Triplex-Forming Peptide Nucleic Acids with Extended Backbones

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Dedication ((optional))

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Abstract: Peptide nucleic acid (PNA) forms a triple helix with double-stranded RNA (dsRNA) stabilized by a hydrogen-bonding zipper formed by PNA's backbone amides (N-H) interacting with RNA phosphate oxygens. This hydrogen-bonding pattern is enabled by the matching ~5.7 Å spacing (typical for A-form dsRNA) between PNA's backbone amides and RNA phosphate oxygens. We hypothesized that extending the PNA's backbone by one -CH_2- group may bring the distance between PNA amide N-H closer to 7 Å, favourable for hydrogen-bonding to the B-form dsDNA phosphate oxygens. Extension of PNA backbone was expected to selectively stabilize PNA-DNA triplexes compared to PNA-RNA. To test this hypothesis, we synthesized triplex-forming PNAs that had the pseudopeptide backbones extended by an additional -CH_2- group in three different positions. Isothermal titration calorimetry measurements of the binding affinity of these extended PNA analogues for the matched dsDNA and dsRNA showed that, contrary to our structural reasoning, extending the PNA backbone at any position had a strong negative effect on triplex stability. Our results suggest that PNA may have an inherent preference for A-form-like conformations when binding double-stranded nucleic acids. It appears that the original six atoms long PNA backbone is an almost perfect fit for binding to A-form nucleic acids.

Introduction

Peptide nucleic acid (PNA) is a DNA analogue where the entire sugar-phosphate backbone is replaced by a neutral and achiral N-(2-aminoethyl)glycine moiety (Figure 1) that has the same number of backbone bonds and the same distance between backbone and nucleobases as DNA.[1] PNA binds to complementary single-stranded DNA and RNA (ssDNA and ssRNA) in an antiparallel orientation, where the PNA amino-end aligns with the 3′-end of the DNA or RNA strand, with high affinity and sequence specificity obeying the Watson-Crick base-pairing rules.[2] The favorable binding of PNA is mostly attributed to the lack of electrostatic repulsion between the neutral PNA and the negatively charged nucleic acids. PNA was originally designed for major groove triple-helical recognition of double-stranded DNA (dsDNA).[1, 3] However, the extraordinarily high affinity of PNA for ssDNA[4] enabled an unprecedented strand-invasion binding mode where PNA displaces the pyrimidine strand of dsDNA and binds to the purine strand to form a 2:1 PNA-DNA strand-invasion triplex.[1] This unexpected discovery shifted the interest away from the PNA-DNA triple helix and most of the subsequent research focused of PNA as an DNA invading ligand.

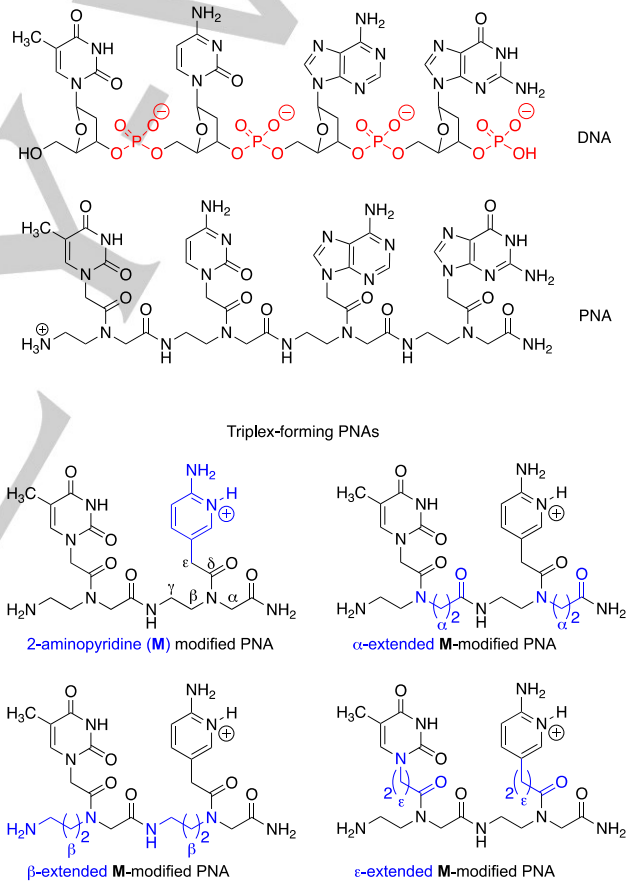


Figure 1. Structural comparison of DNA, PNA and triplex-forming PNAs with regular and extended backbones.

