Peptide Nucleic Acids

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γ-Substituted Peptide Nucleic Acids Constructed from L-Lysine are a Versatile Scaffold for Multifunctional Display**

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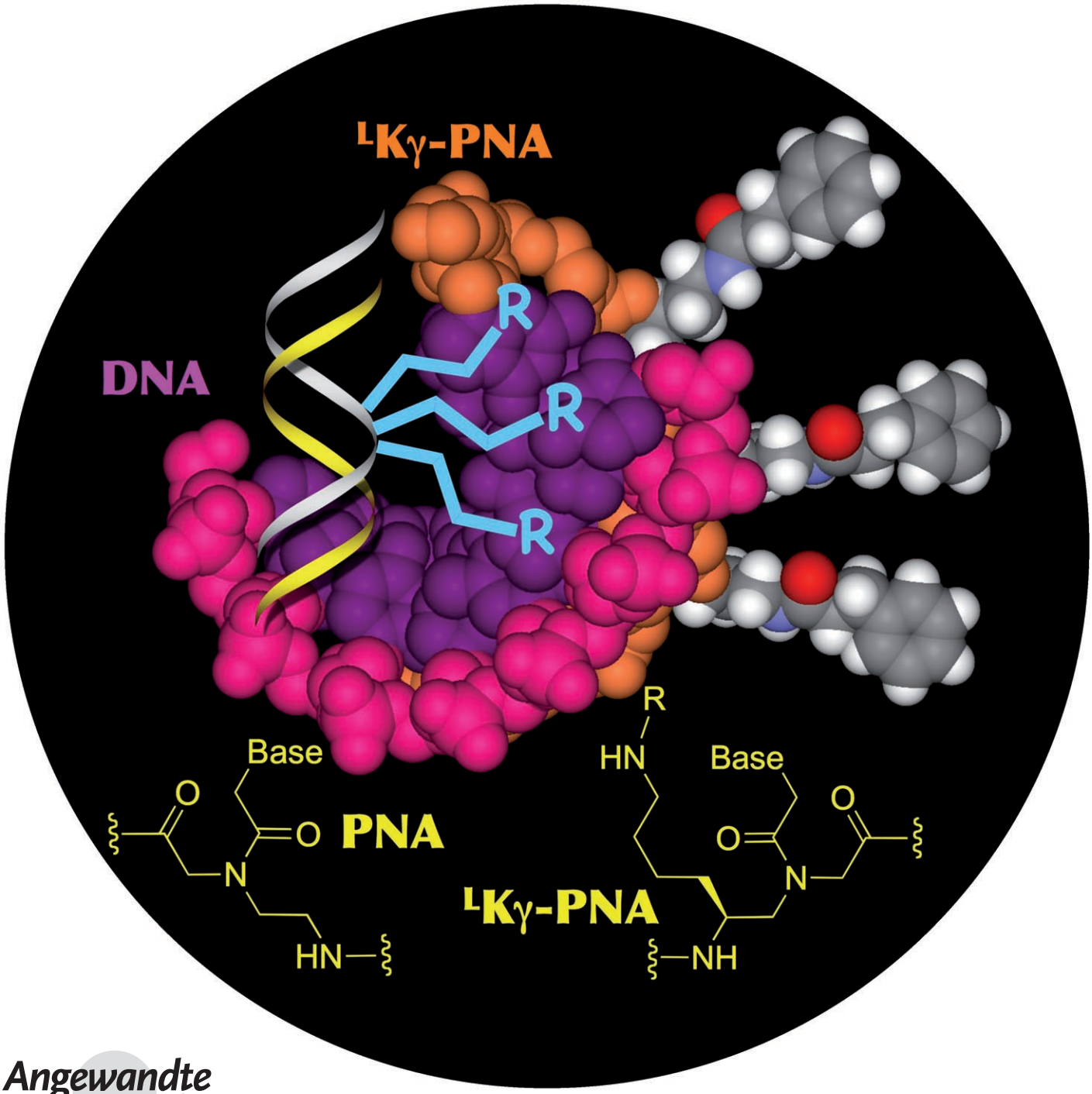
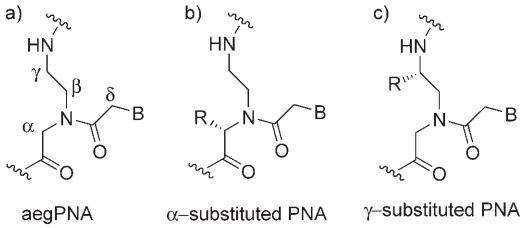


