Impact of Chirality and Position of Lysine-Conjugation in Triplex-Forming Peptide Nucleic Acids

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ABSTRACT

Conjugation with cationic lysine residues improves the biophysical and biological properties of peptide nucleic acids (PNAs). Single lysine is routinely used to improve the solubility and prevent aggregation due to the neutral and hydrophobic amide backbone of PNA. Literature precedents include attachment of lysine at either the N- or the C-terminus. Moreover, conjugation with short lysine peptides (four to eight residues) improves the cellular uptake of PNA akin to more complex cell-penetrating peptides. Herein we report a systematic study of the effect of lysine location (N- vs. C-terminus) and chirality (D- vs L-) on triple-helical binding of PNA to double-stranded RNA and DNA (dsRNA and dsDNA). The results confirmed our earlier findings that conjugation with lysine significantly increased the stability of PNA-dsRNA and PNA-dsDNA triplexes and that PNAs affinity for dsRNA was about an order of magnitude higher than for the same sequence of dsDNA. In contrast, conjugation of PNA with non-charged amino acids decreased the affinity of PNA. Surprisingly, neither the location, nor the chirality of lysine had significant impact on PNA affinity for either dsRNA or dsDNA. The results are consistent with lack of chiral preorganization of single-stranded PNAs, even after conjugation with four D- or L-amino acids. Instead, the positive charge of lysine appears to be the main driving force behind the increased affinity.

INTRODUCTION

Peptide nucleic acid (PNA) is a chimeric biopolymer that combines the molecular recognition elements of DNA nucleobases with a neutral and achiral protein-like backbone (Figure 1).[1] As a neutral nucleic acid mimic, PNA has unique properties compared to traditional oligonucleotide probes allowing for exciting potential applications in biotechnology, diagnostics, and medicine.[2,3] PNA was originally designed as a mimic of DNA for improving the binding properties of triplex-forming oligonucleotides.[4] Typically, the triplex formation is disfavored due to electrostatic repulsion between the negatively charged oligonucleotide and double-stranded DNA (dsDNA). Conversely, the lack of electrostatic repulsion between the neutral amide backbone and the negatively charged phosphates of dsDNA was expected to impart PNA with a high binding affinity.[1,4] Consistent with this expectation, PNA also binds single-stranded DNA and RNA (ssDNA/ssRNA) with high affinity and sequence selectivity.[5,6] An unexpected discovery was the ability of PNA to form a 2:1 PNA-DNA strand-invasion triplex by displacing a pyrimidine rich strand of dsDNA as the so-called P-loop.[1] Cumulatively, the early studies[1,4-6] showed that PNA was a remarkably effective ligand for the molecular recognition of complementary single stranded nucleic acids as well as dsDNA.

