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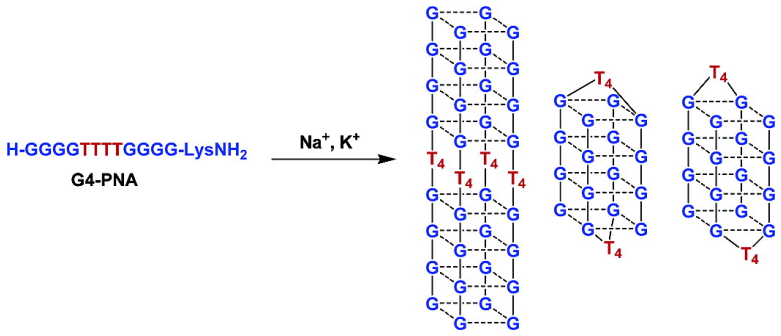

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Quadruplex Formation by a Guanine-Rich PNA Oligomer

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Abstract: A guanine-rich PNA dodecamer having the sequence H-$G_4T_4G_4$-Lys-NH_2 (**G_4-PNA**) hybridizes with a DNA dodecamer of homologous sequence to form a four-stranded quadruplex (Datta, B.; Schmitt, C.; Armitage, B. A. *J. Am. Chem. Soc.* **2003**, *125*, 4111−4118). This report describes quadruplex formation by the PNA alone. UV melting curves and fluorescence resonance energy transfer experiments reveal formation of a multistranded structure stabilized by guanine tetrads. The ion dependency of these structures is analogous to that reported for DNA quadruplexes. Electrospray ionization mass spectrometry indicates that both dimeric and tetrameric quadruplexes are formed by **G_4-PNA**, with the dimeric form being preferred. These results have implications for the use of G-rich PNA for homologous hybridization to G-rich targets in chromosomal DNA and suggest additional applications in assembling quadruplex structures within lipid bilayer environments.

Introduction

The guanine (G) quartet or tetrad structure[1,2] is gaining increasing attention due to its frequent appearance in biologically relevant systems[3−9] and its emergence from combinatorial pools of nucleic acid molecules when subjected to in vitro selection for binding to both small molecule[10] and protein[11−17] targets. The G-quadruplex motif is formed intramolecularly when a single, guanine-rich DNA or RNA strand folds back on itself multiple times to form a secondary structure stabilized by stacked guanine tetrads (Figure 1). Metal ions such as sodium or potassium, or other cations such as ammonium, bind by coordinating to the O6 atoms of the guanine units at the core of the stacked tetrad assembly.[18] Coordination occurs either within the plane of a single tetrad or between tetrad planes and is essential for stabilizing the G quadruplex. Depending on the sequence and length of a G-rich nucleic acid molecule as well as the solution conditions, two- and four-stranded G-quadruplex structures can also form. In addition, mono-, bi-, and tetramolecular quadruplexes can adopt a variety of morphologies distinguished by the relative orientations of adjacent strands forming the structure.[19]

The growing interest in G quadruplexes has led to considerable effort in the design and discovery of small molecule ligands that bind to these structures, with varying affinities and selectivites.[20−30] An alternative strategy for recognizing G quadruplexes relies not on binding to the quadruplex secondary structure but rather on hydrogen bonding to the nucleobases.

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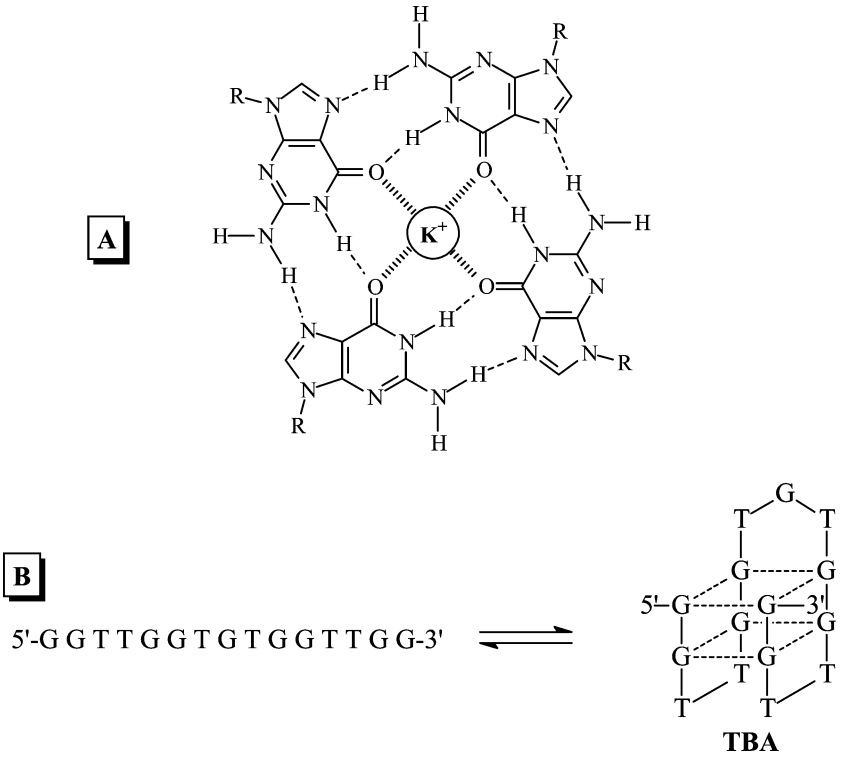

Figure 1. (A) Assembly of four guanine nucleobases into a G-tetrad stabilized by a central potassium ion. (B) Folding of the 15 nucleotide thrombin-binding aptamer (TBA) into an intramolecular G-quadruplex.[17,32−33]

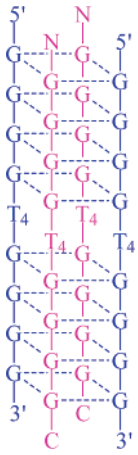

Figure 2. Four-stranded hybrid quadruplex formed between homologous PNA (magenta) and DNA (blue) strands.

While this could be done by designing a complementary oligonucleotide based on the Watson−Crick rules for base pairing, we recently reported an alternative strategy, termed "homologous hybridization", in which a peptide nucleic acid (PNA) having an identical sequence (H-GGGGTTTTGGGG-Lys-NH_2, **G_4-PNA**) to a G-quadruplex forming DNA oligonucleotide (5′-GGGGTTTTGGGG-3′, **G_4-DNA**) was used.[31] While the backbones of the PNA and DNA molecules were chemically different, the thermodynamic product upon mixing the two was a four-stranded hybrid quadruplex (Figure 2).

