GC ends control topology of DNA G-quadruplexes and their cation-dependent assembly

Daša Pavic¹,², Baifan Wang¹, Lea Spindler³,⁴, Irena Drevenšek-Olenik⁴,⁵, Janez Plavec ¹,²,⁶ and Primož Šket ¹,∗

¹Slovenian NMR Center, National Institute of Chemistry, 1000 Ljubljana, Slovenia, ²University of Ljubljana, Faculty of Chemistry and Chemical Technology, 1000 Ljubljana, Slovenia, ³University of Maribor, Faculty of Mechanical Engineering, 2000 Maribor, Slovenia, ⁴Department of Complex Matter, Jozef Stefan Institute, 1000 Ljubljana, Slovenia, ⁵University of Ljubljana, Faculty of Mathematics and Physics, 1000 Ljubljana, Slovenia and ⁶EN-FIST Center of Excellence, 1000 Ljubljana, Slovenia

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ABSTRACT

GCn and GGCnCG, where \( n = (G_2A)_nG_2A \), fold into well-defined, dimeric G-quadruplexes with unprecedented folding topologies in the presence of Na⁺ ions as revealed by nuclear magnetic resonance spectroscopy. Both G-quadruplexes exhibit unique combination of structural elements among which are two G-quartets, A(GGGG)A hexad and GCGC-quartet. Detailed structural characterization uncovered the crucial role of 5′-GC ends in formation of GCn and GGCnCG G-quadruplexes. Folding in the presence of \(^{15}\)NH₄⁺ and K⁺ ions leads to 3′-3′ stacking of terminal G-quartets of GCn G-quadruplexes, while 3′-GC overhangs in GGCnCG prevent dimerization. Results of the present study expand repertoire of possible G-quadruplex structures. This knowledge will be useful in DNA sequence design for nanotechnological applications that may require specific folding topology and multimerization properties.

INTRODUCTION

Guanine-rich DNA sequences have propensity to fold into non-canonical, four stranded structures with G-quadruplexes being most well-known. Their main building block is a G-quartet formed by four guanine residues in planar arrangement held together by eight Hoogsteen-type hydrogen bonds. Cations, coordinated between G-quartets reduce repulsion of negatively charged O6 guanine atoms and are therefore crucial for G-quadruplex formation (1,2). Folding topologies are susceptible to changes in environment like pH, temperature and molecular crowding conditions as well as nature and concentration of cations, which all together add to wide repertoire of structural polymorphs. Among factors contributing to vast structural diversity of G-quadruplex structures are also different possibilities of base pairing alignments in addition to classical Hoogsteen-type hydrogen bonding between guanines in G-quartets. G-rich DNA sequences containing cytosine residues can be stabilized through formation of mixed GCGC-quartets in a major groove (3–6), minor groove (7,8) and slipped arrangement (9,10) or prefer formation of other tetrahelical structures such as AGCGA-quadruplexes (11). When G-tracts are separated by adenine residue, A(GGGG)pentads (12,13), A(GGGG)A hexads (14–16), G(A)G(A)G(A)G heptads (17,18) and mixed GAGA-quartets (11) could be formed.

It is believed that G-quadruplexes have important role in regulation of biological processes since G-rich sequences are over represented in human genome such as telomeres, promoter regions and even in genes connected with neurodegenerative diseases (19–21). In addition, G-quadruplexes also gained great attention in the field of nanotechnology. Their self-assembling ability, programmable control of their shape and size and unique optical and electrochemical properties make them attractive candidates for nanotechnological applications such as nano-electronics (22–25), nanosensors (26,27) and nanodevices (28). G-rich DNA oligonucleotides are able to form long, continuous nanostructures termed G-wires (29). In a recent review by Professors Mergny and Sen, G-wire was defined as an extended DNA nanostructure in one-dimension, formed by the self-assembly of one or more individual DNA oligonucleotides by way of G-quadruplex formation (30). One of the possibilities to assemble G-wires is through multimerization of individual G-quadruplex subunits. G-quadruplexes can form multimers via stacking or interlocking. Typically parallel G-quadruplexes with ‘blunt-ends’ can stack through π–π interactions of terminal G-quartets (31). Another possibility of stacking is through expanded π-
systems such as hexads, heptads and octads, which facilitate association (14,17,32,33). Depending on which side of G-quadruplex stacking occurs, can be further classified as 5′–5′ (head-to-head) (34–38), 3′-3′ (tail-to-tail) (39,40) and 5′-3′ (head-to-tail) stacking (40,41). Potassium ions are known to more efficiently promote stacking in comparison to ammonium or sodium ions (42). Interlocks can be formed via: (i) extra G-quartet(s) formed by slipped G-rich strands from different G-quadruplexes or (ii) extra quartets formed by sticky ends. Similarly as stacking, interlocking can be classified as 3′–3′ (43), 5′–3′ and the most commonly observed 5′–5′ (6,12,13,15,16,40,44–46). A lot of efforts have been put into prediction and programming of multimerization of G-quadruplexes (31,41,47–49). One of the promising approaches for programmed self-assembly is via complementary GC ends, which could form linkages between two successive G-quadruplexes via inter-quadruplex GCGC-quartet formation (6,40,43,50,51). 5′-GC ends commonly form 5′-5′ interlocks (6,40,43,51), while the effect of 3′-GC ends has been reported to be more diverse (40,43,50).

In the presence of Na+ ions, oligonucleotides d(GCG2TG4TG2) and d(GCG2TG4TG2) fold into dimeric, parallel G-quadruplexes composed of four G-quartets connected with thymine residues in propeller loops (40). Both G-quadruplexes exhibit two GC overhangs at their 5′-ends, which enable dimerization via inter-quadruplex GCGC-quartets. 5′-5′ interlocked G-quadruplexes adopted by d(GCG2TG4TG2) undergo further multimerization via 3′–3′ stacking of terminal G-quartets resulting in four assembled G-quadruplexes. In contrast, the 3′-GC ends of a G-quadruplex formed by d(GCG2TG4TG2) prevent multimerization.

