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G Quadruplexes Stabilised by 8-Oxo-2′-deoxyguanosine

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G quadruplexes (GQPs) are four-stranded secondary structures formed of guanosine-rich nucleic acids. They consist of at least two G tetrads composed of four guanosine nucleotides. DNA GQPs have been shown to fold into a variety of different topologies that can be divided into antiparallel and parallel conformations (the latter is depicted in Figure 1 A).[1] The topology of DNA GQPs depends on the sequence and the loop length. For example, sequences with single nucleotide loops favour all-parallel strand orientation.[2] Furthermore, the loop length also determines the stability of GQPs: the shorter the loop, the more stable is the GQP structure.[3] G tetrads interact via π–π stacking interactions between each other, and thus form a very compact and stable fold. Additionally, the GQP structures are stabilised by monovalent cations coordinated by the 6-oxo functionalities of the tetrad-forming guanines in the centre of the GQP, with a preference for potassium and sodium ions.[4]

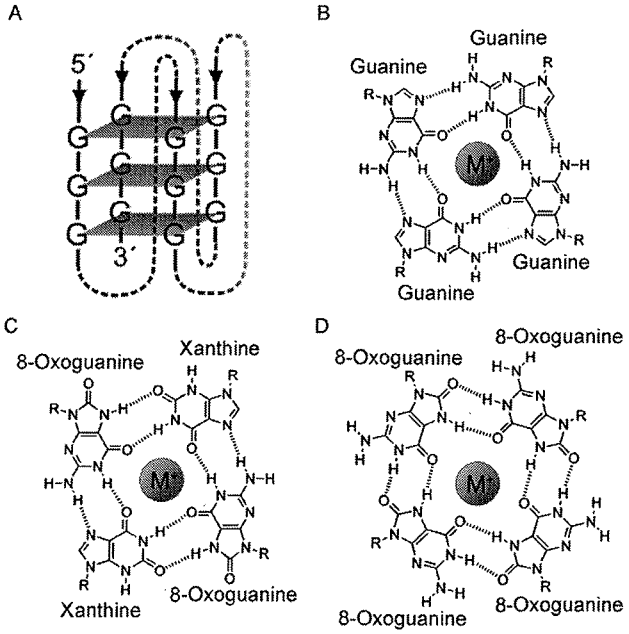

Figure 1. Schematic representation of G quadruplex topology and potential modified tetrads. A) All-parallel, intramolecular G quadruplex. B) Unmodified tetrad composed of four guanine nucleobases. C) Proposed tetrad composed of two 8-oxoguanines and two xanthines. D) Possible assembly of four 8-oxoguanines to a tetrad. Tetrads are stabilised by a monovalent cation.

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Naturally occurring GQPs are found in telomeric regions of DNA, like the $(G_3T_2A)_n$ repeats in humans and other higher eukaryotes,[5] $(G_4T_2)_n$ in *Tetrahymena*, and $(G_4T_4)_n$ in *Oxytricha* and *Stylonchia*.[6] Human telomeric DNA comprises thousands of tandem repeats of the (G_3T_2A) sequence and a single-stranded overhang of the G-rich strand of about 100–200 nucleotides at the 3′ end.[7] It has been shown that under physiological ionic conditions human telomeric sequences form stable quadruplex structures both in solution and in crystal states.[8] Since induction of the quadruplex structures at the telomeres seems to inhibit proliferation of cells, stabilisation of telomeric G quadruplexes by small molecules has become a promising strategy for the development of a novel class of anticancer drugs.[9] In addition, quadruplexes are also frequently found in promoter regions of genomes and their formation influences the expression of associated genes.[10]