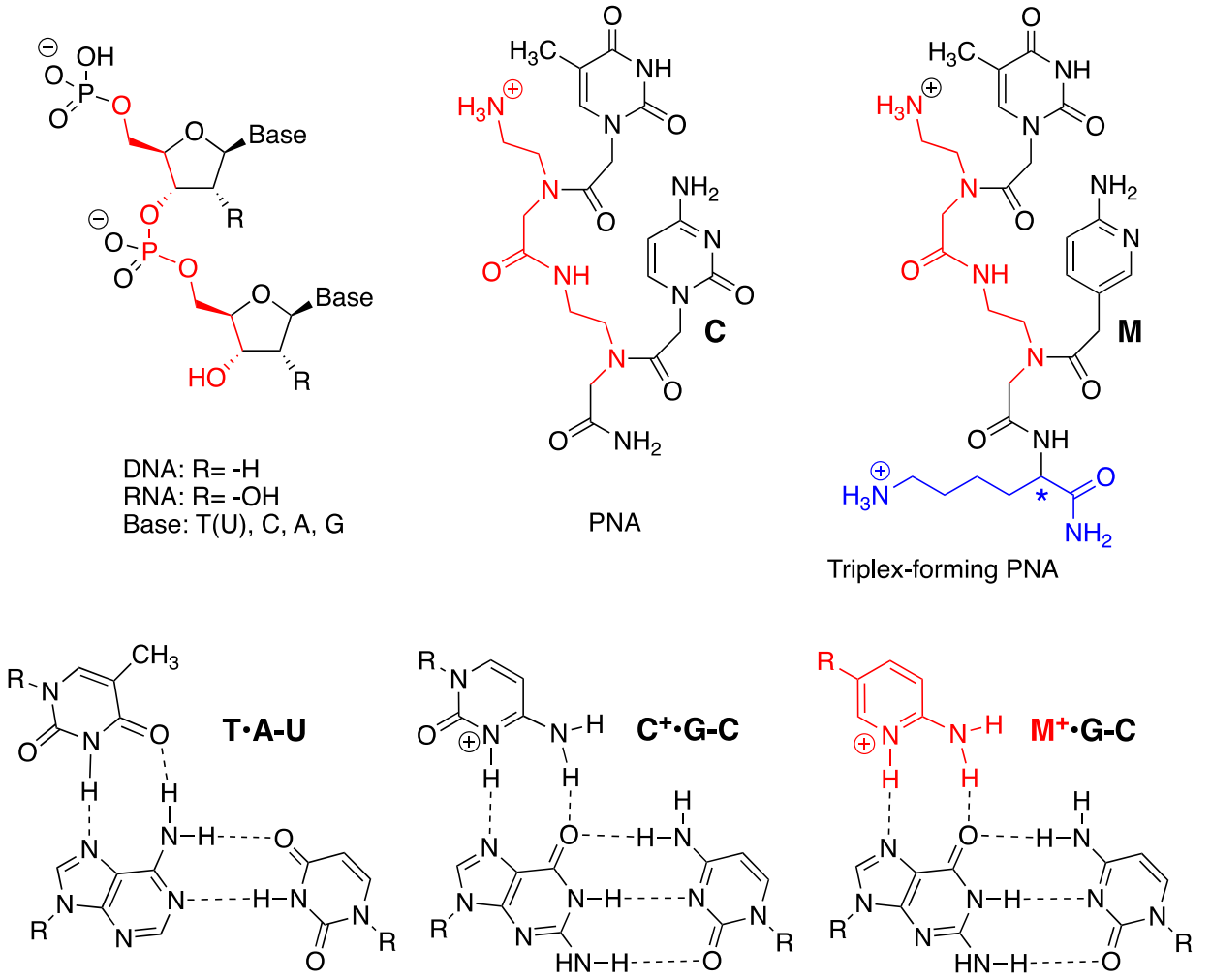

Figure 1. Structures of DNA/RNA, PNA, and lysine-conjugated triplex-forming PNA (above) and Hoogsteen hydrogen bonded base triplets (below).

Interestingly, binding of PNA to dsRNA was not explored prior to a study from our group published in 2010 that found that PNA formed a 1:1 Hoogsteen triple helix with dsRNA with high affinity and sequence selectivity.[7] Using of a more basic nucleobase, 2-aminopyridine (M, pK_a ~ 6.7) instead of cytosine (pK_a ~ 4.5) facilitated formation of protonated M$^+$•G-C triplet (Figure 1), which was important for fast and selective PNA binding to dsRNA at physiological pH

7.4 and salt concentration.[8-10] Interestingly, these studies also suggested that PNA had a much higher affinity for dsRNA than for the same sequence of dsDNA.[8-10]

Recent NMR structural studies from our group provided insights into the surprising PNA preference to bind dsRNA over dsDNA.[11] The NMR solution structure of the PNA-dsRNA triple helix was similar to the crystal structure of PNA-DNA-PNA triplex published earlier.[12] Both adapted A-form-like helical conformations where the ~5.7 Å spacing between the neighboring phosphate oxygens enabled PNA backbone amide N-H hydrogen-bonding to RNA or DNA phosphate oxygens. This hydrogen bonding zipper would not be possible in the B-form dsDNA that has ~7 Å spacing between the neighboring phosphate oxygens. Thus, favorable backbone hydrogen-bonding may be the main driving force for the PNA's preference to bind A-form dsRNA over B-form dsDNA. Taken together, previous studies from our group[8-11] suggested that PNA was an even better ligand for sequence specific recognition of dsRNA than for dsDNA for which it was originally designed.

While the elimination of charge from PNA's backbone was the primary driver of high binding affinity, the neutral backbone also caused problems with PNA's physicochemical properties. By replacing the anionic phosphodiester backbone with neutral amides, PNA became less water soluble than its parent DNA and was prone to aggregation akin to peptides.[13] To address these limitations, the initial design of PNAs placed a lysine at the C-terminus (blue in Figure 1), which introduced the second positive charge in addition to the charge at the N-terminus of PNA.[1] The additional charge was expected to increase solubility and decrease aggregation. It should be noted that the solid phase synthesis protocol forms an amide at the carboxy end of synthetic

PNAs; hence, there is no negative charge on PNAs because of the lack of free carboxylic acid functionality. Later studies found that addition of several lysine residues to both ends of PNA also increased the cellular uptake of PNA.[14-18] This increased uptake was due to endocytosis of lysine-modified PNA similarly to PNAs modified with more complex cell-penetrating peptides such as the TAT peptide from HIV-1 or penetratin peptide from the Antennapedia transcription factor in *Drosophila*.[19] We found that extending the M-modified PNA by conjugation with four L-lysine[10] or D-lysine[9] residues not only improved their cellular uptake, but also increased the stability of PNA-dsRNA triplex. This enhancement of stability was expected, as similar results were reported for the PNA-dsDNA triplex by Nielsen and co-workers[20] using pseudoisocytosine (J) nucleobase modified PNAs. While lysine-modification has an established positive impact on PNA solubility and biological activity, a systematic study of the effect of lysine on triple-helical binding of PNA-amino acid conjugates was lacking.

In the present paper, we report how the lysine position (N- vs. C-terminus), chirality, and cationic character impacted triple-helical binding of M-modified PNAs to dsDNA and dsRNA. Isothermal titration calorimetry (ITC) and UV thermal melting experiments showed that lysine conjugation significantly increased the binding affinity of PNA to both dsRNA and dsDNA. Overall, the stability of PNA-dsRNA triplexes was about tenfold higher than the stability of PNA-dsDNA triplexes. Single lysine was equally stabilizing at either N- or C-terminus. Somewhat unexpectedly, chirality of the amino acid (D- vs L-) had relatively small effect, even when four amino acid residues were conjugated to PNA. Circular dichroism (CD) studies confirmed that terminal conjugation of PNA with amino acids caused little chiral induction and preorganization of the achiral single stranded PNA backbone. Our results provide insights in the previously

observed trend of increased binding affinity through lysine conjugation to PNA and provide guidance for future development of triplex-forming PNAs.