Given the ability of **G_4-DNA** to assemble into a dimeric quadruplex on its own,[34−36] the question naturally arose as to whether the PNA homologue was also capable of forming its own quadruplex. There are numerous examples of synthetic guanosine derivatives forming tetrads and quadruplexes,[37] and during the course of our investigations, Balasubramanian and co-workers reported the formation of a four-stranded structure by a guanine-rich PNA tetramer.[38] Quadruplex formation by chimeric PNA−DNA pentamers[39] as well as by a pure locked nucleic acid (LNA) and chimeric LNA−DNA oligomers was also recently reported.[40,41] We now report a series of optical spectroscopic and mass spectrometric studies that demonstrate formation of both two- and four-stranded G-quadruplexes by **G_4-PNA**. Potential implications of this discovery for antigene regulation of oncogene expression as well as in transmembrane ion transport are discussed.

Experimental Section

Materials. Boc/Z-protected PNA monomers were purchased from Applied Biosystems (Foster City, CA). PNA oligomers were synthesized using standard solid-phase synthesis protocols.[42,43] Fluorescein-5-

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Chart 1. PNA Oligomers Used in This Study (Sequences Written from N-Terminus to C-Terminus)

Name	Sequence	Mass (calc.)	Mass (obs.)
G_4-PNA	H-GGGGTTTTGGGG-Lys-NH_2	3540.4	3541.2
C_4-PNA	H-CCCCAAAACCCC-Lys-NH_2	3256.3	3257.6
N-Fl	Fluorescein-GGGGTTTTGGGG-Lys-NH_2	3898.4	3905.8
N-Cy3	Cy3-GGGGTTTTGGGG-Lys-NH_2	4138.2	4137.7

carboxylic acid was purchased from Molecular Probes, Inc. (Eugene, OR) and used without further purification. Cy3-carboxylic acid was a gift from Dr. Brigitte Schmidt (Carnegie Mellon University) and was coupled to the *N*-terminus of the PNA using HBTU. PNA synthesis was performed on a 100 mg scale using Boc-protected Lys-MBHA resin. All PNA oligomers (Chart 1) were purified using reversed-phase high performance liquid chromatography (RP-HPLC). PNAs were analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry using sinapinic acid as the matrix (Chart 1). PNA stock solutions were prepared in 10 mM sodium phosphate buffer (pH 7.0). Extinction coefficients for PNA monomers were obtained from Applied Biosystems (C = 6600 M^{-1} cm^{-1}; T = 8600 M^{-1} cm^{-1}; A = 13 700 M^{-1} cm^{-1}; G = 11 700 M^{-1} cm^{-1}).

Equipment. UV−vis measurements were performed on a Varian Cary3 Bio spectrophotometer equipped with a thermoelectrically controlled multicell holder. Fluorescence experiments were performed on a PTI spectrofluorimeter with a temperature controlled cell holder. Electrospray ionization (ESI) mass spectrometry (MS) experiments were performed on an LCQ Finnigan quadrapole ion trap mass spectrometer using Xcalibur Ver.1.2. The MALDI-TOF mass spectrometer was an Applied Biosystems Voyager DE sSTR (Framingham, MA). The instrument control and acquisition software version was Voyager version 5.1 with Data Explore, Rev. A.

Sample Preparation. G_4-PNA samples were suspended in a buffer containing 10 mM sodium phosphate (pH 7.0) and 0.1 mM EDTA, and variable amounts of NaCl or LiCl were incubated at 90 °C for 10 min and slowly cooled to 25 °C. Samples for ESI experiments were prepared similarly except that the PNAs were suspended in 50% methanol (no phosphate buffer) and 0.1% acetic acid.

Thermal Analysis. Samples were prepared in a buffer containing 10 mM sodium phosphate (pH 7.0), 0.1 mM EDTA, and variable amounts of NaCl or LiCl. Unless otherwise indicated, samples were heated to 90 °C and equilibrated for 5 min. UV−vis absorbance at 295 nm was recorded every 0.5 °C as samples were cooled and then heated at 1.0 °C/min. All experiments were done in triplicate.

Fluorescence Studies. Samples containing 0.5 μM each of (*i*) ***N*-Fl + G_4-PNA**, (*ii*) ***N*-Fl + *N*-Cy3**, or (*iii*) ***N*-Cy3 + G_4-PNA** were annealed in a buffer containing 10 mM sodium phosphate (pH 7.0), 100 mM NaCl, and 0.1 mM EDTA. Emission spectra were measured with excitation at 450 nm. All of the spectra were recorded at 25 °C with a 4 nm band-pass on both the excitation and emission monochromators. Ten scans were recorded and averaged. The spectrum for sample (*ii*) was corrected for direct excitation of Cy3 by subtracting the spectrum for sample (*iii*). The emission intensity at the donor maximum was measured in donor-only (I_D) and donor−acceptor (I_{DA}) hybrids. The efficiency of energy transfer (E) was calculated by the quenching of donor emission in the presence of the acceptor as

$$E = 1 - I_{DA}/I_D$$

Electrospray Ionization Mass Spectrometry. Samples containing 10 μM PNA were prepared in water/methanol (1:1) with 500 μM of NaCl, KCl, LiCl, or CsCl and 0.1% acetic acid. All experiments were conducted under positive-ion ESI conditions. Typical conditions utilized a source voltage of 3 to 5 kV, capillary temperature of 160 °C, nitrogen sheath gas set at 20−25 arbitrary units, and 20−30 scans acquired (each consisting of 30 microscans). The base pressure in the ion trap was ~1 mTorr helium. PNA samples were admitted by direct infusion with a Hamilton syringe at 5−10 μL/min.