Herein, we utilize nuclear magnetic resonance (NMR) experiments complemented with native PAGE, UV and circular dichroism (CD) spectroscopy in order to study DNA oligonucleotides GCn and GCnCG, where n = G2A4G2A2. Adenine instead of thymine as a nucleobase separating G-tracts within GCn and GCnCG was expected to form additional hydrogen bonds with guanine residues, which can result in formation of A(GGGG) pentad or A(GGGG)A hexad and might thereby lead to additional stabilization of G-quadruplexes. Alternatively, adenines within G-rich oligonucleotides, might induce folding via highly stabilizing GNA type of loop leading to formation of different structures (52). Moreover, adenine residues might facilitate multimerization of G-quadruplexes when compared to other nucleobases (41,50). Additionally, mixed GAGA-quartets could be formed, however with specific requirements for grooves’ dimensions that are critical for their ability to stack on a nearby G-quartet (11). Since both GCn and GCnCG exhibit GC ends, formation of intra-quadruplex GCGC-quartets rather than free overhangs is expected to control dimerization (multimerization) of individual G-quadruplexes. Furthermore, we deemed it essential to explore effect of various monovalent cations since GCGC-quartets stacked on G-quartets have been shown to exhibit specific (non)selectivity with respect to cation and water localization (53). At the outline, spectral features suggested alternative folding topologies and thus insights into the role of GC ends at the 5′- and 3′-ends of DNA sequence on G-quadruplex formation.

MATERIALS AND METHODS

Sample preparation

DNA oligonucleotides GCn d(GCG2AG4AG2) and GCnCG d(GCG2AG4AG2) were synthesized in DMT-off mode using DNA/RNA synthesizer H-8 (K&L Laborger-aete Gbr) and standard phosphoramidite chemistry. GCn and GCnCG containing 8% 13C and 15N site-specifically labelled guanine and adenine residues were synthesized using the same procedure. Deprotection was done at 55°C over night with the use of aqueous ammonia which was later removed under low pressure. Samples were heated at 95°C for 5 min in the presence of LiCl and left to cool at room temperature. Desalting was done on Amicon ultrafilter at pH 10, which was adjusted with the use of LiOH solution. Concentrations of prepared samples were determined by measuring absorption at 260 nm on Varian CARY-100 BIO UV-VIS spectrometer. The molar extinction coefficients were calculated using the nearest neighbor method and were 125 300 M−1 cm−1 and 142 000 M−1 cm−1 for GCn and GCnCG, respectively. NMR samples were dissolved in 90% H2O and 10% of D2O in 100 or 175 mM salt concentration (NaCl, 15NH4Cl or KCl) and 10 mM NaPi or KPi buffer (pH 6.8). Samples containing 100% D2O were prepared by lyophilization of previously prepared NMR samples and subsequently dissolved in 100% D2O.

NMR spectroscopy

All NMR experiments were recorded on Agilent Technologies DD2 600 MHz and VNMR 800 MHz NMR spectrometers at 25°C unless stated otherwise. For suppression of the water signal, the double-pulsed field gradient spin echo (DPFGSE) pulse sequence was used. The translation diffusion coefficients were obtained with the use of pulse field gradient stimulated echo (PFG-STE) pulse sequence. Identification of guanine H1 protons in partially (8%) 13C and 15N site-specifically labelled samples was acquired with 1D 15N-edited heteronuclear single quantum correlation (HSQC) experiment. Aromatic protons of adenines and guanines were identified with the use of 2D 13C-edited HSQC experiment. Non-exchangeable proton resonances were assigned using the 2D Nuclear Overhauser Effect SpectroscopY (NOESY) with mixing times (τm) of 80, 150 and 250 ms recorded on NMR samples in 100% D2O. 2D Total Correlation Spectroscopy (TOCSY) with τm of 80 ms, 2D Double Quantum Filtered Correlation SpectroscopY (DQF-COSY) and 2D 1H–31P COSY were used for cross-checking assignment of 2D NOESY spectra. Exchangeable proton resonances were assigned using 2D NOESY experiments with τm of 150 and 250 ms acquired on samples in 90% H2O, 10% D2O.

UV spectroscopy

Samples for UV melting experiment were prepared by diluting NMR samples with blank solution, which contained 175 mM NaCl and 10 mM NaPi buffer (pH 6.8), to achieve final 0.01, 0.05, 0.15 or 0.2 mM concentration of GCn and GCnCG per strand. The melting experiment was carried out on Varian CARY-100 BIO UV-VIS spectrophotometer
with the Cary Win UV Thermal program using 10, 5, 2 and 1 mm path-length cells, respectively. The temperature was increased/decreased from 15 to 80 or 90°C with the rate of 0.1°C/min. Absorbance was measured at 295 nm. To prevent sample evaporation at higher temperatures the mineral oil and fixed cuvette caps were used. To avert condensation at lower temperatures the stream of nitrogen was applied. Melting temperatures were determined from the first derivative of $A_{295}$ versus temperature plot.

**CD spectroscopy**

Circular dichroism (CD) spectra were recorded on an Applied Photophysics Chirascan CD spectrometer at 25°C from 200 to 320 nm. A blank sample containing only 100 mM NaCl and 10 mM NaPi buffer (pH 6.8) was used for baseline correction. Measurements were carried out in 0.1 mm path-length quartz cells for samples with 1.0 mM concentration per strand.

**Native PAGE**

Native gel electrophoresis was run in a temperature controlled vertical electrophoretic apparatus at 15°C in TBE buffer and 175 mM NaCl. Samples contained 0.1, 0.25 and 0.5 mM concentration of GCn or GCnCG per strand, 175 mM NaCl and 10 mM NaPi buffer (pH 6.8) in 90% H2O and 10% D2O solution. Ficoll was added to the samples prior to loading. Polyacrylamide gel concentration was 15% with 100 mM concentration of NaCl. Thermo Scientific GeneRuler Ultra Low Range DNA Ladder was used as standard. Electrophoresis was run at 50 mV for 16 h. DNA was visualized by Stains-all (Sigma-Aldrich) staining.