In addition to the biological relevance of these structures, which occur and likely function in regulating genetic processes, quadruplex folds represent fascinating examples of supramolecular, higher-order structures that are determined by well-understood inter- and intramolecular interactions. More specifically, in a single tetrad the guanine nucleobases interact via Hoogsteen hydrogen bonds between the O6 and N7 of one guanine as hydrogen acceptor on the one hand, and the 2-amino group and the N1–H group of the other guanine as hydrogen donor on the other hand to form eight hydrogen bonds per tetrad (Figure 1 B).[1a] We have recently shown that it is possible to replace the guanines of one distinct tetrad by the guanine analogues 8-oxoguanine and xanthine, and thereby to program the DNA quadruplex sequence of the human telomeric repeat to adopt a certain topology.[11] By applying this strategy, the stability of the resulting modified G quadruplexes is just slightly impaired. Furthermore, Switzer et al.[12] and Brodbelt and co-workers[13] have reported four-stranded structures composed of isoguanines instead of guanines. They have shown that the formation and architecture of these modified G quadruplexes is highly dependent on the presence of specific cations, and moreover, that mixed structures composed of guanines and isoguanines are possible.

In the present work, we report the construction of artificial guanosine analogues containing quadruplexes, one with a tetrad composed of two xanthines and two 8-oxoguanines and another with a tetrad solely composed of 8-oxoguanosines. For this purpose, we have chosen the model quadruplex GQP1 (Table 1) that is known to fold into an all-parallel, intramolecular structure.[3a,14] Because of three single-nucleotide propeller loops connecting the G tracks, the structure is very stable and able to form even in the absence of monovalent cations (Figure 1 A). For the first modified sequence (GQP8X) we inserted one pair of xanthines and one pair of 8-oxoguanines instead of the guanines forming the upper tetrad. In contrast to the guanine tetrad, the orientation of two hydrogen bonds is inverted in the GQP8X structure (Figure 1 B and C). Nevertheless, these guanine analogues should be able to integrate similarly into the tetrad structure, and thus stabilise the quadruplex formation as it was shown for the quadruplex of the human telomeric repeat.[11] In the second modified sequence (GQP8, Table 1) the four positions engaged in a tetrad are composed of 8-oxoguanines exclusively, and we reasoned that it might be formed through stabilisation by a different hydrogen bonding pattern. Hydrogen bonds could form between O8 and N1–H and between N7–H and O6 (Figure 1 D). In order to account for this geometry the 8-oxoguanines need to be slightly rotated in comparison to the unmodified ones. However, it should be noted that this proposed tetrad is a mere hypothesis, and more detailed structural investigations are necessary to prove its existence.

Table 1. Sequences of deoxyoligonucleotides used in this study.

Name	Sequence[a]
GQP1	d(GGGTGGGTGGGTGGG)
GQP8X	d(8GGTXGGT8GGTXGG)[b]
GQP8	d(8GGT8GGT8GGT8GG)[b]
GQPC	d(TGGTTGGTTGGTTGG)
GQPSCR	d(GTGTGTGTGTGTGTG)

[a] Oligonucleotide sequences are shown in the 5′→3′ direction, underlined bases are supposed to participate in tetrads; [b] 8: 8-oxoguanine, X: xanthine.

The DNA sequences were synthesised through standard phosphoramidite solid phase chemistry and purified by HPLC. In order to judge the stability of the folded structures, we also included the sequence GQP1, which forms a very stable all-parallel quadruplex. As further references we designed two sequences, GQPC and GQPSCR (Table 1). In the first control (GQPC) the guanines in the upper tetrad are replaced by thymine; therefore, quadruplex formation is only possible by utilising two tetrads. Hence, GQPC serves as standard for sequences in which the modified segments are not able to form a defined tetrad. Second, a scrambled sequence composed of thymidines and guanosines (GQPSCR) was included in which quadruplex formation should not occur at all.

