Published in final edited form as:

Arch Biochem Biophys. 2015 December 1; 587: 1–11. doi:10.1016/j.abb.2015.08.017.

Characterization of the structural and protein recognition properties of hybrid PNA-DNA four-way junctions

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Abstract

The objective of this study is to evaluate the structure and protein recognition properties of hybrid four-way junctions (4WJs) composed of DNA and peptide nucleic acid (PNA) strands. We compare a classic immobile DNA junction, J1, vs. six PNA-DNA junctions, including a number with blunt DNA ends and multiple PNA strands. Circular dichroism (CD) analysis reveals that hybrid 4WJs are composed of helices that possess structures intermediate between A- and B-form DNA, the apparent level of A-form structure correlates with the PNA content. The structure of hybrids that contain one PNA strand is sensitive to Mg^{+2} . For these constructs, the apparent B-form structure and conformational stability (T_m) increase in high Mg^{+2} . The blunt-ended junction, *b*4WJ-PNA₃, possesses the highest B-form CD signals and T_m (40.1°C) values vs. all hybrids and J1. Protein recognition studies are carried out using the recombinant DNA-binding protein, HMGB1b. HMGB1b binds the blunt ended single-PNA hybrids, *b*4WJ-PNA₁ and *b*4WJ-PNA₃, with high affinity. HMGB1b binds the multi-PNA hybrids, 4WJ-PNA_{1,3} and *b*4WJ-PNA_{1,3}, but does not form stable protein-nucleic acid complexes. Protein interactions with hybrid 4WJs are influenced by the ratio of A- to B-form helices: hybrids with helices composed of higher levels of B-form structure preferentially associate with HMGB1b.

Graphical Abstract

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Author Contributions.

DI and CCS planned, performed experiments and analyzed data. AMB and AS performed experiments. FT contributed essential materials. AJB Jr. planned and performed experiments, analyzed data and wrote paper.

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Keywords

Four-way junctions; peptide nucleic acids (PNAs); high mobility group proteins (HMG); A-DNA helices; B-DNA helices

Introduction

Peptide nucleic acid (PNA) is a synthetic nucleic acid with a peptide backbone. PNAs were originally developed as gene targeting drugs ¹, DNA mimetics that recognize specific DNA sequences and alter the expression of target genes ². Like DNA and RNA, PNAs possess standard purine and pyrimidine bases. As do peptides and proteins, PNAs contain a polyamide backbone – typically aminoethyl glycine (AEG). The neutral PNA backbone imparts greater stability than the natural sugar phosphate backbone in hybridization to complementary strands of DNA and RNA ^{3,4}. PNA-DNA and PNA-RNA duplexes have higher melting temperature (T_m) values than the corresponding DNA-DNA and DNA-RNA structures ^{3,4}. A schematic of the chemical structures of DNA and PNA monomers is shown in Figure 1A. The polyamide backbone of PNA also confers enhanced nuclease resistance ⁵. Thus PNAs have been investigated as reagents for antisense applications ^{6–10}. The majority of PNA-related gene expression studies focus on nucleic acid targets. However, several studies have used PNAs to modulate gene expression by targeting transcription factors ^{11–17}.

We recently expanded upon the PNA-protein targeting strategy by designing hybrid PNA-DNA four-way junctions (4WJs) that bind the DNA-binding proteins, High Mobility Group B1b (HMGB1b) and Histone H1 ¹⁸. Four-way junctions are immobile versions of Holliday junctions, key intermediates in homologous and site-specific genetic recombination ^{19–21}. HMGB1 serves diverse roles as an architectural nuclear protein and proinflammatory cytokine. Our long-term goal is to develop hybrid 4WJs, composed of DNA and PNA, as high affinity ligands against HMGB1 in order to inhibit unintended proinflammatory signaling. The focus of this study is to elucidate the biophysical and protein (HMGB1b) recognition properties of hybrid 4WJs.

X-ray crystallography and NMR structural analysis have confirmed two structural states of immobilized 4WJs: *i*) an open-x and *ii*) a stacked-x conformer ^{22–27}. In low ionic strength solutions ($< 100 \mu M Mg^{+2}$), the open-x or unstacked conformer is favored ^{22–28}. In high ionic strength solutions ($> 100 \mu M Mg^{+2}$), the helical arms of each duplex stack coaxially to generate a cruciform or stacked-x structure ^{28–31}. Several research labs have confirmed that the stacked-x conformer possesses greater structural heterogeneity than the open-x

conformer; these studies show that the stacked-x conformer can fold into the two isomers (I/II and III/IV) displayed in Figure 1B^{32–35}. The hybrid junctions investigated in this study are based on the immobilized DNA junction, J1. J1 possesses an asymmetric sequence pattern that blocks branch migration^{36–38}. Hydroxyl radical footprinting, NMR and tr-FRET (time resolved-FRET) experiments show that the branch point sequences of J1 are biased towards the formation of the I/II structural isomer (Figure 1B)^{32,39}.

