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Right-Handed Triplex Formed between Peptide Nucleic Acid PNA-T_8 and Poly(dA) Shown by Linear and Circular Dichroism Spectroscopy

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Abstract: The binding of an eightmer of peptide nucleic acid, H-T_8-Lys-NH_2 (=PNA-T_8), to a polynucleotide, poly(dA), was studied by flow linear dichroism (LD) and circular dichroism (CD) spectroscopy. Whereas the single stranded DNA, due to its high flexibility, does not display any measurable LD signal when subjected to shear flow, the complex with PNA does. A titration shows that saturation occurs at a stoichiometry of two PNA thymine bases per DNA adenine base, indicating the formation of a triplex PNA_2–DNA complex. The persistence length of the adduct remains small up to relatively high stoichiometries (above 1:1 T:A) indicating that no significant amounts of PNA:DNA duplex are formed. Instead triplex stretches seem to form surrounded by flexible parts of single stranded poly(dA). Upon approaching the stoichiometry 2:1 of T:A the LD increases dramatically demonstrating that the stiffness of the PNA–DNA triplex arises from base–base contacts preventing bending of the chain. It is also inferred that the main stiffness of duplex DNA very probably has a similar origin and is not primarily a result of the increased phosphate–phosphate repulsion. Circular dichroism spectra support the conclusion that a triplex is formed as the only PNA–DNA complex and that it is a right-handed helix. The wavelength dependence of the reduced linear dichroism shows that the inclination of the bases from perpendicularity relative to the helix axis is small. The base conformation of the poly(dA)[PNA-T_8]$_2$ triplex is very similar to that of the conventional poly(dA)[poly(dT)]$_2$ triplex.

Introduction

Reagents that bind sequence selectively to double stranded DNA are of significant interest in medicinal chemistry and molecular biology since they can provide tools for sequence specific modification of DNA and gene targeted drugs.[1] Oligonucleotides and their analogues capable of forming triple helices have so far been the prime candidates for developing such reagents.[1,2] However, we recently showed that an oligonucleotide analogue

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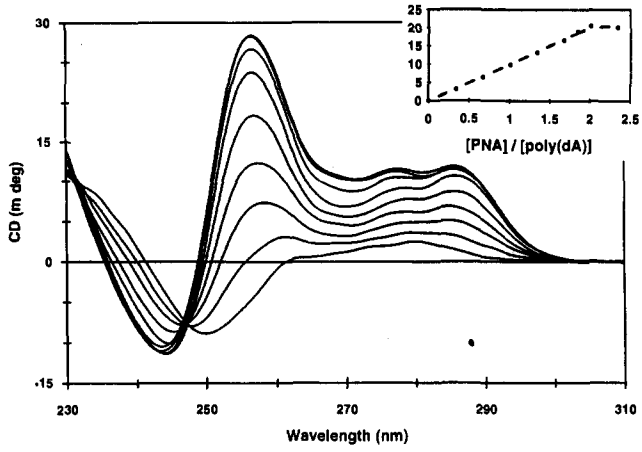

Figure 1. Circular dichroism titration of poly(dA) (constant concentration) with PNA H-T_8-LysNH$_2$ (PNA-T_8). The mixing ratios, [PNA-T_8]/[poly(dA)], are the following from bottom to top at 260 nm: 0.00, 0.33, 0.67, 1.00, 1.33, 1.67, 1.83, 2.00, and 2.33. The CD spectra for the ratios 2.00 and 2.33 were found to be almost identical. The concentration of the poly(dA) was 25 mM, the pathlength was 1 mm, and each spectrum was averaged 10 times.