Later studies revealed the full complexity of PNA binding to dsDNA. At high ionic strength and low concentration, PNA forms a major groove Hoogsteen triple helix (PNA:dsDNA, 1:1) in a parallel orientation, where the PNA amino-end aligns with the 5′-end of the DNA purine strand.[5] However, at low ionic strength, high PNA oligomer concentration, or longer incubation times, the 1:1 triplex converts to a 'P-loop' strand-invasion triplex

(PNA:dsDNA, 2:1).[5] In addition, the binding mode may be influenced by the sequence of PNA. Thymine-rich homopyrimidine PNAs generally prefer invasion complexes, while cytosine-rich PNAs prefer triple helix formation.[6] Interestingly, binding of PNA to double-stranded RNA (dsRNA) was not explored prior to our studies, first published in 2010.[7] An earlier report by Toulme and coworkers[8] suggested that PNA did not bind strongly to dsRNA, but these results were difficult to interpret because the triplex had two consecutive T*C-G mismatches and the binding affinity was not quantified. We found that PNA formed 1:1 Hoogsteen triple helix with dsRNA with high affinity and sequence selectivity.[7] Follow up studies showed that using of 2-aminopyridine (M, Figure 1) nucleobase instead of cytosine enabled fast and selective PNA binding to dsRNA at physiological pH and salt conditions.[9] Moreover, PNA had at least an order of magnitude higher affinity for dsRNA than for the same sequence of dsDNA.[9] In parallel to our studies, others have also optimized RNA binding affinity and sequence scope of triplex-forming PNAs.[10] Taken together, these studies suggested that PNA was an even better ligand for sequence specific recognition of dsRNA than for the dsDNA that it was originally designed for.

The most surprising discovery of our studies was the much higher stability of the M-modified PNA-dsRNA triple helix compared to the PNA-dsDNA complex. Follow-up NMR structural studies from our group[11] showed that the solution structure of the PNA-dsRNA triple helix was similar to the published crystal structure of PNA-DNA-PNA triplex.[12] Both adapted A-form-like helical conformations were the ~5.7 Å spacing between the neighboring phosphate oxygens enabled PNA backbone amide N-H hydrogen-bonding to RNA or DNA phosphate oxygens (Figure 2).[11] 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 a major driving force for the PNA's preference to bind A-form dsRNA over B-form dsDNA.[11] Conversely, these studies suggested a hypothesis that extending the PNA's backbone by one -CH_2- group may bring the distance between PNA amide N-H closer to 7 Å, favorable for hydrogen-bonding to the B-form dsDNA phosphate oxygens. In other words, we hypothesized that PNA with CH_2-extended backbone may bind tighter to B-form dsDNA than to the unmodified PNA.

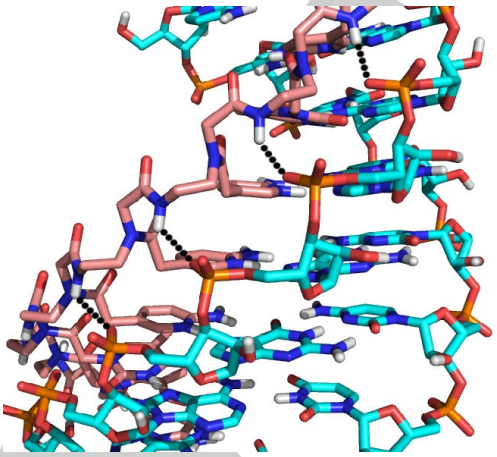


Figure 2. PNA amide to RNA phosphate backbone hydrogen-bonding interactions (black dotted line) stabilizing PNA–dsRNA triplex;[11] the ~5.7 Å spacing between the neighboring phosphate oxygens matches well the distance between the backbone amide N-H in PNA.[11]

In the present paper, we synthesized M-modified triplex-forming PNAs that had the pseudopeptide backbones extended by an additional -CH_2- group in three different positions (Figure 1). We used isothermal titration calorimetry to measure the binding affinity of these extended PNA analogues for the matched dsDNA and dsRNA. Surprisingly and contrary to our structural reasoning, the results showed that extending the PNA backbone at any of these positions had a strong negative effect on binding affinity to either DNA or RNA. Our results suggest that PNA may prefer A-form-like conformations when binding double-stranded nucleic acids and that the original six atoms long PNA backbone (Figure 1) is an almost perfect fit for binding to A-form nucleic acids.