We analysed the formation of quadruplexes first by circular dichroism (CD) studies (Figure 2). CD spectroscopy is a powerful tool for determining the formation of a GQP and also for measuring the stability by thermal denaturation experiments.[15] We applied different buffer conditions for studying the modified sequences: Tris-HCl (10 mM, pH 7.5) was used as buffer, supplemented as indicated with either 100 mM NaCl or KCl. As expected, GQP1 folded into a stable all-parallel quadruplex even in the absence of monovalent cations, and showed a melting temperature of 39 °C (Figure 2 and Table 2). In the presence of sodium and potassium the quadruplex was much more stable with melting temperatures of 63 °C and above 90 °C, respectively. In comparison the control sequence GQPC displayed significantly lower melting temperatures; this is due to the involvement of only two tetrads in quadruplex formation instead of three (Figure 2), and the formation of a quadruplex was only detectable in the presence of potassium. Furthermore, GQPC formed an antiparallel structure in contrast to all other structures observed for GQP1, GQP8X and GQP8. As detailed below, the modified structures showed CD spectra similar to all-parallel structures under all buffer conditions tested, which was indicated by a maximum CD signal at 265 nm and a minimum at 240 nm. From the finding that the sequence GQPC with two thymidines in the loops adopts a different topology it can be concluded that in the modified quadruplexes GQP8X and GQP8 the guanine analogues are not serving as flexible loop nucleotides but instead participate in a tetrad.

In the absence of monovalent cations GQP8X was slightly less stable while GQP8 showed an even increased stability in comparison to the unmodified GQP1 sequence (Figure 2 A). The remarkable stability of the structure formed by the DNA with a potentially formed 8-oxo-dG tetrad GQP8, was only slightly increased from 49 to 53 °C (Table 1) by the addition of sodium, but the control sequence GQP1 was much more stable (Figure 2 B). In the presence of potassium, the CD spectrum of GQP8 showed a broadened peak at 265–270 nm, which might be explained by the presence of more than one distinct structure. Accordingly, the melting curve does not show a well-defined transition. The sequence containing the mixed tetrad, GQP8X, behaved differently. In the absence and in the presence of sodium it only showed moderate stability with melting temperatures of 27 and 42 °C, respectively, whereas potassium stabilised the structure to a much larger extend. However, with potassium GQP8X as well showed a melting curve indicative of two transitions with melting points of approximately 50 and 90 °C.

We next repeated the CD spectra and stability measurements at different pH conditions. If 8-oxo-dG is involved in the formation of a structure, a different pH dependency is expected. Compared to deoxyguanosine, 8-oxo-deoxyguanosine was characterised by the absence of an acidic pK_a.[16] Whereas quadruplexes built by dG should be destabilised by acidic conditions due to protonation at N7, this effect should be less pronounced in 8-oxo-dG-containing oligonu-