termed PNA (peptide nucleic acid), in which the entire deoxyribose phosphate backbone is replaced by a chemically completely different but structurally homomorphous polyamide (peptide) backbone, composed of (2-aminoethyl)glycine units (Figure 1), has retained at least some of the hybridization properties of natural DNA.[3] More specifically, we found that very stable, sequence specific complexes of unprecedently high thermal stability are formed between thymine and cytosine containing PNA and complementary (adenine and guanine containing) oligodeoxynucleotides.[3] Titration experiments with UV absorption spectrophotometry indicated that these consist of two PNA strands and one DNA strand.[3] Furthermore, we have found that a homopyrimidine PNA sequence specifically binds to a double stranded DNA target by strand displacement.[4] *In vitro* molecular biological experiments show that binding of homopyrimidine PNA proximal to a restriction enzyme recognition site inhibits DNA cleavage by the enzyme at this site and that binding of homopyrimidine PNA downstream from an RNA polymerase promoter site causes blockage of transcription at this site.[5,6] Finally, evidence has been obtained indicating that PNA, when microinjected into mammalian cells, exhibits a gene specific antisense effect, probably due to ribosome translation elongation arrest at the PNA binding site on the mRNA.[7]

Thus PNA is from several angles a most interesting DNA analogue for medicinal chemistry.[8] Since the PNA backbone is achiral and contains neither the sugar nor the phosphate moities of the normal DNA backbone, it is also of interest to study the structures of the PNA/DNA complexes, since they could provide various pieces of information about interaction and structure properties of the DNA double helix itself.

In the present study we use circular and linear dichroism spectroscopy to characterize binding stoichiometry, conformation, and chain flexibility of the DNA–PNA complex formed between a single-strand poly(dA) chain and a complementary eightmer of PNA.

Experimental Section

Chemicals. Poly(dA) and poly(dT), purchased from Pharmacia, were dissolved in 5 mM phosphate buffer, containing 100 mM NaCl and 1 mM EDTA, at pH 7.0 and dialyzed several times against 5 mM phosphate buffer at pH 7.0. This buffer was used through out the work. The concentrations were determined spectrophotometrically by using the molar extinction coefficients: $\epsilon_{257} = 8600\ M^{-1}\ cm^{-1}$ for PNA-T_8 and poly(dT). The poly(dA)[poly(dT)]$_2$ triplex was prepared by mixing, in 1:2 concentration ratio, poly(dA) and poly(dT) in the presence of 1 mM $MgCl_2$ followed by simming at 90 °C for 15–20 min and then annealing over night at room temperature. Triple-helical poly(dA)[PNAT_8]$_2$ was obtained by mixing poly(dA) and PNA-T_8 at room temperature and annealing over night without heating. PNA, H-T_8-Lys-NH$_2$, was synthesized as described elsewhere.[3]

Measurements. Circular dichroism (CD), defined as differential absorption of left and right circularly polarized light, was measured on a Jasco 720 spectropolarimeter using either a 1 cm or a 1 mm quartz cell. Linear dichroism (LD) is defined as differential absorption of linearly polarized light, polarized parallel and perpendicular, respectively, to the flow direction in a Wada-type Couette flow cell:[9]

$$LD(\lambda) = A_{\parallel} - A_{\perp}$$

LD is affected by factors such as the contour length and flexibility of DNA, temperature, flow gradient, flow symmetry, and solution viscosity.[9] LD was measured on a Jasco 500A spectropolarimeter, converted from CD to LD mode and calibrated as described before.[9b] All measurements were performed at ambient temperature.

The reduced linear dichroism (LD^r) is a dimensionless quantity, defined as:[9a]

$$LD^r(\lambda) = LD(\lambda)/A_{iso}(\lambda) \quad (1)$$

where $A_{iso}(\lambda)$ is the absorption spectrum of the isotropic sample, i.e. in the absence of flow. LD^r is related to the orientation of the light absorbing transition moments according to:[9a,c]

$$LD^r(\lambda) = SO(\lambda) = \frac{3}{2}S\sum F_i(3\cos^2\alpha_i - 1) \quad (2)$$

where S is an orientation factor $0 \le S \le 1$ describing the degree of orientation of the DNA helix axis relative to the flow direction, $O(\lambda)$ is an optical factor defined by the second sign of equality of eq 2, given by the angles α_i between the helix axis and the transition moments responsible for the absorption of light, and the weight factors $F_i = \epsilon(\lambda)_i/\sum\epsilon(\lambda)_i$ defining their respective contributions to the absorption at the particular wavelength λ.

