Understanding self-assembly at molecular level enables controlled design of DNA G-wires of different properties

Daša Pavc, Nerea Sebastian, Lea Spindler, Irena Drevenšek-Olenik, Gorazd Koderman Podboršek, Janez Plavec & Primož Šket

A possible engineering of materials with diverse bio- and nano-applications relies on robust self-assembly of oligonucleotides. Bottom-up approach utilizing guanine-rich DNA oligonucleotides can lead to formation of G-wires, nanostructures consisting of continuous stacks of G-quartets. However, Gewire structure and self-assembly process remain poorly understood, although they are crucial for optimizing properties needed for specific applications. Herein, we use nuclear magnetic resonance to get insights at molecular level on how chosen short, guanine-rich oligonucleotides self-assemble into G-wires, whereas complementary methods are used for their characterization. Additionally, unravelling mechanistic details enable us to guide G-wire self-assembly in a controlled manner. MD simulations provide insight why loop residues with considerably different properties, i.e., hydrogen-bond affinity, stacking interactions, electronic effects and hydrophobicity extensively increase or decrease G-wire length. Our results provide fundamental understanding of G-wire self-assembly process useful for future design of nanomaterials with specific properties.

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dsDNA is an interesting molecule for nanoscience and nanotechnology, arguably due to its programmable self-assembling properties desired in bottom-up approaches. Simple Watson-Crick base pairing enables rational design of DNA nanostructures, ranging from static 2D to 3D materials, complex constructs as well as dynamic molecular machines with precise and controllable motions with various biotechnological and nanotechnological applications. In comparison to dsDNA, four-stranded DNA exhibits interesting optical and electronic properties, superior resistance to enzymatic degradation as well as mechanical and thermal stability on substrates thus offering diverse applications. An example of such four-stranded DNA structures are G-wires, which are long, G-quadruplex-based nanostructures composed of continuous runs of G-quartets. These planar motifs consist of four guanine residues held together by Hoogsteen-type of hydrogen bonds (Supplementary Fig. 1a). A unique feature of G-quartets is sensitivity to cations, which assist their formation and stacking into G-quadruplexes (Supplementary Fig. 1b) as well as potentially leading to G-quadruplex-based nanostructures. Furthermore, non-guaine residues often form loops, which are another important structural element of G-quadruplexes.

Since the first report in 1994 on G-wires formed by d(G4T2G4), they have been gaining interest due to their potential use as nanosensors, photonic and electronic nanodevices. These long nanostructures can be formed by self-assembly of poly(G) strands as well as short G-rich oligonucleotides. Among proposed mechanisms for the latter are interlocking of G-quadruplexes via slipped G-rich strands (Supplementary Fig. 1c) and via sticky ends, e.g., GC-ends (Supplementary Fig. 1d). Alternatively, G-wires can be formed via π–π stacking of G-quadruplexes (Supplementary Fig. 1e). Fundamental understanding of self-assembly process and structure of G-wires remains poor, which is however needed to achieve precise controllable as well as tunable formations and therefore tailoring of materials with the desired properties. The first step towards better understanding was achieved only recently, where solution-state atomic force microscopy (AFM) complemented with molecular dynamics (MD) simulations were used to show that d(G1T4G3) can self-assemble into three different structural types of G-wires via interlocking of slipped G-rich strand mechanism. However, study of G-wire self-assembly still remains challenging since spontaneous multimerization makes difficult to “catch” individual building blocks (i.e., G-quadruplexes) in order to characterize them and monitor their further assembly in a controlled manner.

Herein, we offer detailed insights into the self-assembly process of d(G2A4G2G2) G-wire formation at molecular level. Previously, we showed that GC ends attached to either 5′-end or both 5′ and 3′ ends of oligonucleotide d(G2A4G2G2) hindered multimerization. In general, such type of oligonucleotides, i.e., G2X(G2)XG2 (where X = T, A, TC and n = 0, 1, 2, and 4) with 5′-GC and/or 3′-GC ends, were subject of many studies for their multimerization ability. Varying factors such as sample preparation and cation concentration led us to solution conditions, where single G-wire building blocks (G-quadruplexes) are dominant, thus enable their structural determination by nuclear magnetic resonance (NMR). We believe that beside steric availability of terminal G-quartets, hydrophobic properties of stacking interfaces are important in aqueous solutions and thus have a key role in self-assembly of stacked G-wires. Therefore, G-wires lengths can be manipulated by designing oligonucleotides with different loop residues near terminal G-quartets. Significant changes might be expected already by substituting adenine for thymine and cytosine residues, whereas considerably different, i.e., in terms of hydrogen-bond affinity, stacking properties, electronic effects and hydrophobicity, c3 linkers and abasic residues could influence lengths of G-wires even more. For visualization and characterization of G-wires we used several complementary methods including dynamic light scattering (DLS), AFM, scanning and transmission electron microscopies (SEM, TEM).