RESULTS

We used ITC to measure the binding affinity of control **PNAC** (no amino acid) and amino acid-PNA conjugates, **PNA1**-**PNA9** (Figure 2) for both dsRNA and dsDNA under physiologically relevant buffer conditions. Association constants and thermodynamic parameters for binding of single lysine-modified PNAs and PNAs with three C-terminal and one N-terminal amino acid modification (referred to as [3+1] modified PNA) are presented in Table 1 and Figures 3 and 5.

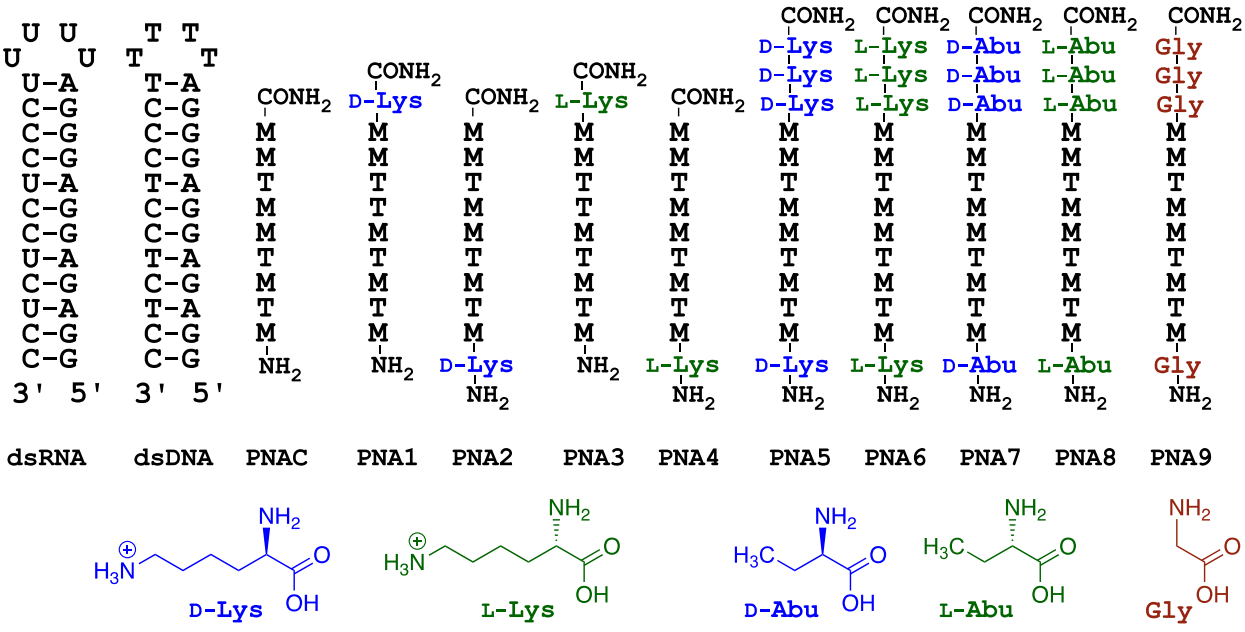

Figure 2. Sequences of dsRNA and dsDNA hairpins and complementary PNAs. The conjugation with D- or L-amino acids is highlighted in blue and green, respectively.

Table 1. Binding affinity ($K_a \times 10^6$ M^{-1}) of amino acid-PNA conjugates to complementary dsRNA and dsDNA.

PNA	Lys modification	dsRNA[a]	ΔH (kJ/mol)	-TΔS (kJ/mol)	dsDNA[a]	ΔH (kJ/mol)	-TΔS (kJ/mol)
PNAC	none (control)	24 ± 1	-254 ± 9	212 ± 9	2.4 ± 0.1	-136 ± 5	100 ± 5
PNA1	D-Lys, C-term	44 ± 2	-282 ± 2	239 ± 1	4.2 ± 0.4	-156 ± 3	118 ± 3
PNA2	D-Lys, N-term	44 ± 1	-290 ± 10	249 ± 10	4.3 ± 0.6	-145 ± 3	108 ± 4
PNA3	L-Lys, C-term	44 ± 1	-255 ± 7	210 ± 6	3.9 ± 0.5	-138 ± 4	100 ± 4
PNA4	L-Lys, N-term	44 ± 2	-296 ± 5	253 ± 5	4.0 ± 0.6	-157 ± 6	120 ± 6
PNA5	[3+1], D-Lys	92 ± 3	-180 ± 3	135 ± 3	9.3 ± 0.4	-191 ± 6	152 ± 5
PNA6	[3+1], L-Lys	88 ± 2	-190 ± 10	144 ± 10	9.1 ± 0.3	-180 ± 7	140 ± 6
PNA7	[3+1], D-Abu	4.4 ± 1.7	-220 ± 10	180 ± 9	0.5 ± 0.2	-140 ± 24	100 ± 23
PNA8	[3+1], L-Abu	3.8 ± 0.7	-196 ± 8	158 ± 7	0.6 ± 0.1	-140 ± 18	100 ± 18
PNA9	[3+1], Gly	11 ± 1	-187 ± 2	98 ± 2	1.1 ± 0.3	-140 ± 16	104 ± 17

[a] Association constants ($K_a \times 10^6$ M^{-1} ± 1 standard deviation) were measured at 25 °C in 50 mM potassium phosphate buffer (pH 7.4) containing 2 mM $MgCl_2$, 90 mM KCl, 10 mM NaCl.