The π–π^* transitions of the DNA bases that are responsible for the LD of the polynucleotides around 260 nm are polarized at various angles in the plane of the bases. If the base planes were all perfectly perpendicular to the helix axis, α_i would all be equal to 90° for the π–π^* transitions so that $O(\lambda) = -1.5$ and, hence, LD$^r(\lambda)$ would be constant and not dependent on wavelength. However, when the bases are inclined from perpendicularity (defined by the roll and tilt angles)[9c–e] various degrees of wavelength dependency of the optical factor $O(\lambda)$, and of LD$^r(\lambda)$, are anticipated. For a wormlike (very long) chain the orientation factor, at low degrees of orientation, is proportional to the persistence length, P_∞, of the chain:[9a,f]

$$S = P_\infty\,(^2/_3L)\,K\beta^2 \quad (3)$$

with L the contour length of the chain, K the drain parameter, and β the Peterlin gradient parameter. The increase in LD upon interaction between poly(dA) and PNA-T, exploited here for the probing of the complex

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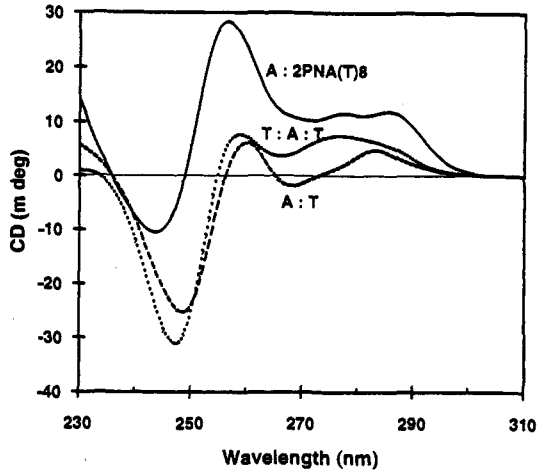

Figure 2. CD spectrum of poly(dA)[PNA-T_8]$_2$ compared with CD spectra of duplex poly(dA)poly(dT) and triplex poly(dA)[poly(dT)]$_2$. Conditions as in Figure 1.

formation, is a result of increased S as the persistence length increases as stiff triple-helical segments are introduced into the flexible poly(dA) chain.

Results

Figure 1 shows the CD spectrum of poly(dA), at fixed concentration, in the presence of H-T_8-Lys-NH_2 (=PNA-T_8) at various concentrations. Except for the terminal lysine, PNA is inherently achiral and exhibits by itself only a very weak CD. Therefore, any changes observed in the stronger CD spectrum of poly(dA) in the presence of PNA must be due to either changed CD of poly(dA) itself or to additional CD, from helically stacked PNA thymine bases, in either case as a result of formation of poly(dA)–PNA-T_8 complexes. The CD titration clearly shows that 1 mol of poly(dA) (in adenine bases) is saturated by 2 mol of PNA-T_8 (in thymine bases) indicating a triplex structure of the complex. Furthermore, isosbestic points at 232 and 248 nm are observed during the titration suggesting that just two species are present; poly(dA) and triplex poly(dA)[PNA-T_8]$_2$. A confirmation of this conclusion is provided by LD (vide infra).

The CD spectrum of the 1:2 complex of poly(dA)[PNA-T_8]$_2$ is compared with that of a poly(dA)·poly(dT) duplex and a poly(dA)[dT]$_2$ triplex in Figure 2. The CD spectrum of the poly(dA)·2PNA-T_8 mixture gives a sign pattern similar to that of poly(dA)·poly(dT) duplex, suggesting that it has a right-handed helical structure. The shape of the spectrum shows further resemblance to the CD spectrum of poly(dA)·2poly(dT) supporting the theory that it is a triplex. The positive maxima at 257, 277, and 285 nm, a negative minimum at 244 nm, and crossover points at 232 and 247 nm are thus characteristics of the poly(dA)[PNA-T_8]$_2$ complex.

