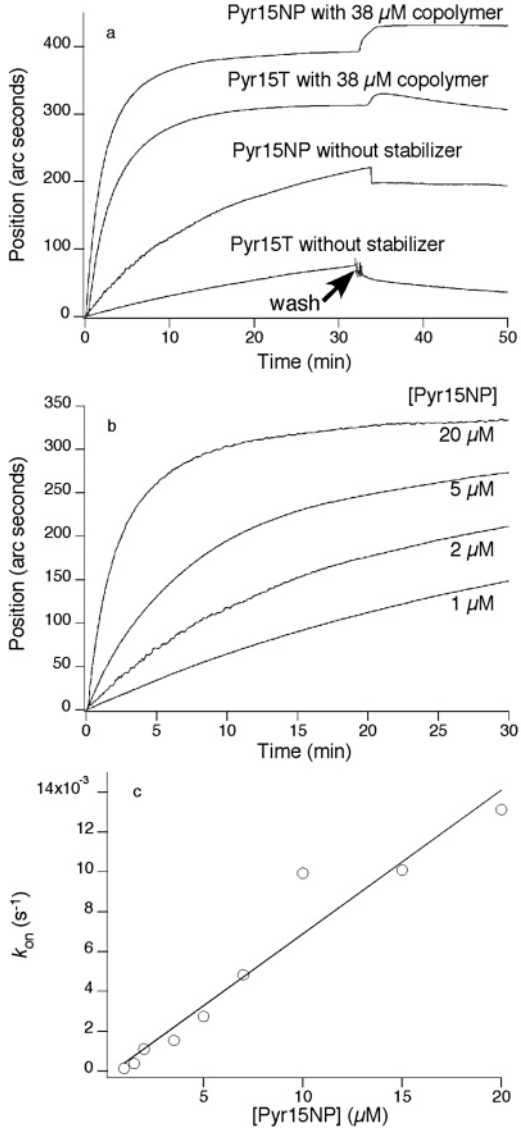

Figure 5. Kinetic analyses for triplex formation involving Pyr15T or Pyr15NP at pH 6.8 in buffer A (see Materials and Methods in the Supporting Information) with or without the PLL-*g*-Dex copolymer. (a) Typical sensorgrams for triplex formation at 25 °C and pH 6.8 after injecting 2.0 μM TFO (Pyr15T or Pyr15NP) with or without 0.038 mM PLL-*g*-Dex copolymer into the Bt-Pyr23T·Pur23A-immobilized cuvette are shown. (b) A series of sensorgrams for triplex formation between Pyr15NP and Pur23A·Pyr23T at 25 °C and pH 6.8 without the copolymer. The Pyr15NP solutions, diluted in buffer A to achieve the indicated final concentrations, were injected into the Bt-Pyr23T·Pur23A-immobilized cuvette. Binding of Pyr15NP to Bt-Pyr23T·Pur23A was monitored over time. (c) The on-rate constants, k_{on}, obtained from part b were plotted against the respective concentrations of Pyr15NP. The plot was fit to a straight line ($r^2 = 0.97$) by linear least-squares methods.

constant for the triplex. Note that both intrinsic effects from the copolymer and the PN modification were apparent in both association and dissociation curves for Pyr15NP in the presence of the copolymer. Combination of the copolymer and the PN modification, thus, permitted rapid formation of a durable triplex.

To explore these kinetic effects more quantitatively, we analyzed a series of association and dissociation curves as a function of TFO concentration. As shown in Figure 5b, increasing concentrations of Pyr15NP produces a gradual change in the association curves. On-rate constants (k_{on}) obtained from the analysis of each association curve are shown in Figure 5c plotted against Pyr15NP concentrations. The association rate constant (k_{assoc}) was determined from the slope of the fitted line obtained by a linear least-squares method.[19,28,35,41,42] The off-rate constant (k_{off}) was obtained from analysis of each dissociation curve (Figure 5a; data not shown). Because k_{off} is usually independent of the solution concentration, the dissociation rate constant (k_{dissoc}) was determined by averaging k_{off} for several concentrations.[19,28,35,41,42] The binding constant, K_a, was calculated from $K_a = k_{assoc}/k_{dissoc}$. All kinetic parameters obtained under various conditions were summarized in Table 1. Magnitudes of K_a determined from the kinetic study (Table 1) were consistent with those obtained from EMSA (Figure 2). Either the copolymer or the PN modification increased K_a by nearly 70-fold. However, their kinetic contributions were quite distinct. The copolymer increased k_{assoc} by about 50-fold, while decreasing k_{dissoc} by only 1.5-fold. In contrast to the copolymer, the PN modification decreased k_{dissoc} by ~35-fold, while it moderately increased k_{assoc} by ~2-fold. The combination of the copolymer and the PN modification resulted in a more than 4000-fold increase in K_a. By comparing the kinetic parameters, we note that k_{assoc} and k_{dissoc} obtained for Pyr15NP with the copolymer closely coincide with the mathematical products of values individually obtained for either Pyr15T with the copolymer or Pyr15NP without the copolymer. Consequently, results clearly demonstrate that mutual kinetic influences successfully cooperate to stabilize triplex formation without generating negative interference.