**Structure calculations**

The structures of GCn and GCnCG were calculated by the simulated annealing (SA) simulations based on NOE-derived distance restraints. Distance restraints for exchangeable and nonexchangeable protons were obtained from 2D NOESY spectra ($\tau_m$ 150 ms), recorded on samples with 10% and 100% D2O, respectively. Spectra were recorded at 25°C, 175 mM NaCl and 10 mM NaPi buffer (pH 6.8). In case of GCn, volumes of C2 H2′-H2′ (1.9 Å) and C2 H5−H6 cross-peaks (2.45 Å) were used as references, for exchangeable and nonexchangeable protons, respectively. In case of GCnCG, average volume of C2 and C13 H5-H6 cross-peaks (2.45 Å) was used as reference for exchangeable and nonexchangeable protons. NOEs were classified as strong (1.8−3.6 Å), medium (2.5−5.0 Å) and weak (3.5−6.5 Å). SA simulations were performed using the CUDA version of pmemd module of AMBER 14 program suites (54,55) and Cornell et al. force field basic version parm99 (56) with the bsc0 (57), $\chi$ OL4 (58), $\epsilon/\xi$ OL1 (59) and $\beta$OL1 (60) refinements. The initial extended single-stranded DNA structure was obtained using the leap module of AMBER 14 program suites. A total of 200 structures were calculated in 80 ps of NMR restrained simulated annealing (SA) simulations using the generalized Born implicit solvation model (61,62). The cut-off for non-bonded interactions was 999 Å and the SHAKE algorithm (63) for hydrogen atoms was used with the 0.4 fs time steps. For each SA simulation, a random velocity was used. The SA simulation was as follows: in 0–2 ps, the temperature was raised from 300 K to 1000 K and held constant at 100 K for 38 ps. Temperature was scaled down to 500 K in the next 24 ps and reduced to 100 K in the next 8 ps and was further reduced to 0 K in the last 8 ps. Restraints used in the calculation were NOE-derived distance restraints (force constant 20 kcal mol$^{-1}$ Å$^{-2}$), hydrogen bond (force constant 40 kcal mol$^{-1}$ Å$^{-2}$), torsion angle $\chi$, $\epsilon$ (region $170^{\circ}$−$290^{\circ}$) and sugar pucker phase angle restraints (force constant 200 kcal mol$^{-1}$ rad$^{-2}$). Based on the intensity of respectively H8−H1' 2D NOEY cross-peaks, glycosidic torsion angles of G1 in GCn and GCnCG were restrained to the syn-region ($30^{\circ}$−$90^{\circ}$), while glycosidic torsion of other residues of GCn and GCnCG were restrained to the anti-region ($200^{\circ}$−$280^{\circ}$). Torsion angle $\epsilon$ of each residue was constrained to the allowed range in nucleosides, nucleotides, deoxynucleotides, and deoxypolyribonucleotides (64). The phase angle of pseudorotation of A10 residue in GCnCG and GCn were restrained in the range from $-10^{\circ}$ to $+40^{\circ}$, corresponding to North-type conformation (64). The phase angle of pseudorotation of G4, G6, G7, G8, G11, G12, C13 residues in GCnCG and G1, C2, G3, G4, G7, G8, G9, G11 and G12 residues in GCn were restrained in the range from $-140^{\circ}$ to $185^{\circ}$, corresponding to South-type conformation (64). For the rest of residues in GCn and GCnCG, their phase angle of pseudorotation were not restrained. NOE-derived distance restraints were used after the first 3000 steps. All structures were minimized with a maximum of 10 000 steps of energy minimization and a family of 10 structures was selected based on the smallest constraints violations and lowest energy.

**RESULTS**

**GCn and GCnCG fold into symmetric, dimeric G-quadruplexes**

Folding of oligonucleotides GCn and GCnCG into well-defined G-quadruplex structures in aqueous solution is achieved in the presence of NaCl (Figure 1). 1D $^1$H NMR spectrum of GCn reveals six sharp signals in the region between δ 11.2 and 11.7 ppm corresponding to imino protons of guanine residues involved in Hoogsteen-type hydrogen bonds. Observation of six sharp signals that correspond to 12 imino protons in three G-quartets indicates that GCn adopts a symmetric G-quadruplex fold. A downfield sharp signal at δ 13.23 ppm is characteristic for H1 proton of guanine residue involved in Watson−Crick (WC) base pair. Additional weak and broad signal is observed at δ 11.1 ppm. Its broad nature may be due to location of respective atom in flexible region of G-quadruplex structure or due exposure to exchange with bulk solvent. 1D $^1$H NMR spectrum of GCnCG exhibits the same number of signals in the imino region corresponding to Hoogsteen and WC base pairing as observed for GCn despite additional 3′−GC end, which suggests formation of similar folds for both oligonucleotides. A great similarities between GCn and GCnCG are also reflected in aromatic regions of 1D $^1$H NMR spectra with two additional signals for GCnCG at 6.740 and 7.48 ppm corresponding to G14 and C13 residues in the latter (vide infra).
Translational diffusion coefficients (Dt) at 25°C and 1.0 mM oligonucleotide concentrations are $1.6 \times 10^{-10}$ and $1.5 \times 10^{-10} \text{ m}^2 \text{s}^{-1}$ for GCn and GCnCG, respectively. Observed values are in accordance with formation of dimeric G-quadruplexes formed by GCn and GCnCG. Similar size of both G-quadruplexes is nicely reflected in mobility on native polyacrylamide gel, where it can be clearly seen that GCn and GCnCG have the same gel mobility as approximately 12 base pairs (bp) long reference dsDNA (Supplementary Figure S1). Intermolecular nature of GCn and GCnCG G-quadruplexes is further supported by UV spectroscopy, which demonstrated concentration-dependence of temperature of mid-transition (T_{1/2}) in unfolding processes (Supplementary Figure S2A, B). G-quadruplexes display high thermal stability with T_{1/2} of 64 and 68°C at 0.2 mM concentration per strand for GCn and GCnCG, respectively (Supplementary Figure S2A, B). From the experimentally determined slopes of van’t Hoff plots, molecularities n = 2 were determined, which is in perfect agreement with dimeric nature of GCn and GCnCG G-quadruplexes (Supplementary Figure S2C, D).