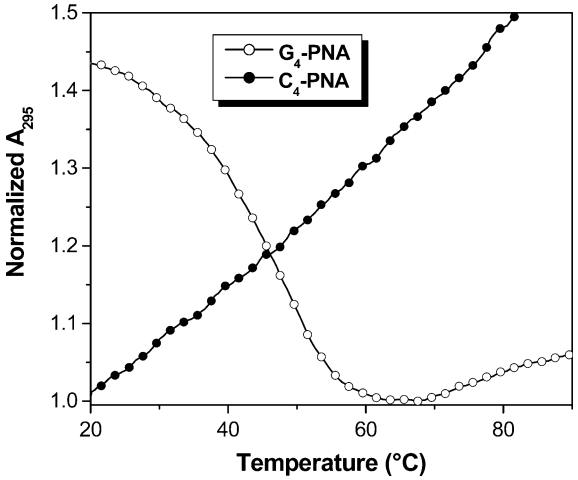

Figure 3. UV−vis melting profiles of **G_4-PNA** (5.0 μM) (○) and **C_4-PNA** (5.0 μM) (●) recorded at λ = 295 nm in 10 mM NaPi (pH 7.0), 0.1 mM EDTA with 100 mM NaCl. Data are normalized to the lowest A_{295} value.

Results

UV−Hypochromicity and Cation Dependence. G_4-PNA was annealed in sodium phosphate buffer as described in the Experimental Section. While DNA quadruplexes are often studied using circular dichroism (CD) spectropolarimetry, this did not turn out to be possible for **G_4-PNA** because no significant CD signal was observed. This, of course, does not rule out formation of a quadruplex: unlike DNA, the PNA backbone is achiral and the only asymmetric center in **G_4-PNA** is the α-carbon of the *C*-terminal lysine residue. The relatively weak electronic interaction between the nucleobases and the lysine could be insufficient to produce a measurable CD signal, although this might not be the case for all guanine-rich PNAs. For example, the PNA tetramer H-GGGT-Lys-OH, recently reported to self-assemble into a four-stranded quadruplex, exhibits a CD spectrum reminiscent of antiparallel DNA G-quadruplexes.[38] Alternatively, multiple **G_4-PNA** quadruplex structures might coexist in solution and give different CD signals that largely cancel one another.

G_4-PNA was instead characterized by UV thermal denaturation ("melting") monitored at 295 nm. This wavelength has previously been used to follow intra- and intermolecular G-quadruplex formation.[44] **G_4-PNA** shows a smooth transition with substantial hypochromicity (>30%) (Figure 3). In contrast, a sequence that is unable to form G-quartets, **C_4-PNA** (Chart 1), does not exhibit a transition at the same wavelength.

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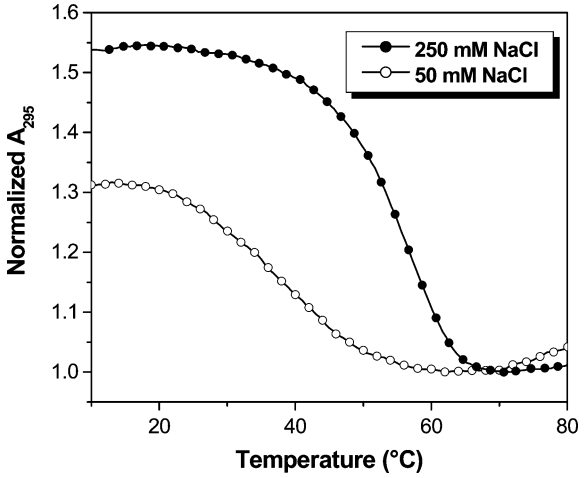

Figure 4. UV−vis melting profiles of **G_4-PNA** (5.0 μM) in 50 mM NaCl (○) and 250 mM NaCl (●) recorded at $\lambda = 295$ nm in 10 mM NaPi (pH 7.0), 0.1 mM EDTA.

Formation of a G-tetrad can also be characterized by monitoring its dependence on ionic strength and the presence of specific alkali metal ions.[45] The proximity of four negatively charged strands in DNA quadruplexes results in a marked dependence of quadruplex stability on the ionic strength of the medium. A higher ionic strength provides more cations for screening the negative charges on the DNA backbones, resulting in a more stable quadruplex. This effect should not be significant for a pure PNA structure, due to the uncharged polyamide backbone. In fact, a PNA−PNA duplex bearing terminal positive charges is only weakly stabilized by increased ionic strength.[46]

Figure 4 illustrates the effect of ionic strength on the stability of the **G_4-PNA** structure. Surprisingly, a 5-fold increase in sodium chloride concentration significantly stabilizes the structure, leading to an increase in melting temperature (T_m) of 18 °C. A larger hypochromicity is observed at the higher ionic strength, as well, suggesting better stacking of the bases.

UV melting curves were recorded at different NaCl concentrations for **G_4-PNA** alone and hybridized to the complementary strand **C_4-PNA** (Chart 1) to form a PNA−PNA duplex. As shown in Figure 5, the plot of ΔT_m versus log [Na^+] follows a linear, positive relationship in both cases, but a much steeper dependence is observed for **G_4-PNA** alone. This is consistent with binding of the sodium ions at specific sites within a **G_4-PNA** quadruplex, whereas, for the duplex, the effect of the salt is evidently restricted to screening of the relatively weak electrostatic repulsions between the cationic termini of the complementary PNA strands. Interestingly, the ionic strength dependence for the pure PNA quadruplex was significantly greater than that observed for the four-stranded hybrid quadruplex formed by **G_4-PNA** and **G_4-DNA** (Figure 5). The reason for this difference is unclear at this time, although it is possible that sodium ions bind with different affinities to the two quadruplexes.

These results are indicative of a specific interaction between the added ions and the PNA structure rather than a general electrostatic screening effect. In DNA and RNA quadruplexes, the large negative electrostatic potential at the center of the

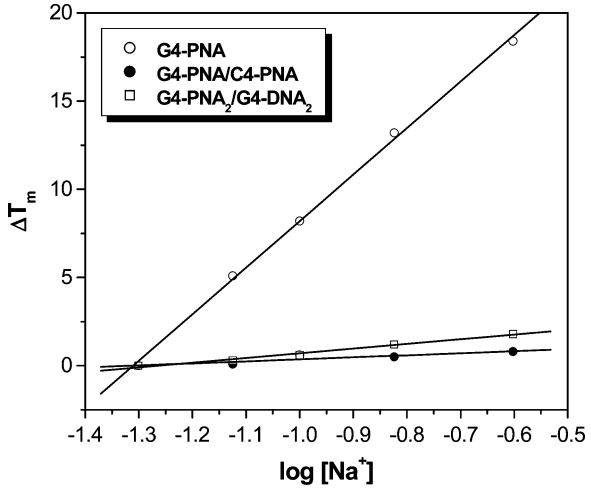

Figure 5. Plot of ΔT_m (relative to the lowest [Na^+]) versus log [Na^+] of **G_4-PNA** (5.0 μM, ○), **G_4-PNA+C_4-PNA** (5.0 μM each, ●), and **G_4-PNA+G_4-DNA** (5.0 μM each, □) in 10 mM NaPi (pH 7.0), 0.1 mM EDTA. Sodium concentration was varied by adding NaCl. Lines represent linear fits to each data set.