Peptide nucleic acids (PNAs), which contain an achiral, uncharged aminoethylglycine (aeg) backbone, bind natural nucleic acids through Watson–Crick base pairing with high affinity and selectivity (Scheme 1a).[1–3] PNA oligomers are not enzymatically degraded,[4] do not require high salt concentration for binding,[2] and are useful in nucleic acid detection systems[5] and as antisense molecules.[6]

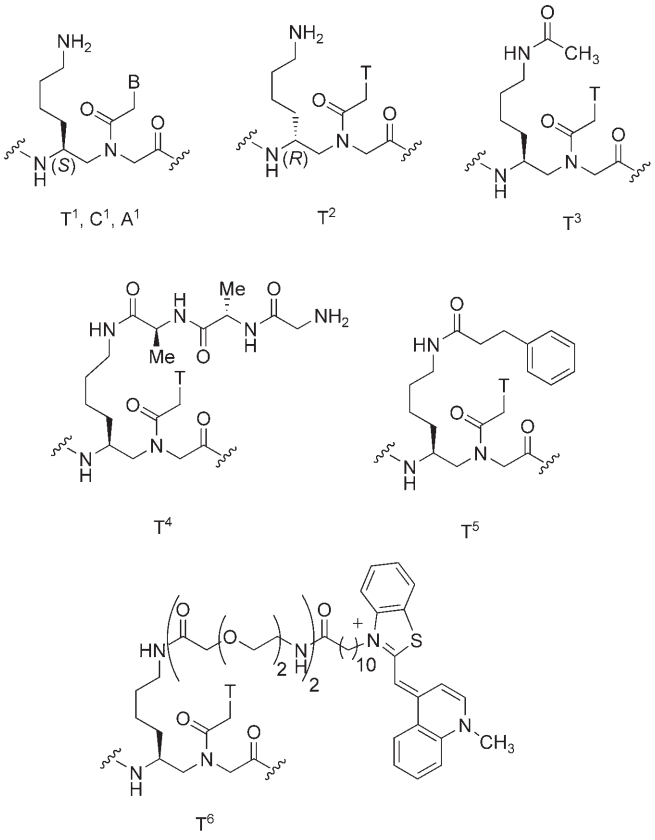


Scheme 1. aegPNA and backbone positions for side chains.

PNA research has focused on improving the favorable binding properties of aegPNAs[7] while eliminating some of the inherent weaknesses (e.g. solubility and bioavailability).[8] Common strategies include conjugating moieties to PNA termini,[9] utilizing PNA/DNA chimeras,[10] and synthesizing PNA derivatives with backbone modifications.[11] Although there are currently a large number of PNA derivatives and variations, one challenging area of research has been the strategic placement of side chains on the aegPNA scaffold. In this regard, PNAs synthesized from chiral amino acids in which the side chains extend from the α-carbon atom have been examined (Scheme 1b).[12] The presence of positively charged side chains at the α-carbon atom can result in improvements in cellular uptake and duplex stability.[13] However, neutral or negatively charged side chains from the α position destabilize PNA/DNA duplexes, limiting their utility. Some research has focused on appending functionality onto these side chains, but in these cases, the modified PNA/DNA duplexes are less stable than unmodified aegPNA/DNA duplexes.[14] Recent studies also show that having multiple α side chains positioned on adjacent PNA residues is detrimental to duplex stability, although such arrangements improve the specificity of binding.[15] A strategy that improves upon aegPNA binding properties while providing a facile means of conjugating functionality directly to PNA residues has the potential to positively impact many applications that employ aegPNA and stimulate development of new PNA-based applications.

