



## A study of the interactions that stabilize DNA frayed wires

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### ABSTRACT

Oligodeoxyribonucleotides (ODNs) with long, terminal runs of consecutive guanines, and either a dA or dT tract at the other end form higher-order structures called DNA frayed wires. These aggregates self-assemble into species consisting of 2, 3, 4, 5, ... associated strands. Some of the remarkable features of these structures are their extreme thermostability and resistance to chemical denaturants and nucleases. However, the nature of the molecular interactions that stabilize these structures remains unclear. Based on dimethyl sulfate (DMS) methylation results, our group previously proposed DNA frayed wires to be a unique set of nucleic-acid assemblies in which the N7 of guanine does not participate in the guanine–guanine interactions. To probe the hydrogen bonding involved in the stabilization of d(A<sub>15</sub>G<sub>15</sub>) frayed wires, we used Raman spectroscopy in which the DNA sample is held in photonic crystal fibers. This technique significantly enhances the signals thus allowing the use of very low laser power. Based on our results for d(A<sub>15</sub>G<sub>15</sub>) and those of incorporating the isoelectronic guanine analog pyrazolo[3,4-d]pyrimidine or PPG, into a frayed wire-forming sequence, we provide evidence that these structures are based on the G-quadruplex model. Furthermore, from the Raman spectrum, we observed markers that are consistent with the presence of deoxyguanosine residues in the *syn* conformation, this suggests the presence of anti-parallel G-quadruplexes. To identify the species that contain *syn* guanine residues, we used circular dichroism and gel electrophoresis to study an ODN in which all of the guanine residues were brominated, d(A<sub>15</sub><sup>8-Br</sup>G<sub>15</sub>). In the presence of potassium, d(A<sub>15</sub><sup>8-Br</sup>G<sub>15</sub>) forms what appears to be an anti-parallel dimeric G-quadruplex. To our knowledge, this is the first report of a DNA sequence having all its guanine residues replaced by 8-bromo-guanine and maintaining its ability to form a G-quadruplex structure.

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### 1. Introduction

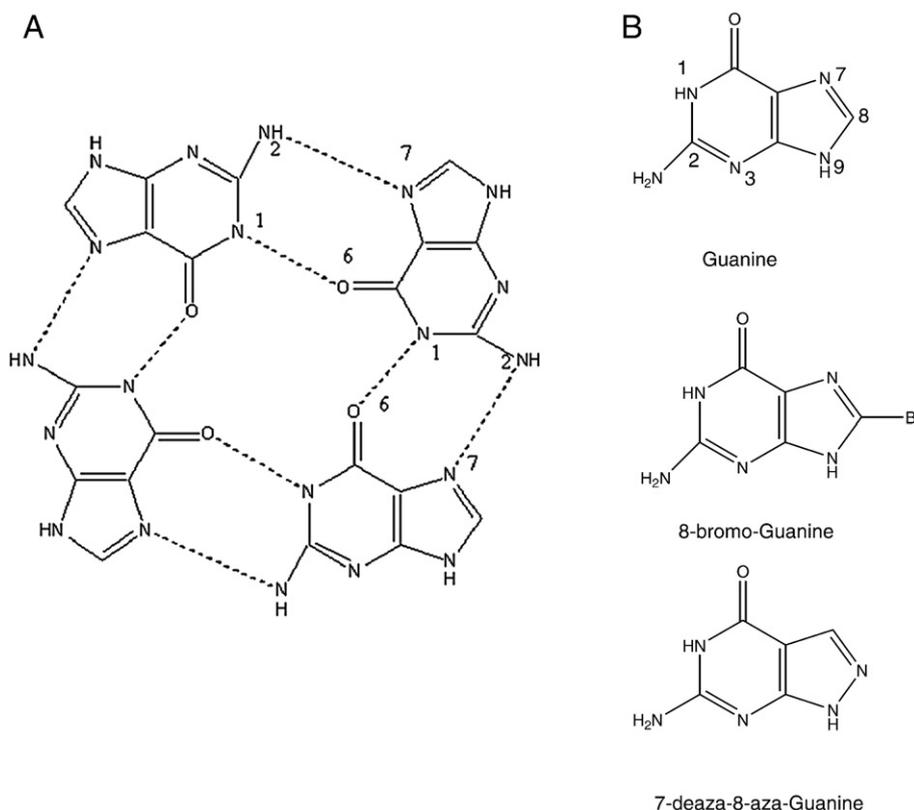
DNA molecules with long runs of consecutive guanine residues can form four-stranded helices called G-quadruplexes in the presence of monovalent cations, especially K<sup>+</sup> and Na<sup>+</sup>. The interaction of the four strands is stabilized by Hoogsteen hydrogen bonding between the guanine residues, resulting in a motif called G-quartets (Fig. 1A) [1]. Although G-quadruplexes are highly polymorphic by nature [2], the basic structural motif, the G-quartet, remains conserved. These four-stranded structures can be comprised of a single folded G-rich oligodeoxyribonucleotide (ODN) incorporating three loops; two ODNs with two loops; or an assembly of four individual strands with no loops. Parallel G-quadruplexes are those with all strands sharing the same polarity; a G-quadruplex is said to be anti-parallel if at least one of the strands is oriented in an opposing direction in relation to the other three strands. The glycosidic angles of parallel G-quadruplexes are all in the *anti* conformation, whereas the

