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A PNA_4 Quadruplex

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Guanine-rich sequences of nucleic acids can self-recognize via cyclic interactions at their Hoogsteen sites to form quartets, called G-tetrads.[1] Quadruplex DNA (Q-DNA) is a quadruple helical nucleic acid structure containing stacked G-tetrads. Q-DNA has potential as a structural element for the construction of nanoscale assemblies.[2] We wanted to create an unnatural analogue of Q-DNA that lacks the negatively charged backbones but utilizes the self-recognition property of guanine. Peptide nucleic acids (PNAs) are synthetic analogues of DNA where the sugar−phosphate backbone of DNA has been replaced by a neutral polyamide backbone.[3] The pseudopeptide backbone has proven to be a good structural mimic of the sugar−phosphate backbone forming duplexes[4] and triplexes.[5] PNA can also form hybrid quadruplexes only if provided with a DNA template.[6] Here we describe the first quadruplex composed entirely of PNA (Q-PNA).

We have investigated the self-assembly of the PNA sequence **pTG3** (Figure 1A) comprising three tandem guanine residues. A lysine was appended to the C-terminus to enhance solubility in water. A thymine residue was included at one terminus to prevent uncontrolled aggregation that is sometimes observed with terminal guanines.[7] **pTG3** was synthesized using solid-phase synthesis using Fmoc chemistry (Supporting Information).

To form a PNA complex, a solution of 200 μM **pTG3** was heated at 90 °C for 10 min, then cooled at a rate of 0.5 °C/min to 5 °C and incubated at 5 °C for 8 h. The solution was subjected to electrospray ionization mass spectrometry (ESI-MS) to study the molecularity of the PNA species and seek evidence for Q-PNA. ESI-MS has been used to observe noncovalent intermolecular complexes of DNA[8] and PNA−DNA hybrids.[9] **pTG3** was analyzed by positive ion nanoelectrospray ionization mass spectrometry (nano-ESI-MS). Analysis at a cone voltage of 60 V and source temperature of 30 °C showed peaks corresponding to a triply charged species at *m*/*z* 1716.9 and a doubly charged species at *m*/*z* 2575.2 (Supporting Information).[10] The associated molecular weight (MW) for these peaks was 5148 ± 2 Da, consistent with tetramer formation by **pTG3** (MW of **pTG3** = 1287 ± 0.5 Da). Peaks corresponding to $(M_4 + 2H + Na)^{3+}$, $(M_4 + 2H + K+)^{3+}$, and $(M_4 + H + 2K)^{3+}$ were also seen (Figure 2A). Therefore ESI-MS supports that **pTG3** forms tetramers.

For small molecules with significant potential to form hydrogen bonds, the observation of noncovalent complexes by ESI-MS can sometimes be the result of a nonspecific interaction, due to the nature of the electrospray process. To address this, we combined solution-phase H/D exchange with ESI-MS to ascertain whether the noncovalent complexes are formed in solution via a specific mode of interaction or whether they were formed nonspecifically in vacuo.[11] H-bonded protons exchange slowly with the solvent as compared to non-H-bonded protons. Therefore, a comparison of the number of exchangeable protons for the monomer with that of the tetrameric complex would provide insight into the structure of the complex and the associated noncovalent interactions. H/D

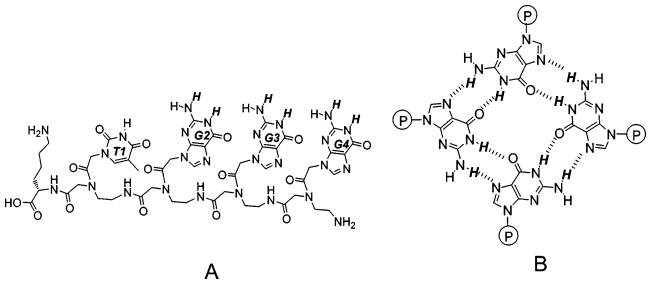

Figure 1. Structure of peptide nucleic acid pTG3. Exchangeable protons are indicated as H, and those involved in H-bonding are shown in bold italics.