We initiated a program investigating the γ position on the aegPNA backbone for its potential as an effective conjugation point (Scheme 1c).[16] In particular, L-lysine γ-PNA (^{L}Kγ-PNA) is ideally suited to support a broad range of functional groups without interfering with the binding to DNA or RNA. Since our initial report, other researchers have similarly found that side chains extending from the γ position of aegPNA are tolerated.[17] We herein present an extensive analysis of ^{L}Kγ-PNA to demonstrate its improved binding affinity to DNA and RNA compared with aegPNA, regardless of the group that is conjugated to the amine of the lysine side chain. The advantage of this approach is that the primary amine of the Lys-derived side chain can be easily modified to display many different groups. Furthermore, this basic strategy opens the possibility of using PNAs as a scaffold for the multifunctional display of groups in well-ordered, three-dimensional arrays.

Several moieties were attached to the γ side chain to gauge its stability when incorporated into aegPNA oligomers (Scheme 2). Compared with an aegPNA oligomer, PNA oligomers with ^{L}Kγ-PNA containing a primary amine on the side chain (T^1, C^1, A^1) show increased melting temperatures



Scheme 2. ^{L}Kγ-PNA monomers. T^1, C^1, A^1, and T^2 were synthesized by using Cbz-protected side chains. T^3, T^4, T^5, and T^6 were synthesized by using Fmoc-protected side chain amines, which were deprotected during PNA synthesis on the solid support and coupled to carboxylic acids. Cbz = benzyloxycarbonyl; Fmoc = 9-fluorenylmethoxycarbonyl.

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(T_m) when complexed with the complementary antiparallel DNA (Table 1, PNA molecules 1–4). This increase in T_m was consistent for different nucleobase monomers (thymine, adenine, and cytosine) and demonstrated an additive effect when multiple thymine monomers were incorporated (Table 1, PNA **8**). Because of the important role that chirality and flexibility can have in PNA/DNA duplex formation, the stereochemistry of ^{L}Kγ-PNA was examined. Oligomers incorporating D-lysine γ-PNA (T^2) showed greatly reduced binding affinity with antiparallel DNA. Modeling based on the NMR-solved structure of complexed PNA/DNA[18] suggests that the side chains with *S* stereochemistry will orient along the periphery of the duplex, whereas side chains with the *R* stereochemistry are directed to the interior of the duplex (Figure 1). To determine whether nonspecific charge–charge interactions contribute to binding, the sequence specificity was examined. When PNA **2** is annealed to DNA with a single base mismatch across from the γ-PNA residue T^1, a difference in T_m was observed equal to or greater than aegPNA mismatches (see the Supporting Information).

Table 1: Thermal melting data[a] for PNA decamers bound to complementary antiparallel DNA.

PNA	Sequence[b]	T_m [°C]
1	H_2N-G T A G A T C A C T-Lys	49.7
2	H_2N-G T A G A T^1 C A C T-Lys	51.4
3	H_2N-G T A G A^1 T C A C T-Lys	53.1
4	H_2N-G T A G A T C^1 A C T-Lys	51.8
5	H_2N-G T A G A T^2 C A C T-Lys	36.4
6	H_2N-G T A G A T^3 C A C T-Lys	51.2
7	H_2N-G T A G A T^4 C A C T-Lys	51.7
8	H_2N-G T^1 A G A T^1 C A C T^1-Lys	54.5
9	H_2N-G A C T T T A C G A-Lys	42.4
10	H_2N-G A C T^5 T^5 T^5 A C G A-Lys	49.3
11	H_2N-C C T C T T C C T C-Lys	34.5
12	H_2N-C C T C T^6 T C C T C-Lys	44.6
13	H_2N-C C T C T^6 T C C T^6 C-Lys	46.4