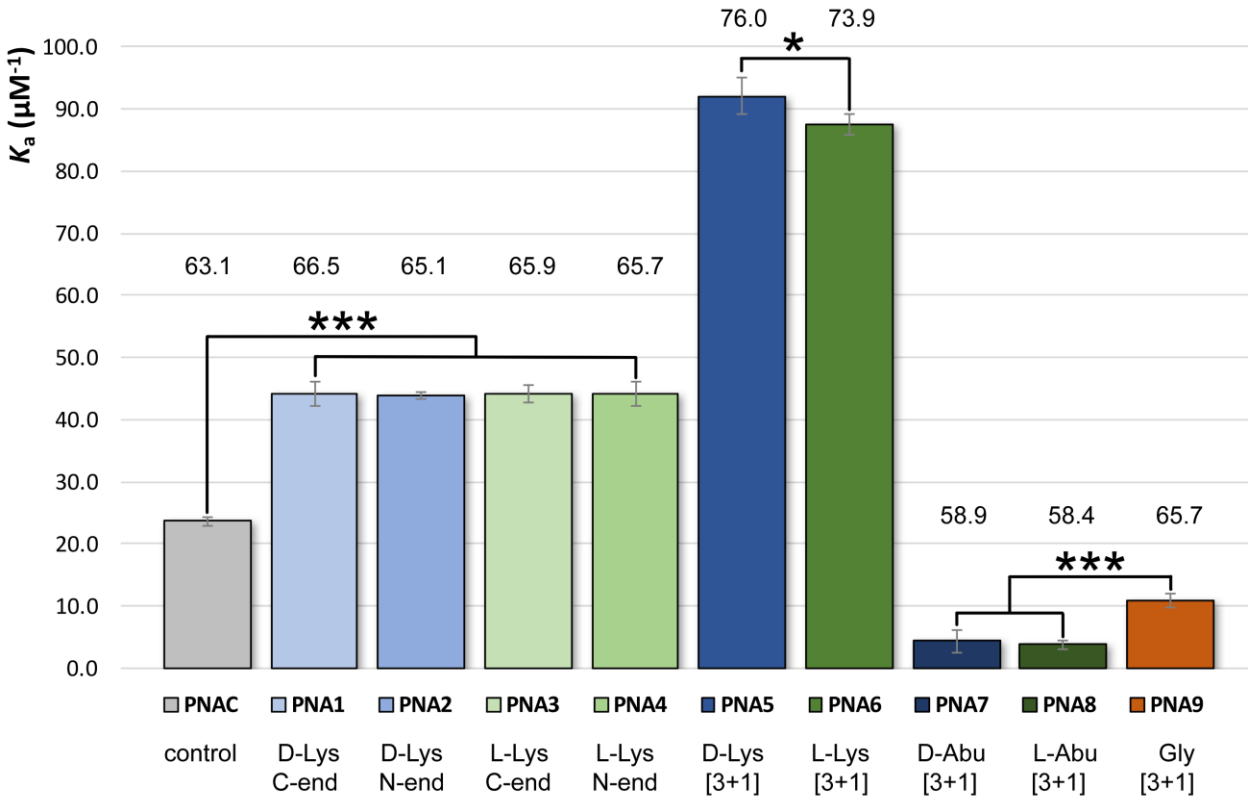

Figure 3. Binding affinity ($K_a \times 10^6$ M^{-1}) of amino acid-PNA conjugates for complementary dsRNA. Error bars = ± 1 standard deviation in K_a measured by ITC, * denotes P ≤ 0.1, ** denotes P ≤ 0.5, and *** denotes P ≤ 0.01. The numbers above the bars are UV thermal melting temperatures of 1:1 PNA-dsRNA triplexes (15 μM) measured at 300 nm.

Addition of a single lysine to **PNAC** doubled the binding affinity of this triplex-forming PNA for dsRNA (Figure 3). However, neither the location (C- vs. N-terminus), nor the chirality (D-Lys vs. L-Lys) had significant impact on binding as the affinity of **PNA1**-**PNA4** for dsRNA was virtually identical. Addition of three more lysines further doubled the affinity of [3+1] modified PNAs for dsRNA. Statistical analysis suggested that D-Lys modified **PNA5** had a slightly higher affinity than the L-Lys modified **PNA6**, when binding to dsRNA, albeit the difference was relatively small (P ≤

0.1, Figure 3). To obtain insights in the impact amino acid chirality on preorganization of PNA backbone, we prepared [3+1] modified **PNA7** and **PNA8** having D- and L-α-aminobutyric acids (Abu), which we expected to act as non-charged mimics of lysine, and **PNA9** having achiral glycine modifications. Somewhat surprisingly, conjugation with the neutral amino acids resulted in significant decrease of binding affinity, even when compared to the non-conjugated PNA control (**PNAC**). In contrast to lysine conjugated PNAs, there was no statistically significant difference between the affinity of D-Abu **PNA7** and L-Abu **PNA8**, and both were significantly lower than the affinity of glycine-conjugated **PNA9**.

The trends in PNA-dsRNA duplex stabilities observed by ITC were also confirmed by UV thermal melting at 300 nm (Figure 4, Table S4). At this wavelength (300 nm) M nucleobase has unique and relatively strong absorbency while the absorbency of native nucleobases is low. Therefore, UV melting curves at 300 nm report specifically on triplex melting that is not obscured by overlapping DNA or RNA hairpin melting. Overall, the UV melting temperatures nicely followed the same trend as association constants obtained by ITC. **PNA9** was a notable exception giving melting temperature similar to **PNA1**-**PNA4**, despite having significantly lower K_a. At this point we do not have a compelling explanation for the higher than expected T_m of **PNA9**. However, it should be noted that because the thermodynamic parameters (ΔG, ΔH, etc.) of various PNA-dsRNA triplexes may have different temperature dependency, we can expect some variations in thermodynamic stability of complexes measured by ITC at 25 °C and measured by UV at the melting temperatures (~40 °C).[21] In the present study, we relied on ITC as the primary method to determine stabilities of the various triplexes.