A remarkable thermal stability of the poly(dA)[PNA-T_8]$_2$ complex was shown by the CD measurements (Figure 3). The CD signal at 258 nm showed only a slight decrease up to 50 °C which could indicate a minor structural change such as, for example, a conformational redistribution due to larger amplitudes of thermal motion in the complex. The temperature dependence in Figure 3B gives a midpoint of melting at about 75–80 °C, but melting appears not to be complete even at 80 °C. This melting interval is significantly higher than the T_m found for an oligonucleotide d(A)$_8$[PNA-T_8]$_2$ triplex by UV-melting experiments (52 °C) under somewhat different conditions.[3a] However, when the latter type of experiment was performed on poly(dA)-[PNA-T_8]$_2$, T_m = 79.5 °C was obtained, which is consistent with the results in Figure 3B.

Flow linear dichroism (LD) spectra of poly(dA) in the presence of various amounts of PNA-T_8 are shown in Figure 4A. Poly(dA) alone exhibits no measurable LD signal at our flow conditions due to its very high flexibility (short persistence length) which prevents it from orienting significantly in the flow gradient. However, upon addition of PNA-T_8, a strong negative LD signal appears in the adenine/thymine absorption region with a maximum at 260 nm (Figure 4A). Just as with CD, saturation is distinctly obtained at a poly(dA)/PNA-T_8 ratio of 1:2 (Figure 4B). The LD intensity at 260 nm is not proportional to the PNA-T_8 concentration which contrasts with the results of the CD titration. At low PNA-T_8 concentration the increase per added amount of PNA-T_8 is small, but close to the saturation point the LD intensity increases sharply, indicating that the stiffness of the complex increases abruptly at this point (see Discussion).

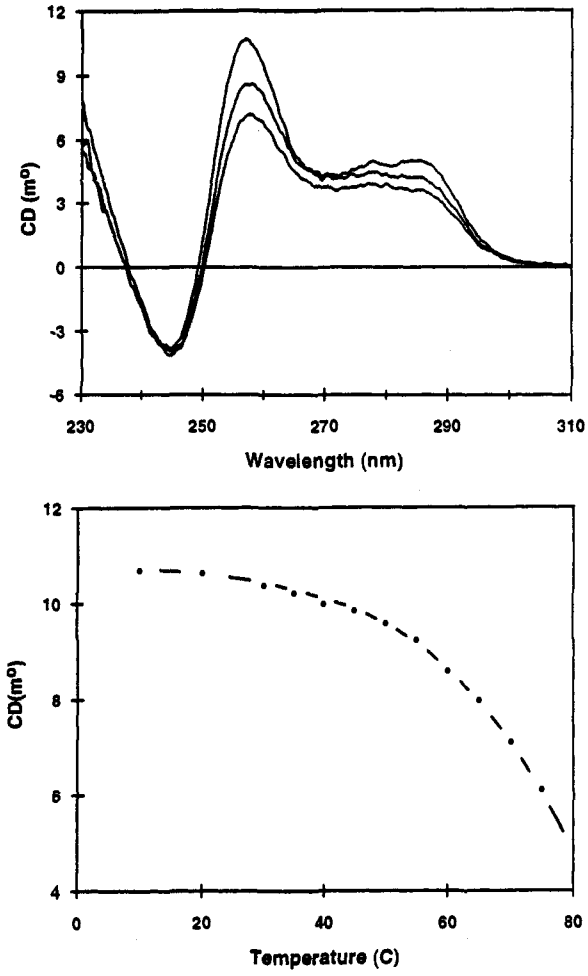

Figure 3. Temperature dependence of circular dichroism of poly(dA)-[PNA-T_8]$_2$ complex. (A, top) CD spectra at 10 °C (top spectrum), 60 and 70 °C. (B, bottom) CD signal at 258 nm gradually decreases with increasing temperature and complete melting is not observed before 80 °C.

The reduced linear dichroism spectrum, LD$^r(\lambda)$, of the poly(dA)[PNA-T_8]$_2$ complex is compared with that of the poly(dA)-[poly(dT)]$_2$ triplex in Figure 5. The spectra are scaled to −1.0 at 260 nm. The wavelength dependence of LD^r in the 240–290 nm region is very similar, indicating a similar tilt and roll conformation of the bases in the two triplex complexes (see Discussion).