corresponding anti-parallel structures exhibit a mixture of *anti* and *syn* conformations. The biological significance of four-stranded structures appears to lie in the number guanine-rich sequences across the human genome (~370,000) [3,4]; these sequences may form intramolecular quadruplexes. Guanine-rich sequences are found in telomeres [5], immunoglobulin-coding regions [6], and promoter regions [7].

G-rich DNA sequences can also form higher-order structures such as G-wires [8], interlocked-G-quadruplexes [9], and DNA frayed wires (FW). The formation of these structures appears to depend on the presence of terminally-positioned guanine-tracts in the primary DNA sequence of the parent strands. ODNs with the general sequence d(G<sub>x</sub>N<sub>y</sub>) or d(N<sub>y</sub>G<sub>x</sub>) where x ≥ 10 and y ≥ 6, where N is any nucleobase (but not G or C), spontaneously self-assemble to form these higher-order structures [10,11]. In the Frayed wire structure, the non-guanine portion remains single stranded and can participate in Watson–Crick base-pairing without detrimentally affecting the overall structure [12]. Thus, DNA FWs are comprised of two conformationally independent domains: the arms (the non-guanine portion) and the stem, which is made up the guanine residues [13]. Once resolved by gel electrophoresis, the aggregates appear as a ladder pattern of bands [12,14,15] that differ from each other by one

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**Fig. 1.** (A) G-quartet; (B) chemical structures of (from top to bottom): guanine base; 8-bromo-guanine; and 7-deaza-8-aza guanine (PPG).

parent strand; that is, the aggregates consist of 2, 3, 4, 5, ... interacting parent strands [16].

DNA FWs possess unusual features such as extreme thermostability, resistance to chemical denaturants and to nuclease digestion [14,15,17]; however little is known about the intermolecular interactions that stabilize them. The circular dichroism (CD) spectrum of DNA FW closely resembles that of parallel-stranded G-quadruplexes [18]. Scanning force microscopy (SFM) measurements showed that the average height of DNA FW was approximately 2 nm [19]. This is consistent with the height of G-wires, which are G-quadruplex-based super-structures [20]. Intriguingly however, probing the N7 of guanines in DNA FW with dimethyl sulfate (DMS) revealed that N7 is actually accessible [11,16]. Because one of the hallmarks of G-quadruplex formation is the inaccessibility of N7 of guanines involved in a G-quartet [21], this observation raised the possibility that guanine–guanine interactions of DNA FWs differ from those found in G-quadruplexes.

In this report we have used modified guanine residues and employed biophysical techniques including Raman spectroscopy and CD to provide evidence of N7 involvement in the formation of DNA FW. Furthermore, we report, for the first time, that a DNA sequence with all of its guanines replaced with 8-bromo-guanine (<sup>8-Br</sup>dG) forms what appears to be a dimeric G-quadruplex.

## 2. Materials and methods

### 2.1. Oligonucleotides

The cartridge-purified oligonucleotides d(A<sub>15</sub>G<sub>15</sub>) and d(A<sub>15</sub><sup>8-Br</sup>G<sub>15</sub>) were purchased from Cortec DNA Service Laboratories Inc. (Kingston, Ontario, Canada). Their concentrations were estimated spectrophotometrically, using extinction coefficients at 260 nm calculated from the nearest-neighbor model [22] for d(A<sub>15</sub>G<sub>15</sub>) (408,150 M<sup>-1</sup> cm<sup>-1</sup>), and an extinction coefficient of 400,500 M<sup>-1</sup> cm<sup>-1</sup> was used for

d(A<sub>15</sub><sup>8-Br</sup>G<sub>15</sub>). The Glen–Pak purified 7-deaza-8-aza-dG (pyrazolo [3,4,-d]pyrimidine) oligonucleotide d(A<sub>15</sub><sup>PPG</sup>G<sub>15</sub>) was graciously supplied by Glen Research (Sterling, VA, USA). The extinction coefficient used for this oligonucleotide was 387,400 M<sup>-1</sup> cm<sup>-1</sup> which was provided by Nanogen Inc. (San Diego, CA, USA). The DNA samples were dissolved in 10 mM Tris–HCl (pH 7.5) plus 100 mM of either KCl or NaCl for experimentation.