Results and discussion

d(G2A4G2G2) forms G-wires. We probed d(G2A4G2G2) oligonucleotide for G-wire formation by slow cooling (annealing) its 1.0 mM solution in 100 mM KCl (see “Method 1” in “Methods” section). A broad hump between 6 9.7 and 11.4 ppm in 1D 1H NMR spectrum at 25 °C was tentatively assigned to guanine H1 protons of G-wires (Fig. 1a). In agreement, DLS revealed translational diffusion coefficient D of 0.33 × 10⁻⁷ m² s⁻¹, which corresponds to G-wires with 43 nm effective length (Fig. 1b). AFM images at 0.2 μM d(G2A4G2G2) concentration (see “Methods” section, Fig. 1c and Supplementary Fig. 2a) show rod-like structures with 2.0 nm average height as typical for G-wires. Length analysis shows a log-normal distribution with 22.1 nm mean value and 65 nm maximum detected length. At higher, 2 μM d(G2A4G2G2) concentration AFM shows an interconnected network of G-wires with 2.4 nm height (Supplementary Fig. 2b, c). Furthermore, SEM and TEM images at 1.0 mM d(G2A4G2G2) concentration show randomly distributed G-wires and their bigger deposits (Fig. 1d–f and Supplementary Figs. 3 and 4). Comparative to SEM, TEM offers more discernible images of shorter G-wires (Fig. 1e, f and Supplementary Fig. 4). The width of G-wires is between 10 and 20 nm (Fig. 1g), which is greater than solution diameter of G-quadruplexes (~2.8 nm), but in agreement with previously observed width by AFM. Various observed lengths of d(G2A4G2G2) G-wires by SEM/TEM, AFM, and DLS can be explained by different surface and solution behavior as well as sample concentration. These data demonstrate that d(G2A4G2G2) is suitable model for in depth mechanistic study of G-wire self-assembly.