Discussion

Circular Dichroism. The CD titration (Figure 1) shows clearly that two PNA-T_8 bases complex one poly(dA) base. The change (insert of Figure 1) is approximately proportional to the amount of added PNA-T_8 all the way up to the saturation point. This suggests the binding to be very strong. However, the two isosbestic

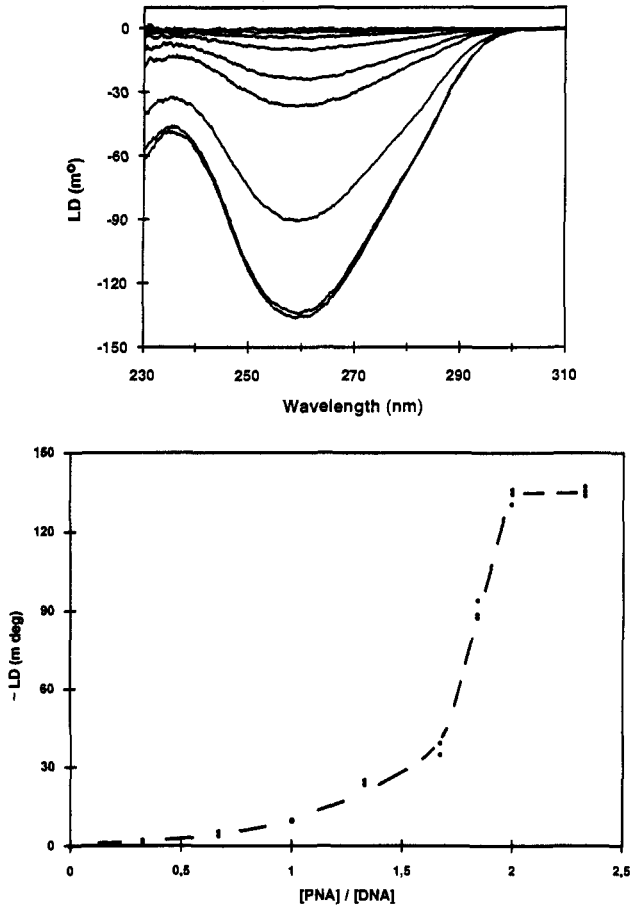

Figure 4. Flow linear dichroism spectra of PNA-T_8 + poly(dA) mixtures. The mixing ratios are the same as those in Figure 1. (A, top) Increasing the PNA/DNA ratio gives an increasingly stronger negative LD band with a maximum at 258 nm. The LD spectra for the ratios 2.00 and 2.33 are almost identical. Shear gradient was 3600 s^{-1} and pathlength was 1 *mm*. The conversion factor between ellipticity units of the Jasco instrument and dichroism in absorbance units, θ (deg), is 33.0 × ΔA (absorbance units). (B, bottom) Negative LD signal at 258 nm plotted versus the PNA/DNA ratio (three experiments). LD increases rapidly when approaching the stoichiometry of two PNA thymines per DNA adenine. The LD is saturated at this stoichiometry.

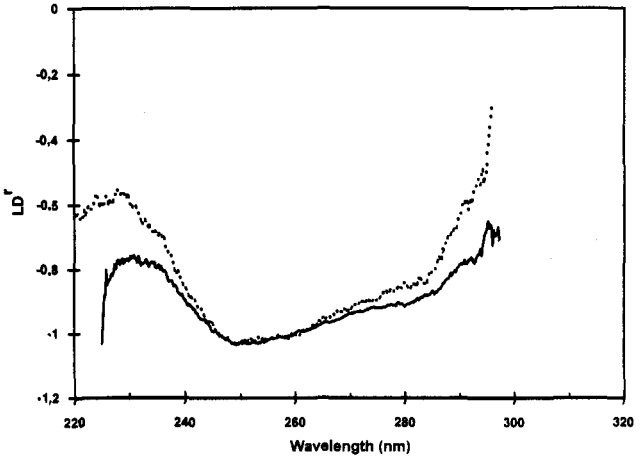

Figure 5. Reduced linear dichroism spectra, $LD^r(\lambda)$, of complex poly(dA)[PNA-T_8]$_2$ (—) and of triplex poly(dA)[poly(dT)]$_2$ (- - -). LD^r spectra of both systems are scaled to −1 at the LD maxima at 258 nm.