In this study, the biophysical and protein-binding characteristics of six hybrid PNA-DNA junctions are investigated. The DNA and PNA strands of each hybrid 4WJ are shown in black and magenta in Figure 2A. The branch point sequences are highlighted in bold text. Three hybrids, 4WJ-PNA₁, 4WJ-PNA₃ and 4WJ-PNA_{1,3}, contain shorter PNA strands relative to the DNA strands (12 vs. 16) in order to minimize synthetic problems associated with longer PNA sequences (Figure 2A). In our previous study we show that the DNA overhangs, present in 4WJ-PNA₁ and 4WJ-PNA₃, do not prohibit the formation of immobilized junctions because the non-bonded regions are not located close to the junction branch point¹⁸. To avoid overhanging DNA residues, three hybrids were designed with shorter (blunt) DNA strands: *b*4WJ-PNA₁, *b*4WJ-PNA₃ and *b*4WJ-PNA_{1,3}.

Circular dichroism (CD) analysis reveals that the hybrid 4WJs are composed of helices that possess structures that are intermediate between A- and B-form DNA. Moreover, the apparent level of A-form helices is correlative with the PNA content. The influence of ionic strength (Mg^{+2}) on the secondary structure and conformational stability (T_m) of each hybrid 4WJ is described in the Discussion section. The protein recognition characteristics of each hybrid are evaluated using the recombinant DNA-binding protein, HMGB1b. HMG proteins bind preferentially to bent and cruciform DNA within the minor groove in a non-sequence specific manner. We focus on HMGB1b because the b-box subunit is responsible for important extracellular functional roles of HMGB1^{40–42}. A schematic of the 3-D structure of HMGB1b is displayed in Figure 2B^{43,44}. Electrophoretic mobility shift assays (EMSAs) reveal that HMGB1b binds 4WJs composed of a single PNA strand with similar affinity to the DNA control, J1. HMGB1b binds hybrid junctions with multiple PNAs with relatively lower affinity. Models that describe the basis of HMGB1b binding interactions with hybrid 4WJs are presented in the Discussion.

Materials and Methods

Synthesis of PNA oligomers

Fmoc-protected PNA monomers A, C, G, and T were purchased from Panagene and used without further purification. Fmoc-Lys(Boc)-OH was obtained from Novabiochem and used without further purification. PNA oligomers were synthesized manually by standard Fmoc solid-phase peptide synthesis protocol using Rink amide resin (0.62 mmol/g), HBTU (O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate) as activating reagent and N,N-diisopropylethylamine as base. 30 mg of resin was first swollen for 1 h with dichloromethane and downloaded overnight with Fmoc-Lys(Boc)-OH (5.7 mmol) to give a final loading of 0.19 mmol/g. The unreacted amino sites were then blocked using Ac₂O/DIEA/NMP (1:2:2). The Fmoc group of the Rink-amide resin and the growing peptide chain was removed by treatment with 20% of piperidine in DMF (2x8 min). Upon

completion of the last monomer coupling, PNA oligomers were cleaved from the resin using TFA/m-cresol (9:1) and precipitated with diethyl ether. The crude PNAs were purified by reversed phase HPLC with UV detection at 260 nm, using a C-18 column (2.2 x 25 cm, 300 Å; Grace Vydac Co., Hesperia, CA), and eluting with H₂O + 0.1% TFA (eluent A) and CH₃CN + 0.1% TFA (eluent B). Elution gradient: 0–100% CH₃CN in 40 min and flow rate = 7 mL/min. The resulting pure products were collected, lyophilized, and characterized by MALDI-TOF, which gave positive ions consistent with the final products.

PNA1: H-CAATCCTGAGCA-K-NH₂ (MW = 3364.4); found m/z = 3387.21 (M+Na⁺)

PNA3: H-ATTCGGACTATG-K-NH₂ (MW = 3410.4); found m/z = 3433.14 (M+Na⁺).

MALDI-MS spectra and HPLC chromatographs that indicate high purity end PNA products are included in supporting information. PNA concentrations were determined spectrophotometrically using the following molar extinction coefficients at 260 nm: A = 13700, C = 6600, G = 11700, and T = 8600 M⁻¹ cm⁻¹.