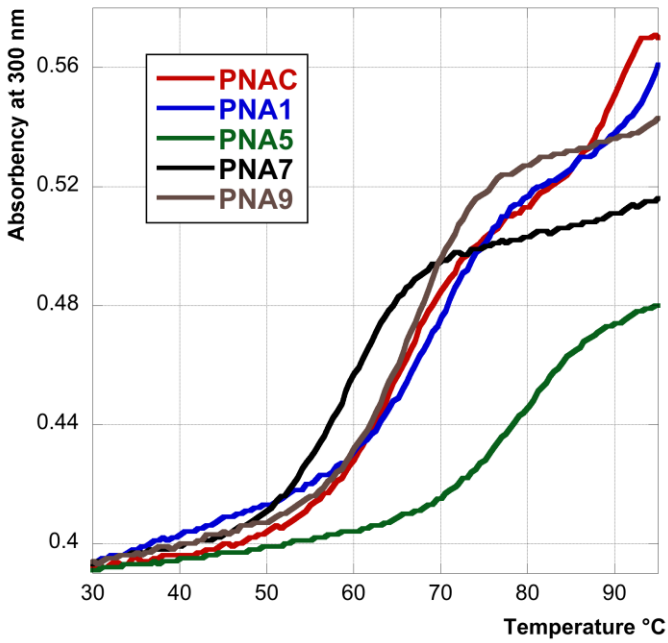

Figure 4. UV thermal melting curves of selected 1:1 PNA-dsRNA triplexes (15 μM) measured at 300 nm.

Binding of PNAs to dsDNA (Figure 5) followed essentially the same trends as for dsRNA. As we have observed previously,[8-11] PNA binding to dsDNA was about tenfold weaker than to dsRNA (Table 1, also c.f., Figure 3 and Figure 5). As in the RNA series, neither the location (C- vs. N-terminus), nor the chirality (D-Lys vs. L-Lys) had significant impact on binding as the affinity of **PNA1**-**PNA4** for dsDNA was practically identical. Addition of one lysine, and three more lysines, each doubled the affinity of PNA for dsDNA; however, statistical analysis showed no significant difference between the D-Lys modified **PNA5** and the L-Lys modified **PNA6**. Conjugation with the neutral D- and L-Abu significantly decreased the binding affinity of PNA for dsDNA, similar to what was observed for dsRNA. UV thermal melting temperature confirmed the overall trends in the DNA series (Figure S38, Table S4) with **PNA9** giving again higher than expected T_m.

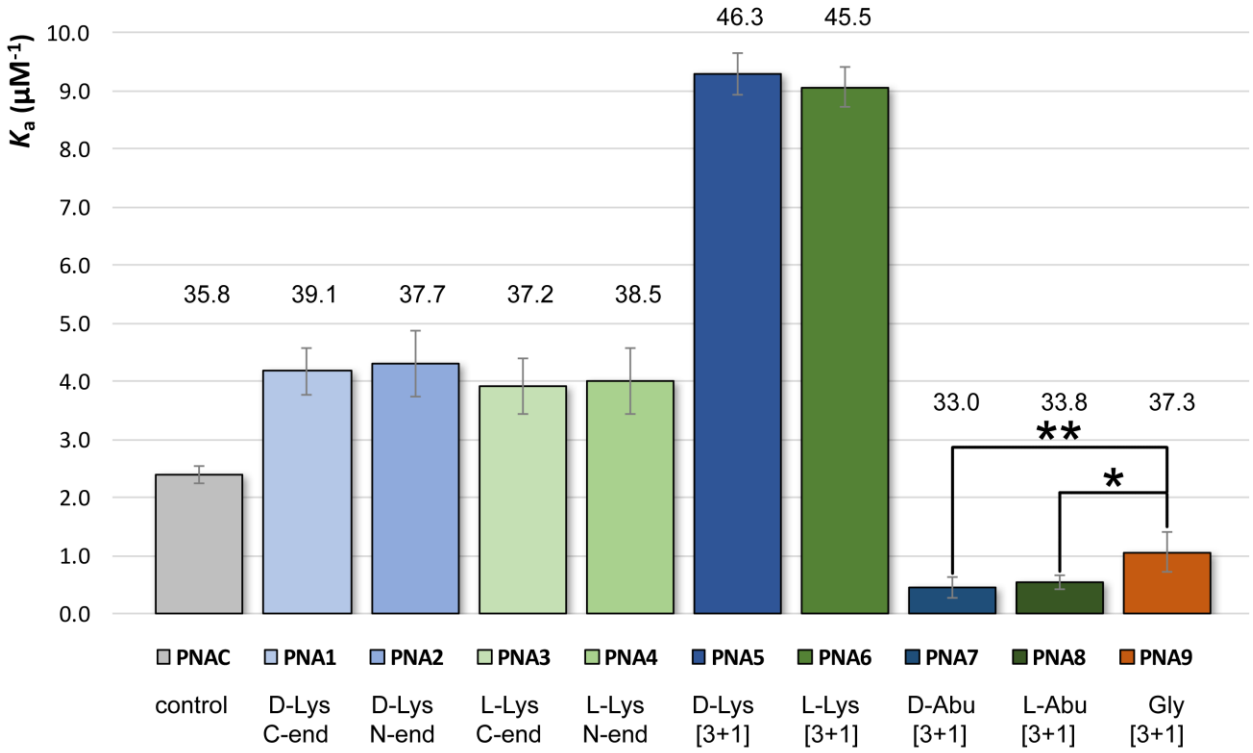

Figure 5. Binding affinity ($K_a \times 10^6$ M^{-1}) of amino acid-PNA conjugates for complementary dsDNA. Error bars = ± 1 standard deviation in K_a measured by ITC, * denotes P ≤ 0.1, ** denotes P ≤ 0.5, and *** denotes P ≤ 0.01. The numbers above the bars are UV thermal melting temperatures of 1:1 PNA-dsRNA triplexes (15 μM) measured at 300 nm.