Structural rearrangement is the crucial step in self-assembly of d(G2A4G2G2) G-wires. By lowering temperature to 0 °C and varying KCl concentration (100–3 mM) we determined solution conditions where Q1k and Q2k, first and second G-quadruplexes in d(G2A4G2G2) G-wire self-assembly are predominant structures (see “Method 2” in “Methods” section; Fig. 2 and Supplementary Figs. 5 and 6). We used fast cooling (quenching) of d(G2A4G2G2) in 3 mM KCl to shift equilibrium towards Q1k (see “Method 3” in “Methods” section). 1D 1H NMR spectrum of Q1k exhibits eight sharp signals between δ 10.77 and 11.67 ppm, which correspond to H1 protons of guanines in G-quartets (Fig. 2a). Eight H1 proton signals together with D of 1.55 × 10⁻⁷ m² s⁻¹ and mobility on a native polyacrylamide electrophoresis (PAGE) gel indicate that Q1k is a dimeric G-quadruplex composed of four G-quartets (Fig. 2a and Supplementary Figs. 7 and 8). Unambiguous assignment of H1 and H8 guanine proton resonances revealed two unusual chemical shifts δ of 10.77 and δ at 6.28 ppm, where the latter indicates presence of A(GGGG)A hexad. Unambiguous assignment of H1 and H8 guanine proton resonances revealed two unusual chemical shifts δ of 10.77 and δ at 6.28 ppm, where the latter indicates presence of A(GGGG)A hexad. Unambiguous assignment of H1 and H8 guanine proton resonances revealed two unusual chemical shifts δ of 10.77 and δ at 6.28 ppm, where the latter indicates presence of A(GGGG)A hexad. Unambiguous assignment of H1 and H8 guanine proton resonances revealed two unusual chemical shifts δ of 10.77 and δ at 6.28 ppm, where the latter indicates presence of A(GGGG)A hexad.
at 240 nm, maximum at 270 nm and shoulder at 300 nm is in agreement with the mixed antiparallel/parallel folding topology of Q1k (Fig. 2a). Q1k is transformed into Q2k as followed at 0 °C, where the rate of Q2k formation varies with KCl concentrations (Fig. 2b and Supplementary Figs. 5 and 6). Quenching of d(G2AG4AG2) in 60 mM KCl shifts equilibrium towards Q2k and thus enables its NMR structural analysis (see Method 4 in Methods section). Compared to Q1k, 1D 1H NMR spectrum of Q2k exhibits upfield shifted H1 signals as well as lower Dt of 1.28 × 10^{-10} m^2 s^{-1} and slower mobility on native PAGE gel, which suggest formation of bigger structure for the latter G-quadruplex (Fig. 2c and Supplementary Figs. 8 and 11). NMR spectroscopic markers δG2H1 at 10.45 and δG1H8 at 5.96 ppm together with cross-peaks in 2D NOESY spectrum show that Q2k exhibits the same combination of structural elements as Q1k (Fig. 2c and Supplementary Figs. 12 and 13). Changes in chemical shifts of H1 protons are the most pronounced for G7 and G10 (ΔδG7H1 of 0.44 and ΔδG10H1 of 0.91 ppm), indicating 3′-3′ stacking of individual G-quadruplex building blocks (Fig. 2c and Supplementary Figs. 9 and 12). 3′-3′ stacking in Q2k is further supported by H-D exchange experiment, where H1 protons of the outer G2-G4-G2-G4 quartet disappear immediately after dissolving in D2O. The apparent rate of exchange is slower for remaining G-quartets, where their H1 signals are still observable in 1D 1H NMR spectrum after 11 days (Fig. 2c). Comparison of Q1k and Q2k CD spectra reveals highly similar shapes with increase in shoulder around λ300 nm for the latter, which can be attributed to 3′-3′ stacking (Fig. 2a, c).

We observed a new set of signals in 1D 1H NMR spectra of d(G2AG4AG2) 6 days after addition of 3 mM KCl at 0 °C corresponding to Q2t (Supplementary Fig. 14a, c). Equilibrium is shifted towards formation of Q2t when 3 mM KCl is added into solution of d(G2AG4AG2) at 25 °C (Fig. 2d and Supplementary Fig. 14b, d). Q2t becomes dominant structure at 25 °C, when d(G2AG4AG2) is annealed in 15 mM KCl (see Method 5 in Methods section; Fig. 2e). In 1D 1H NMR spectrum of Q2t H1...
and H8 of guanines, including G2H1 and G1H8 resonate within narrower chemical shift range in comparison to Qk-type, indicating considerably different structure (Fig. 2e and Supplementary Fig. 15). $D_t$ of $1.14 \times 10^{-10}$ m$^2$ s$^{-1}$ and mobility on native PAGE gel suggest that Q2t is also composed of two stacked G-quadruplexes (Fig. 2e and Supplementary Figs. 8 and 16). Perusal of 2D NOESY spectra reveals formation of G1-G4-G1-G4 and G2-G5-G2-G5 quartets in Q2t, which together with G6-G9-G6-G9 and G7-G10-G7-G10 quartets establish all-parallel folding topology consisting of eight G-quartets with A3 and A8 residues in propeller loops (hereupon referred as loop residues at position 3 and 8) (Fig. 2e and Supplementary Fig. 17). 3'-3' stacking in Q2t is confirmed by inter-quadruplex G7H1-G7H8 and G10H1-G10H8 NOEs (Supplementary Fig. 17b). Q2t displays CD