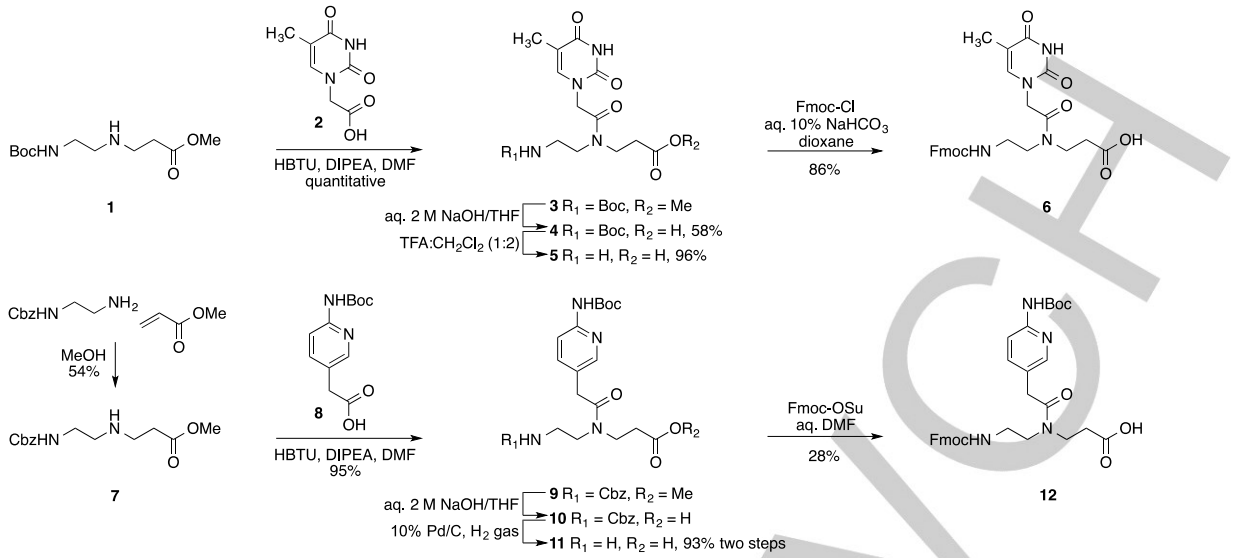
Results

Synthesis of PNA monomers with extended backbones

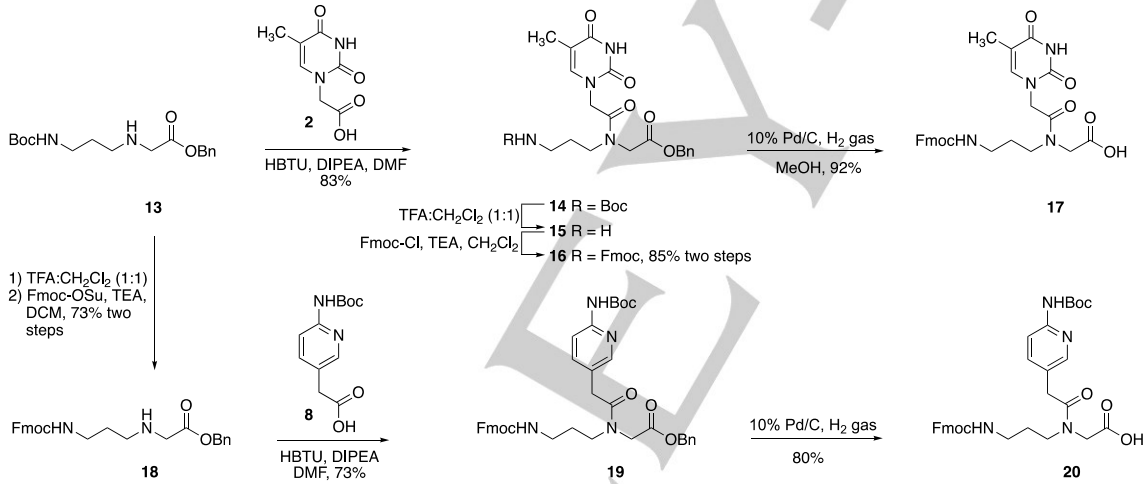
For the synthesis of backbone extended PNAs, we used Fmoc solid-phase peptide synthesis as in our previous studies on 2-aminopyridine (M) modified triplex-forming PNAs.[9] We modified the previously reported routes for Boc-protected T and C monomers[13] to give the corresponding Fmoc-protected T and M monomers. Synthesis of PNA monomers having α-extended backbones started with the known Boc-protected aminoethyl-β-alanine **1** (Scheme 1).[14] Coupling of **1** with commercially available thymine acetic acid **2** using *N,N,N′,N′*-tetramethyl-*O*-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) in the presence of *N,N*-diisopropylethylamine (DIPEA) gave compound **3**. The methyl ester and Boc protecting groups were cleaved using aqueous sodium hydroxide and trifluoroacetic acid (TFA), respectively, to give the thymine amino acid **5**, which was treated with 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl) to give the target α-extended thymine monomer **6**.