To further assess the degree of preorganization of PNA's backbone by chiral amino acid conjugation, we recorder CD spectra of PNA single strands in the absence of RNA or DNA targets. We observed only weak CD signals at ~200 nm likely corresponding to peptide bond absorbance. The CD signal had the expected opposite polarities, positive signal for conjugates of D-amino acids and negative signal for conjugates of L-amino acids, and was stronger for [3+1] modified PNAs (Figure 6) than for singly modified PNAs (Figures S35-S37).

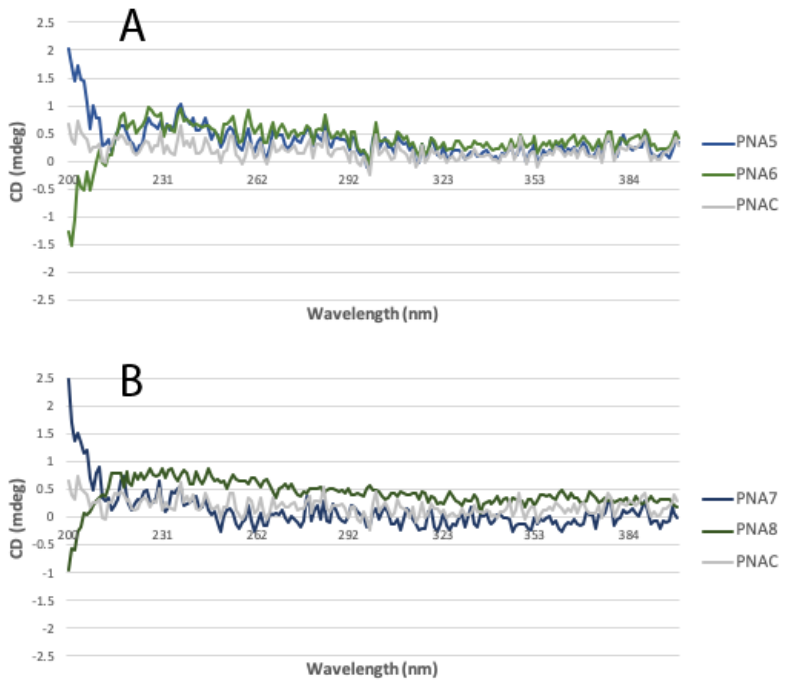

Figure 6. CD-spectra of single-stranded PNAs (A) comparison of **PNA5** and **PNA 6**; and (B) comparison of **PNA 7** and **PNA 8**. **PNAC** is included as a control.

DISCUSSION

PNA is an achiral DNA analogue having the sugar-phosphate backbone replaced with a neutral *N*-(2-aminoethyl)glycine moiety.[1] In the original design, the lack of chirality and charge were considered as key advantages expected to simplify the synthesis and improve DNA binding affinity by eliminating the electrostatic repulsion, respectively. However, the neutral amide backbone also decreased the solubility and caused unfavorable aggregation of PNA. To overcome this limitation, the original design placed an L-lysine residue at the C-terminus.[1] The expectation was that having a positive charge at each end of PNA (the N-terminus already has a charge due to a terminal amino group) will increase the solubility and disfavor aggregation of PNA. Triplex-forming PNAs, having only pyrimidine nucleobases, are more soluble than duplex-

forming PNAs containing a mixture of purines and pyrimidines. Nevertheless, most of currently used PNA designs, regardless of desired binding mode, retain some kind of lysine conjugation.

In a related approach to optimize PNA ligands, lysine has been used as a chiral building block to impart the internal *N*-(2-aminoethyl)glycine backbone unites of PNA with chirality.[22-24] This effectively induces chirality in PNA-PNA duplexes, as well as in single-stranded NAs.[22,23] These studies also reported that D-lysine-modified PNAs formed more stable right-handed PNA-DNA duplexes than L-lysine-modified PNAs.[22,23] Another motivation for conjugation of several lysine residues to PNA has been the improvement of cellular uptake of PNA, which is a long-standing challenge for in vivo applications of PNA technology.[25] The research teams led by Corey[14,18] and Gait[15-17] showed that conjugation of PNA with short oligolysine peptides (four to eight amino acids) enabled efficient delivery of PNA in several cell lines. Studies from our group,[9,10] showed that 2-aminopyridine (M) and lysine modifications had a mutually reinforcing enhancement of cellular delivery of PNA. Recent designs of PNAs as potential therapeutic molecules have also placed three lysines at both terminus of PNA.[26] Collectively, these results demonstrate the benefits of lysine conjugation; however, a systematic comparison of the impact of conjugation position (N- vs. C-terminus) and lysine chirality on triple helix formation had not been done.