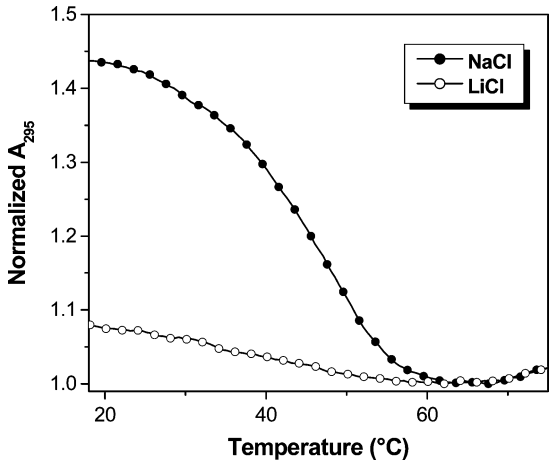

Figure 6. UV−vis heating profile of **G_4-PNA** (5.0 μM) in 100 mM NaCl (●) and 100 mM LiCl (○) recorded at $\lambda = 295$ nm in 10 mM NaPi (pH 7.0), 0.1 mM EDTA.

structure due to the guanine O6 oxygen atoms creates a reasonably high affinity binding site for various cations, including sodium, potassium, and strontium, which significantly stabilize the structure. Smaller ions, most notably lithium, destabilize these quadruplexes, and this type of ion dependence provides strong support for a quadruplex structural motif.

Figure 6 compares the UV melting curves recorded for **G_4-PNA** in the presence of 100 mM NaCl or LiCl. A clear transition is observed only in the case of the sodium salt, indicative of quadruplex formation by the PNA. The experiment cannot be performed in KCl due to extensive precipitation of the PNA, perhaps due to the formation of extended wirelike structures that are kinetically favored over smaller soluble structures. For many G-rich DNA sequences, potassium has been found to stabilize four-stranded linear quadruplexes better than sodium.[47] These linear quadruplexes can rearrange to form wirelike structures stabilized by enhanced stacking interactions.

Quadruplex Composition: (A) Fluorescence Resonance Energy Transfer. Guanine quadruplexes can assemble from

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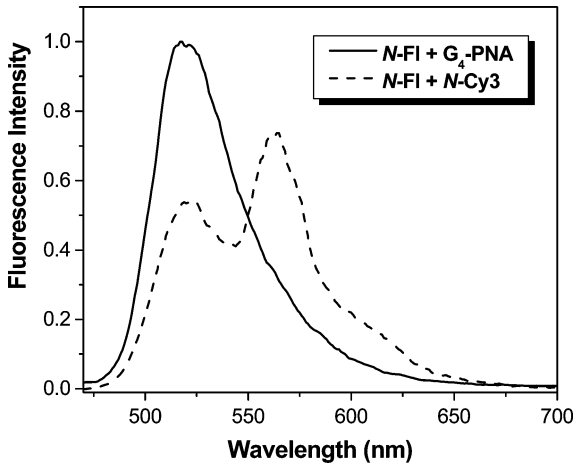

Figure 7. Fluorescence spectra of 0.5 μM ***N*-Fl** + 0.5 μM **G_4-PNA** (solid line) and 0.5 μM ***N*-Fl** + 0.5 μM ***N*-Cy3** (dashed line) recorded at 25 °C in 10 mM sodium phosphate buffer (pH = 7.0) with 100 mM NaCl and 0.1 mM EDTA. The latter was corrected for direct excitation of **Cy3** as described in the Experimental Section. Samples were excited at 450 nm.

one, two, or four strands and exhibit a diverse set of morphologies.[19] To investigate the molecularity of the quadruplex formed by **G_4-PNA**, we first used fluorescence resonance energy transfer (FRET) experiments.[31] Fluorescein (Fl) and Cy3 labeled **G_4-PNA** (Chart 1) were prepared using solid-phase synthesis protocols. The Fl and Cy3 fluorophores function as energy donor and acceptor groups, respectively. In the FRET experiments, three samples were prepared containing equimolar amounts of (*i*) ***N*-Fl** with **G_4-PNA**, (*ii*) ***N*-Cy3** with **G_4-PNA**, and (*iii*) ***N*-Fl** with ***N*-Cy3**. As shown in Figure 7, 52% energy transfer was observed based on quenching of ***N*-Fl** in the presence of ***N*-Cy3**. Since 1 equiv each of ***N*-Fl** and ***N*-Cy3** is used, only half of the complexes containing an ***N*-Fl** PNA strand should also have an ***N*-Cy3** PNA strand. The maximum FRET efficiency is thus 50%, implying that the actual FRET efficiency in the present case is practically 100%. This result demonstrates that multiple PNA strands are present in the quadruplex, although it does not distinguish between two- and four-stranded structures.

(B) Electrospray Ionization Mass Spectrometry. Electrospray ionization mass spectrometry (ESI-MS) has been used to probe noncovalent complexes in nucleic acids such as those involved in formation of DNA G-quadruplexes as well as interaction of quadruplexes with antitumor drugs and metal ions in the gas phase.[48−50] Molecules participating in the noncovalent interactions are identified depending on their mass/charge ratios. In the present case the FRET experiments suggest that the **G_4-PNA** quadruplex may be composed of either two or four strands, both of which will exhibit identical mass/charge (*m*/*z*) ratios. For example, a dimeric quadruplex (of mass 2M) that displays an *m*/*z* peak attributed to $[2M]^{n+}$ could also arise from a four-stranded quadruplex forming $[4M]^{2n+}$ ions. These quadruplexes cannot be distinguished simply based on their *m*/*z* values. However, the binding of metal cations by G-tetrads may be used to probe the number of strands involved. In particular, a quadruplex formed by either two or four strands with an odd number of cations (Y^+) bound would exhibit different *m*/*z* values. For example, a dimeric quadruplex with *x* protons and three metal cations bound would display *m*/*z* of $[2M + xH^+ + 3Y^+]^{n+}$, but this *m*/*z* may also arise from $[4M + 2xH^+ + 6Y^+]^{2n+}$ ions. However, ions formed by a four-stranded quadruplex with three metal ions bound, namely $[4M + 2xH^+ + 3Y^+]^{(2n+1)+}$, cannot also be assigned to a dimeric quadruplex, since that would require a nonintegral number of Y^+ ions to be associated. Therefore, observation of $[4M + 2xH^+ + 3Y^+]^{(2n+1)+}$ ions would indicate formation of a four-stranded **G_4-PNA** quadruplex.