Four-way junction oligonucleotides

The sequences for J1 are: 101^{*}, 5'-CGCAATCCTGAGCACG-3'; 102, 5'-CGTGCTCACCGAATCG-3'; 103, 5'-GCATTCGGACTATGGC-3' and 104, 5'-GCCATAGTGGATTGCG. The sequences for 4WJ:PNA₁ are: PNA1, H-CAATCCTGAGCA-K-NH₂; 102, 5'-CGTGCTCACCGAATCG-3'; 103^{*}, 5'-GCATTCGGACTATGGC-3' and 104, 5'-GCCATAGTGGATTGCG. The sequences for *b*4WJ:PNA₁ are: PNA1, H-CAATCCTGAGCA-K-NH₂; *b*102, 5'-TGCTCACCGAATCG-3'; 103^{*}, 5'-GCATTCGGACTATGGC-3' and *b*104, 5'-GCCATAGTGGATTG. The sequences for 4WJ:PNA₃ are: 101^{*}, 5'-CGCAATCCTGAGCACG-3'; 102, 5'-CGTGCTCACCGAATCG-3'; PNA3: H-ATTCGGACTATG-K-NH₂ and 104, 5'-GCCATAGTGGATTGCG. The sequences for *b*4WJ:PNA₃ are: 101^{*}, 5'-CGCAATCCTGAGCACG-3'; *nb*102, 5'-CGTGCTCACCGAAT-3'; PNA3: H-ATTCGGACTATG-K-NH₂ and 104, 5'-GCCATAGTGGATTGCG. The sequences for 4WJ:PNA_{1,3} are: PNA1, H-CAATCCTGAGCA-K-NH₂; 102, 5'-CGTGCTCACCGAATCG-3'; PNA3: H-ATTCGGACTATG-K-NH₂ and 104^{*}, 5'-GCCATAGTGGATTGCG. The sequences for *b*4WJ:PNA_{1,3} are: PNA1, H-CAATCCTGAGCA-K-NH₂; *db*102, 5'-TGCTCACCGAAT-3'; PNA3: H-ATTCGGACTATG-K-NH₂ and *b*104^{*}, 5'-CATAGTGGATTG. Fluorescein labeled strands are denoted with an asterisk (101^{*}, 103^{*}, *nb*104^{*} and *b*104^{*}). Each fluorescent strand was purified via HPLC; each non-labeled DNA strand was purified via denaturing polyacrylamide gels. All DNA was purchased from Integrated DNA Technologies (IDT). Each junction was formed by lyophilizing a mixture of a fluorescein labeled strand (25 μM) with 5-fold excess of the unlabeled strands (125 μM). The pellet was in 50 mM Tris-HCl (pH 7.5) and 1.0 mM MgCl₂, incubated at 95°C for 2 minutes, followed by cooling to room temperature for 12 – 16 hours. To determine purity of each 4WJ, samples were loaded onto 15% mini-PROTEAN native polyacrylamide gels (BioRad) and run for 1 – 5 hours (4°C). The gel running buffer was composed of 0.5 X TBE•MgCl₂ buffer (45 mM Tris, 45 mM boric acid, 1.0 mM EDTA and 1 mM MgCl₂), pH 7.6. The gels were subsequently scanned with a Typhoon 9400 Phosphorimager.

Protein expression and purification

HMGB1b from rat was expressed from pHB1-*Escherichia coli* B121(DE3)pLysS in accordance to the methods described by Chow *et al.*⁴⁵ The protein was purified via FPLC using an Econo-Pac CM cartridge (Bio-Rad). The crude protein fraction was loaded onto the CM cartridge in the presence of low salt buffer: 50 mM Tris-HCl (pH7.0), 50 mM NaCl and eluted with high salt buffer 50 mM Tris-HCl (pH7.0), 500 mM NaCl using a linear gradient. The purity of HMGB1b was monitored by resolution of each sample on 12% SDS-polyacrylamide (29:1 acrylamide:bisacrylamide) gels in Tris-Tricine buffer (150 V for 45 minutes) followed by staining for 12 hours with Coomassie Brilliant Blue G-250⁴⁶. The concentration of HMGB1b was determined by methods described previously by Pace *et al.*⁴⁷. An image (SDS gel) displaying the purity of the proteins is included in the supporting material.

Circular dichroism analysis

CD spectra were recorded using Jasco J-815 spectrometer. A 2.0 μM solution of each 4WJ was prepared in CD analysis buffer: 20 mM HEPES, 30 mM NH_4Cl , 200 mM KCl, 2 mM DTT and 10% glycerol. The low Mg^{+2} CD buffer contained 100 μM MgCl_2 ; the high Mg^{+2} CD buffer contained 2 mM MgCl_2 . All spectra were measured in a 0.1 cm path-length quartz cuvette; spectra were recorded from 320 to 200 nm in 1.0 nm increments at 4°C. The thermal denaturation profile of each 4WJ was monitored under the same buffer conditions (low and high Mg^{+2}) at a fixed wavelength (maxima near 275 nm) from 4 to 100°C. The T_m values were calculated by the fitting the average value of at least three independent scans to a sigmoidal curve equation using GraphPad Prism. All of the CD data displayed in Figures 4 – 6 and listed in Tables 1 and 2 were based on at least three independent assays.