### 2.2. Sample preparation

Prior to electrophoresis or circular dichroism scans, all DNA samples were prepared to a final concentration of 5 μM DNA strands in 10 mM Tris–HCl (pH 7.5) and either 20 or 100 mM KCl. Samples were heated to 95 °C for 5 min and then left to cool slowly to room temperature. Samples were then left at 10 °C overnight. For Raman spectroscopy measurements, d(A<sub>15</sub>G<sub>15</sub>) was prepared with a final concentration of 2 mM of DNA strands in 10 mM Tris–HCl (pH 7.5), and 100 mM KCl. Samples were heated for 5 min at 95 °C and left to cool slowly to room temperature.

### 2.3. Circular dichroism spectroscopy

CD spectra were collected at 10 °C in 1.0-nm increments from 220–300 nm on an Aviv model 62A DS circular dichroism spectrometer (Lakewood, NJ, USA) using a cuvette with a 0.1-cm pathlength. The CD spectra presented are the average of three consecutively measured scans.

### 2.4. Electrophoresis

Native polyacrylamide gel electrophoresis was carried out on a 10% polyacrylamide gel containing 50 mM Tris–borate buffer (pH 7.5) and 20 mM KCl. The gels were run at 10 V/cm, at 10 °C. Oligonucleotides were radiolabelled with [γ-<sup>32</sup>P]ATP using T4 polynucleotide kinase

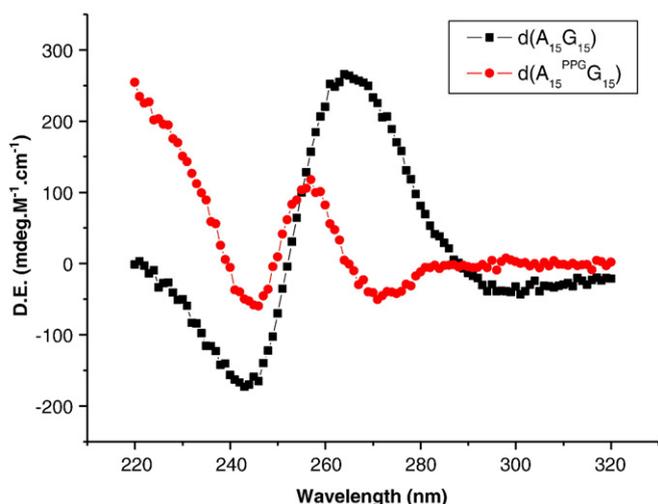
(Fermentas). Band patterns of the resolved gels were visualized on a STORM model 840 imager (GE Healthcare).

### 2.5. Raman spectroscopy

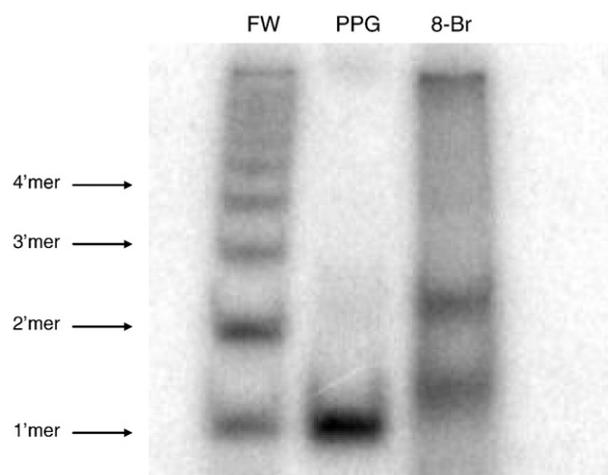
A hollow-core photonic crystal fiber (PCF) model HC800, purchased from Crystal Fiber (Denmark), was prepared as described [23]. The PCF is a novel optical structure and it features a hollow core surrounded by a cladding composed of silica and air [24]. A sample volume of 10  $\mu\text{L}$  (2 mM DNA strands) was transferred to a well and one end of the fiber was immersed in the solution. The fiber and well were then placed on a JY Horiba LabRam (Boston, MA, USA) system for Raman excitation and collection. The Raman system includes a HeNe laser emitting at a wavelength of 633-nm. A laser power of 4 mW was used. The beam is directed on to the sample by a dielectric filter used as a dichroic mirror with a drop-off Stokes edge of  $< 150 \text{ cm}^{-1}$ . Light is coupled into the core of the PCF through a 100 $\times$  objective with a numerical aperture of 0.90. Back scattered radiation from the PCF's entire length is then collected through the same objective. The collected radiation is refocused and passed through the dielectric filter after which it is directed through an adjustable slit aperture into the spectrometer. The slit aperture was kept at 500  $\mu\text{m}$  throughout the experiment. The spectrometer is 300 mm in length and it incorporates a 1200 lines/mm grating, which provides a resolution of  $2.4 \text{ cm}^{-1}$ /pixel. The detection was carried out using a 16-bit Peltier cooled  $1024 \times 256$  pixel CCD. Acquisition times of 10 s were consistently used and the signal was averaged over 10 cycles. Additionally, the system is equipped with a motorized XY stage with a resolution of 0.1  $\mu\text{m}$  which allows optimal focusing into the PCF.