Discussion

The PLL-*g*-Dex copolymer increased K_a for triplex formation at pH 6.8 and 25 °C by approximately 2 orders of magnitude (Figures 2 and 5 and Table 1). The copolymer also increased the T_m for the triplex (Figure 3). These results indicate that the PLL-*g*-Dex copolymer significantly stabilizes the triplex at neutral pH, consistent with our previous results.[33−36] Entropic loss due to counterion condensation and electrostatic repulsion caused by excess accumulation of phosphate anions on triplex formation usually produces triplex instability.[43−45] Therefore, cationic substances, such as polyamines, are employed to stabilize triplex formation.[46,47] Spermine, bearing four positive charges, stabilizes the triplex under low ionic strength.[46,47] However, its stabilizing effect is drastically reduced by increasing ionic strength to physiological levels due to competitive replacement of spermine with coexisting cations.[47] Our previous study[33−36] and the present results described above demonstrate that the PLL-*g*-Dex copolymer maintains its stabilizing efficacy even under physiological ionic strength through polyvalent electrostatic interactions. Reduced counterion condensation, causing a net increased entropy change, may increase K_a.[39,48,49]

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Table 1. Kinetic Parameters for Triplex Formation Involving Pyr15T or Pyr15NP with or without 0.038 mM PLL-*g*-Dex Copolymer in Buffer A (see Materials and Methods in the Supporting Information) at 25 °C, Obtained from Analysis of the IAsys Surface Affinity Capture Assay

TFO	PLL-*g*-Dex copolymer	k_{assoc} (M^{-1} s^{-1})	k_{assoc} (relative)	k_{dissoc} (s^{-1})	k_{dissoc} (relative)	K_a (M^{-1})	K_a (relative)
Pyr15T	−	$(6.31 \pm 0.18) \times 10^2$	1.0	$(1.17 \pm 0.14) \times 10^{-2}$	1.0	$(5.41 \pm 0.91) \times 10^4$	1.0
Pyr15T	+	$(2.88 \pm 0.23) \times 10^4$	45.6	$(7.8 \pm 3.4) \times 10^{-3}$	0.66	$(3.69 \pm 1.32) \times 10^6$	68.2
Pyr15NP	−	$(1.19 \pm 0.13) \times 10^3$	1.89	$(3.36 \pm 0.18) \times 10^{-4}$	0.029	$(3.54 \pm 0.61) \times 10^6$	65.4
Pyr15NP	+	$(5.27 \pm 0.85) \times 10^4$	83.5	$(2.26 \pm 0.18) \times 10^{-4}$	0.019	$(2.33 \pm 0.61) \times 10^8$	4310

As shown in the CD spectra (Figure 4), the copolymer does not change observed triplex highly ordered structures despite stable association, whereas structural changes have been commonly observed for complexes between DNA and cationic homopolymers, such as poly(L-lysine).[50−52] Preservation of triplex highly ordered structures may be important for conserving sequence specific interactions between TFO and the target duplex. Increased K_a by the copolymer resulted largely from the increase in k_{assoc} rather than decrease in k_{dissoc} (Table 1), consistent with our previous study.[35,36] The reason the copolymer increases k_{assoc} rather than decreasing k_{dissoc} is unclear. It may be plausible that the copolymer facilitates nucleation of TFO with a target duplex to accelerate triplex formation. Our recent observation[53−55] that the copolymer considerably accelerates strand exchange reactions between double-stranded DNA and its complementary single-stranded DNA may support this contention. Nucleation complexes comprising three DNA strands are likely involved in transition states for both triplex formation and strand exchange reactions. It seems that the copolymer is capable of stabilizing not only mature triplex but also three-stranded nucleation complexes by reducing counterion condensation effects.