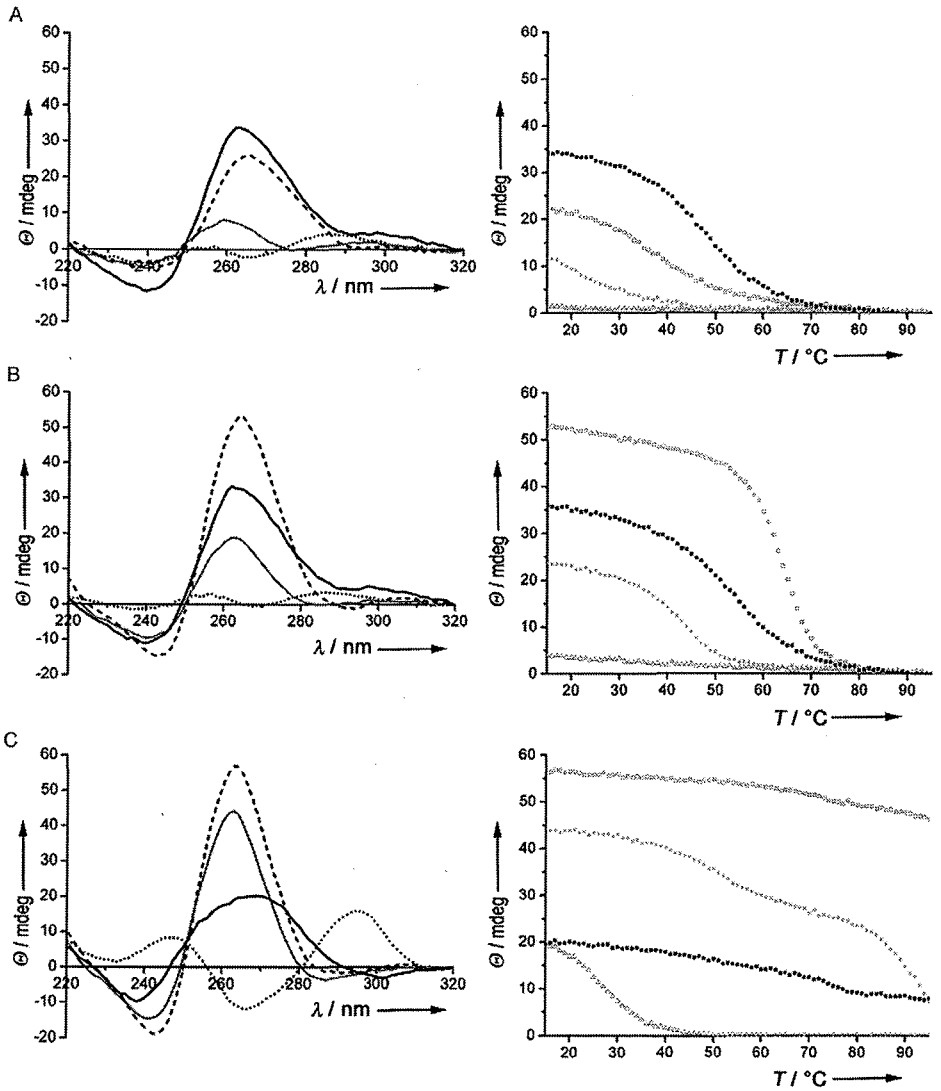

Figure 2. CD spectra and thermal denaturation studies of GQP oligonucleotides. Measurements were carried out with 5 μM oligonucleotide in Tris-HCl (10 mM, pH 7.5). A) Buffer only, B) NaCl (100 mM), C) KCl (100 mM). CD spectra (left-hand panels): GQP1 (-----), GQPC (•••••), GQP8X (——) and GQP8 (——). CD melting experiments (right-hand panels): GQP1 (□), GQPC (△), GQP8X (■), GQP8 (■).

Table 2. Melting temperatures of GQP1 quadruplexes. Standard deviations were determined by repetition of example measurements and were found to be less than 1 °C.

Name	Buffer	Na^+	K^+
GQP1	39 °C	63 °C	> 90 °C
GQPC	_[a]	_[a]	28 °C
GQP8X	27 °C	42 °C	~50 and ~90 °C[b]
GQP8	49 °C	53 °C	_[a]

[a] Melting point not detectable due to absence of a stable structure. [b] Not well-defined or more than one transition in melting curve.

cleotides. As shown in Figure 3, the stability of GQP8 indeed showed only very little pH dependency whereas the unmodified GQP1 was drastically destabilised at pH 5 and 6.

In order to analyse whether the sequences are folding into intramolecular quadruplexes, we performed an electrophoretic mobility shift assay (EMSA) in a native polyacrylamide gel (Figure 4). The scrambled GQPSCR oligonucleotide did not fold into a quadruplex structure and as a result could be used as a control of an unfolded sequence. In native gel-shift experiments, intramolecularly folded quadruplexes migrate farther and intermolecular quadruplexes migrate less compared to an unfolded sequence.[4] As shown in the EMSA, in the presence of sodium both control sequences (GQPSCR and GQPC) migrated to the same position in the gel representing unfolded oligonucleotides (Figure 4, lanes 1 and 3). This is in accordance to the CD spectroscopy in which GQPC did not show structure formation in Na^+ solution.