Fig. 2 Initial steps of d(G2AG4AG2) G-wire self-assembly. a NMR data, CD spectrum and folding topology of Q1k. In 2D NOESY spectrum ($\tau_m$ 150 ms) the intra-nucleotide H8(i)-H1(n) cross-peaks are labeled with corresponding residue numbers, where their observed intensities reveal syn conformation along glycosidic bond for G1 and G2 and anti for the remaining residues. Black lines show sequential walk, which is followed from G2 up to G10 residue via H8(i)-H1(n) and H1(n)-H8(i) connectivities as characteristic for anti-anti step. G1 and G2 residues in syn conformation are presented with rectangles surrounded with black in Qk folding topology. Arrows indicate progression of strand from 5'→3' end. b Q1k→Q2k transformation at 0 °C in 100 mM KCl. c Folding topology of Q2k and corresponding NMR and CD data. Similarly to Q1k, sequential walk in Q2k is followed from G2 up to G10 residues in 2D NOESY spectrum ($\tau_m$ 150 ms). Deuterium exchange experiment supports 3'→3' end. d Q1k→Q2k→Q2t transformation at 25 °C in 3 mM KCl. e Structural differences between Q2t and Qk/Q2k are reflected in CD, 1D $^1$H and 2D NOESY ($\tau_m$ 150 ms) spectra. Crosses in 2D NOESY denote weak crosspeaks. Simplified scheme of these structures is presented in Fig. 3. Samples were assembled via method 3, method 2, method 4, method 2, and 5 (see "Methods" section).
**Design of G-wires of different lengths.** We expect that loop residues might play an important role in determining the properties of G-wires, e.g., length. Therefore, we systematically substituted adenines with thymines and cytosines at positions 3 and/or 8 (Fig. 4a). All modified oligonucleotides are capable of G-wire formation, as indicated by a broad hump observed in H1 region of their 1D 1H NMR spectra as well as ladder pattern with smearing on native PAGE gel (see “Method 1” in “Methods” section; Supplementary Figs. 21 and 22a). Modified oligonucleotides follow the same folding pathway as parent d(G2AG4AG2) (Fig. 3) as we demonstrated by adding KCl to final 3 mM concentration into solution of d(G2TG4AG2), d(G2GG4C2), d(G2AG4TG2), d(G2AG4CG2), d(G2TG4TG2) and d(G2GG4CG2) oligonucleotides and following changes by NMR at 25 °C. 1D 1H NMR spectra recorded immediately after addition of KCl exhibit multiple sets of signals between δ 10.5 and 12.3 ppm, likely corresponding to H1 protons of guanines in G-quartets of Q1k, Q2k and Q2t, whereas only one set of signals is dominant in 1D 1H NMR spectra one day later (Supplementary Fig. 23). Furthermore, G-wires formed by modified oligonucleotides exhibit highly similar CD spectra as parent d(G2AG4AG2) G-wire, which supports formation of Qt-type building blocks in all cases (Supplementary Fig. 24). DLS revealed D1 between 0.25 and 0.44 × 10−9 m2 s−1 for modified oligonucleotides indicating formation of rather long G-wires (Fig. 4a and Supplementary Fig. 22b). Using theory for cylindrical scatterers,36 the D1 correspond to G-wires with effective lengths between 28 and 67 nm (Fig. 4a and Supplementary Fig. 22c). Based on our determined G-quadruplex model and with a typical stacking distance between G-quartets of 0.34 nm, the number of stacked Qt-type building blocks (n) in Qnt-type G-wires ranges from 20 to 49 (Figs. 3 and 4a).

The difference in length of d(G2AG4AG2) and the longest d(G2AG4CG2) G-wires is clearly observed in AFM images, where the latter exhibit 31.6 nm mean length with values up to 120 nm (Fig. 4b and Supplementary Fig. 25a). For d(G2AG4CG2) we were also able to obtain AFM images of G-wires deposited on mica, which was not pre-treated with MgCl2 where structures have higher mean length of 40.6 nm and values up to 160 nm indicating that substrate pre-treatment allows adhesion of the smaller G-wires, which are not observed otherwise (Supplementary Fig. 25b,c). In agreement, SEM and TEM images of d(G2AG4CG2) show G-wires with average length values up to 180 nm, which are clearly longer compared to parent ones (Fig. 4e–f and Supplementary Figs. 26 and 27).

**The type of loop residues influences thermal stability of G-wires.** We evaluated thermal stability of G-wires by following their unfolding from 5 to 85 °C by NMR melting experiment.
1D\textsuperscript{1}H NMR spectra of G-wires acquired between 5 and 25 °C exhibit a broad hump in H1 region, while broad signals on top of a hump start to appear at 35 °C, which indicates beginning of G-wires dissociation into shorter structures. Above 55 °C a set of sharp signals is observed in addition to broader signals, which likely correspond to Q2t. At 85 °C only sharp signals are observed in 1D\textsuperscript{1}H NMR spectra of all G-wires indicating that solely Q2t is stable at this temperature, as later confirmed by NMR melting experiment on a sample with 25 mM KCl, where only Q2t and Q4t are present in solution at room temperature (Supplementary Fig. 30).