Table 1. Observed and Expected Masses of **G_4-PNA** (M) Ions and K^+ Adducts and Possible Molecularities of PNA Complexes

entry	species	*m*/*z* obsd	*m*/*z* calcd	possible molecularity
1	$[M + 2H^+]^{2+}$	1771.0	1771.2	monomer
2	$[M + 3H^+]^{3+}$	1181.1	1180.7	monomer
3	$[M + H^+ + K^+]^{2+}$	1790.0	1790.7	monomer/dimer/tetramer
4	$[M + 2K^+]^{2+}$	1809.0	1810.2	monomer/dimer/tetramer
5	$[2M + 3H^+ + K^+]^{4+}$	1780.9	1780.7	dimer/tetramer
6	$[2M + 2H^+ + 2K^+]^{4+}$	1789.8	1790.2	dimer/tetramer
7	$[2M + H^+ + 3K^+]^{4+}$	1799.9	1799.8	dimer/tetramer
8	$[4M + 5H^+ + 3K^+]^{8+}$	1785.5	1785.5	tetramer
9	$[4M + 3H^+ + 5K^+]^{8+}$	1795.4	1795.0	tetramer
10	$[4M + H^+ + 7K^+]^{8+}$	1805.0	1804.5	tetramer

Ions could not be detected in ESI-MS experiments performed in the same buffer conditions used for the optical spectroscopy experiments described above. However, an aqueous methanol solvent with added chloride salts allowed various complexes to form and be detected. For example, in the presence of NaCl, triply charged **G_4-PNA** molecules ($[M + 3H^+]^{3+}$) were observed along with multiple Na^+ adducts of a single **G_4-PNA** molecule (Supporting Information, Figure S1). Ions of a dimeric (or tetrameric) **G_4-PNA** were not observed. Thus, nonspecific adducts of Na^+ with **G_4-PNA** single strands predominate over **G_4-PNA** quadruplexes under these conditions.

In the presence of KCl, $[M + 2H^+]^{2+}$ and $[M + 3H^+]^{3+}$ ions are observed (entries 1 and 2, Table 1). More importantly, ions arising from K^+ adducts of **G_4-PNA** are also observed. As shown in Figure 8 and Table 1, notable among these are ions arising from an odd number of K^+ ions associating with a dimeric **G_4-PNA** quadruplex, such as $[2M + 3H^+ + K^+]^{4+}$ (entry 5) and $[2M + H^+ + 3K^+]^{4+}$ (entry 7).[51] Notably, these ions cannot arise from a single **G_4-PNA** molecule alone, since that would require a nonintegral number of K^+ ions in the complex. A similar strategy has been previously used to identify complexes composed of identical polypeptide chains.[52,53] In this context, the ions attributed to $[2M + 3H^+ + K^+]^{4+}$ and $[2M + H^+ + 3K^+]^{4+}$ (indicated with double asterisks in Figure 8) may also arise due to the tetrameric quadruplex adducts $[4M + 6H^+ + 2K^+]^{8+}$ and $[4M + 2H^+ + 6K^+]^{8+}$, respectively. Ions that can *only* be assigned to K^+ adducts of a tetrameric species, namely $[4M + 3H^+ + 5K^+]^{8+}$ (entry 9) and $[4M + H^+ + 7K^+]^{8+}$ (entry 10), are in fact also observed (Figure 8, peaks indicated with single asterisks), but in much lower relative

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(51) We note that some of the peaks in the mass spectra are rather broad and might contain sodium adducts in addition to the potassium adducts assigned in Table 1. However, these species should have low abundance, since only trace amounts of sodium ions are present and potassium ions should be bound more tightly by the PNA G-tetrads.
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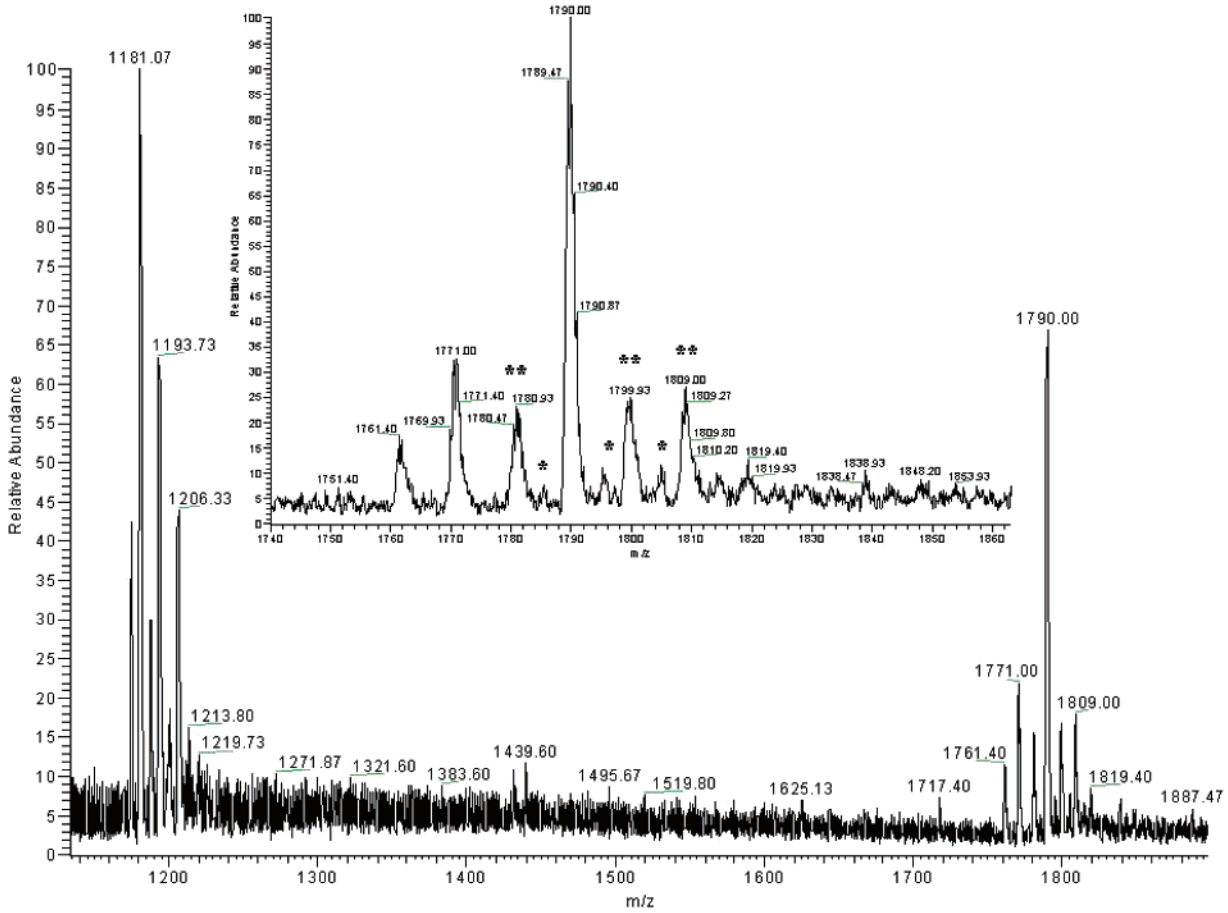