### 3. Results

On the basis of their reactivity with dimethyl sulfate, we previously proposed that the non-covalent interactions between the guanine residues in DNA FW differ from those stabilizing the G-quartet motif [11,16]. In the results presented here we were interested in further investigating the involvement of the N7 of guanine in DNA FW. To this end, we studied the behavior of  $d(\text{A}_{15}\text{G}_{15})$ , an ODN in which all guanines were replaced with 7-deaza-8-aza-dG (pyrazolo[3,4-d]pyrimidine) or PPG (Fig. 1B), a base that is isoelectronic with guanine. The CD spectra of these two ODNs display a difference in the intensity of the positive band (Fig. 2); and in their electrophoretic mobility in buffers containing 100 mM KCl (Fig. 3). The CD spectrum of the



**Fig. 2.** CD spectra of  $d(\text{A}_{15}\text{G}_{15})$  (black squares) and  $d(\text{A}_{15}^{\text{PPG}}\text{G}_{15})$  (red diamonds) in Tris-HCl, pH 7.5 and 100 mM KCl. The DNA strand concentration equals 5  $\mu\text{M}$ . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



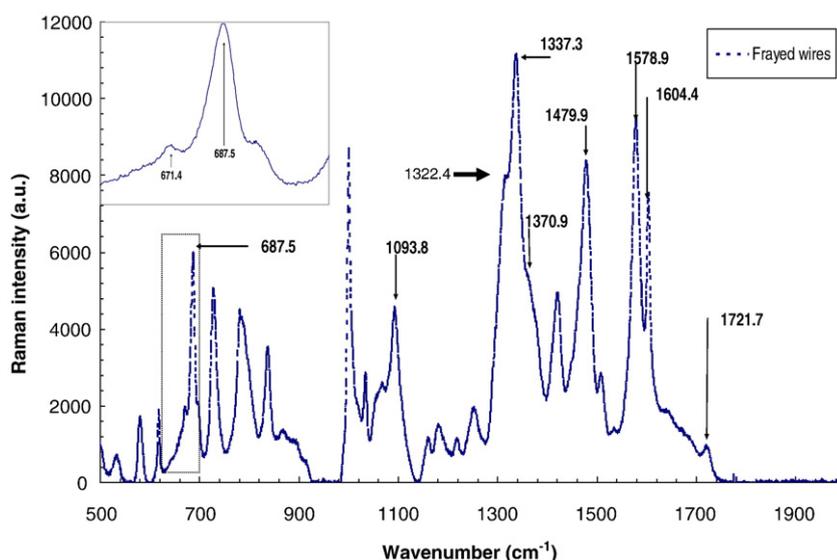
**Fig. 3.** Autoradiograms of  $^{32}\text{P}$ -labeled oligonucleotides following electrophoresis on native 10% polyacrylamide gels. DNA samples were prepared in Tris 10 mM (pH 7.5) and contained either 20 mM KCl for FW ( $d(\text{A}_{15}\text{G}_{15})$ ) and 100 mM KCl for PPG ( $\text{A}_{15}^{\text{PPG}}\text{G}_{15}$ ) and 8-Br ( $\text{A}_{15}^{8\text{-Br}}\text{G}_{15}$ ).

FW-forming sequence  $d(\text{A}_{15}\text{G}_{15})$  displays a positive peak  $\sim 265 \text{ nm}$ ; consistent with parallel-stranded G-quadruplex formation [25]. The modified ODN,  $d(\text{A}_{15}^{\text{PPG}}\text{G}_{15})$  displays a less intense band  $\sim 255 \text{ nm}$ . Fig. 3 compares the electrophoretic mobilities of the unmodified with the modified sequences. As anticipated, electrophoresis of  $d(\text{A}_{15}\text{G}_{15})$  results in a ladder of bands; while only one band, corresponding to a single-stranded species, was observed for  $d(\text{A}_{15}^{\text{PPG}}\text{G}_{15})$ , (Fig. 3). We observed the same electrophoretic pattern of the PPG-containing ODN after incubation at  $4^\circ\text{C}$  for 14 days in the presence of various concentrations of KCl, NaCl, and  $\text{NH}_4\text{Cl}$  (data not shown).

Fig. 4 shows the Raman spectrum of  $d(\text{A}_{15}\text{G}_{15})$ . In this figure, markers that identify Hoogsteen hydrogen bonding present in G-quartets [26–28] can be seen. The mode at  $1479.9 \text{ cm}^{-1}$ , an indicator of N7 hydrogen bonding can be clearly identified (Fig. 4). In addition, there are other bands characteristic of Hoogsteen-type hydrogen bonding. These include:  $1721.7 \text{ cm}^{-1}$ , an O6 marker;  $1604.4 \text{ cm}^{-1}$ , an N1 marker; and  $1578.9 \text{ cm}^{-1}$ , the C2– $\text{NH}_2$  group marker [26,28–30]. These modes indicate that the DNA assemblies in DNA FW are in fact involved in Hoogsteen hydrogen bonding.