It should be mentioned that the thermal stability of these structures greatly depends on the concentration of KCl, i.e., at 85 °C, Q2t is still observed in 100 mM KCl, but is completely unfolded in the presence of 25 mM KCl. When cooling the sample with 25 mM KCl back from 85 to 65 °C, H1 region of 1D\textsuperscript{1}H NMR spectrum exhibits signals that correspond to Q2t and in a lesser amount to Q1k indicating that at higher temperature the equilibrium is shifted towards formation of Q2t (Supplementary Fig. 30). Interestingly, Q2k is not observed at 65 °C, likely due to immediate structural rearrangement of Q2k to Q2t, which is faster at higher temperatures as shown before at 0 and 25 °C (Supplementary Figs. 14c, d and 30). Returning back at 25 °C we observe H1 protons signals corresponding to Q2t and Q4t as well as to Q1k and Q2k, which indicates the same folding and refolding pathway of d(G\textsubscript{2}AG\textsubscript{4}AG\textsubscript{2}). Reversibility of self-assembly process was also observed for all studied G-wires.

UV melting curves obtained from 5 up to 95 °C on G-wires exhibit one transition, which actually corresponds to unfolding of Q2t, which is the last stable G-quadruplex in unfolding of G-wires as demonstrated before by NMR melting experiments (Supplementary Figs. 28–31).

Since UV melting curves did not reach a clear plateau at 95 °C, mid-transition temperatures (T\textsubscript{1/2}) are only estimations, where most of them are in the range between 83 and 92 °C (Supplementary Fig. 31d). The most stable are d(G\textsubscript{2}AG\textsubscript{4}TG\textsubscript{2}) G-wires where T\textsubscript{1/2} exceeds 92 °C (Supplementary Fig. 31d). Plot, where G-wires are arranged by increasing T\textsubscript{1/2} reveals that their thermal stability greatly depends on the type of loop residue at position 8 (Supplementary Fig. 31e). G-wires with T and A at position 8 are the most and the least thermally stable, respectively, which correlates with previously observed data on single G-quadruplexes\textsuperscript{37–39}.

Why can the type of loop residues affect the length of G-wires?

In aqueous solution nonpolar molecules tend to self-assemble due to the hydrophobic effect. It was shown that 5'‐quartets are more hydrophobic compared to 3'‐quartets, which might add to 5'‐5' stacking being the preferred mode of G-quadruplex multimerization\textsuperscript{40}. For substitutions with the same type of loop residues at positions 3 and 8 lengths of G-wires increase as follows: d(G\textsubscript{2}TG\textsubscript{4}TG\textsubscript{2}) < d(G\textsubscript{4}AG\textsubscript{4}AG\textsubscript{2}) < d(G\textsubscript{4}CG\textsubscript{4}CG\textsubscript{2}) (Supplementary Fig. 22c). In accordance, the hydrophobicity of nucleobases decreases in the same order, i.e., T > A > C\textsuperscript{41}. Therefore, our observations suggest that hydrophobicity of loop residues affects nearby G-quartets and in this way influence length of G-wires. In complete agreement, folding of oligonucleotides with c3 linker (li) and abasic residue (ab), which are

(Supplementary Figs. 28 and 29).
more and less hydrophobic, respectively compared to T, A, and C leads to formation of the shortest (15 nm) and the longest (>90 nm) G-wires as confirmed by NMR and DLS (Fig. 5a–c and Supplementary Fig. 32). However, one must be aware that nucleotides also possess different rotational degrees of freedom, i.e., the c3 linker exhibits significantly greater rotational freedom compared to deoxyribose in the backbone, which can impact the equilibrium distribution of the different guanine-rich DNA folds and consequently affect length of resulting G-wires.