Electrophoretic mobility shift assays

To ensure maximum complex (4:1) formation, 500 nM of each 4WJ was incubated with HMGB1b at 4°C for 30 minutes in binding buffer. The binding buffer was composed of 20 mM Tris-HCl (pH 7.5), 10 mM NaCl, 1mM MgCl and 10% (w/v) glycerol. The junction and protein are expressed in terms of molar ratio of protein to junction (P/J). P/J binding interactions were evaluated over a molar ratio range of 3.2:1 (1.6 μM :0.05 μM) to 79:1 (40.0 μM :0.05 μM). Lane 1 corresponds to 0.05 μM of 4WJ, lanes 2 – 8 represent each 4WJ incubated with each protein at protein/DNA ratios of 3.2:1, 6.4:1, 12.8:1, 25.6:1, 47.8:1, 63.4:1 and 79:1. Each sample was loaded onto 15% Mini-PROTEAN TBE precast native polyacrylamide gels (BioRad). EMSAs were run using a buffer composed of 0.5 X TBE• MgCl_2 buffer (45 mM Trisma, 45 mM boric acid, 1.0 mM EDTA and 1 mM MgCl_2), pH 7.6, at 4°C for 6 – 8 hours. The gels were subsequently scanned with a Typhoon 9400 Phosphorimager.

Results

Four-way junctions

The six hybrid 4WJs of interest are shown in Figure 2A. To determine if each construct forms stable immobilized junctions, samples are prepared under standard conditions

(Materials and Methods) and assayed by native polyacrylamide gel electrophoresis. J1 is loaded in lanes 2 and 9 to ensure a more accurate comparison of each hybrid 4WJ *vs.* the DNA control throughout the gel. As displayed in Figure 3, four of the six hybrid junctions migrate with similar mobility to that of J1. The blunt single-PNA junctions, *b*4WJ-PNA₁ and *b*4WJ-PNA₃, display electrophoretic patterns that are similar to their derivative structures (lane 4 *vs.* 3 and lane 6 *vs.* 5). The mobility patterns of the multi-PNA hybrids, 4WJ-PNA_{1,3} and *b*4WJ-PNA_{1,3}, are slower than J1 (lanes 7 and 8 *vs.* 9) reflecting the loss of charge due to the neutral PNA backbone(s) in these constructs.

The presence of fast running (i.e. high mobility) bands in each sample, including the DNA control, indicates that immobilized junctions dissociate to a certain extent during gel analysis. However, the large amount of high mobility bands in the PNA1-derived junctions (lanes 3 and 4) is an area of concern. It is plausible that these bands represent contaminating structures that promote the assembly of larger structures that mimic immobilized 4WJs. To prove that the high mobility bands are not contaminating junction structures, a series of different combinations of double and triple strands of J1 (i.e. 101–102, 102–103, 101–102–103 *etc.*) are evaluated against each hybrid 4WJ (see Figures 2 – 4 in Ref [48]⁴⁸). These controls are analogous to those used by Kallenbach and Seeman to characterize J1⁴⁹. As shown in Figure S1A, the double and triple DNA strand combinations do not form slow-running species. Hence, the high mobility bands present in lanes 3 and 4 (Figure 3) presumably represent dissociated fragments of immobilized PNA1 junctions.

CD analysis of the secondary structure of hybrid 4WJs

The CD spectra of B- and A-form nucleic acid helices possess distinctive signatures; the B-form helices found in duplex DNA and 4WJs have a minimum and maxima at 250 and 280 nm in their CD bands^{50–54}. By contrast the CD bands of A-form helices of duplex DNA and RNA show a minima near 205 nm and a maxima between 260 and 270 nm^{53,55}. Figure 4 compares the CD spectra of single- and multi-PNA junctions *vs.* J1 (black line). Panels A and B display scans run in low Mg⁺² (100 μM) and high Mg⁺² (2 mM). The CD spectrum of J1 is consistent with a full complement of B-form DNA helical arms having spectral minima and maxima at 250 and 280 nm. The CD spectra of each hybrid junction, however, display global helical conformations that are intermediate between A- and B-form DNA. Each hybrid possesses a prominent A-form band at 205 nm that is absent in the DNA control, J1. Moreover, the position of the maxima for each hybrid is shifted either slightly or significantly from 280 nm to shorter wavelengths *vs.* J1. The single-PNA junctions: 4WJ-PNA₁, *b*4WJ-PNA₁, 4WJ-PNA₃ and *b*4WJ-PNA₃ display moderate shifts (2 – 8 nm) from 280 nm (Figure 4). The multi-PNA junctions, 4WJ-PNA_{1,3} and *b*4WJ-PNA_{1,3}, exhibit more significant shifts (~15 nm) from 280 nm (Figure 4).

To determine if the A- and B-form CD signatures are unique to hybrid 4WJs and not the result of PNA structural isomers *per se*, the CD spectra of the hybrid 4WJs are compared to the spectra of PNA duplexes. The report by Wittung *et al.* clearly shows that the CD signatures of PNA duplexes are strongly influenced by the sequence and the amino acid at the C-terminus⁵⁶. According to their results, PNA duplexes have min/maxima at the following wavelengths: 220 nm (min), 240 nm (max), 255 nm (min) and 275 nm (max). The

CD signals at 220 and 240 nm are unique to PNA duplexes, whereas the signatures above 250 nm resemble those of B-form DNA. As shown in Figure 4, the CD spectra of each hybrid do not conform to those of PNA duplexes. Each spectrum in Figure 4 is based on at least three independent assays.