**Folding topology with unique combination of structural elements**

Unambiguous assignment of imino and aromatic proton resonances of guanine and adenine residues was achieved by the use of 1D $^{15}$N-edited and 2D $^{13}$C-edited HMQC spectra acquired on $^{15}$N, $^{13}$C residue-specific partially (8%) labelled GCn and GCnCG (Supplementary Figures S3 and S4). Proton assignment was completed based on connectivities in 2D NOESY, TOCSY, $^1$H-$^3$P COSY and DQF-COSY spectra (Figure 2, Supplementary Figures S5-S9). G1, G7, G8, G9, G11 and G12 residues are involved in formation of G-quartets, while G6 participates in WC base pair with C2. Broad signals at δ 11.1 ppm are assigned to G4H1 of GCn and GCnCG. G3H1 protons of GCn and GCnCG do not give observable signals, indicating that they are not involved in base pairing. No signal is observed for G14H1 proton of GCnCG. Interestingly, G1H8 protons of GCn and GCnCG resonate at δ 6.28 and 6.27 ppm, respectively, which is outside the range typical for guanine aromatic protons. On the other hand, A10H8 protons (δ 8.92 and 8.99 ppm for GCn and GCnCG, respectively) are observed downfield relative to the other aromatic protons.

Intensities of intra-nucleotide H8-H1’ cross-peaks in 2D NOESY ($r_m$ 80 ms) spectra revealed syn conformation along glycosidic bond for G1 and anti conformation for all other residues in GCn and GCnCG (Supplementary Figure S10). In addition to NOEs characteristic for anti-anti sequential steps, G1 displays G1H8−C2H1’ connectivity typical for syn-anti step of sequential walk (Figure 2). Interruption of sequential walk occurs only between G4 and A5, which implies unusual inter-nucleotide conformation in loop regions of G-quadruplexes adopted by GCn and GCnCG. Head-to-head stacking of bases characteristic for G1(syn)-C2(anti) step is nicely reflected in CD spectra of GCn and GCnCG with a shoulder at around 300 nm. The maxima at 270 and 265 nm in CD spectra of GCn and GCnCG, respectively, as well as minima at 237 nm are characteristic for head-to-tail stacking of nucleobases, which is in agreement with anti-anti sequential steps (Supplementary Figure S11). CD spectrum of GCnCG exhibits additional shoulder at 285 nm, which is attributed to stacking interactions of 3′-GC ends with a nearby G-quartet.

Perusal of imino-aromatic regions of 2D NOESY spectra reveals formation of G1−G7−G1−G7, G8−G11−G8−G11 and G9−G12−G9−G12 quartets (labelled with green, violet and red, respectively in Figure 3C and Supplementary Figure S12C). These correlations together with A10H8-G11H8 NOE interactions substantiate formation of sheared G8-A10 base pairs (Figure 3D). Positions of A10 residues are further defined by NOEs with G1 (G1H8−A10H2, G1H1′−A10H2, G1H1′−A10H8, G1H4’−A10H1, G1H4’−A10H2, G1H5’−A10H2) and C2 (C2H4’−A10H2) residues in GCn and GCnCG G-quadruplexes. It is interesting to note that A10 residues adopt N-type sugar conformation as indicated by the absence of H1’-H2’ and the presence of an intense H3’−H4’.
cross-peaks in 2D DQF-COSY spectra of GCn and GCnCG (Supplementary Figure S9).

G6–C2 base pair formation in WC geometry is supported by G6H1–C2H41 and G6H1–C2H42 NOEs. Downfield chemical shifts of C2H41 and C2H42 protons (δ 8.72 and 8.75 ppm for GCn, 8.81 and 8.96 ppm for GCnCG) confirm association of G6–C2 base pairs into G6–C2–G6–C2 quartet through their major groove edges (Figure 3E). Chemical shift of G4H1 protons (δ 11.1 ppm for GCn and GCnCG) suggests formation of symmetric G4–G4 N1-carbonyl base pairs, which is the only symmetric G-G base pair geometry with imino protons involved in hydrogen bonds (Figure 3F). 3′-GC ends in G-quadruplex adopted by GCnCG are less well defined in comparison to other parts of the structure, where C13 residues exhibit no stacking interactions, while G14 residues fold back as evident by G14H8–G12H8, G14H2'/H2'–G12H8 and G14H8–G11H8 cross-peaks in 2D NOESY spectra.

Diversity of structural elements and unique G-quadruplex folds adopted by GCn and GCnCG are nicely reflected in dispersion of 31P NMR resonances. 1D 31P spectra exhibit 11 and 13 resolved signals, which are dispersed between δ −1.4 and 0.5 ppm for GCn and GCnCG, respectively (Supplementary Figure S8). A10P and G11P resonate downfield relative to the other phosphorous atoms, which suggests unusual backbone conformation associated with formation of A(GGGG)A hexad.

Hexad is the most stable structural element

Local structural flexibility and unfolding were evaluated by analysis of a set of 1D 1H NMR spectra acquired between 25 and 80°C on GCn (Supplementary Figure S13). As expected from its location in more solvent-exposed region of the structure, decrease in intensity of G4H1 signal is observed already at 30°C. Partial dissociation of G4–G4 N1-carbonyl base pair is reflected in changes in local environment as suggested by better dispersion of signals corresponding to C2H41, C2H42 and A5H8. At 40°C, complete dissociation of symmetric G4–G4 N1-carbonyl base pair occurs. Its destacking is reflected in downfield shift of neighboring G6H1 proton resonance. At the same temperature, intensities of G6H1, G9H1 and G12H1 proton signals start to decrease, which is well correlated with their location in the outer quartets of G-quadruplex. With opening of terminal G9–G12–G9–G12 quartet, G8H1 and A10H2 protons of the adjacent A10–(G8–G11–G8–G11)–A10 hexad became deshielded. Complete dissociation of G9–G12–G9–G12 and G6–C2–G6–C2 quartets occurs at 75°C, where signals corresponding to G9H1, G12H1, G6H1, C2H41 and C2H42 protons broaden to baseline. Interestingly, at 75°C signals belonging to residues within hexad are still detectable, indicating its high thermal stability. At 80°C signals corresponding to GCn G-quadruplex are no longer visible.