The synthesis of α-extended M monomer had to be modified because of the Boc protecting group in M nucleobase. Reaction of benzyloxycarbonyl (Cbz) protected ethylene diamine[15] with methyl acrylate gave the orthogonally protected PNA backbone **7** that was coupled with Boc protected 2-aminopyridine acetic acid **8**. The methyl ester and Cbz protecting groups were cleaved using aqueous sodium hydroxide and hydrogenation, respectively, to give the M amino acid **11**, which was treated with *N*-(9-fluorenylmethoxycarbonyloxy) succinimide (Fmoc-OSu) to give the target α-extended M monomer **12**.

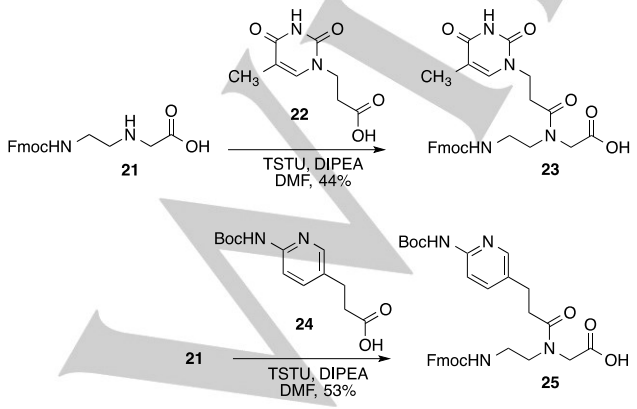
Synthesis of PNA monomers having β-extended backbones started with the known Boc-protected 3-aminopropylglycine **13** (Scheme 2).[16] Following the same synthetic strategy as in Scheme 1, HBTU mediated coupling with thymine acetic acid **2** gave, after protecting group exchange, the target β-extended thymine monomer **17**. For the synthesis of β-extended 2-aminopyridine monomer **20**, the Boc protection on backbone **13** was changed to Fmoc, followed by coupling with 2-aminopyridine acetic acid **4**. Finally, deprotection of benzyl ester by hydrogenation gave the target β-extended M monomer **20**. It is important to note that limiting the quantity of palladium catalyst and reaction time was important to minimize loss of Fmoc protection from **17** and **20** in the last hydrogenation step. The PNA monomers having ε-extended backbones were synthesized by *O*-(*N*-succinimidyl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate (TSTU) mediated coupling of the known nucleobase propionic acid derivatives (**22**[17] and **24**[18] in Scheme 3) with the previously synthesized PNA backbone **21**.[19]



Scheme 1. Synthesis of PNA monomers having α-extended backbones derived from β-alanine.



Scheme 2. Synthesis of PNA monomers having β-extended backbones derived from 3-aminopropylglycine.



Scheme 3. Synthesis of PNA monomers having ε-extended backbones derived from nucleobase propionic acids.