Analysis of the change in apparent A- and B-form content in low and high Mg^{+2}

The CD data are analyzed further to: *i*) determine the apparent levels of A- and B-form helices within the global helical structure of each spectrum and *ii*) investigate the influence of ionic strength on secondary structure. The apparent level of A- and B-form helices are estimated by using the signals at 205 and 250 nm to represent the apparent population of A- and B-form helices. The signals between 265 nm and 280 nm are excluded because these bands represent heterogeneous A- and B-form conformers. Figure 5 displays the apparent fraction of A-form (AF_A) and B-form (AF_B) helices recorded in low and high Mg^{+2} . The hatched bars represent apparent helical fractions calculated in low Mg^{+2} ; the solid bars represent the apparent helical fractions calculated in high Mg^{+2} . As expected, the secondary structure of the DNA control J1 consists predominantly of B-form helices. For J1, the AF_B values exceed 0.85 in low and high Mg^{+2} (Figure 5A). The structural features of the single-PNA junctions display two clear trends: *i*) the AF_B values are sensitive to Mg^{+2} and *ii*) the blunt hybrids possess larger AF_B values than their root structures. As shown in Figure 5A, the AF_B value of each single-PNA construct increases in high Mg^{+2} (solid bars); three of four hybrids possess AF_B values > 0.5 . A comparison of the trends of PNA1- vs. PNA3-derived hybrids reveals, that the secondary structure of the PNA3-derived hybrids is more sensitive to Mg^{+2} . 4WJ-PNA₃ and b4WJ-PNA₃ display an approximate 2-fold increase of AF_B levels in high Mg^{+2} , with the AF_B value for b4WJ-PNA₃ being nearly identical to J1. The corresponding change(s) in apparent A-form fractions for each construct is displayed in Figure 5B. The large AF_A values (> 0.9) for the multi-PNA hybrids reflect the CD signatures displayed in Figure 4. Moreover, the structure of the multi-PNA junctions is not sensitive to high ionic strength.

CD analysis of the thermal unfolding temperature (T_m) of hybrid 4WJs

The CD wavelength scans clearly show that ionic strength strongly influences the secondary structure of certain (i.e. single-PNA) hybrid junctions. We are interested in determining if a similar correlative links ionic strength to the conformational stability of hybrid 4WJs. The T_m values for each junction measured in low and high Mg^{+2} are listed in Table 1. The T_m values show that the majority of hybrid junctions (five of six) display an increase in stability in high Mg^{+2} . The T_m values for these hybrids range from 1.4°C (b4WJ-PNA_{1,3}) to 5.9°C (4WJ-PNA₃). One hybrid, 4WJ-PNA_{1,3}, is slightly less stable in high Mg^{+2} ($T_m = -0.6$). The relative increase in T_m for the majority of hybrid 4WJs may be attributed to the excess solvated Mg^{+2} ions, $Mg[(H_2O)_6]^{+2}$, that form a stabilizing solvent shell around the junction lattice.

A comparison of the T_m values of the hybrid junctions vs. J1 reveals that the majority of the hybrids (five of six) are, somewhat surprisingly, less stable than J1. The T_m values for each hybrid vs. J1 in low and high Mg^{+2} are listed in Table 2. As expected, the thermal unfolding values are reduced significantly in low Mg^{+2} ; the T_m value for each hybrid vs. J1 in low

Mg⁺² range from -9.6°C (4WJ-PNA₃) to -3.0°C (b4WJ-PNA₃). Upon shifting to high Mg⁺², the T_m values of the hybrids vs. J1 range from -7.7°C (b4WJ-PNA_{1,3}) to 1.3°C (b4WJ-PNA₃).

Comparison of the T_m values of hybrids with DNA overhang vs. blunt ends

A direct comparison of the T_m values of the hybrids with DNA overhangs vs. blunt-ended hybrids supplies a more direct method to evaluate the efficiency of the DNA end-shortening strategy. The T_m values for each hybrid junction are listed in Table 3; the corresponding CD temperature melt scans are displayed in Figure 6. As shown in Table 3, the end-shortened strategy is most effective for the single-PNA hybrids. In low Mg⁺², the T_m values for b4WJ-PNA₁ and b4WJ-PNA₃ are 0.6° and 6.6°C higher than their derivatives 4WJ-PNA₁ and 4WJ-PNA₃. In high Mg⁺², the T_m values for b4WJ-PNA₁ and b4WJ-PNA₃ are 1.5° and 5.5°C higher than their derivative structures. As shown in Figure 6A, the PNA1-derived junctions display cooperative unfolding transitions in low and high Mg⁺². The denaturation profiles of the PNA3-derived junctions do not possess a clear cooperative transition in low Mg⁺² (Figure 6B). Upon shifting to high Mg⁺², the denaturation profiles of 4WJ-PNA₃ and b4WJ-PNA₃ display more discrete transitions between folded and unfolded states (Figure 6B). For the multi-PNA hybrids, the thermal unfolding data shows that end-shortening does not enhance the stability of the b4WJ-PNA_{1,3} vs. 4WJ-PNA_{1,3}. As shown in Table 3, the T_m value for b4WJ-PNA_{1,3} is -4.7°C lower than 4WJ-PNA_{1,3} in low Mg⁺²; the T_m for b4WJ-PNA_{1,3} is -2.7°C lower in high Mg⁺². With respect to the denaturation curves, both multi-PNA hybrid displays a clear cooperative transition between folded and unfolded states in low and high Mg⁺² (Figure 6C). The denaturation scans are based on an average of at least three independent assays.