[a] Solutions of DNA/PNA (1:1) were prepared in pH 7.0 buffer solution consisting of 10 mM sodium phosphate, 0.1 mM EDTA, and 150 mM NaCl. Strand concentrations were 5 μM in each component. Estimated error is ±0.5 °C. [b] Sequences are written from the N terminus to the C terminus. PNA molecules **1**–**8** were bound to DNA 3′-CATCTAGTGA-5′, **9** and **10** were bound to DNA 3′-CTGAAATGCT-5′, and **11**–**13** were bound to DNA 3′-GGAGAAGGAG-5′. The ^{L}Kγ-PNA monomers are underlined for clarity.

Several oligomers containing ^{L}Kγ-PNA were synthesized with a range of functional groups appended to their primary amines to test whether bulky or nonionic groups could be tolerated. When an oligomer containing an acetamide group on the γ-Lys side chain (Scheme 2, T^3) was annealed to the target DNA, the T_m was also higher than the analogous aegPNA oligomer, further suggesting that the increase in duplex stability is independent of positive charge on the side chain. This increase in stability is consistent with results from Dragulescu-Andrasi et al., who recently showed that γ substitution with a serine-derived side chain in PNA monomers enhances binding affinity by preorganization of the PNA oligomer.[17a] The circular dichroism (CD) spectrum of PNA **8** suggests a definite helical conformation in the absence of DNA (Figure 2). Considering the interest that PNA/peptide conjugates have generated,[19] a small peptide chain (Ala, Ala, Gly) was synthesized through Fmoc chemistry from the side chain T^4; this oligomer (Table 1, PNA **7**) also showed a consistent increase in T_m.

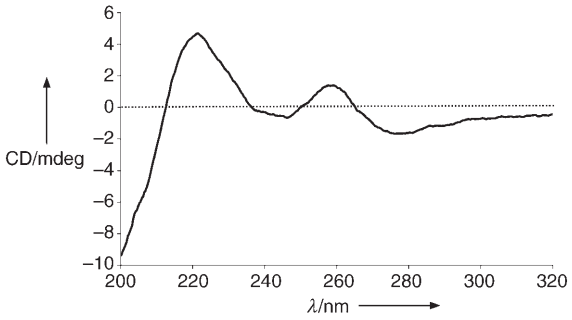

Figure 2. CD spectrum of PNA **8** (25.0 μM). The spectrum was measured at 25 °C and represents an average of eight scans collected at 100 nm min^{-1}.

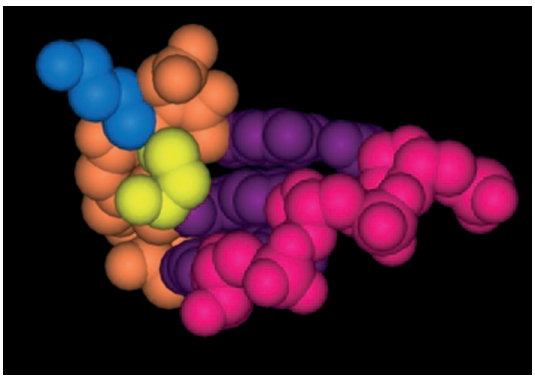

Figure 1. A model of Lys γ-PNA/DNA duplex (purple = base pairs, orange = PNA backbone, fuscia = DNA backbone). The side chain at the γ position derived from L-(*S*)-lysine projecting away from the duplex is shown in blue. The side chain at the γ position derived from D-(*R*)-lysine projecting into the minor groove is shown in yellow.