Similar to the PLL-*g*-Dex copolymer, the PN modification increases triplex K_a at pH 6.8 and 25 °C by about 2 orders of magnitude (Figures 2 and 5 and Table 1). This PN stabilization effect was also demonstrated by T_m measurements (Figure 3). These results and others[23,26] indicate that PN modification significantly stabilizes the triplex at neutral pH. Note that the increase in K_a by the PN modification results primarily from the decrease in k_{dissoc} rather than increase in k_{assoc} (Table 1), in sharp contrast with the kinetic effects of the PLL-*g*-Dex copolymer (vide infra). The PN modification stabilization mechanism should be, therefore, distinct from that of the copolymer. While PN modification demonstrates a higher increase in triplex T_m ($\Delta T_m = 35$ °C) compared to the copolymer ($\Delta T_m = 18$ °C), both provided similar K_a and ΔG at 25 °C (Figure 2 and Table 1). The PN modification may affect the temperature dependence of ΔG under constant pressure, that is, $-\Delta S$ [i.e., $(\delta \Delta G/\delta T)_P = -\Delta S$], for the triplex equilibrium differently from the copolymer. In fact, our previous isothermal titration calorimetry study showed that the magnitude for negative ΔS at 25 °C for triplex formation involving Pyr15NP was significantly smaller than that involving Pyr15T.[28] Because the source of the negative ΔS for triplex formation was proposed to be due to TFO conformational restraints involved in triplex formation,[56] the smaller negative ΔS value for PN TFO suggests that PN TFO in its free state may be more rigid than the corresponding PO TFO. This PN TFO rigidity causes a smaller entropic loss upon triplex formation, leading to increased K_a. As the copolymer stabilizes triplexes by reducing counterion condensation, the extent of this condensation for PN TFO was seemingly identical to that for PO TFO (increase in K_a by the copolymer was nearly 70-fold for both PO TFO and PN TFO, Table 1). The extent of such counterion condensation reflects a highly ordered DNA structure,[57,58] so that the PN modification may not alter polyelectrolyte features of the oligonucleotide and its complex with DNA. This is supported by the CD spectra (Figure 4) showing that the PN modification does not alter the triplex highly ordered structure. However, the detailed stabilization mechanism for the PN modification remains to be elucidated.

The combination of the copolymer and PN modification increases triplex K_a at pH 6.8 by about 4 orders of magnitude (Figures 2 and 5 and Table 1). This cooperative effect was also confirmed by T_m measurements (Figure 3). The increase in K_a results mainly from increased k_{assoc} by the copolymer and decrease in k_{dissoc} by PN modification (Table 1). Note that k_{assoc} and k_{dissoc} obtained by synergistic effects of both copolymer and PN modification closely coincided with the mathematical products of values obtained individually (Table 1). These results also clearly demonstrate that neither interference nor negative cooperation between their mutual kinetic effects complicated the synergism.

Different combinations of various methods have attempted to produce cooperative stabilization. Simple combinations of two stabilizing methods had not yet yielded the desired cooperative effects. For example, each of the 2′,4′-bridged nucleic acid modifications[16−21] and PN modifications[22,23,26,28] increased T_m for both duplex and triplex significantly. However, thermal stability for both duplex and triplex produced by these combinations was weaker than that by the 2′,4′-bridged nucleic acid modification alone.[29] That is, strand interference and no cooperativitiy of the two stabilizing methods occurred. Both the 2′,4′-bridged nucleic acid and PN modification seemingly constrain nucleotide conformation, so that their cooperative is difficult. Our previous kinetic studies revealed that these modifications decrease k_{dissoc} to increase K_a,[19,21,28] demonstrating that these modifications contribute similarly to the kinetics of nucleic acid assembly. Negative cooperativity between two triplex-stabilizing methods was also reported for combinations

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of acridine-modified TFO with a triplex-binding ligand.[30] By contrast, we report synergistic stabilizing effects of copolymer addition and the PN modification. Taken together, the observed kinetic parameters can be quite useful indicators for selecting rational combinations of stabilizing methods.

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Supporting Information Available: Experimental procedures and further details. This material is available free of charge via the Internet at http://pubs.acs.org.

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