**GCn and GCnCG form intertwined G-quadruplex structures**

High-resolution G-quadruplex structures of GCn and GCnCG (Figure 4) were calculated using 748 and 804 NOE derived distance-restraints together with 149 and 135 torsion angle restraints, respectively in addition to 32 hydrogen bond restraints for both G-quadruplexes (Supplementary Table S1). Family of 10 refined structures was selected based on the lowest energy and the lowest number of restraint violations (Supplementary Figure S14). Overall pairwise heavy atom RMSDs for family of 10 refined structures are 1.01 ± 0.31 Å and 1.39 ± 0.36 Å for GCn and GCnCG, respectively. NMR based structural calculations resulted in a symmetric, dimeric, intertwined G-quadruplexes of GCn and GCnCG, which can be viewed as structures composed of two units—an antiparallel unit at the 5′- and a parallel unit at the 3′-end. The two units are connected with two phosphodiester linkages between G7 and G8 residues. Nucleobases in both G-quadruplexes exhibit extensive stacking interactions with partial overlap between their six- and five-membered rings (Figure 4A and Supplementary Figure S15).

The 5′-units with antiparallel topologies of GCn and GCnCG consist of two stacked quartets (G1–G7–G1–G7 and G6–C2–G6–C2) and two lateral loops formed by G3–G4–A5 residues. Loops are interconnected by symmetric G4–G4 N1-carbonyl and A5–A5 N1-amino

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Figure 2. Aromatic-anomeric regions of 2D NOESY spectra (τenvelope 150 ms) with marked sequential walks for (A) GCn and (B) GCnCG. The intranucleotide H8/H9(i)–H9′(i+1) NOEs are labeled with residue numbers. Dashed lines show reverse inter-nucleotide H8(i)–H8′(i) NOEs. Cross-peaks in 2D DQF-COSY spectra of GCn and GCnCG (Supplementary Figure S9).
Figure 3. (A) Imino-aromatic region of 2D NOESY spectrum (τ_m 250 ms) of GCn. Cross-peaks coloured in green, violet and red indicate formation of G1−G7−G1−G7, G8−G11−G8−G11 and G9−G12−G9−G12 quartets, respectively. Inter-quartet NOE correlations are presented in black. (B) Folding topologies of GCn (left) and GCnCG (right) G-quadruplexes. Hatched rectangles indicate G1 in syn conformation, while all other residues adopt anti conformation. Dotted lines indicate symmetric A5-A5 N1-amino base pair revealed by MD simulations (vide infra). Dashed black lines indicate formation of GGCG- and G-quartets. (C) Imino-amino region of 2D NOESY spectrum (τ_m 150 ms) of GCn. NOE cross-peaks coloured in blue and violet support formation of major groove GGCG-quartet and A(GGGG)A hexad, respectively. Schematic representation of (D) A(GGGG)A hexad, (E) major groove GGCG-quartet, (F) symmetric GG N1-carbonyl base pair and (G) symmetric AA N1-amino base pair. Protons and observed NOE interactions crucial for formation of A(GGGG)A hexad and major groove GGCG-quartet are coloured in violet (C) or in blue (D), respectively.

base pairs (Figure 3F, G). Structural calculations suggest formation of A5−A5 base pair in GCn and GCnCG, which could be a consequence of favorable stacking interactions with lower G6−C2−G6−C2 quartet and upper symmetric G4−G4 N1-carbonyl base pair. The formation of A5−A5 base pair could not be verified experimentally due to dynamic nature or exposure to solvent. The 3′-units with parallel topologies consist of A10−(G8−G11−G8−G11)−A10 hexad and G9−G12−G9−G12 quartet in both GCn and GCnCG structures. Strands of the 3′-unit form single nucleotide (A10) double-chain reversal loops, which are part of A10−(G8−G11−G8−G11)−A10 hexad. The hexad is sandwiched between G9−G12−G9−G12 quartet and G1−G7−G1−G7 quartet (from the 5′-unit). In the case of GCnCG, the 3′-unit contains additional 3′-GC ends, which are rather flexible as seen in structural ensemble of 10 refined structures (Supplementary Figure S14). Consequently, G-quartets are less well defined in G-quadruplex of GCnCG (RMSD 0.62 ± 0.22 Å) compared to GCn (RMSD 0.40 ± 0.11 Å), which is reflected in distortion of planarity for G9−G12−G9−G12 and G8−G11−G8−G11 quartets (Supplementary Figure S14).

The antiparallel strands in the 5′-units of GCn and GCnCG form alternating narrow and wide grooves. The dimensions of narrow grooves formed by pairs of intra-strand
Figure 4. High-resolution structures of (A) GCn and (B) GCnCG G-quadruplexes. Stacking interactions between bases are shown for GCn in (A). Structural details and stacking (GCGC- and G-quartets, A(GGGG)A hexad, GG and AA base pairs) are presented with different colours for clarity.

G1–G7 and C2–G6 residues are extremely small with average width of 1.9 ± 0.9 Å and 3.1 ± 0.7 Å for GCn and GCnCG, respectively. Narrow grooves are bridged by G3–G4–A5 lateral loops. This trinucleotide arrangement is also known as GNA (G, guanine; N, any nucleotide; A, adenine) type of loop, which is known to form stable structures. Wide grooves formed by pairs of inter-strand G1–G7 and C2–G6 residues exhibit average width of 14.4 ± 0.5 Å and 14.6 ± 0.4 Å for GCn and GCnCG, respectively. The homo-purine G4–G4 and A5–A5 base pairs are formed diagonally on the wide grooves side in the 5′-units of the GCn and GCnCG G-quadruplexes. In contrast, the 3′-units with parallel topology of GCn and GCnCG exhibit four medium grooves of similar size with dimensions of 11.0 ± 0.3 Å for GCn and 11.4 ± 0.4 Å for GCnCG.