To gain further insights into how loop residues affect G-wire lengths we performed NMR-derived structural calculations of $d(G_2A_2G_2C_2)$, $d(G_2T_2G_2C_2)$, $d(G_2A_2G_2A_2)$, $d(G_2C_2G_2C_2)$ and $d(G_2aG_2aG_2aG_2)$ Q4t G-quadruplexes (Fig. 6a–c and Supplementary Fig. 33). Analysis of intra-quadruplex and inter-quadruplex groove dimensions reveals that $d(G_2aG_2aG_2aG_2)$ Q4t exhibits the smallest size deviation with the greatest periodicity (Fig. 6c). Contrary, bigger deviations of intra- and inter-quadruplex groove dimensions are observed for $d(G_2G_2G_2A_2G_2)$, $d(G_2T_2G_2T_2)$, $d(G_2T_2A_2G_2)$, and $d(G_2C_2G_2C_2)$ Q4t. It seems that more hydrophobic loop residues tend to interact with each other within the structure, which affects the dimensions of the grooves and consequently reduces the flexibility and distorts the planarity of G-quartets and thus makes formation of G-wires more difficult. In addition, structural calculations show that T, A, and C residues can stack on the terminal 5’-quartets and thus interfere with further stacking (Fig. 6b–d and Supplementary Fig. 33). Incorporation of ab residues at only one position, i.e., 3 or 8 lead to formation of generally long G-wires as well (Fig. 5b, c).

Interestingly, oligonucleotides with li residues only at one position yield quite long G-wires as well, which can be attributed to reduced hydrophobic interactions of loop residues and stacking on terminal 5’-quartets compared to bulkier residues. Herein, using NMR we revealed the exact mechanism of how short, guanine-rich oligonucleotide self-assemble into G-wire at molecular level. The crucial step of this sophisticated mechanism includes structural rearrangement of kinetically favored G-quadruplex building block into thermodynamic one. Furthermore, we showed that properties of resulting G-wires, i.e., length and thermal stability can be tailored by changing the type and functionalization needed for specific applications. Our discoveries

![Image](image_url)
Fig. 5 C3 linker and abasic nucleotide in loops influence the length of G-wires even more. a Chemical structures of c3 linker (li) and abasic residue (ab). b A table showing $D_t$ obtained from DLS measurements and calculated effective lengths and number of stacked Qt-type building blocks. c In plot, oligonucleotides are arranged by increasing effective length of resulting G-wires (from left to right). For visualization in plot we used 92 and 91 nm as effective lengths of the longest two G-wires $d(G_2liG_4abG_2)$ and $d(G_2AG_4abG_2)$, whose lengths exceed the validity region of the used model. Samples were assembled via method 1 (see “Methods” section).

Fig. 6 Hydrophobicity of loop residues and their stacking ability might explain differences in lengths of G-wires. Q4t structures formed by a $d(G_2liG_4liG_2)$, b $d(G_2TG_4TG_2)$, c $d(G_2AG_4AG_2)$, d $d(G_2CG_4CG_2)$, and e $d(G_2abG_4abG_2)$. Plots in a–e show mean value (490 measurements for each groove) with corresponding dispersion presented with error bars, for each set of groove distance measurements. We used different colors for data in plots (a–e) for 1–4 Qt-type building blocks as well as for 3′-3′ and 5′-5′ stacking interfaces as presented in a $d(G_2liG_4liG_2)$ Q4t.
will have implication not only for DNA nanotechnology in the fields of bioinspired materials and medicine, but also provide deeper understanding of fundamental properties of G-quadruplex aggregates with biological significance.

Methods

Oligonucleotide synthesis and purification. All oligonucleotides (isotopically unlabeled and with residue-specific 10% 13C and 15N-labeled guanine residues as well as with incorporated c3 linkers (Spacer Phosphoramidite C3, Glen Research) and abasic residues (dSpacer CE Phosphoramidite, Glen Research)) were synthesized on K&A Laborgeraete GbR DNA/RNA Synthesizer H-8 in DMT-off mode using standard phosphoramidite chemistry. 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 ultrafiltration tubes at pH10, which was adjusted with the use of LiOH solution. Varian CARY-100 and CARY 3500 BIO UV–VIS spectrophotometers were used to measure absorption of desalted samples at 260 nm, which was used to calculate concentrations of stock solutions.

Sample preparation for NMR. NMR samples were prepared by dissolving desalted oligonucleotides in 350 μL of 90% H2O/10% D2O solution, in the presence of different concentrations of KCl. KPi buffer (pH 6.8) was added to final 10 mM concentration to samples used for NMR and UV melting experiments. Oligonucleotide concentration in the sample was 3–100 μM. Methods 1, 2, 3, and 4 were used with Q2k G-quadruplex used for D2O exchange experiment was prepared by lyophilization of previously prepared NMR sample folded via method 4 and subsequent dissolving in 100% D2O.