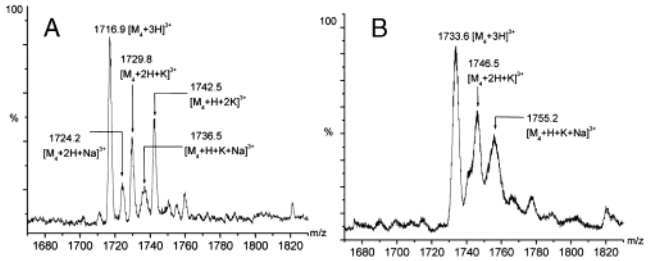

Figure 2. Partial nano-ESI-MS spectrum of 200 μM **pTG3** (A) before and (B) after D_2O exchange.

exchange and ESI-MS have been used in this way to probe protein conformation[12] and also DNA G-quadruplex formation.[13]

A 200 μM solution of **pTG3** tetramer in water was lyophilized and resuspended in D_2O to deuterate exchangeable protons. Nano-ESI-MS of this sample showed peaks now shifted to *m*/*z* 1733.6 (from 1716.9) corresponding to $[M_4 + 3H]^{3+}$ (Figure 2B). The average MW of a tetramer with *m*/*z* 1733.6 is 5198 ± 3 Da, and the MW for the corresponding monomer is 1300 ± 0.75 Da. This means that on average, H/D exchange occurs at 13 sites per **pTG3** monomer out of a possible 19. This suggests that the remaining six exchangeable protons per **pTG3** monomer are present in a protected environment in the tetrameric complex, $(\mathbf{pTG3})_4$, and therefore do not exchange. In fact, these protected protons were found to be exchange inert over 2 weeks at 4 °C. Such slow exchange is consistent with the observations made for H-bonded guanine imino protons in tetramolecular DNA quadruplexes by NMR spectroscopy.[14]

In a complementary back-exchange experiment, **pTG3** was heated to 90 °C in D_2O to disrupt the noncovalent complex, lyophilized, and resuspended in D_2O three times to give the fully deuterated form of **pTG3**. Figure 3A shows the spectrum of the complex formed from fully deuterated **pTG3**. A triply charged species with *m*/*z* 1742 was observed where the average MW of the corresponding tetramer was 5224 ± 2 Da and the corresponding monomer was 1306 ± 0.5 Da, confirming deuteration at all 19 possible sites. The sample was freeze-dried, resuspended in H_2O, and analyzed by ESI-MS after a 2 h incubation. The spectrum (Figure 3B) showed a triply charged peak at *m*/*z* 1724.6 corre-

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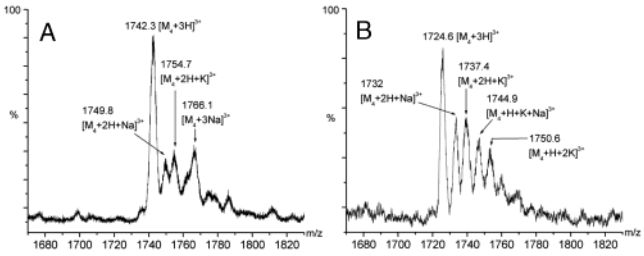

Figure 3. Partial nano-ESI-MS spectrum of 200 μM **pTG3** (A) where all the exchangeable sites are deuterated and (B) after H_2O exchange.