There are also Raman bands that are diagnostic for the conformation of the glycosidic bond of the bases. In a previous study using Raman spectroscopy we showed that the structures contain guanine residues in the *anti* conformation consistent with parallel-stranded tetramolecular G-quadruplexes [17]. In the present study, we were able to observe previously unseen Raman markers using a novel technique to measure the Raman spectra [23]. In agreement with our previous study, the Raman spectrum of  $d(\text{A}_{15}\text{G}_{15})$  (Fig. 4) provided clear modes that identify guanines in the *anti* conformation ( $687.5$  and  $1337.3 \text{ cm}^{-1}$ ) [26,28]. However, there were also weak modes arising from guanine residues in the *syn* conformation at  $671.4 \text{ cm}^{-1}$  (Fig. 4, inset) and  $1322.4 \text{ cm}^{-1}$  (Fig. 4) [28]. This suggests that DNA FW may be comprised of modest amounts of anti-parallel G-quadruplex species since the glycosidic angles of the guanine residues in such structures are a mixture of *anti* and *syn* [28,29].

The glycosidic bond of bromo-guanine residues (Fig. 1B) preferentially adopts the *syn* conformation due to steric constraints resulting from the presence of the bulky bromine at the C8 position. The use of bromo-guanine has been especially useful for the identification of *syn* conformations of guanine residues in G-quadruplex-forming sequences [31]. The incorporation of 8-bromodeoxyguanosine (8-Br) at positions that are *syn* increases the stability of the folded G-quadruplex; whereas one observes a decrease in stability with 8-Br placed in positions that are



**Fig. 4.** Raman spectrum of  $d(A_{15}G_{15})$  frayed wires. Raman spectra ( $500\text{--}2000\text{ cm}^{-1}$ ) of  $d(A_{15}G_{15})$  with a total concentration of 2 mM of DNA strands in solution containing 10 mM Tris-HCl (pH 7.5) and 100 mM KCl.

*anti*. Based on the CD and Raman results of  $d(A_{15}G_{15})$ , it appears as though the majority of the guanine residues are in the *anti* conformation. However, due to the presence of Raman markers indicating the existence of guanines adopting the *syn* conformation, we wanted to investigate whether  $d(A_{15}^{8\text{-Br}}G_{15})$  would form frayed wires.

Fig. 3 displays the electrophoretic mobility of samples of  $d(A_{15}^{8\text{-Br}}G_{15})$  incubated in a buffer containing 100 mM KCl in relation to  $d(A_{15}G_{15})$  DNA FW and  $d(A_{15}^{\text{PPG}}G_{15})$  single strands. The lane containing  $d(A_{15}^{8\text{-Br}}G_{15})$ , shows two distinct bands: a fast moving band sharing the same mobility of the fastest moving band in  $d(A_{15}G_{15})$  and that of the single band in  $d(A_{15}^{\text{PPG}}G_{15})$ ; and a second band that exhibits a mobility similar to the dimeric (2'mer) species of DNA FW. Both bands appear to have a slightly lower mobility than the corresponding bands of the DNA FW sample; this may arise because of the higher molecular weight  $d(A_{15}^{8\text{-Br}}G_{15})$ .

In order to see whether the slow moving band of  $d(A_{15}^{8\text{-Br}}G_{15})$  is a G-quadruplex, we collected CD spectra of this ODN incubated in buffers containing various chloride salts (Fig. 5A). It is well established that potassium and sodium ions are effective inducers of G-quadruplexes; while  $\text{Li}^+$  and  $\text{Cs}^+$  are not [21,32]. The CD spectrum of  $d(A_{15}^{8\text{-Br}}G_{15})$  in the presence of 100 mM KCl shows two positive peaks; a shoulder around 260 nm and a major band at  $\sim 300$  nm indicating the presence of anti-parallel G-quadruplexes [25]. In the presence of 1 M KCl, the CD spectrum is identical to the spectrum observed in 100 mM KCl (data not shown). In the presence of sodium ions the intensity of the band at 300 nm is somewhat lower than the intensity observed in the presence of potassium ions; in buffers containing  $\text{Li}^+$  or  $\text{Cs}^+$  the intensity of this band is significantly diminished, indicating a clear dependency on the type of cation in solution for the presence of that particular band.