Stability of triple helices formed by PNAs having extended backbones

Using the backbone extended PNA monomers (**6**, **12**, **17**, **20**, **23** and **25**), commercially available T monomer (Link Technologies), and M monomer prepared following our previously described procedures,[9] we synthesized two PNA sequences, nine and twelve nucleobases long. The PNA sequences were similar to ones we had used in our previous studies on triple-helical recognition of dsRNA and dsDNA (Figure 3).[9] The modified PNAs had extended backbones at either all (PNA1 and PNA4), or selected positions (marked blue in Figure 3). The synthesis, purification and isothermal titration calorimetry (ITC) measurements of triple-helical binding affinity of backbone-extended PNAs to dsRNA and dsDNA were done following our previously reported procedures.[20] ITC results (Table 1) showed that PNA1 and PNA2 having regular backbone had the expected high binding affinity to dsRNA (rHRP1).

Table 1. Binding affinity (K_a × 10^6 M^{-1}) of backbone-modified PNAs to complementary dsRNA (rHRP1 and rHRP2) and dsDNA (dHRP1 and dHRP2).

PNA[a]	Backbone	dsRNA[b]	ΔH (kJ/mol)	-TΔS (kJ/mol)	dsDNA[b]	ΔH (kJ/mol)	-TΔS (kJ/mol)
PNA1	regular	**42 ± 3**	-235 ± 24	192 ± 24	**3.0 ± 0.04**	-153 ± 27	116 ± 27
PNA2	regular	**73 ± 4**	-278 ± 42	233 ± 42	**21 ± 0.4**	-296 ± 26	254 ± 26
PNA3α	α-extended	**9.1 ± 0.4**	-320 ± 13	280 ± 13	**1.5 ± 0.02**	-203 ± 27	168 ± 27
PNA3β	β-extended	**8.9 ± 0.4**	-334 ± 2	294 ± 1	**1.4 ± 0.01**	-161 ± 32	125 ± 32
PNA4[c]	regular	**15 ± 0.6**	-120 ± 6	79 ± 6	**1.1 ± 0.01**	-78 ± 5	43 ± 5
PNA5ε [c]	ε-extended	**0.9 ± 0.02**	-76 ± 1	42 ± 1	**N/B**[d]	N/A	N/A
PNA6ε	ε-extended	**1.2 ± 0.02**	-133 ± 2	98 ± 2	**N/B**[d]	N/A	N/A

[a] Association constant K_a × 10^6 M^{-1} ± Standard Deviations are averages of three ITC measurements using a Malvern MicroCal iTC200 in 2 mM $MgCl_2$, 90 mM KCl, 10 mM NaCl, 50 mM potassium phosphate buffer pH 7.4 at 25 °C. [b] Unless noted otherwise, binding to complementary rHRP1 or dHRP1. [c] Binding to complementary rHRP2 or dHRP2. [d] (N/B) No binding, K_a < 10^4 M^{-1}; (N/A) Not available.

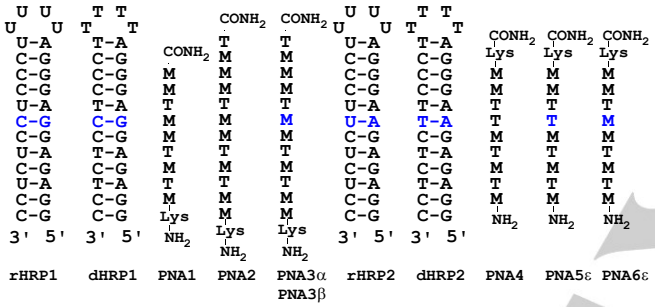


Figure 3. Sequences of dsRNA and dsDNA hairpins and modified PNAs. The variable base pair and the position of single modification is highlighted in blue.

Consistent with our previous studies, the PNA affinity dropped about an order of magnitude when binding to dsDNA (dHRP2).[9] PNA4, which differs from PNA1 by having T at the variable position (blue X in Figure 3), showed a similar trend. Somewhat surprisingly, PNA1 built of all T and M monomers having either α- or β-extended backbones did not show any binding (K_a <10^4) to either rHRP1 or dHRP1. We obtained the same result, no binding to either complementary dsRNA or dsDNA, with PNA1 that had all positions, or only all Ms modified with ε-extended linker from the main backbone to nucleobases, and PNA4 that had only all ε-extended Ts (these data are not shown in Table 1).