To determine the viability of using γ-PNA coupled to bulky substituents that are located in close proximity, three adjacent thymine γ-Lys residues (Scheme 2, T^5) were coupled to hydrocinnamic acid (Table 1, PNA **10**). The oligomer showed a significant increase in T_m compared with the aegPNA decamer (PNA **9**). The corresponding single-base-mismatch studies also showed slightly increased mismatch discrimination for ^{L}Kγ-PNA **10** compared with its aeg analogue (see the Supporting Information).

In some cases, backbone-modified PNAs can bind DNA or RNA selectively.[20] Therefore, the binding of ^{L}Kγ-PNA to RNA was examined. Two ^{L}Kγ-PNA oligomers, one composed of T^1 and the other containing multiple, bulky substituents T^5, were annealed to complementary RNA (Table 2). Both of the ^{L}Kγ-PNA molecules showed increases in duplex stability compared with aegPNAs that are very similar to the increase observed when ^{L}Kγ-PNA is annealed to DNA. A ^{D}Kγ-PNA oligomer (containing T^2) exhibited a large decrease in

Table 2: Thermal melting data[a] for PNA decamers and complementary antiparallel RNA.

PNA	Sequence[b]	T_m [°C]
1	H_2N-G T A G A T C A C T-Lys	54.8
2	H_2N-G T A G A T^1 C A C T-Lys	57.3
5	H_2N-G T A G A T^2 C A C T-Lys	42.1
9	H_2N-G A C T T T A C G A-Lys	52.6
10	H_2N-G A C T^5 T^5 T^5 A C G A-Lys	56.6

[a] Solutions of RNA/PNA (1:1) were prepared in pH 7.0 buffer consisting of 10 mM sodium phosphate, 0.1 mM EDTA, and 150 mM NaCl. Strand concentrations were 5 μM in each component. Estimated error is ±0.5 °C. [b] Sequences are written from the N terminus to the C terminus. PNA molecules **1**, **2**, and **5** were bound to RNA 3′-CATCTAGTGA-5′; PNA molecules **9** and **10** were bound to RNA 3′-CTGAAATGCT-5′. The ^{L}Kγ-PNA monomers are underlined for clarity.

thermal stability when bound to complementary RNA. This finding was also consistent with the binding affinity of ^{D}Kγ-PNA oligomers to DNA. These results strongly indicate that ^{L}Kγ-PNA binding properties are common to both DNA and RNA.

PNA oligomers incorporating a fluorescent molecule, such as thiazole orange,[21] have been used as detection probes.[22] Expanding on our previous PNA-beacon strategy,[16] a thiazole orange carboxylic acid derivative was coupled to the γ-Lys side chain (Scheme 2, T^6) in a polypyrimidine PNA oligomer. Thiazole orange emits very low fluorescence in its unbound state. However, when present in a nucleic acid duplex, thiazole orange base stacking limits its bond rotation and the molecule emits a fluorescent signal.[23] When an oligomer containing the T^6 residue (PNA **12**) was unbound or in the presence of noncomplementary DNA, there was a very low background fluorescence (Figure 3). However, when annealed to the complementary DNA, PNA **12** gave a large increase in fluorescent intensity (about two orders of magnitude). This increase was consistent with the increase seen when thiazole orange was coupled to the N terminus of the same oligomer (fluorescent intensity approximately 42-times greater than at 530 nm).[24] Because the ^{L}Kγ-PNA residue is located in the middle of the sequence, base stacking on the end of the duplex is not possible (even taking into account the length of the linkers). Therefore, bond rotation of thiazole orange must be restricted due to intercalation[22b] or some other type of noncovalent association. Because aegPNA lacks possible positions where further functional groups can be easily appended, most aegPNA oligomers only have one functional group attached per oligomer, usually to the N terminus. By using the ^{L}Kγ-PNA, multiple fluorophores can be incorporated into a single-molecular probe, increasing sensitivity of the probe without negatively affecting the T_m of the PNA/DNA duplex. When the oligomer containing two T^6 residues (PNA **13**) was combined with the complementary DNA, a similar increase in fluorescence intensity was observed. Although the effect of having two thiazole orange molecules attached to PNA **13** did not double the fluorescent intensity, there was a 30% larger fluorescent intensity compared with the oligomer with one thiazole orange fluorophore attached (Figure 3).