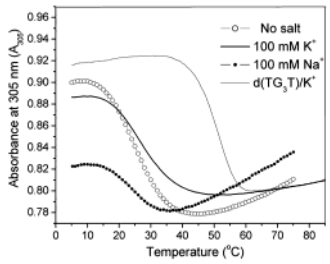

Figure 4. UV melting profiles at 305 nm of 200 μM **pTG3** in the absence and presence of various ions in phosphate buffer at pH 7.4.

sponding to a tetramer with MW 5171 ± 3 Da. The average MW of the associated monomer was 1293 ± 0.75 Da showing that on average only 13 sites per monomer had back-exchanged. This confirms that **pTG3** forms a tetramolecular complex, (**pTG3**)$_4$, in solution via a specific mode of interaction, where six protons per strand are present in a protected environment. This is consistent with the formation of three G-tetrads in (**pTG3**)$_4$ where two exchangeable protons per guanine (shown in bold italics in Figure 1A) are involved in Hoogsteen hydrogen bonds (Figure 1B).[15]

UV melting experiments were then performed on (**pTG3**)$_4$. DNA duplexes show a positively sloped sigmoidal curve when the UV absorbance at 260 nm (A_{260}) is plotted against temperature (T). Q-DNA melting is characterized by an *inverse* sigmoidal thermal melting curve observed at 295 nm.[16] This inverted denaturation profile is highly characteristic of quadruplexes. At higher strand concentrations, quadruplex melting may be followed at 305 nm.[16] The UV melting profile of 200 μM **pTG3** at 305 nm in water (pH 7), in the absence of salt, showed an inverse sigmoidal curve with a $T_{1/2}$ of 25 ± 1 °C. In 100 mM sodium phosphate buffer (pH 7.4), 200 μM **pTG3** showed a transition with a comparable $T_{1/2}$ of 24 ± 1 °C. Changing the medium to 100 mM potassium phosphate buffer (pH 7.4) increased the $T_{1/2}$ slightly to 28 ± 1 °C.[17] The analogous DNA sequence d(GGGT) did not show a simple transition because of aggregation at the 5′ end leading to the formation of an interlocked quadruplex. Hence, d(TG3T) was used as the DNA control. d(TG3T)$_4$ under identical conditions showed a transition with a relatively steeper curve at a higher $T_{1/2}$ of 51 ± 1 °C (Figure 4). This observation suggests that Q-PNA is not as stable or cooperative as Q-DNA and shows little additional stabilization upon the addition of K^+ or Na^+ unlike Q-DNA.[18] Stabilization of Q-DNA by cations is due to two factors: (a) electrostatic screening of the negatively charged DNA strands and (b) coordination by eight guanine O6 functional groups between tetrads.[18b] For Q-PNA, cation stabilization can only result from coordination between tetrads. Given that noncovalent PNA complexes are slightly destabilized by cations,[19] it is not so surprising that the overall stabilization of Q-PNA by cations is small.

To address strand polarity in the quadruplex, circular dichroism (CD) measurements were carried out (Supporting Information). Q-PNA showed a CD spectrum similar to that of antiparallel DNA quadruplexes.[20] Indeed, an antiparallel arrangement would align the positively and negatively charged termini of the PNA strands more favorably than a parallel arrangement.

This communication describes the first four-stranded motif based solely on PNA. The PNA_4 motif offers some core structural features of the G-quadruplex, without the negatively charged backbone. We envisage that this novel structure may find application as a design element in nanoscale assemblies.

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Supporting Information Available: Synthesis, dependence of $T_{1/2}$ on [**pTG3**], ^{1}H NMR in H_2O/D_2O, CD, and MS analysis of triplex (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) Modest intensity peaks at 1931.5 Da $[M_3 + 2H]^{2+}$ were also observed corresponding to a triplex. Detailed analysis confirmed that these were due to dissociation of (**pTG3**)$_4$ (Supporting Information).

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(15) ^{1}H NMR showed a broad peak at 10−11 δ ppm corresponding to H-bonded Imino protons on the guanines, which were inert to D_2O exchange (Supporting Information)

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(17) UV melting studies of varying strand concentrations in phosphate buffer pH 7.2, 100 mM K^+ revealed that as [**pTG3**] increased, not only did $T_{1/2}$ increase, but the transition became more pronounced (Supporting Information), confirming the intermolecular nature of the complex. The transition investigated by UV is a two-state process where the tetramer melts into single strands.

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