A single incorporation of either α- or β-extended backbone modification at a central M position in PNA3α and PNA3β (blue M in Figure 3) caused approximately an order of magnitude drop of binding affinity, which was similar to the effect of mismatched base triplets in our earlier studies.[9] A similar result was obtained with PNA5ε and PNA6ε having a single ε-extended linker to either T or M nucleobase at the variable position (blue in Figure 3). Overall, no extension of the PNA backbone at any of the positions was tolerated in either PNA-dsRNA or PNA-dsDNA triplexes.

Circular Dichroism Spectroscopy

To obtain more insight into structural features of triplexes formed by M-modified PNAs with dsRNA and dsDNA, we measured Circular Dichroism (CD) spectra of triplexes formed upon PNA binding (red lines in Figure 4).

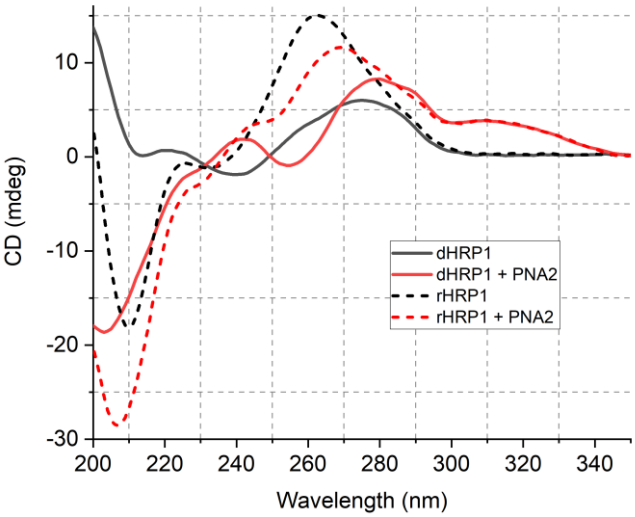


Figure 4. Comparison of CD spectra of hairpins alone (dHRP1 and rHRP1) and triple helices (red lines) formed by PNA2 with (A) dsRNA and (B) dsDNA. The samples (10 μM of each PNA2 and dHRP1 or rHRP1) were measured after 30 min. incubation in 50 mM potassium phosphate buffer containing 2 mM $MgCl_2$, 90 mM KCl, 10 mM NaCl at pH 7.4 and 25° C.

The CD spectra of DNA hairpin dHRP1 (solid black line in Figure 4) exhibited positive Cotton effects at 275 nm and 220 nm and negative Cotton effects at 240 and 214 nm, which were typical for the expected B-form helical structure.[21] The CD spectra of RNA hairpin rHRP1 (dashed black line in Figure 4) displayed a positive Cotton effect at 262 nm and a negative Cotton effect at 210 nm, which were typical for the expected A-form helical structure.[21] The addition of PNA caused changes in CD spectra that indicated the formation of new triple helical structures. The CD spectra of triplex formed between the unmodified PNA2 and RNA hairpin rHRP1 showed a slight change in CD spectra (dashed red line in Figure 4) with a positive Cotton effect at 268 nm and a shoulder peak at 245 nm and a strong negative Cotton effect at 207 nm. Thus, consistent with the strong binding (Table 1), triplex formation between dsRNA and the unmodified PNA2 did not

significantly change the A-form structure of RNA duplex. Consistent with weaker binding, the CD spectra of triplex formed between the unmodified PNA2 and DNA hairpin dHRP1 showed more notable changes in positive peaks at 279 and 242 nm and new negative peaks at 253 and 203 nm (solid red line in Figure 4). These data suggested that the B-form helical structure of DNA adopted a new conformation when it formed a triplex with PNA2, which had some characteristics of the A-form triplex structure formed by PNA2 and dsRNA (cf. red lines in Figure 4, see also Figure S37).

Interestingly, both triplex structures showed new broad peaks between 300 and 350 nm. These are likely due to the absorbance of 2-aminopyridine[22] and indicative of the structural organization of M-nucleobases in the Hoogsteen triplex. The CD spectra of triplexes formed between PNA3α and PNA3β, having either a single α- or β-extended backbone modification, were similar to CD spectra of triplexes formed by unmodified PNA2 (Figures S38-S39). Consistent with ITC results, fully modified PNAs with uniformly extended backbones did not cause any changes of the CD spectra of either RNA or DNA hairpins.