Electrophoretic Mobility Shift Assays (EMSAs) of 4WJ:HMGB1b binding interactions

Finally, EMSAs are used to evaluate the binding affinity of the previously uncharacterized hybrid 4WJs: b4WJ-PNA₁, b4WJ-PNA₃, 4WJ-PNA_{1,3} and b4WJ-PNA_{1,3} toward the recombinant DNA-binding protein, HMGB1b. Xin *et al.* used fluorescence binding and analytical ultracentrifugation analysis to show that HMGB1b binds J1 with a stoichiometry of four to one (4:1), presumably favoring the open configuration⁵⁷. The complex typically migrates as a single band without intermediate binding species. In our previous study, EMSAs were used to measure the binding constants of 4WJ-PNA₁ and 4WJ-PNA₃ toward HMGB1b. This report showed that the protein binds 4WJ-PNA₃ with a nearly identical affinity to J1 ($K_D \sim 6.0 \mu\text{M}$) and 4WJ-PNA₁ slightly less ($K_D \sim 16.0 \mu\text{M}$)¹⁸. Moreover, these data show that the insertion of a PNA strand into the immobilized lattice of J1 does not abolish protein recognition. In this study, the effect(s) of DNA length (i.e. blunt hybrids) and insertion of additional PNA strands (i.e. multi-PNA hybrids) are monitored.

The EMSA data are displayed in Figure 7. Panels A – E represent: J1, b4WJ-PNA₁, b4WJ-PNA₃, 4WJ-PNA_{1,3} and b4WJ-PNA_{1,3}. In each gel, lane 1 contains the 4WJ control (without protein); lanes 2 – 8 report on the mobility of 4WJ in the presence of increasing amounts of protein. The protein and junction are expressed in terms of molar ratio of protein to junction (P/J). As shown in Figure 7 (panel A), HMGB1b binds J1 with high affinity. The protein displays initial binding to J1 at a P/J molar ratio of 3.2:1 (lane 3). Complete binding

of HMGB1b to J1 is achieved upon increasing the P/J ratio to 6.4:1 (lane 4). Tight binding is indicated by the absence of any free J1. As shown in panel B, HMGB1b binds *b*4WJ-PNA₁ with high affinity as well. The protein displays initial binding interactions with *b*4WJ-PNA₁ at a P/J ratio of 3.2:1 (lane 3). Complete binding of HMGB1b to *b*4WJ-PNA₁ is achieved at a P/J ratio of 12.8:1 (lane 5). HMGB1b binds *b*4WJ-PNA₃ with slightly lower affinity than J1 and *b*4WJ-PNA₁. In this case, the protein displays initial binding toward *b*4WJ-PNA₃ at a P/J ratio of 6.4:1 (lane 5). Complete binding of HMGB1b to *b*4WJ-PNA₃ is achieved upon increasing the amount of protein to 12.8:1 (lanes 6 – 9). The EMSA profiles of the multi-PNA hybrids, 4WJ-PNA_{1,3} and *b*4WJ-PNA_{1,3} are shown in panels D and E. HMGB1b binds 4WJ-PNA_{1,3} beginning at a P/J ratio of 12.8:1 (panel D, lane 5) and binding appears to increase as the P/J molar ratio increases beyond 24:1 (lanes 6–8). In this case, protein binding is detected by the reduction of free 4WJ-PNA_{1,3} and the appearance of broadened/smeared bands, indicative of metastable 4:1 complexes (lane 8). The binding affinity profile of *b*4WJ-PNA_{1,3} displays a similar pattern to 4WJ-PNA_{1,3}. HMGB1b:*b*4WJ-PNA_{1,3} binding is reflected by a reduction of free *b*4WJ-PNA_{1,3} and the appearance of broadened/smeared bands (lanes 6 – 8). These data indicate that HMGB1b binds the multi-PNA constructs but does not form stable 4:1 complexes. Each EMSA represents at least three independent runs/junction.

Discussion

The current study is the first to focus on hybrid 4WJs composed of blunt DNA ends and multiple PNA strands. The overall structural features of each hybrid junction are determined via gel electrophoresis. Despite the substantial reduction in electrostatic charge, single- and multi-PNA 4WJs have similar migration patterns to J1. The multi-PNA junctions migrate slightly slower than J1 and single-PNA junctions. The less pronounced difference in electrophoretic mobility is expected because DNA-PNA gel shift assays are sensitive to conformational properties in the resulting hybrid nucleic acid complex as opposed to differences in mass or charge of the complex. For example, Peffer *et al.* showed that the difference in electrophoretic mobility of a large DNA duplex (217 nucleotides) *vs.* the resulting PNA-DNA triplexes (227 nucleotides) was strongly dependent on the shape of the triplex⁵⁸. More specifically, PNA-DNA triplexes that possessed a significant bend migrated more slowly than linear triplexes. In our study, each 4WJ possesses a similar size and global conformation (stacked-x) that migrates in nearly a charge independent manner.