Figure 8. Positive-ion electrospray mass spectrum of **G_4-PNA** in 500 μM KCl. Inset: Expansion of high *m*/*z* region. Dimeric and tetrameric species are indicated with (**) and (*), respectively, and assignments are given in Table 1.

abundance than the other ions. Since ESI-MS abundances can quantitatively reflect the amounts of different complexes in solution,[54] these observations indicate that **G_4-PNA** forms both dimeric and tetrameric quadruplexes, although dimeric structures appear to be favored under these conditions.

To further study the **G_4-PNA** quadruplexes, ESI-MS experiments were performed on a sample containing varying relative concentrations of **G_4-PNA** and ***N*-Fl**. The fluorescein label on the latter strand leads to a higher mass than that for **G_4-PNA**, meaning heterodimeric and homodimeric species will be resolved in *m*/*z*. In addition, four distinct tetrameric complexes are possible, containing zero, one, two, or three **G_4-PNA** strands. In Figure 9A, **G_4-PNA** and ***N*-Fl** were mixed in a 3:1 ratio. The major peak ("a") can be assigned to either a two- or four-stranded **G_4-PNA** homoquadruplex having two or four bound potassium ions (Table 2). Similarly, the peak at 1799.7 is due to two- or four-stranded structures having three or six bound potassium ions. Peaks for heteroquadruplexes ("b", 1:1 or 2:2 hybrids) or ***N*-Fl** homoquadruplexes ("c") are very weak.

When the ratio of the two strands was 1:1, peaks due to both homo- and heteroquadruplexes are observed (Figure 9B). Peak "b" is assigned to a 1:1 or 2:2 heteroquadruplex having two or four bound potassium ions, while peak "c" corresponds to the two- or four-stranded ***N*-Fl** homoquadruplex having two or four bound potassium ions. The minor peak labeled "d" is assigned to a four-stranded heteroquadruplex having five bound potassium ions. Finally, when **G_4-PNA** and ***N*-Fl** were mixed in a 1:3 ratio, the **G_4-PNA** homoquadruplex and heteroquadruplex structures decrease in abundance relative to the ***N*-Fl** quadruplex (Figure 9C, compare peaks "a" and "b" with "c"). Importantly, no peaks corresponding to 3:1 or 1:3 heteroquadruplexes were observed at any PNA ratio. Since the two PNAs have identical sequences, lack of a heterotetramer of 3:1 or 1:3 composition is further evidence for the dimeric quadruplex being more stable under the ESI conditions and, therefore, the major complex in solution.

The idea of cations of a specific size stabilizing the G-quartets formed by **G_4-PNA** was tested further by using Cs^+, which does not effectively stabilize G-tetrads.[9,45] We performed an ESI-MS experiment on **G_4-PNA** in the presence of Cs^+ to examine its behavior with PNA G-quartets. As shown in the Supporting Information (Figure S2), **G_4-PNA** does not form any significant adducts with Cs^+. This result clearly shows that the stabilizing effect of metal ions on dimeric and/or tetrameric **G_4-PNA** quadruplexes is selective for K^+ over Cs^+, analogous to DNA quadruplexes.

Finally, we note that the solution injected into the mass spectrometer was quite different from that used for the optical spectroscopy experiments described above: the samples used for ESI-MS contained no sodium phosphate buffer but had 50% MeOH to promote volatilization. To see if the change in solution conditions affected the optical spectroscopy, we performed UV melting and FRET experiments in conditions consistent with