In the present study, we found that conjugation of lysine to M-modified triplex-forming PNAs strongly enhanced their affinity for dsRNA and dsDNA. Addition of one lysine doubled the binding affinity of PNAs while adding another three lysine residues in the [3+1] modified PNAs further doubled the binding affinity for both dsRNA (Figure 3) and dsDNA (Figure 5). These results were consistent with our earlier findings that lysine conjugation enhances the stability

of PNA-dsRNA triplex.[9,10] Nielsen and co-workers[20] also reported that increasing the number of lysine residues from one to four in a psuedoisocytosine (J)-modified PNA strongly stabilized the corresponding PNA-dsDNA triplex. Somewhat unexpectedly, we found that the location (N- vs. C-terminus) and the chirality of lysine (D- vs L-) did not have significant effect on the stability of either PNA-dsRNA or PNA-dsDNA duplex. D-Lysine appeared to be slightly more stabilizing in RNA series (Figure 3), but this effect only accounted for a difference in binding affinity of <3.5% and was not present in the DNA series (Figure 5).

Lack of significant effect of lysine chirality on triple helix stability was somewhat surprising because conjugation of D- or L-lysine to C-terminus of PNA strands induced opposite chirality in the PNA-PNA double helix.[27] Although the effect was dependent on PNA sequence and strong induction of helicity was only observed for PNAs having a G-C or C-G base pair at the lysine bearing terminus,[28] we expected that D-lysine, which induces the right-handed preorganization of double helical nucleic acid structures[22,23,29] and, therefore, favors the natural conformation of triplex, would be more stabilizing than L-lysine, which induces the opposite chirality. While the small difference between D-modified **PNA5** and L-modified **PNA6** (Figure 3) was consistent with this logic, the impact was negligible. Interestingly, the above studies on amino acid-PNA conjugates[27,28] also found that alanine, phenylalanine, isoleucine, and glutamic acid showed chiral induction similar to lysine; however, the absolute stereochemistry of amino acid did not always correlate with the induced helicity of PNA-PNA duplex. We found that **PNA7** and **PNA8** that were conjugated with four D- and L-α-aminobutyric acids ([3+1] design), respectively, produced the same phase and degree of helicity as their lysine-modified counterparts, but also had the lowest binding affinity among all PNAs studied; decreasing affinity by ~75% when

compared to unmodified **PNAC**. Interestingly, comparison of binding of **PNAC** and **PNA9** to both dsRNA and dsDNA shows that by merely extending the PNA backbone with amino acid residues, binding affinity decreases by about 50% as judged by ITC. However, this was trend was not observed in UV thermal melting experiments with **PNA9** having a slightly higher melting temperature than **PNAC**. Overall, only lysine conjugation strongly enhanced the affinity of triplex-forming PNAs. Conjugation by the non-charged α-aminobutyric acids (**PNA7** and **PNA8**) or even achiral glycine (**PNA9**) caused significant drop in affinity (Figures 3 and 5). The changes in binding affinity did not correlate with chirality of amino acids suggesting a lack of significant preorganization of PNA's backbone.

The very small signal at 200 nm in CD spectra of single-stranded PNAs (Figure 6) supported this conclusion. Virtually no signal was observed at 260 nm indicating that the PNA nucleobases remained disordered even with four amino acid modifications. This lack of signal may be attributed to triplex-forming PNA having only six-membered pyrimidine-like nucleobases, which makes them intrinsically poorer at π-stacking than traditional purine containing PNAs. The small signal at 200 nm is likely due to amide bond absorbance of the amino acid residues. Taken together, the binding (Figures 3 and 5) and CD (Figure 6) data suggested that amino acid conjugation did not induce significant conformational preorganization of PNA's backbone.

Lack of significant effect of lysine location (N- vs. C-terminus) was initially surprising because Alberti et al.[30] reported that DNA triplex formation proceeds through a directional nucleation-zipping mechanism from the 5´- to the 3´-end of the triplex. Hence, we expected differences in binding affinity of N- vs. C-conjugated PNAs. However, more recently Nishizawa and co-

workers[31] reported that PNA-dsRNA triplex formation follows a non-directional nucleation–zipping mechanism. Moreover, conjugation of L-lysine to N-terminus induced opposite helicity in PNA-PNA duplexes compared to conjugation of L-lysine to C-terminus.[28] Taken together with the weak impact of amino acid chirality on binding affinity, our findings that lysine conjugation to either the N- or C-terminus has the same effect on triplex stability are not unexpected and fully consistent with Nishizawa and co-workers report.[31] An important conclusion is that this allows direct comparison of literature data in cases where PNAs bear lysine residues at either N- or C-terminus.

Analysis of thermodynamic parameters showed that the triplex formation was enthalpically-driven (Figures S31-S34) where large favorable enthalpy was compensated by unfavorable entropy. This is generally expected for formation of nucleic acid double and triple helices.[31,32] An interesting observation was that PNA-dsRNA triplexes (but not PNA-dsDNA triplexes) where PNAs were conjugated with four amino acids (**PNA5**-**PNA9**) were less enthalpically stabilized and, consequently, had less unfavorable entropy (Figures S31 and S32) than PNAs bearing no (**PNAC**) or single lysine (**PNA1**-**PNA4**). The effect was independent of the large difference between binding affinity of **PNA5**-**PNA6** and **PNA7**-**PNA9**. This trend was unexpected, suggesting that enthalpic gains and binding affinity have a complex relationship in triplex formation.