5′-GC ends and not GNA loops determine overall folds of GCn and GCnCG G-quadruplexes

Importance of 5′-GC in formation of GCn and GCnCG G-quadruplexes was evaluated by switching their respective positions in a sequence. Interestingly, d(CG2TGG4G2) forms completely different G-quadruplex structures with no WC GC base pairing (Supplementary Figure S16), which clearly demonstrates key role of WC GC pairing for stabilization and formation of intertwined G-quadruplexes.

In order to evaluate importance of GNA trinucleotide loop in formation of GCn and GCnCG G-quadruplexes, we have substituted A5 for thymine within G3–G4–A5. Results showed that A5T substitution in d(GCG2TGG4G2) leads to formation of a structure similar to GCn G-quadruplex (Supplementary Figure S16). Therefore, it appears that G3–G4–A5 loop element is not determining factor for structures shown in Figure 3B.

3′–3′ stacking of GCn G-quadruplexes induced by 15NH4+ and K+ ions

GCn and GCnCG are folded into well-defined G-quadruplexes in the presence of 15NH4+ and K+ ions (Figure 5). Signals for H1 protons of GCn folded in the presence of 15NH4+ (Figure 5B) and K+ (Figure 5C) ions resonate upfield with respect to the same protons of GCn folded in the presence of Na+ ions (Figure 5A). Uplift H1 proton signals suggest additional stacking interactions, which are also reflected in upfield shifts of some aromatic protons in 1D 1H NMR spectra of GCn G-quadruplex in the presence of 15NH4+ and K+ ions. Lower Dt values obtained at 25°C and 1.0 mM oligonucleotide concentration for GCn folded in the presence of 15NH4+ and K+ ions (1.3 × 10−10 m2 s−1) in comparison to Dt value in the presence of Na+ ions (Dt 1.6 × 10−10 m2 s−1) are in agreement with formation of twofold bigger G-quadruplex structures.

Most of H1 and H8 proton resonances of GCn G-quadruplex in the presence of 15NH4+ ions, were unambiguously assigned with the use of 1D 15N-edited and 2D 13C-edited HSQC spectra recorded on 15N and 13C residue-specific partially (8%) labeled oligonucleotides (Supplementary Figure S17). The remaining H1 and H8/H6 proton resonances were assigned based on connectivities in 2D NOESY spectra (Supplementary Figure S18). H8 protons of G1 and A10 residues display upfield (δ 5.93 ppm) and downfield (δ 8.92 ppm) shifts, respectively, relative to the other aromatic protons. Intra- and inter-nucleotide connectivities in sequential walk are observed from G1 to G4 and...
from G6 to G12 (Supplementary Figure S18A). Sequential walk is interrupted within G4–A5–G6 segment indicating their unusual inter-nucleotide conformation in the loop region. All residues in the GCn G-quadruplex folded in the presence of $^{15}$NH$_4^+$ ions adopt anti conformation along glycosidic bond except G1, which exhibits syn conformation. Analysis of imino-aromatic and imino-imino regions of 2D NOESY spectrum (Supplementary Figure S18B, C) reveals formation of G1–G7–G1–G7 and G9–G12–G9–G12 quartets and A10–(G8–G11–G8–G11)–A10 hexad (Supplementary Figure S18D), which is consistent with GCn G-quadruplex folded in the presence of Na$^+$ ions. The 5′-unit of GCn in the presence of $^{15}$NH$_4^+$ ions exhibits two WC GC base pairs as indicated by G6H1 signal at 8.12 ppm (Figure 6A). Lower intensity of G6H1 signal in the presence of $^{15}$NH$_4^+$ ions suggests that WC GC base pairs are not as well protected from the exchange with solvent or are more flexible than in GCn G-quadruplex folded in the presence of Na$^+$ ions. Further association of WC GC base pairs into GCGC-quartet is not observed in the presence of $^{15}$NH$_4^+$ ions since cross-peaks characteristic for its formation were not detected in 2D NOESY spectrum. Another difference observed in the 5′-unit of GCn G-quadruplex folded in the presence of $^{15}$NH$_4^+$ ions is the absence of symmetric G4–G4 N1-carbonyl base pair. In the 3′-unit stacking of terminal G-quartets is induced by the presence of $^{15}$NH$_4^+$ ions, which leads to dimerization of two dimeric GCn G-quadruplexes (Figure 6A). 3′–3′ stacking is supported by inter-residue NOE observed between G12H8–G12H1 protons (Supplementary Figure S18B). The 3′–3′ stacked GCn G-quadruplexes are able to form in the wide range of DNA concentration, as nicely demonstrated with a set of 1D $^1$H NMR spectra, recorded with DNA concentration ranging from 3.0 to 0.09 mM per strand in the presence of 175 mM $^{15}$NH$_4^+$ ions (Supplementary Figure S19A). Interestingly, the 3′–3′ stacked GCn G-quadruplexes are preserved even at low DNA concentration (0.09 mM), which indicates their high stability.