It has been shown that primary alcohols induce G-quadruplex formation [33,34]. We attempted to promote the apparent dimer formation from the single-stranded  $d(A_{15}^{8\text{-Br}}G_{15})$  present in 100 mM KCl (Fig. 3) by adding ethanol to the solution. Fig. 5B compares the CD spectra of  $d(A_{15}^{8\text{-Br}}G_{15})$  in buffer only (10 mM Tris), 40% (v/v) ethanol with and without 10 mM KCl. The CD spectrum of  $d(A_{15}^{8\text{-Br}}G_{15})$  in the presence of 40% ethanol without any added KCl closely resembles to that of  $d(A_{15}^{8\text{-Br}}G_{15})$  in 10 mM KCl. Addition of 10 mM KCl to a buffer containing 40% (v/v) ethanol caused an increase in the intensity of the CD band  $\sim 300$  nm. Finally, in the absence of potassium or ethanol, the CD spectra of  $d(A_{15}^{8\text{-Br}}G_{15})$  are devoid of the 300 nm peak and only one visible peak is present around 260 nm (Fig. 5B). We suggest

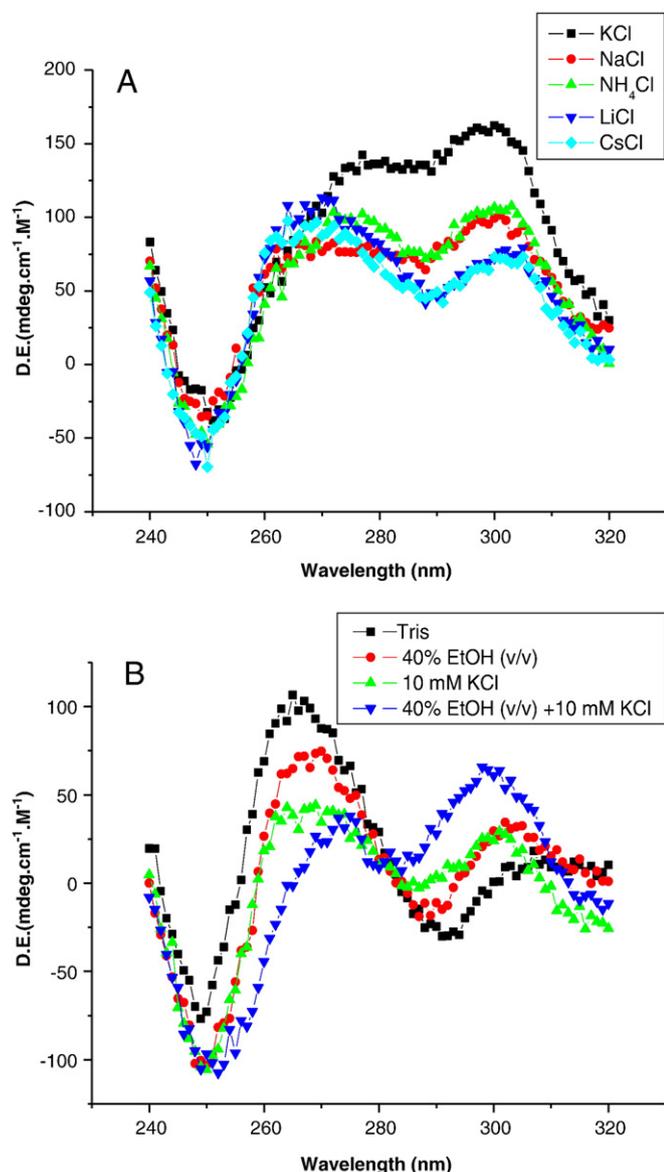
that the CD bands at  $\sim 260$  and  $\sim 300$  nm arise from the single-stranded species of  $d(A_{15}^{8\text{-Br}}G_{15})$  and anti-parallel dimeric G-quadruplexes respectively.

#### 4. Discussion

In this report, we employed two types of chemically-modified guanosine residues in order to gain a better understanding of the factors affecting the formation and stabilization of DNA frayed wires. In general, modifying the guanine residue in ODNs known to form G-quadruplexes disfavors the formation of these four-stranded structures [35].

In standard G-quadruplexes, the N7 of the interacting guanines participate in Hoogsteen hydrogen bonding and cannot be methylated by DMS. DNA frayed wires, a set of self-assembled structures that arise from ODNs that contain long, terminal runs of guanine residues, are stabilized by guanine-guanine interactions that were thought, up until recently, to be distinct from G-quadruplexes. In a previous study we showed N7 of guanines in  $d(T_{15}G_{15})$  to be readily available for methylation by DMS [11,16]. This observation led us to propose that DNA frayed wires might be stabilized by an interaction distinct from G-quartet motif (Fig. 1A). To probe the existence of Hoogsteen hydrogen bonding and to assess the role that N7 plays in the formation of  $d(A_{15}G_{15})$  DNA FW, we employed a novel technique to enhance the Raman signal and replaced the guanine residues in the DNA FW-forming ODN  $d(A_{15}G_{15})$  with an isoelectronic guanosine analog, 7-deaza-8-aza (PPG).