The CD data suggests that the helical structure(s) of each hybrid 4WJ are intermediate between A- and B-form DNA. The structural heterogeneity of the hybrid PNA-DNA junctions is consistent with the findings by Eriksson *et al.*⁶⁰. This report showed that the 3-D structure of a PNA-DNA duplex possesses characteristics of A- and B-form DNA⁶⁰. In this study, both single- and multi-PNA hybrids display an ~4-fold increase in the amplitude at 205 nm *vs.* J1. The minima at 205 nm provide strong evidence that the hybrid junctions possess a significant population of A-form structure.

Our studies also indicate that the structure of single-PNA 4WJs is more sensitive to ionic strength. The AF_B values for each single-PNA junction are < 0.5 in low Mg⁺². Upon shifting to high Mg⁺², the AF_B values of these constructs (with the exception of 4WJ-PNA₁)

increase beyond 0.5. Although CD does not provide high-resolution information, logic suggests that the structural dimensions (i.e major and minor groove) of the single-PNA junctions are similar to J1 based on the large ratio of DNA to PNA strands (3:1). For duplex DNA, the preference of B- vs. A-form helices in high activity solvents is well known^{61–63}. Taking this into account, the suspected widened major groove of the single-PNA hybrids more readily accommodates solvated Mg^{+2} ions to promote the formation of B-form helices. The multi-PNA junctions presumably have a narrow major groove (associated with A-DNA structure) that does not readily accommodate solvated Mg^{+2} ions. For the single-PNA hybrids, high ionic strength conditions may also favor B-form helices by: *i*) reducing the repulsion between the DNA phosphate backbones, particularly at the branch point and *ii*) more effectively hydrating the phosphate backbones of the DNA strands. Support for this hypothesis is provided by molecular dynamics (MD) simulations data that show B-DNA helices are preferred in high activity solvent(s) due to favorable solvation free energy values^{64,65}. The corresponding solvation free energy values are considerably smaller for A-DNA helices⁶⁴.

Finally, the protein recognition properties of the previously uncharacterized junctions: *b*4WJ-PNA₁, *b*4WJ-PNA₃, 4WJ-PNA_{1,3} and *b*4WJ-PNA_{1,3} are evaluated with HMGB1b. The protein binds *b*4WJ-PNA₁ and *b*4WJ-PNA₃ with similar affinity to J1. With respect to the multi-PNA hybrids, HMGB1b recognizes both constructs but does not consistently form discrete 4:1 complexes. One plausible explanation for the reduced level of the protein affinity for these hybrids is linked with significant alterations of the helix rotation angle. To date, one 3-D crystal structure of a DNA junction composed of four asymmetric strands has been solved²⁶. The rotation angle (J_{twist}) between the two helices of this structure is 56.5°, a value that is strikingly close to the J_{twist} value of 60° that was determined using electrophoresis, FRET and atomic force microscopy analysis^{29,66–69}. The EMSAs are run in high ionic strength conditions (1 mM Mg^{+2}). Hence, we presume the junctions are stacked-x conformers that possess J_{twist} values near 60°. Figure 8 displays a model of HMGB1b binding to single- and multi-PNA hybrids. The model displays the predominant structural isomer - I/II. As shown in Figure 8A, the single-PNA 4WJs (and J1) possess J_{twist} angles (~60°) that facilitate strong protein binding interactions. Hence, HMGB1b forms stable 4:1 complexes with this class of hybrid 4WJs. As shown in panel B, the multi-PNA junctions may possess J_{twist} values that deviate significantly from 60°. A phenomenon that is likely the result of the flexible PNA strands within the junction lattice. This viewpoint is supported by NMR studies of PNA-DNA duplexes that show each hybrid possesses heterogeneous base stacking arrangements that are attributed to the high degree of flexibility of the PNA backbone^{60,70}.

Conclusion

PNA-DNA four-way junctions are composed of helices that are intermediate between A- and B-DNA; the apparent level(s) of A-form structure correlate with PNA content. The single-PNA constructs favor B-DNA structures and possess enhanced T_m values in high Mg^{+2} . Future studies will be conducted with to determine if different di- and monovalent cations generate similar results. The single-PNA junction, *b*4WJ-PNA₃, possesses larger apparent B-form structure (AF_B) and T_m values than the DNA control, J1. HMGB1b also binds *b*4WJ-

PNA₃ with high affinity. These results clearly show that the insertion of a PNA strand and DNA end-shortening are effective means to stabilize 4WJs without comprising protein recognition. Future studies will focus on developing novel strategies to shift/constrain the topology of the multi-PNA toward conformations that favor HMGB recognition.