In the presence of K$^+$ ions unambiguous assignment of imino and aromatic protons of G1 and G6 (Supplementary Figure S20) revealed similar chemical shifts as observed for the same protons in the presence of $^{15}$NH$_4^+$ ions (Figure 5B, C). In addition to the same Dt values obtained in the presence of both cations, comparable 1D $^1$H and 2D NOESY NMR spectra confirmed similar GCn G-quadruplexes in the presence of $^{15}$NH$_4^+$ and K$^+$ ions (Figure 6A). Dt values obtained at 25°C and 1.0 mM oligonucleotide GCnCG concentration folded in the presence of Na$^+$, $^{15}$NH$_4^+$ and K$^+$ ions (1.5 × 10$^{-10}$ m$^2$s$^{-1}$) are the same, which suggest formation of dimeric G-quadruplexes. Therefore, 3′-GC ends in GCnCG irrespective of monovalent cation nature are not involved in inter-quadruplex GCGC-quartets but rather prevent formation of bigger G-quadruplex based structures. Contrary to differences observed for GCn G-quadruplexes formed in the presence of various monovalent cations, comparison of 1D $^1$H (Figure 5D–F) and 2D NOESY NMR spectra of GCnCG G-quadruplexes folded in the presence of Na$^+$, $^{15}$NH$_4^+$ and K$^+$ ions reveals minor structural differences (Figure 6B). The 5′-unit of GCnCG G-quadruplexes in the presence of $^{15}$NH$_4^+$ and K$^+$ ions is characterized by less defined WC GC base pairs, which do not associate into GCGC-quartet. Additionally, no G4-G4 base pair was observed in the presence of $^{15}$NH$_4^+$ and K$^+$ ions. The molecularity of GCnCG G-quadruplexes is preserved in DNA concentration ranging from 3.0 to 0.09 mM per strand in the presence of 175 mM $^{15}$NH$_4^+$ ions (Supplementary Figure S19B). Even at the highest DNA concentration no 3′–3′ interlocking was observed.

**DISCUSSION**

In the presence of Na$^+$ ions GCn and GCnCG form symmetric, dimeric G-quadruplexes, with unique combination of structural elements. GCn and GCnCG G-quadruplexes are composed of two G-quartets, A(GGGG)A hexad, major groove GCGC-quartet and symmetric GG N1-carbonyl and AA N1-amino base pairs. Only few high-resolution G-quadruplex structures containing major groove GCGC-quartets (3–6,46) and only one with A(GGGG)A hexad (14) in solution have been reported so far. Interestingly, 11 out of 12 residues in GCn are involved in hydrogen bonds. The only non-hydrogen bonded residues are...
G3. They are part of two lateral loops, which are interconnected by symmetric G4-G4 N1-carbonyl and A5-A5 N1-amino base pairs. In case of GCn, additional nucleobases at the 3′-end (C13 and G14) are not involved in hydrogen bonds. Both, GCn and GCnCG G-quadruplexes can be viewed as structures composed of two units with 5′-antiparallel and 3′-parallel topologies. G-quadruplex composed of 5′-antiparallel and 3′-parallel units was till now described only for crystal structure of oligonucleotide d(G3CG4AG3A2G3A) derived from B-raf gene in the presence of K+ ions (65). In addition, this G-quadruplex contains distorted GCGC-quartet and similar arrangements of adenines as observed within A(GGGG)A hexad in GCn and GCnCG G-quadruplexes. Existence of GCn and GCnCG structures in solution undoubtedly demonstrates that G-quadruplexes composed of two distinct units with different strand orientations and various hydrogen bonding patter are not only a consequence of crystallization conditions in solid state. The two DNA strands intertwine in order to fold into G-quadruplexes, which is another interesting structural feature of GCn and GCnCG G-quadruplexes. Dimeric, intertwined G-quadruplexes were described before for oligonucleotides d(G3CG4AG3A2G3A) and d(CG3CG3CGCGAG3AG4) (65–67).

Diverse structural elements of GCn and GCnCG G-quadruplexes are easily distinguished by NMR since involved residues display characteristic spectroscopic properties. A10H8 protons exhibit downfield shifts of δ 8.92 and 8.99 ppm for GCn and GCnCG, respectively. Such downfield shifts for adenine H8 protons were previously observed for adenine residues involved in a sheared G-A base pair within pentad (12) or heptad (18). Interestingly, not all H8 protons of adenines involved in formation of pentad, hexad or heptad display such downfield chemical shift (13,14,16–18). Another interesting feature of GCn and GCnCG G-quadruplexes are G1 residues, which display upfield shifts of their aromatic and anomeric protons (δ 6.28 and 4.36 ppm for GCn, δ 6.27 and 4.35 ppm for GCnCG, respectively). Such upfield shifts of aromatic and anomeric protons were observed for guanines stacked above G-(A-G) triad (12), which is in good agreement with location of G1 above A10-G8–G11–G8-G11-A10 hexad in GCn and GCnCG G-quadruplexes. Interestingly, only G1 residues in GCn and GCnCG G-quadruplexes exhibit syn conformation along the glycosidic bond. Furthermore, G1 residues occupy unusual position at the interface of two G-quadruplex units within the GCn and GCnCG G-quadruplexes. Although G1 residues are usually located in the outer G-quartets, their positions in the middle of G-quadruplex were observed before (12,68–70). Additionally, unusual upfield chemical shifts were observed for H4′ protons of N nucleotides in trinucleotide GNA segments when forming loops (52,71,72) or participating in mixed AG hexads (14) or heptads (17,18). This is in perfect agreement with our observations since G4H4′ are involved in G3–G4–A5 (lateral loops) and G9H4′ in G8–G9–A10 (A10 form single nucleotide double-chain reversal loops).

Figure 6. Folding topologies of (A) GCn and (B) GCnCG G-quadruplexes in the presence of 15NH4+ and K+ ions. Hatched rectangles indicate syn conformation of G1 residues, while all other residues adopt anti conformation. Dashed black lines indicate formation of G-quartets. 15NH4+ and K+ ions lead to formation of bigger structures.
quences, which are similar to GCn and GCnCG but with thymines separating G-tracts instead of adenines (40). It seems that adenine residues can affect ability for multimer formation. Both, GCn and GCnCG possess G2A segments with tendency to form GNA loop (52,71,72). Surprisingly, A5 for thymine substitution still leads to formation of G3-G4-T5 lateral loop and therefore excludes importance of GNA loop in folding of GCn and GCnCG G-quadruplexes. Second adenines (A10) in front of the shortest G-tracts within oligonucleotides GCn and GCnCG form A(4GGG)A hexads, which is in perfect agreement with observation that adenine before the shortest G-tract will participate in a pentad or in our case hexad formation (45).