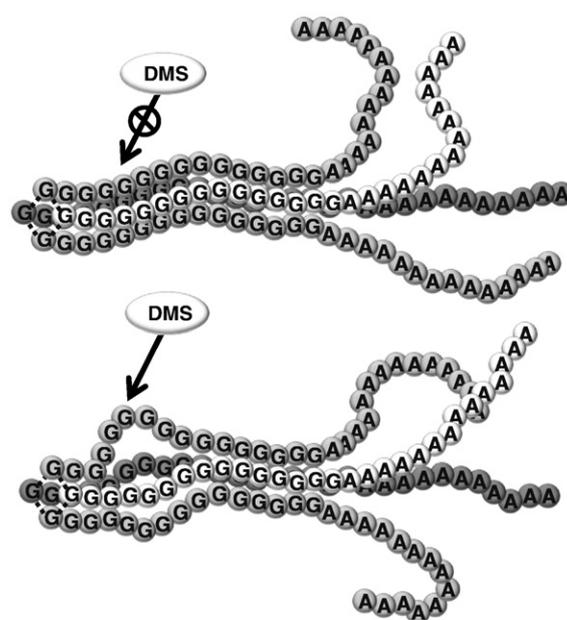
The instrumentation we used to measure the Raman spectra employs hollow-core photonic crystal fiber as the sample holder. The co-propagation of the Raman pump laser with the solutions in the hollow core enhances the backscattered signal by approximately two orders of magnitude for given power using 5-cm long fiber strand. The results provide evidence for the presence Hoogsteen hydrogen bonding in  $d(A_{15}G_{15})$  DNA FW in Raman markers such as:  $1479.9\text{ cm}^{-1}$  (N7),  $1721.7\text{ cm}^{-1}$  (O6),  $1604.4\text{ cm}^{-1}$  (N1), and  $1578.9\text{ cm}^{-1}$  (C2-NH<sub>2</sub>). For further analysis on the role played by N7 of the guanines in  $d(A_{15}G_{15})$ ,  $d(A_{15}^{\text{PPG}}G_{15})$  oligonucleotides were examined using gel electrophoresis and CD. Electrophoresis results show that this modified oligonucleotide migrates as a single-stranded species in the presence of KCl (Fig. 3). Week-long incubations in the presence of various concentrations of KCl did not lead to the formation



**Fig. 5.** (A) CD spectra of  $d(A_{15}^{8-Br}G_{15})$  in Tris-HCl, pH 7.4 and 100 mM XCl; X = K (black squares), Na (red diamonds),  $NH_4$  (green upright triangles), Li (blue inverted triangles), and Cs (light-blue diamonds). (B) CD spectra of  $d(A_{15}^{8-Br}G_{15})$  in Tris (10 mM), pH 7.5; only (black squares), with 40% (v/v) ethanol (red diamonds), with 10 mM KCl (green upright triangles), with 40% (v/v) ethanol and 10 mM KCl (blue inverted triangles). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of aggregated structures (data not shown). Furthermore, the CD results show a large decrease in the intensity of the band at 265 nm and a shift to 260 nm, a typical signal from parallel-stranded G-quadruplexes. These results, coupled with those from the Raman measurements, indicate that DNA FWs are based on the G-quartet motif and are, therefore, G-quadruplex super-assemblies.

Although these results are in direct contradiction to our previous findings using DMS, it is believed that those methylation results can be rationalized by the possibility that DNA frayed wires are not only polydisperse, but the individual  $n$ -mers (e.g.  $[d(A_{15}G_{15})]_n$ ) are also heterogeneous. Fig. 6 is a schematic diagram showing two possible tetrameric  $d(A_{15}G_{15})$  frayed wire species. In the top drawing, the guanine residues from all four parent strands are involved in self-interacting hydrogen bonding, which would limit the methylation reaction of DMS with N7 of the guanines. In the lower drawing some



**Fig. 6.** A schematic diagram describing the heterogeneity of tetrameric forms of  $d(A_{15}G_{15})$  frayed wires. In the upper schematic the N7 group of the guanine residues are inaccessible to methylation by DMS because they are all participating in the hydrogen bonds. The lower schematic shows a four-stranded DNA frayed wire in which the interaction is stable but not all of the hydrogen bonds are formed. Guanine residues that are not involved in Hoogsteen interactions are accessible to methylation by DMS.

guanine residues form guanine loops (or bulges) that do not take part in Hoogsteen hydrogen bonds and therefore the N7 of those guanines are susceptible to methylation by DMS. This partial methylation rationalizes reaction of DMS with DNA FW. Furthermore, the presence of these guanine loops does not contradict previous data on DNA FW that show that these structures are resistant to thermal denaturation and enzymatic degradation. DNA sequences that contain four or more consecutive guanines, i.e.  $d(TGGGGT)$ , and that form tetramolecular G-quadruplexes are resistant to thermal denaturation. Consistent with this, we do not observe a temperature-dependent melting curve with  $d(A_{15}G_{15})$  or  $d(T_{15}G_{15})$  frayed wires. Although these structures may represent a heterogeneous mixture of aggregated parent oligonucleotides, it is reasonable to assume that more than four of the fifteen guanine residues partake in G-quartet formation resulting in the extreme thermostability that we have observed in these structures. In the case of DNA FWs' resistance to nuclease digestion, we believe that having guanine bulges in these structures would also confer nuclease resistance due to the requirement for these enzymes to "dock" to the non-canonical stems that are comprised of G-quartets. This preliminary step is most likely unfavorable since these bulges are expected to be present at non-terminal positions in the particular species.