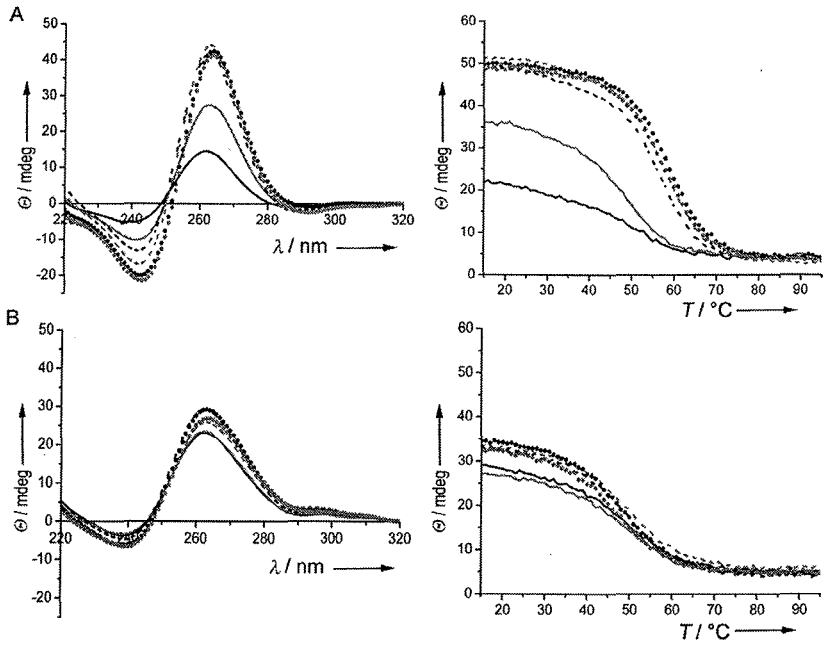

Figure 3. CD spectra and thermal denaturation studies of GQP1 and GQP8 oligonucleotides at different pH values. Measurements were carried out with 5 μM oligonucleotide in Tris-HCl (10 mM). A) GQP1, B) GQP8. CD spectra (left-hand panels and CD melting experiments (right-hand panels): pH 5.0 (——), pH 6.0 (——), pH 7.0 (-----), pH 8.0 (-----), pH 9.0 (●), pH 10.0 (●).

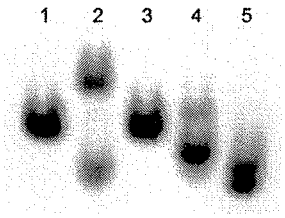

Figure 4. Electrophoretic mobility shift assay (EMSA) of GQP oligonucleotides in a native polyacrylamide gel (12%) with TBE (1×) and NaCl (100 mM). Lane 1: GQPSCR; lane 2: GQP1; lane 3: GQPC; lane 4: GQP8X; lane 5: GQP8.

Under the conditions of the gel-shift experiment, the unmodified control sequence GQP1 seems to form both an intramolecular species represented by a band below the controls and some intermolecular fraction above the control bands. Hence, it seems that GQP1 does not fold into a very defined sequence. Support for this observation was also obtained by ^{1}H NMR spectroscopy of GQP1 (see below). The EMSA of the modified GQP8X and GQP8 sequences show intramolecular species exclusively as they migrate faster than the unfolded controls; this excludes the formation and stabilisation of intermolecular structures and aggregates.

In order to further investigate the nature of the proposed 8-oxo-dG tetrad in the oligonucleotide GQP8, we carried out ^{1}H NMR spectroscopy studies. The unusual tetrad should be identified by differences in the imino proton regions in comparison to the unmodified sequence GQP1. Despite screening of conditions, such as buffers, pH, presence of Na^+, K^+, and Mg^{2+} both sequences did not yield well-resolved NMR spectra; this is likely due to the co-existence of multiple conformations. However, the spectra of GQP1, but not of GQP8, improved at increased temperatures (Figure 5); again this demonstrates the remarkable thermostability of this parallel propeller structure.