The influence of adenine on multimerization ability was observed before (31,39,41,50). For short G-rich oligonucleotides d(GGG2XG2CG) and d(GCG2XG2), where X = A, T or TC, it was demonstrated that sequences with X = A most readily form long and organized structures in the presence of Na+/Na+/Mg2+ ions on mica substrate (50). For d(GGG2AG2-CG) the formation of G-wire was also observed in solution (43). Oligonucleotides d(GCG2AG2-CG) and d(GCG2AG2) have similar nucleotide sequence as GCn and GCnCG, but lack one AG4 segment. Our NMR study of GCn and GCnCG clearly shows that only dimeric G-quadruplexes were present in Na+ ion containing solution. A5 in GCn and GCnCG are part of lateral loops and A10 residues participate in hexad hydrogen bond alignments. Therefore, the role of adenines in formation of longer structures is not straightforward. It should be noted that surface organization of G-quadruplexes could differ from their behavior in solution, due to molecular crowding effect reached during evaporation process on surface (50). Furthermore, in tetrameric G-quadruplexes formed by d(T2AG3), bulky adenine bases prevent formation of G-quadruplexes with a slipped strands, which are essential for assembly of the interlocking G-quadruplex dimer as observed for d(G1T) (39,44). When varying Gs in the central quartet of unimolecular, parallel-stranded G-quadruplex formed by d(G1T2G1T2G1), adenine was demonstrated to induce dimer as well as multimer formation, while variations with cytosines or thymines led to monomeric or dimeric structures, respectively (41). On the other hand, by varying nucleotide sequence in loops of monomeric G-quadruplexes composed of four G2 or four G1 tracts, adenine and cytosine residues display different multimerization mechanisms compared to thymines and mutations that mimic abasic sites, which shifted equilibrium towards higher-order structures (31).

Contradictory data were reported for the role that 3′-GC ends play in elongation of G-quadruplex based nanostructures. For example, d(GCG2AG2-CG) is able to multimerize into G-wires on surface as well as in solution, where the mechanism of interlocking includes 3′-GC ends (43). For oligonucleotides d(GCG2XG2), where X = A, T or TC, it was shown that G-wires growth on surface through connectivities formed by GC ends was equally effective as growth through stacking of terminal G-quartets in sequences where 3′-GC was removed (50). On the other hand, for d(GGG2TG1TG2CG) and d(G2TG1TG2CG) no interactions through 3′-GC ends were observed in solution (40). In the case, if free 3′-GC ends of GCnCG would enable inter-quadruplex connections, two major groove GCGC-quartets would link two GCnCG G-quadruplexes. Major groove GCGC-quartets are characterized by alternating narrow and wide grooves. The 3′-GC ends are part of the 3′-unit with parallel topology of GCnCG G-quadruplex, which exhibits medium grooves with average dimensions of 11.4 ± 0.4 Å. It may be assumed that different groove dimensions of 3′-unit with parallel topology and GCGC-quartet might prevent interconnections through 3′-GC ends.

The role of cations on G-quadruplex stabilization is well known as well as their effect on folding topologies (1,2,73). Structural rearrangement of G-quadruplexes can be triggered by change in nature of cations present in solution (74,75). For example, cation-dependent polymorphism of human telomeric sequence is likely one of the most well described (76). On the other hand, change of cations exhibits only minor effect on structure of G-quadruplexes formed by telomeric sequences derived from Oxytricha nova d(G1T1G4) and d(G1T4G1), which retain the same general fold in the presence of Na+, 15NH4+ and K+ ions (77,78). For GCn and GCnCG folded in the presence of 15NH4+ and K+ ions, structural rearrangements of the 5′-units with antiparallel topologies were observed compared to the G-quadruplexes folded in the presence of Na+ ions. Two G6-C2 WC base pairs are formed in the presence of 15NH4+ and K+ ions instead of major groove G6-C2-G6-C2 quartet observed in the presence of Na+ ions. Symmetric G4-G4 N1-carbonyl base pair are observed only in G-quadruplexes of GCn and GCnCG in the presence of Na+ ions. Such structural transition of GCGC-quartet induced by change of Na+ with 15NH4+ and K+ ions was previously observed for d(G1C1T1G4) G-quadruplex (9,53). In the 3′-unit with parallel topology of GCn and GCnCG G-quadruplexes, G9-G12-G9-G12 quartet and A10-(G8-G11-G8-G11)-A10 hexad including 3′-GC ends in the case of GCnCG were preserved even in the presence of 15NH4+ and K+ ions. Interestingly, in GCn G-quadruplexes 15NH4+ and K+ ions promoted dimerization of two dimeric G-quadruplexes via 3′-stacking. Dimerization of G-quadruplexes triggered by different cations was observed before. For example stacking through common U-quartet of d(U1G1T) tetramolecular G-quadruplex was induced by change of Na+ ions to 15NH4+ and K+ ions (38). Among monovalent cations, K+ ions are known to most likely enhance multimerization of ‘blunt-end’ G-quadruplexes through stacking interactions as was demonstrated by ESI-MS (42).

In summary, we have shown that GCn and GCnCG form a novel six-layered dimeric G-quadruplex structures in solution of Na+ ions. In the presence of larger cations, such as 15NH4+ and K+, dimerization via 3′-3′ stacking interactions of terminal G-quartets was observed, which is precluded with 3′-GC ends. Our results might be useful for design of DNA oligonucleotides with ability to form G-quadruplexes with unique folding topologies as required for a specific nanotechnological application. In the designing process of G-rich oligonucleotides, introduction of adenine residues at critical position within DNA sequence might lead to additional hydrogen bonding or to formation of completely different structures. Furthermore, position of cytosines within the sequence is crucial due to their tendency to participate in formation of WC GC base pairs,
which may disrupt the anticipated structure. 5’-GC residues in GCₙ and GCₙCG form G- and GCGC-quartets within G-quadruplexes and do not promote inter-quadruplex association. To the best of our knowledge, GCₙ and GCₙCG G-quadruplexes exhibit unique combination of structural elements observed in solution for the first time.

DATA AVAILABILITY

Atomic coordinates and lists of chemical shifts for GCₙ and GCₙCG have been deposited with 6SX6 and 6SYK accession numbers, respectively.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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