points at 247 and 232 nm indicate that just two species are contributing to the CD spectra. Since the complex formation should fulfill the kinetic scheme

$$\mathrm{DNA} + 2\mathrm{PNA} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{DNA\text{–}PNA} + \mathrm{PNA} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \mathrm{DNA\text{–}(PNA)_2}$$

the CD titration shows that the population of duplex DNA–PNA is very small compared to that of DNA–PNA_2 and, hence, that $k_{+2}/k_{-2} \gg k_{+1}/k_{-1}$. In other words, the triplex is solely responsible for the strong induced CD intensity and the complex formation is totally cooperative in the sense that two PNA:s immediately close up in a pair on either side of an $(A)_8$ stretch of poly(dA) to concomitantly form $(TAT)_8$ stretches. As we shall see from the LD experiment, there is no evidence for any (positive) cooperativity among the T8 stretches on the same "strand" but these are relatively evenly spread and readjust themselves (slide) along the poly(dA) strand to allow newcomers to bind,

The CD spectrum of the poly(dA)[PNA-T_8]$_2$ triplex (Figure 2) shows a resemblance to the duplex poly(dA)·poly(dT) and an even stronger resemblance to the triple helical poly(dA)[poly(dT)]$_2$. We consider this to be strong evidence in favor of the PNA/DNA complex being a right-handed triple helix and having a conformation with respect to base pair stacking that is similar to that of a T–A–T triple helical DNA. Still, the intensity of the positive near-ultraviolet CD bands is significantly stronger than that for the pure T–A–T triplex. Since the bases are evidently stacked in similar ways, with their planes preferentially perpendicular to the helix axis for both A(PNA-T)$_2$ and the conventional AT_2 triplex (see below), this difference in CD spectra could indicate that the helical winding angle may differ somewhat between the two types of triplexes.

The poly(dA)[PNA-T_8]$_2$ triplex exhibits a remarkable thermal stability (Figure 3, spectra A and B). A small reduction of CD up to 50 °C could be due to a minor conformational redistribution within the triplex structure. The estimated inflection point of melting, around 75–80 °C, is much higher than that of the poly(dA)·poly(dT) duplex and the poly(dA)[poly(dT)]$_2$ triplex and is also higher than the melting point of 52 °C observed for the d$(A)_8$-[PNA-T_8]$_2$ complex.[3] The lower melting point of the latter triplex, compared to the one with a polynucleotide strand, is most likely the effect of instability as a result of breathing at the ends of this short oligonucleotide complex, well-known from NMR studies on oligonucleotide duplexes.[10] The high T_m for poly(dA)[PNA-T_8]$_2$ was confirmed by UV photometry as well.

Linear Dichroism. The appearance of a substantial LD signal (Figure 4A) shows that the poly(dA)[PNA-T_8]$_2$ complex is rigid since it can be oriented, in contrast to the very flexible poly(dA) chain (cf. eq 3). The shape of the LD titration curve (Figure 4B) indicates that the persistence length of the poly(dA)-PNA complex, as reflected by its orientability, is not linearly dependent on either the [PNA]/[poly(dA)] or the $[PNA]^2/[DNA]$ ratio. Rather, a dramatic increase in the LD signal, indicating a corresponding increase in rigidity, is observed as saturation is approached. This result suggests a non-cooperative binding distribution such that, at low and intermediately high binding ratios, the PNA-T_8 stretches are rather scattered over the poly(dA) template, with flexible "hinges" of uncomplexed template in between. However, as saturation is closely approached rearrangement occurs so that no single-stranded flexible regions are left uncovered. In addition to intrinsic rigidity of each triplex [PNA-T·dA·PNA-T]$_8$ stretch, base stacking between proximal PNAs will then contribute extra rigidity to the chain.