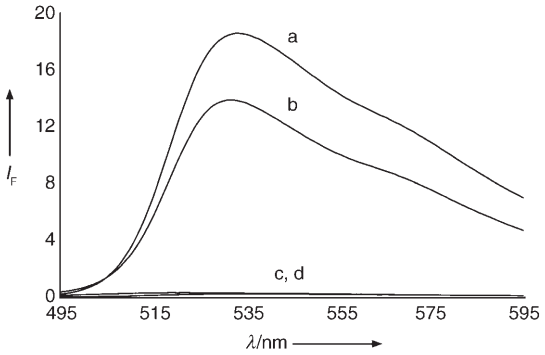

Figure 3. Fluorescence intensity (I_f) of ^{L}Kγ-PNAs with thiazole orange attached to the side chains: a) PNA **13** combined with the complementary DNA; b) PNA **12** combined with the complementary DNA; c) PNA **13** by itself; and d) PNA **12** by itself.

Herein, we have shown that ^{L}Kγ-PNA is a versatile scaffold to attach a range of functional groups without compromising the ability of PNAs to bind to complementary nucleic acid sequences. In addition to this versatility, the chemistry to introduce different side chains onto ^{L}Kγ-PNAs can be performed during solid-phase synthesis of the PNA oligomer by judicious selection of orthogonal protecting groups. We hope that these basic properties of ^{L}Kγ-PNAs can be used to impact the areas of diagnostics and therapeutics where aegPNA is currently used, and stimulate creative new uses of PNAs as a scaffold for multifunctional display.

Experimental Section

^{L}Kγ-PNA monomer synthesis and characterization is described either in the Supporting Information or was described previously.[16] Analytical TLC was carried out on Sorbent Technologies TLC plates precoated with silica gel (layer thickness of 250 mm). Flash column chromatography was performed by using a Biotage Flash12+ apparatus on Biotage Si 25M columns. Fmoc-8-amino-3,6-dioctanoic acid was purchased from Peptides International, Inc. BocLys(Cbz)-OH (Boc = *tert*-butoxycarbonyl) was purchased from Advanced ChemTech. Unless otherwise noted, all other commercially available reagents and solvents were purchased from Aldrich and used without further purification. Dichloromethane, THF, and *N*,*N*-dimethylformamide was passed through a column of activated alumina.[25] Unless otherwise indicated, all reactions were performed under an inert atmosphere of N_2. Glassware was dried in an oven at 180 °C for at least 2 h prior to use. PNA oligomers were made by manual solid-phase synthesis on 4-methylbenzhydrylamine (MBHA) resin as previously described.[7] Oligomers were purified by reverse-phase HPLC and characterized by electrospray or MALDI mass spectrometry. The oligomers were quantified by using UV absorbance at 80 °C.

Thermal denaturation studies were conducted by annealing oligomer samples while monitoring the absorbance at $\lambda = 260$ nm by using a UV/Vis spectrophotometer. The buffered oligomer samples were heated to 91 °C for 5 minutes and then cooled to 15 °C at a rate of 1 degree per minute. The T_m was calculated as the maximum for the first derivative of the melting curve. CD experiments were performed on a Jasco J-175 spectropolarimeter. Samples in buffered solutions were identical to the thermal denaturation studies.

Fluorescence studies were carried out on a Jobin Yvon Fluoromax 3, by using a duplex concentration of 0.5–2 μM. Samples were prepared either by annealing oligomers under the same conditions as were used for the thermal denaturation studies or without annealing. Results were the same under both conditions. Samples were excited

by using at a wavelength of 470 nm, and fluorescence was monitored at 495–595 nm.

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