In this study we have shown that modified quadruplex units containing both 8-oxo-dG and deoxyxanthosine nucleotides as well as 8-oxo-dG alone support the formation of stable quadruplex structures, and that the stability of these structures is more or less dependent on the presence of monovalent cations, depending on the composition of the modified tetrad. One possible reason for the different behaviour towards the tested metal ions could be an altered tetrad architecture leading to different channel geometries associated with an altered tendency for cation coordination. Another contribution to this phenomenon could be the fact that 8-oxoguanosine favours the *syn* conformation of the glycosidic bond.[17] Thus, the 8-oxoguanines would adopt a disfavoured *anti* conformation since all nucleosides in the natural all-parallel quadruplex structure are oriented in this manner.[1a] This might be an explanation for the slightly decreased melting temperatures of GQP8X und GQP8 in some cases. The polymorphic structures observed by CD in potassium buffers and in the NMR spectroscopy experiments might also represent distinct quadruplex species that differ in nucleobase orientation, glycosidic bond conformation, etc. More detailed structural investigations will be needed to ultimately prove the formation of the suggested tetrads shown in Figure 1. However, we have shown that the 2'-deoxyguanosine analogues 8-oxo-dG and deoxyxanthosine stabilise the quadruplex fold, and have demonstrated that building blocks other than the natural guanines can contribute to the formation of these interesting supramolecular structures.

Experimental Section

The unmodified DNA sequences were purchased from Metabion (Martinsried, Germany) in HPLC grade while modified DNA sequences were synthesised by standard phosphoramidite chemistry with DMT on protocol by using 8-oxoguanosine and deoxyxanthosine phosphoramidites,