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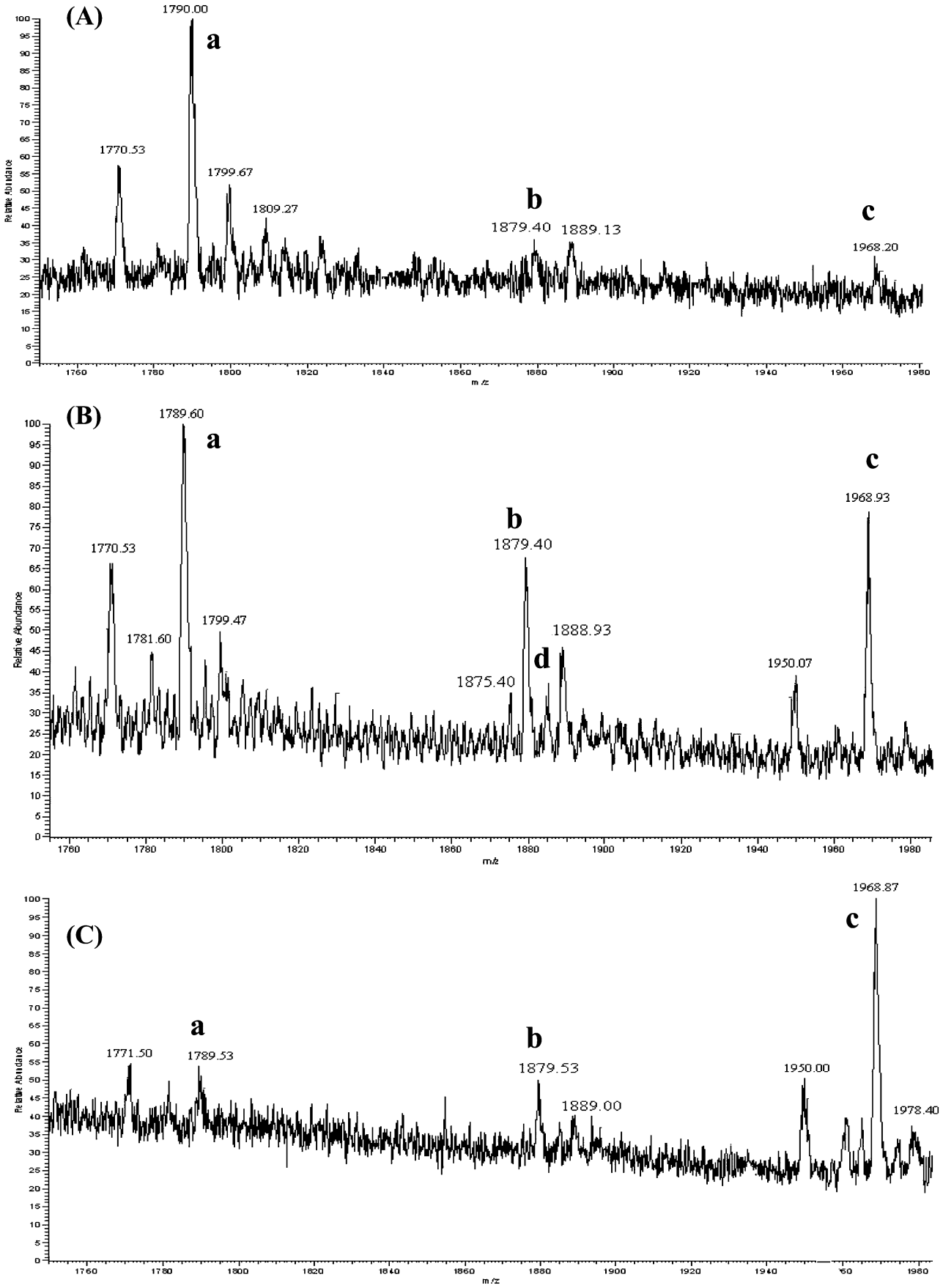

Figure 9. Comparative electrospray mass spectra of **G_4-PNA** (**M**) and ***N*-Fl** (**L**) in varying proportions (A) **M:L** = 1.5:0.5; (b) 1.0:1.0; (C) 0.5:1.5. Labeled ions are (a) $[2M + 2H^+ + 2K^+]^{4+}$, (b) $[M + L + 4H^+ + 2K^+]^{4+}$, (c) $[2L + 6H^+ + 2K^+]^{4+}$, and (d) $[2M + 2L + 7H+ + 5K^+]^{8+}$. (See Table 2 for other assignments.)

Table 2. Observed and Expected Masses of **G_4-PNA** (**M**) and ***N*-Fl** (**L**)[a] Adducts with K^+

species	*m/z* observed	*m/z* calculated
$[M + 2H^+]^{2+}$	1770.5	1771.2
$[2M + 3H^+ + K^+]^{4+}$	1781.6	1780.7
$[2M + 2H^+ + 2K^+]^{4+}$ [b]	1790.0	1790.2
$[2M + H^+ + 3K^+]^{4+}$	1799.7	1799.8
$[L + 4H^+]^{2+}$	1950.1	1950.2
$[2L + 6H^+ + 2K^+]^{4+}$	1968.9	1969.3
$[M + L + 4H^+ + 2K^+]^{4+}$	1879.4	1879.7
$[M + L + 3H^+ + 3K^+]^{4+}$	1888.9	1889.3
$[2M + 2L + 7H^+ + 5K^+]^{8+}$	1885.3	1884.5

[a] Note that the fluorescein dye on ***N*-Fl** contributes a charge of −2 to the PNA. [b] There is also contribution from $[M + H^+ + K^+]^{2+}$.

ESI-MS. The quadruplex melting temperature retains its strong dependence on ionic strength, while FRET is also still observed and is practically quantitative (Supporting Information, Figures S3 and S4).

To probe further the effect of methanol on the PNA quadruplexes formed in sodium-containing buffer, we measured UV melting curves for **G_4-PNA** in solutions containing increasing amounts of methanol (Figure S5). Interestingly, the melting temperature and hypochromicity increased significantly with added methanol, indicating the methanol stabilizes PNA quadruplexes. This seems to contradict the fact that no quadruplexes were observed in the mass spectra recorded under these conditions. One possible explanation for this discrepancy is that the methanol promotes formation of extended G-wirelike structures that are sufficiently soluble to avoid precipitation but are heterogeneous in both size and charge, leading to the lack of a single observable peak.

Overall, the results from the ESI-MS together with the UV and fluorescence experiments clearly show that **G_4-PNA** (*i*) is capable of quadruplex formation and (*ii*) likely forms both dimeric and tetrameric quadruplexes, the ratio of which depends on the solution conditions.

Discussion

The results described above demonstrate the ability of a guanine-rich PNA to assemble into multistranded complexes stabilized by G-tetrads. These structures are stabilized by Na^+ and K^+, but destabilized by Li^+ and Cs^+. This dependence on the specific cation mirrors that observed for DNA guanine quadruplexes,[45] which are generating much interest for their suspected role in regulating gene expression and as ligands isolated from combinatorial polynucleotide libraries for binding to small molecule and protein targets.

The quadruplex formed by **G_4-PNA** does not exhibit a significant CD spectrum. This is in contrast to the PNA tetramer HO-Lys-TGGG-H, which was recently reported to assemble into a four-stranded quadruplex and exhibited a weak but clearly measurable CD spectrum.[38] The lack of a CD spectrum for **G_4-PNA** could indicate that the quadruplex has little or no helicity, but it is also possible that the lone L-lysine residue on the C-terminus of the PNA is insufficient to impart a *preferred* helicity to the structure, meaning that approximately equal amounts of right- and left-handed quadruplexes are present in solution. Complementary PNA strands form duplexes for which the helicity depends strongly on the appended amino acid as well as the base pair adjacent to the amino acid.[55−57] The lack of backbone chirality in PNA oligomers renders the induction of chirality in their secondary (and higher) structures weak, in most cases. G-rich PNAs based on chiral backbones in conjunction with high-resolution structural characterization should help address the issue of PNA quadruplex helicity in greater detail.