Method 1. This method can be used in order to check the ability of selected oligonucleotide for G-wire formation. Annealing in the presence of relatively high concentration of KCl leads to thermodynamically favored structures, G-wires. Method 1 consists of annealing of oligonucleotide in 100 mM KCl and (10 mM KPi buffer (pH 6.8)) for NMR and UV melting experiments. The annealing procedure includes heating the samples in water bath at 90 °C for 10 min, followed by slow cooling in water back to room temperature (~8 h).

Method 2. This method enables observation of G-quadruplexes, which are formed at the beginning of d(G2A3G2A) G-wire formation. Before addition of cations, sample solutions as well as KCl solution were cooled to 4 °C in order to decrease the rate of multimerization induced by addition of KCl. Different amounts of cooled KCl solution to achieve final 100, 80, 40, 20, 10, and 3 mM KCl concentrations were added to cooled solutions on which we monitored changes by NMR on a new sample. Therefore, to demonstrate the formation of next G-quadruplex in G-wire formation, i.e., Q2t. In order to increase KCl concentrations (3 upto 100 mM) and is always transformed into Q2k. Six days after addition of KCl, we prepared one sample at 25 °C and added KCl to kinetically governed G-quadruplexes (i.e., Q2k is forming slowly) over few days, which is needed to get high-quality NMR spectra immediately after sample preparation. At the same time, Q1k is stable and abasic residues (dSpacer CE Phosphoramidite, Glen Research)) were synthesized. Sample with Q2k was measured on the 4th day following addition of KCl. Meanwhile, sample was stored in the fridge (~4°C). To get DOSY of Q2k, we annealed the sample using annealing procedure described in “Method 1” section.

Phase diagram sample preparation. In order to get phase diagram, we have prepared five samples with different oligonucleotide concentration, i.e., 0.1, 0.25, 0.5, 1.0, and 2.0 mM oligonucleotide concentration. We added KCl to oligonucleotide solution to final 3 mM KCl concentration followed by annealing, using the same annealing procedure as described in “Method 1” section. We continued with titration of KCl to final 15, 40, 60, 80, 100, and 300 mM concentrations. At each KCl concentration we have acquired 1D 1H NMR spectra.

NMR spectroscopy. NMR experiments were recorded on Agilent Technologies DD2 600 MHz and VNMRS 800 MHz NMR spectrometers equipped with triple resonance/gradient probes or Bruker AVANCE NMR 600 MHz spectrometer equipped with quadruple-resonance cryogenic probe at 0 and 25 °C unless stated otherwise. The double-pulsed field gradient spin echo (DPFGE) and excitation sculpting (ES) pulse sequences were used for suppression of water signal. The translation diffusion coefficients were obtained on Bruker spectrometer with the use of stimulated echo sequence using bipolar gradients (STEBp). Guanine H1 protons were identified with the use of 1D 1H-edited heteronuclear single quantum correlation (HSQC) experiment on 13C and 15N site-specifically labeled samples. Guanine H8 protons were assigned with the use of 2D 13C-edited HSQC experiment on 13C and 15N site-specifically labeled samples. 2D Nuclear Overhauser Effect Spectroscopy (NOESY) with mixing times (τm) 150 and 250 ms were used for structural determination of Q1k, Q2k, and Q2t G-quadruplexes.

PAD experiments. We analyzed the effect of different KCl concentrations on G-quadruplex multimerization by monitoring changes with a set of 1D 1H NMR spectra acquired every 15 min (for 17 h) with the use of pre-acquisition delay (PAD) on VNMRS 800 MHz NMR spectrometer.

UV spectroscopy. UV melting experiments were performed on Varian Cary 3500 UV–VIS spectrophotometer with the Cary Win UV thermal program in 1.0 mm path-length cells on samples prepared via method 1 in 10 mM KPi buffer (pH 6.8). A blank sample containing only 100 mM KCl and 10 mM KPi buffer (pH 6.8) was used for baseline correction. The temperature was increased/decreased between 5 and 95 °C with the rate of 0.1 °C min−1. Absorbance was measured at 295 nm. Mineral oil and fixed cuvette caps were used to prevent evaporation of the samples at higher temperature. At lower temperature, we applied the stream of nitrogen. First derivative of A295 versus temperature plot was used for estimation of mid-transition temperatures (T1/2).