Acknowledgments

This work was supported by the Mississippi INBRE, funded by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20GM103476 and startup funds from The University of Southern Mississippi.

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Highlights

Immobilized four-way junctions assembled from PNA and DNA strands contain mixed A- and B-form helices.

The apparent level of A-form helical content of hybrid PNA-DNA four-way junctions is correlative with PNA content.

The secondary structure of hybrid PNA-DNA junctions composed of single PNA strands is sensitive to Mg^{+2} .

The DNA-binding protein, HMGB1b, readily recognizes hybrid PNA-DNA junctions.

Figure 1.

A–B: A) Chemical structure of DNA and PNA monomeric units. B) Schematic of open-x and stacked-x conformers.

Figure 2.
A–B: A) Native and hybrid junctions. B) 3-D structure of HMGB1b displaying the helix-turn-helix DNA binding motif.

Figure 3. Nondenaturing polyacrylamide gel of 4WJs. Lane 1) 101, 2) J1, 3) 4WJ-PNA₁, 4) *b*4WJ-PNA₁, 5) 4WJ-PNA₃, 6) *b*4WJ-PNA₃, 7) 4WJ-PNA_{1,3}, 8) *b*4WJ-PNA_{1,3} and 9) J1.

Figure 4. CD spectra highlighting A- and B-form signals for J1 vs. single-PNA and multi-PNA 4WJ in low and high Mg^{+2} . Panel A) represents each single- and multi-PNA hybrid vs. J1 in low Mg^{+2} . Panel B) represents each single- and multi-PNA hybrid vs. J1 in high Mg^{+2} .

Figure 5.
Bar graphs displaying the apparent fraction of B- and A-form helices for each junction in low and high Mg^{+2} . Panel A) represents the fraction of B-form helices (AF_B). Panel B) represents the fraction of B-form helices (AF_A).

Figure 6.

CD thermal denaturation scans of overhang vs. blunted 4WJs in low and high Mg^{+2} . Panels A – C) display the denaturation of: 4WJ:PNA₁ vs. *b*4WJ:PNA₁, 4WJ:PNA₃ vs. *b*4WJ:PNA₃ and 4WJ:PNA_{1,3} vs. *b*4WJ:PNA_{1,3}.

Figure 7.
EMSA of HMGB1b binding with: A) J1, B) *b*4WJ:PNA₁, C) *b*4WJ:PNA₃, D) 4WJ:PNA_{1,3} and E) *b*4WJ:PNA_{1,3}.

Figure 8.

A–B: Binding models of HMGB1b binding to the I/II structural isomer of J1 and hybrid PNA-DNA junctions. Panel A displays J1 and single-PNA 4WJs. Panel B displays J1 and multi-PNA 4WJs.

Table 1

Thermal unfolding temperatures of 4WJs in low and high Mg^{+2} .

Junction	J1	4WJ:PNA ₁	b4WJ:PNA ₁	4WJ:PNA ₃	b4WJ:PNA ₃	4WJ:PNA _{L3}	b4WJ:PNA _{L3}
T _m (°C) low Mg^{+2}	39.5	32.4	32.90	29.9	36.5	35.7	31.0
T _m (°C) high Mg^{+2}	40.1	34.1	35.6	35.8	41.4	35.1	32.4
T _m (°C) (high – low)	0.6	1.7	2.7	5.9	4.9	– 0.6	1.4

Table 2

Unfolding temperatures of hybrids 4WJs in low and high Mg⁺² vs. J1.

Junction	J1	4WJ:PNA ₁	b4WJ:PNA ₁	4WJ:PNA ₃	b4WJ:PNA ₃	4WJ:PNA _{1,3}	b4WJ:PNA _{1,3}
T _m (°C) low Mg ⁺²	39.5	32.4	32.90	29.93	36.52	35.67	31.01
T _m low Mg ⁺² (°C) (J1 – hybrid)	-	- 7.1	- 6.6	- 9.6	- 3.0	- 3.8	- 8.5
T _m (°C) high Mg ⁺²	40.1	34.1	35.6	35.8	41.4	35.1	32.4
T _m high Mg ⁺² (°C) (J1 – hybrid)	-	- 6.0	- 4.5	- 4.3	1.3	- 5.0	- 7.7

Table 3

Unfolding temperatures of overhang vs. blunt 4WJs in low and high Mg⁺².

Junction	4WJ:PNA ₁	b4WJ:PNA ₁	4WJ:PNA ₃	b4WJ:PNA ₃	4WJ:PNA _{1,3}	b4WJ:PNA _{1,3}
T _m (°C) low Mg ⁺²	32.4	32.9	29.9	36.5	35.7	31.0
T _m (°C) (overhang – blunt)		0.5		6.6		– 4.7
T _m (°C) high Mg ⁺²	34.1	35.6	35.8	41.4	35.1	32.4
T _m (°C) (overhang – blunt)		1.5		5.6		– 2.7