Although the individual absorption bands of the different transition moments of the DNA bases in the 230–290 nm region cannot be resolved but overlap each other extensively,[9c] the fact that the strongly negative LD^r of DNA in this region is relatively constant is an indication that the bases are not markedly inclined from a perpendicular orientation relative to the DNA helix axis.[9c,d] The $LD^r(\lambda)$ spectrum of the poly(dA)[PNA-T_8]$_2$ triplex may be compared with that of the poly(dA)[poly(dT)]$_2$ triplex (Figure

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5). The wavelength dependence of LD^r is similar for the two triplexes and somewhat resembles that of DNA. The base tilt of the poly(dA)·[PNA-T_8]$_2$ triplex is concluded to be similar to that of the poly(dA)[poly(dT)]$_2$ triplex. We have also studied the LD of a DNA probe, 4′,6-diamidino-2-phenylindole (DAPI), upon binding to the two triplexes poly(dA)·[PNA-T_8]$_2$ and poly(dA)·[poly(dT)]$_2$ (results not shown); the orientation of the probe, at roughly 45° in both cases, suggests that the two triplexes also have a similar helical pitch.

T–A–T Cooperativity. Both CD and LD consistently show that PNA-T_8 binds pairwise in a triplex fashion to the poly(dA) chain. This conclusion indicates that the free-energy gain by hydrophobic interactions of the second PNA-T_8 in the major groove of a duplex (dA)-(PNA-T_8) structure more than compensates for the fact that only 1 set of Watson–Crick + 1 set of Hoogsteen base-pair hydrogen bonds are formed per pair of PNAs, in contrast to the 2 Watson–Crick sets of bonds that would have been the result if the two PNAs had instead preferred to bind in a duplex manner. We believe an origin of this cooperativity of binding 2 T to each A may be an anticipated hydrophobic nature of the central part of each PNA-T_8 stretch in combination with the positive charges that it carries at its ends: each [PNA-T_8]-$(dA)_8$-[PNA-T_8] unit might in this way behave like a micell, e.g., providing a hydrophobic interior and a polar surface. Some support for this speculation is the distinct saturation of LD only at 2 PNAs per DNA base. If the triplex had formed preferentially as long sequences, favored by base-stacking between adjacent PNA-T_8 units, one would have expected an earlier stiffening of the chain and an earlier rise in LD. The late stiffening could indicate that the positive end groups of the PNA units make these distribute evenly along the poly(dA) template. The fact that saturation is reached at stoichiometry 2, finally, means that the PNA can redistribute itself over the poly(dA) so as to eliminate gaps of 7 or fewer bases into which PNA cannot bind.

DNA Stiffness. The flow LD titration experiment (Figure 4B) unambiguously demonstrates that the poly(dA) chain becomes stiff only very close to the point when it is stoichiometrically saturated by PNA-T_8 in a T–A–T complex. An important byproduct of this result is a conclusion regarding stiffness that could be extended to duplex DNA and stacked nucleic acids in general: the basic stiffness toward bending is a result of steric interactions when the bases in the triplex (or duplex) in a coin-pile manner come so close to each other that they prevent bending of the chain. This conclusion may seem trivial regarding the well-known great difference in persistence length between single-stranded and double-stranded DNA. However, electrostatic effects are also important and have dominated current discussions of flexibility of nucleic acids.[11] The negative phosphate charge density is doubled in duplex DNA, and the spatial distribution of the negative charges defines a potential that is related to the effect on persistence length of DNA by the presence of electrolytes. For example, flow LD experiments on long DNA show that P_∞ is strongly reduced (eq 3), from the range of 100 nm at low ionic strength (mM) to 35–60 nm at salt concentrations of 0.1–1.0 M NaCl. It is in this limit of high ionic strength, at which the phosphate–phosphate repulsion is minimized, that one would expect to observe a P_∞ value that is representative for the stiffness according to a "coin-pile", steric mechanism. We conclude in any case that the persistence-length increase, responsible for the flow LD increase in Figure 3, is entirely of such a steric nature since there is no increase (but rather a decrease) in phosphate–phosphate repulsion as PNA binds to poly(dA).

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