In addition to the Raman markers that characterize Hoogsteen hydrogen bonding networks, we also observed two weak modes arising from *syn* dG conformations in the Raman spectrum at 671.4 and  $1322.4\text{ cm}^{-1}$  (Fig. 4, inset). This raised the possibility that a small fraction of the frayed wire aggregates may be anti-parallel-stranded G-quadruplexes comprised of guanine residues in both *syn* and *anti* conformations despite the fact that DNA FWs consistently provide a CD spectrum that is comparable with those of parallel-stranded G-quadruplexes (Fig. 2). In order to determine whether such anti-parallel species exist in DNA FW, we introduced another modified oligonucleotide:  $d(A_{15}^{8-Br}G_{15})$ . It is well known that bromination of guanine at the C8 position creates a steric constraint that

favors the *syn* over the *anti* conformation [31]. Samples of  $d(A_{15}^{8-Br}G_{15})$  incubated with KCl appear as two bands after electrophoresis; a fast moving band corresponding to single-stranded species, and slower moving band that is thought to be a dimer (Fig. 3). In the absence of any added KCl, only the faster moving band is observed (data not shown). The observations that only KCl induced the appearance of the slower moving band and that KCl led to the creation of a positive peak at  $\sim 300$  nm in the CD spectrum (which is characteristic of anti-parallel G-quadruplexes [25]), led us to propose that  $d(A_{15}^{8-Br}G_{15})$  forms a dimeric, anti-parallel G-quadruplex. Note that in the absence of any added cations, the CD spectrum of  $d(A_{15}^{8-Br}G_{15})$  has a single positive peak at 260 nm, which is characteristically related to parallel-stranded G-quadruplexes. However, in this case the CD band is red-shifted by about 5 nm. The band observed at 300 nm CD is also shifted in the presence of KCl (Fig. 5A). From the data presented here, we propose that the two CD bands can be assigned to the two species that are resolved by electrophoresis. The CD absorption at 260 nm corresponds to the single-stranded species, while the CD band at 300 nm arises from the formation of the dimeric G-quadruplex species.

The effect of co-solvents on G-quadruplex formation and stability has been gaining increased attention in an attempt to better understand such processes under conditions that mimic the cellular environment [36–38]. Vorlickova et al. demonstrated that sequences that would not otherwise form G-quadruplexes in the presence of KCl or NaCl did so in aqueous solutions containing 40% (v/v) ethanol [33]. Smirnov and Shafer rationalized this observation by shedding light on the relationship between the solvent dielectric constant and G-quadruplex stability [34]. They showed that, in general, a decrease of the solvent dielectric constant results in increased thermostability, illustrating the importance of electrostatics on the stability of these non-canonical structures [34]. This behavior is the opposite of that observed with DNA duplexes [34]. The addition of 0.1 or 1.0 M KCl results in similar CD spectra of  $d(A_{15}^{8-Br}G_{15})$  (data not shown). We found that buffers containing 40% (v/v) ethanol provided CD spectra similar to those containing 10 mM KCl, and that buffers containing both 40% (v/v) ethanol and 10 mM KCl led to an enhancement of the CD band at 300 nm. It appears that having 15 consecutive 8-Br-guanines limits the formation of higher-order DNA structures and leads to the formation of a dimeric anti-parallel G-quadruplex structure. To our knowledge, this is the first report of G-quadruplex formation from an ODN with all guanine bases brominated at the C8 position. Finally, our attempts to gather the Raman vibrational modes of  $d(A_{15}^{8-Br}G_{15})$  were hampered by the presence of fluorescence that consistently mask the markers sought.

In conclusion, DNA FWs are a set of G-quadruplex-based structures. They are composed of primarily of parallel-stranded and some anti-parallel G-quadruplexes. The anti-parallel species are believed to be most likely sub-species of the dimeric form of the polydisperse network of DNA FW structures. From our results, it would appear that DMS methylation is not a good assay for the characterization of guanine interactions in DNA FW because of their polymorphic and apparent heterogeneous nature. The incorporation of 7-deaza-8-aza guanine residues is a powerful tool for establishing the hydrogen bonding network that differentiates G-quadruplexes from other nucleic-acid-based structures. A simpler method would be to exploit the unique characteristic of G-quadruplexes in their selectivity of cations. Collecting CD spectra of DNA samples in potassium-containing buffers and comparing them to those in buffers containing lithium or cesium ions would provide a clear indication of the presence of G-quadruplexes.

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