As mentioned above, the PNA tetramer HO-Lys-TGGG-H assembles into a four-stranded quadruplex.[38] Four-stranded structures are also detected unambiguously in the ESI-MS data for **G_4-PNA**. Assigning other peaks to dimeric structures is complicated by the fact that any such peak could also arise from a tetrameric structure with double the charge. However, two pieces of evidence suggest that dimeric quadruplexes are formed by **G_4-PNA** and may, in fact, be the preferred molecularity in the presence of potassium ions. First, the peaks in Figure 8 and Table 1 that can only arise from tetrameric complexes are of very low relative abundance. There is no obvious explanation for why these quadruplexes, having five or seven bound potassium ions, would be significantly less stable than similar structures having four, six, or eight potassium ions. A more likely explanation is that the species having greater abundances correspond to dimeric structures.

The second piece of evidence in support of dimeric quadruplexes formed by **G_4-PNA** comes from the mass spectra acquired after mixing **G_4-PNA** and ***N*-Fl**. The results shown in Figure 9 and Table 2 clearly demonstrate that hybrid structures are formed, most notably one complex having an empirical stoichiometry of 1:1 (e.g., $m/z = 1879.4$). If this were actually due to a four-stranded quadruplex, then one would expect to see peaks corresponding to 1:3 and 3:1 stoichiometries as well, but no such species were detected. Thus, we conclude that **G_4-PNA** preferentially assembles into a dimeric quadruplex, although a small amount of tetrameric structures are observed in the presence of potassium. Depending on the strand concentration, ionic strength and specific cations present, the ratio of dimeric/tetrameric quadruplexes will likely change. In fact, quadruplex polymorphism is well-known for various guanine rich DNA sequences,[45] including the DNA analogue of **G_4-PNA**.[58−60] In addition to variations in the number of strands in these structures, the alignment of the PNA strands, namely parallel versus antiparallel, remains to be determined and will likely require high-resolution structural analysis. Figure 10 illustrates plausible tetrameric and dimeric quadruplex structures for **G_4-PNA**.

Our interest in PNA quadruplexes was originally stimulated by our recent discovery that homologous G-rich DNA and PNA strands can assemble to form a hybrid tetrameric quadruplex consisting of two DNA and two PNA strands.[31] This expanded the DNA recognition repertoire of PNA beyond Watson−Crick and Hoogsteen interactions that are used to assemble duplex and triplex structures, respectively, and suggested the use of PNA to target G-rich DNA sequences within chromosomal

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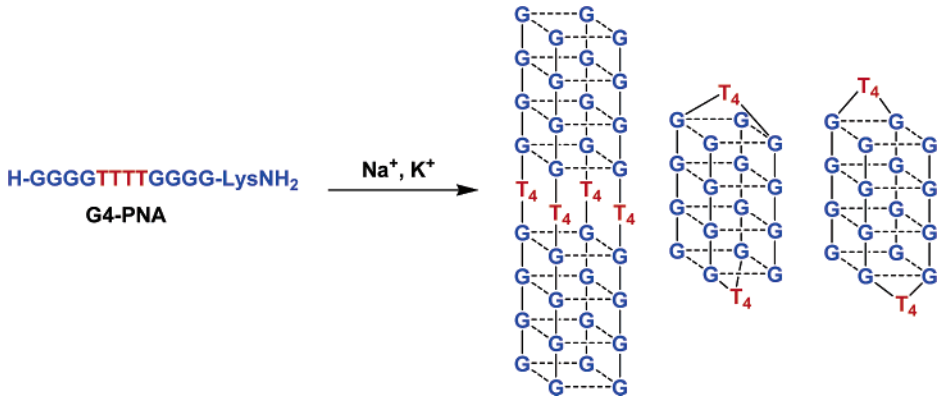

Figure 10. Possible four- and two-stranded G-quadruplex structures adopted by **G_4-PNA**.

DNA. One example of such a target site that is of considerable interest biologically and therapeutically is the nuclease hypersensitivity element (NHE) that lies upstream of the P1 promoter for the c-MYC oncogene. This region of the DNA is highly enriched in guanine and is known to fold into an intramolecular quadruplex in vitro.[6,61−63] Indirect evidence also suggests that the quadruplex can form in vivo, even in the presence of the complementary C-rich strand.[3,5] If a PNA could be designed that targeted the G-rich strand via homologous hybridization, then, by definition, the same PNA strand would be complementary to the C-rich strand. This would permit the same PNA to hybridize to both strands at the target site and could greatly improve the potency for antigene activity.

There are two potential obstacles that must be avoided or overcome in order for such a strategy to be effective. First, any quadruplex structure adopted by the antigene PNA alone should be of sufficiently low thermodynamic and kinetic stability as to be readily disrupted in order for the PNA to hybridize to the DNA target. The fact that the PNA_2−DNA_2 structure we reported previously is more stable than the two isolated quadruplexes suggests that any inherent structure for the PNA will not be insurmountable, at least from a thermodynamic perspective. Second, the PNA should be able to hybridize to the G-rich DNA strand in a 1:1 stoichiometry (or 2:1, depending on the length of the PNA), rather than the 2:2 stoichiometry we reported previously. This is because, in most cases, there will only be one DNA strand to target. The apparent ability of **G_4-PNA** to form dimeric quadruplexes with itself indicates that similar structures should be possible for PNA−DNA homologous hybrids. While strand invading PNA-peptide conjugates[64−66] and pseudocomplementary PNAs[67−69] should be applicable to a broader range of targets within duplex DNA, G-rich PNA domains might be used in combination with these motifs to allow enhanced hybridization to biologically significant guanine-rich sites in genomic DNA.

Finally, we note that our findings might be relevant to recent work directed toward assembling G-quadruplex structures within lipid membranes.[70] One potential application of such structures is to transport potassium ions across membranes, perhaps leading to antibiotic activity. The nonpolar nature of PNA could facilitate cooperative assembly of membrane-spanning four-stranded quadruplexes.

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Supporting Information Available: ESI-MS data for **G_4-PNA** in NaCl and CsCl and UV melting and FRET experiments performed in the ESI-MS solvent. This material is available free of charge via the Internet at http://pubs.acs.org.

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