Our previous studies[9] showed that M-modified PNAs formed triple helices and not strand invasion complexes with dsRNA. To check if the complexes formed in the present study did not change to strand invasion or other structures over longer incubation time, we recorder CD spectra of complexes formed by PNA2 and rHRP1 and dHRP1 at various time points (0 min to 20 h). Spectra of PNA2-rHRP1 remained virtually identical over the entire incubation period (Figure S40). Spectra of PNA2-dHRP1 underwent only minor changes during the first 10 min (Figures S41-S42). It is conceivable that the larger conformational changes of DNA duplex upon PNA binding (as indicated in Figure 4) required somewhat longer time to reach completion. Neither, PNA2-rHRP1 nor PNA2-dHRP1 CD spectra showed any changes that could indicate transitions to structures other than the triple-helix over the longer incubation time.

Discussion

PNA's amide backbone appears to be more flexible than the sugar-phosphate backbone of natural nucleic acids. PNA-PNA formed a novel fold, a wide (28 Å) helix with A-form-like base stacking conformation.[23] The PNA-DNA[24] and PNA-RNA[25] helices adopted intermediate structures resembling the conformations of B-form DNA and A-form RNA, respectively. PNA-DNA-PNA formed an A-form-like triplex stabilized by PNA Hoogsteen strand backbone amide N-H hydrogen-bonding to DNA phosphate oxygens.[12] Our recent NMR structural studies showed that PNA formed a similar triple helix with dsRNA, which was stabilized by the same PNA backbone amide N-H to DNA phosphate oxygen interactions (Figure 2).[11] These hydrogen-bonding zippers, which were enabled by the ~5.7 Å spacing between the neighboring phosphate oxygens matching the spacing of PNA amide N-H, would not be possible in the B-form dsDNA that has ~7 Å spacing between the neighboring phosphate oxygens. Consequently, we hypothesized that extending the PNA's backbone by one -CH_2- group may bring the distance between PNA amide N-H closer to 7 Å, which would enable hydrogen-bonding to the B-form dsDNA phosphate oxygens and stabilize the PNA-dsDNA triplex. Contrary to our expectations, our experiments revealed strong destabilization of either PNA-dsDNA or PNA-dsRNA triplexes by all backbone extensions examined.

Extension of PNA backbone at various positions had been studied before our work, but not in the context of PNA-dsDNA triplex. Buchardt and co-workers[13a] synthesized PNA analogues with extended (2-aminoethyl)-β-alanine (α-extended in Figure 1) and (3-aminopropyl)glycine (β-extended in Figure 1) instead of the original (2-aminoethyl)glycine backbone and reported in 1993 that PNAs containing one modified monomer formed 2:1 complexes with complementary ssDNA with significantly lower affinity than the original PNA. In a follow-up study,[13b] the same group showed that PNAs built entirely of the monomers with extended backbones did not bind to complementary ssDNA at all. Incorporation of a single monomer with extended linker between PNA backbone and a nucleobase (ε-extended in Figure 1) also reduced the affinity of PNA for complementary ssDNA.[13b] In contrast, PNAs built entirely of such ε-extended monomers showed some, albeit weak binding to ssDNA.[13b] These results suggested that maintaining the correct backbone distance between the nucleobases was critical for tight binding, while the length of the linker from backbone to nucleobase was more tolerant to changes. The stoichiometry of PNA to DNA in Buchardt and co-workers study was 2:1. Hence, the first molecule of PNA must form a Watson-Crick hydrogen-bonded duplex with the target DNA, before the second PNA molecule forms a Hoogsteen hydrogen-bonded triplex. This complexity obscures the impact of extended backbones on triple helix formation by PNA. In other words, the low binding affinity may have been due to of unfavorable duplex formation even if the triplex formation would be favored by the extended backbones. Our studies confirmed that the extension of PNA backbone was also not tolerated in the PNA-dsDNA triple helix.