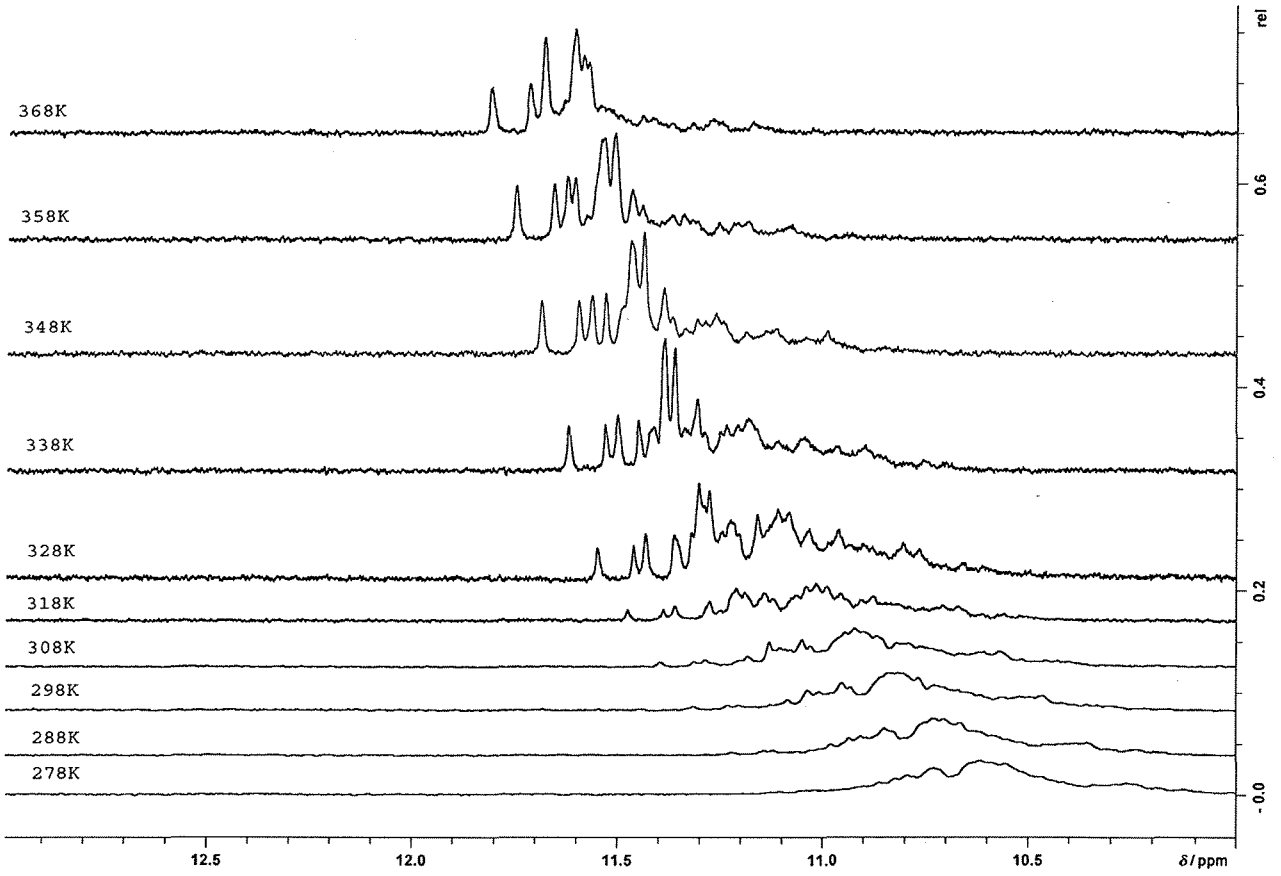

Figure 5. Temperature-dependent NMR spectra of imino protons of GQP1 (300 μM) in K^+ (100 mM), pH 7.5, in H_2O (90%) and D_2O (10%).

which were purchased from Chemgenes (Wilmington, MA, USA) and Berry and Associates (Dexter, MI, USA), respectively. Synthesised oligonucleotides were deprotected and cleaved with DBU (1 M) and ammonia. The oligonucleotides were purified on a C18-RP-HPLC column by using a binary gradient of acetonitrile and triethylammonium acetate buffer (0.1 M, pH 7.0). Acetonitrile concentration was increases from 5 to 75% in 27 min with a flow rate of 2 mL min^{-1}. After the first purification step, the DMT group was removed with AcOH (80%) in H_2O and again purified by RP-HPLC. Purified oligonucleotides were analysed by ESI-MS (GQP8X: calcd 4835.1 g mol^{-1}, found 4835.9 g mol^{-1}; GQP8: calcd 4865.2 g mol^{-1}, found 4865.8 g mol^{-1}).

For CD studies, oligonucleotide samples were prepared at 5 μM concentration with Tris-HCl (10 mM, pH 7.5) and appropriate metal ions (100 mM NaCl or KCl) were added. Oligonucleotides were annealed by first being heated at 95 °C for 2 min and then slowly cooled to 15 °C over 2 h. CD spectra were recorded on a Jasco 815 spectrometer in cuvettes with a 1 cm path-length, resolution of 0.1 nm, band-width of 1.0 nm and speed of 500 nm min^{-1} at 20 °C. Blank spectra of samples containing buffer were subtracted from the test samples. Each spectrum was accumulated five times, averaged and zero-corrected at 320 nm. For thermal denaturation studies the samples were heated from 15 to 95 °C with a heating rate of 1 °C min^{-1}. The CD signal at 265 and 290 nm was recorded every 1 °C, and the melting temperature was obtained by determining the temperature at the half-maximum decrease of the signal.

For the EMSA experiments the oligonucleotide strands were radioactively labelled and purified by using G25 columns. For native PAGE analysis the ^{32}P-labelled oligonucleotides were mixed with 1 vol loading buffer (1 × TBE, 100 mM NaCl, 40%, v/v, glycerol) and were heat-denatured at 95 °C for 5 min. After being cooled slowly to room temperature, the samples were analysed on a native polyacrylamide gel (12%) at 20 °C containing NaCl (100 mM) in the TBE running buffer and gel. Visualisation was performed by using phosphor imaging.

For the NMR spectroscopy experiments, DNA samples were lyophilised and redissolved in a buffer containing potassium phosphate (20 mM, pH 7.0) and KCl (70 mM). The sample was heated to 95 °C for 5 min and cooled slowly to room temperature. NMR spectra were acquired on a Bruker Avance III 600 MHz spectrometer equipped with a TCI-H/C/N triple resonance cryoprobe. The sample (final concentration 0.3 mM) was dissolved in H_2O, supplemented with 10% (v/v) D_2O as field lock. The spectra were acquired with 32000 data points by using 128 accumulated scans, and processed with an exponential line broadening window function. Solvent suppression was achieved by the WATERGATE sequence.[18]

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Keywords: cation coordination · DNA structures · DNA quadruplexes · hydrogen bonds · supramolecular chemistry

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