Finally, our results confirmed earlier observations[8-11] that PNA binds about an order of magnitude stronger to dsRNA than to dsDNA (Table 1, also c.f., Figure 3 and Figure 5). Currently, the best explanation for this difference is that favorable backbone hydrogen-bonding

(PNA amide N-H to RNA phosphates) is the main driving force for the PNA's preference to bind A-form dsRNA over B-form dsDNA.

CONCLUSIONS

Conjugation with cationic lysine residues enhanced the binding affinity of PNA for dsRNA and dsDNA. In contrast, conjugation with non-charged α-aminobutyric acid or glycine residues decreased the affinity of PNA. The location (N- vs. C-terminus) and the chirality of amino acids (D- vs L-) did not have significant effect on binding affinity. D-Lysine appeared to be slightly more stabilizing than L-lysine in the RNA series, but the effect was small and not present in the DNA series. CD spectra of single-stranded PNAs did not show any evidence of significant chiral preorganization, even after conjugation with four D- or L-amino acids. Taken together, these results suggest that the conformational preorganization is not a major player in stabilization of PNA triple helices with dsRNA and dsDNA. The positive charge of lysine appears to be the main driving force behind the increase in affinity. Interestingly, our previous studies[10] showed that the increased affinity due to electrostatic attraction of positively charged lysine to negatively charged dsRNA did not compromise sequence selectivity of PNA binding. The orientation of the charged side chain due to chirality of lysine may have a minor effect in the PNA-dsRNA triplex, but not in the PNA-dsDNA duplex. Collectively, our results provide insights into the impact of lysine conjugation on stability of PNA triplexes with dsRNA and dsDNA, which will be useful for future design of triplex-forming PNAs.

MATERIALS AND METHODS

The PNAs and amino acid-PNA conjugates used in this study (Figure 2) were synthesized on Expedite 8909 synthesizer at 2 μmol scale on NovaSyn TG Sieber resin (Novabiochem) using methods previously developed in our group.[8-10,33] Commercial PNA-T-monomer was purchased from Link Technologies. M monomer was synthesized using the synthetic route reported by our group.[8] PNAs were cleaved from the solid support using 0.6 mL of 20% m-cresol in TFA for 2 h using two-syringe pull-push method. Crude PNA (separated in three Eppendorf tubes, 200 μL in each) was precipitated by the addition of chilled diethyl ether (~1.0 mL) followed by the centrifugation (15000 rpm). The crude PNA, white solid, was dissolved in purified water (~1.0 mL) and analyzed by LC-MS. HPLC purification of the crude PNAs was done on a Shimadzu LC-20 instrument using a semi-preparative Supelco Discovery Wide Pore C18 column (4.6 x 150 mm) and a linear gradient of acetonitrile in water containing 0.1% formic acid. The purity and identity of the PNA sequences were confirmed by LC-MS (ESI) analysis on a Shimadzu LCMS 2020 single quadrupole instrument using an analytical Supelco Discovery Wide Pore C18 column (2.1 x 250 mm) and a linear gradient of acetonitrile in water containing 0.1% formic acid (Figures S1-S10, Table S1). PNA was quantified as previously reported.[33]. RNA and DNA hairpins were purchased crude from Dharmacon and Eurofins, respectively, and purified prior to use on reverse phase HPLC using a gradient of acetonitrile in 50 mM aqueous triethylammonium/acetate buffer as previously reported.[33]

Isothermal titration calorimetry experiments were done on a MicroCal iTC200 instrument at 25 °C in 50 mM potassium phosphate buffer (pH 7.4) containing 2 mM $MgCl_2$, 90 mM KCl, 10 mM

NaCl. In a typical ITC experiment, 2.45 μL aliquots of 90 μM PNA solution were sequentially injected from a 40 μL rotating syringe (750 rmp) into 200 μL of 10 μM RNA or DNA hairpin solution. All ITC experiments were run in triplicate. The average binding affinity for the PNAs were analyzed by single-factor ANOVA in Microsoft Excel to evaluate statistical significance. P-values are given in Supporting Information, Tables S2 and S3. Representative ITC titration traces are given in Supporting Information, Figures S11-S30.

Circular dichroism experiments were done on an JASCO J-1100 CD spectrometer using 18 μM PNA in ITC buffer and a 2 mm pathlength cuvette at room temperature and averaging 10 scans from 200-400 nm with a scan speed 100 nm/min and 1 nm bandwidth for all experiments.

UV thermal melting experiments were done on a Shimadzu UV-2600 spectrophotometer equipped with a TMSPC-8 temperature controller. Each PNA was analyzed in triplicate from a 345 μL solution of buffer containing RNA (5.25 nmols) and PNA (5.25 nmols) distributed in 110 μL aliquots to three separate wells. Experiments were done in phosphate buffer (2 mM $MgCl_2$, 90 mM KCl, 10 mM NaCl, 50 mM potassium phosphate at pH 7.4). Absorbance vs. temperature profiles were measured at 300 nm. The temperature was increased at a rate of 0.5 °C per minute. The melting temperatures were obtained using Shimadzu LabSolutions Tm Analysis software version 1.31.

ASSOCIATED CONTENT

Supporting Information. HPLC purification and LC-MS characterization of PNA oligomers; ITC results and representative titration images; statistical analysis of ITC results; graphical representation of enthalpy and entropy of PNA binding; and CD spectra.

The following files are available free of charge.

Supporting Information (PDF)

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ABBREVIATIONS

ABU, aminobutyric acid; CD, Circular dichroism; ITC, isothermal titration calorimetry; PNA, peptide nucleic acid.

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