Backbone modified PNAs are little studied in PNA-dsRNA triple helices.[26] However, while this work was in progress, Chen and co-workers reported observations similar to our results that replacement of a single T in PNA octamer with a monomer having ε-extended linker to the nucleobase resulted in strong destabilization of a PNA-dsRNA triplex.[27] Taken together with previous studies, our results suggest that the (2-aminoethyl)glycine backbone is uniquely suited for recognition of DNA and RNA either in double or triple-helical structures. Most interestingly, it appears that PNA strongly prefers A-form triple-helical complexes that may be stabilized by favorable hydrogen-bonding of PNA backbone amide N-H to RNA or DNA phosphates. This notion is supported by notable changes from B-form to A-form signals in CD spectra of dsDNA induced by triple-helical binding of PNA (Figure 4B). Conversely, triple helical binding of PNA to dsRNA caused little change in the CD spectrum typical for the A-form RNA (Figure 4A). The hydrogen-bonding between amide and phosphate backbones appears to be an additional benefit rather than the main driving factor for A-form preference, as attempts to fit the hydrogen-bonding pattern to B-form DNA by extending the backbone did not give stable triplexes. Hence, PNA backbone must have subtle, but strong inherent conformational preference for A-form helical structures.

The formation of double and triple helical nucleic acid complexes is usually an enthalpy-driven process and PNA having the higher affinity towards dsRNA or dsDNA is expected to have the largest favorable negative enthalpy compensated by unfavorable entropy change. Most of our ITC experiments (Table 1) are consistent with this trend. However, there were notable exceptions. First, PNA6ε

with ε-extended M nucleobase (X = M) had higher negative enthalpy and entropy than PNA4, while its affinity towards dsRNA was much lower than that of PNA4. In contrast, the enthalpy change for PNA5ε with extended T nucleobase (X = T) was less negative and consistent with the expected lower affinity. This difference may be caused by non-specific interactions of the positively charged M nucleobase with the negatively charged RNA backbone. Second, both α- and β-extensions in PNA3α and PNA3β caused a favorable change in enthalpy (albeit small) when binding to dsRNA, while in the DNA series the enthalpy change was unfavorable, as expected for weaker binding. At this point, we do not have a compelling explanation for these discrepancies, but clearly, the thermodynamic reasons behind destabilization caused by extension of the PNA backbone is likely different in the A-form RNA than in the B-form DNA.

Conclusion

Contrary to our hypothesis, the extension of M-modified triplex-forming PNA's pseudopeptide backbone by an additional -CH_2- group did not increase the binding affinity for dsDNA compared with dsRNA. Instead, we observed strong destabilization of either PNA-dsDNA or PNA-dsRNA triplexes. Our results suggest that PNA may have an inherent preference for A-form-like conformations when binding to double-stranded nucleic acids. It appears that the original six atoms long (2-aminoethyl)glycine PNA backbone is an almost perfect fit for binding to A-form nucleic acids.

Experimental Section

PNA sequences were synthesized using standard 2-μmol scale Fmoc protocol on an Expedite 8909 DNA synthesizer using NovaSyn TG Sieber support and Fmoc chemistry as previously reported.[9, 20] For experimental details on synthesis and characterization (NMR) of modified PNA monomers, PNA synthesis and purification, and LC-MS characterization of PNA oligomers (Table S1), see Supporting Information.

Isothermal titration calorimetry experiments were performed on a MicroCal iTC200 instrument at 25 °C in phosphate buffer containing 2 mM $MgCl_2$, 90 mM KCl, 10 mM NaCl, 50 mM potassium phosphate. In a representative experiment, 2.45 μL aliquots of 150 μM PNA solution were sequentially injected from a 40 μL rotating syringe (750 rmp) into 200 μL of 20 μM RNA hairpin solution. For ITC titration traces, see Supporting Information, Table S2 and Figures S13-S36.

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Keywords: Peptide Nucleic Acid • PNA • Triple Helix • Modified Backbone • Isothermal Titration Calorimetry

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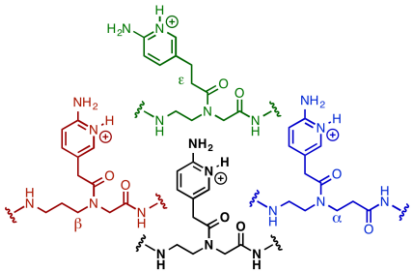

Peptide nucleic acids (PNA) form higher stability triple helices with double-stranded RNA than with DNA. Structural considerations suggested that extending the PNA backbone by one carbon atom may reverse this preference in favor of DNA. However, experiments disproved this hypothesis. The studies suggested that PNA has an inherent preference for forming A-form triple helical structures and, hence, has higher affinity for RNA than DNA.