Circular dichroism (CD) spectroscopy. CD spectra were measured on Applied Photophysics Chirascan CD spectrometer at 25 °C from 200 to 320 nm. A blank sample containing only KCl (3, 15, 60, or 100 mM concentrations, depending on the sample type) was used for baseline correction. CD spectra were measured on 1.0 mM samples prepared via method 1, 3, 4, and 5 in 0.1 mm path-length quartz cells.

Native PAGE. A temperature controlled vertical electrophoretic apparatus and TBE buffer were used for native PAGE gel (15%) electrophoresis. The temperature of cooler was 5 °C for both gels. G-wire samples were deposited on gel at 0.25 mM concentration per strand whereas of Q1k, Q2t, and Q2t G-quadruplexes were deposited on gel at both 0.10 and 0.25 mM concentration per strand (1 and 2.5 nmol). Prior to loading, samples were diluted with blank solution containing the same concentration of KCl as used in individual sample. PAGE were run in the presence of 0 and 25 mM KCl in the gel as well as running buffer for G-quadruplex and G-wire samples, respectively. Prior to loading Ficol was added to the samples. We used Thermo Scientific GeneRuler Ultra Low Range DNA Ladder (from 300 to 10 bp) as standard for size. Electrophoresis with G-quadruplex and G-wire samples were run at 150 V for 2.5 h and 3 h, respectively. DNA was visualized by Stains-All (Sigma–Aldrich) staining.

Structure calculations. We calculated the structures of Q4t G-quadruplexes formed by d(G2A3G2A) oligonucleotide using the simulated annealing (SA) simulations based on NMR-derived restraints. The SA simulations were performed using CUDA version of pmemd module of AMBER 20 program suits and force field with restraints as described previously. Using the leap module of AMBER 20 program suits we generated the initial extended single-stranded DNA structure. We folded Q2t G-quadruplex using hydrogen bond restraints (force constant 10 kcal mol−1 Å−2) and NOE-derived distance restraints for 3′-3′ stacking interface (force constant 40 kcal mol−1 Å−2) and 40-Å distance restraints for 3′-3′ stacking interface (force constant 40 kcal mol−1 Å−2) from 2D NOESY spectra (τm 150 and 250 ms), restrained on sample for 600 ns in the leap method 5. An intensity of respective intra-residue NH H1 NOE cross-peaks, glycosidic torsion angles of all residues were restrained (force constant 80 kcal mol−1 Å−2) to region (200–280°). The
remaining torsion angles and phase angles of pseudorotation were not restrained. GQ G-quadruplex structure was built by 5’-5’ stacking of two Q2t G-quadruplexes and additional base-pairing required for the propensity model. The bases of Q4t were generated using the loop module of AMBER 20 program suits. To remove any potential clashes generated when manually building Q4t, the model underwent unrestrained energy minimization with 10,000 steps of steepest descent method. We calculated also the models for Q4t G-quadruplexes formed by d(G2A4G4), d(G2A2G4G4), and d(G2A2G4G4) oligonucleotides using the same restraints and folding procedure as described for d(GA4GA4) Q4t. Field parameters for c3 linker and abasic residues were derived from RESP ESP charge Derive (RED.S) Server. We calculated a total of 100 structures for all five Q4t models in 500 ps long NMR-restrained SA simulations using generalized Born implicit solvent model and random starting velocities. The cut-off for non-bonded interactions was 5000 Å and the SHAKE algorithm was used for hydrogen bonds.

**DLS measurements.** DLS measurements were performed using a frequency doubled Nd:YAG (532 nm) laser as the light source and a ALV-7002 Multiple tau digital real time correlator. Experiments were conducted at room temperature using samples with 1.0 mM oligonucleotide concentration. The experimental setup was fitted to the obtained intensity autocorrelation function $g_2(t)$. The slow diffusion mode (diffusion coefficient $D_{slow}$) was assigned to large, unclustered structures, whereas the fast diffusion mode (diffusion coefficient $D_{fast}$) was assigned to small, clustered structures.

**Sample preparation for AFM.** Sample solution for AFM imaging was prepared by diluting 1.0 mM d(GA4GA4) solution with 100 mM KCl to very low oligonucleotide concentrations (2 and 0.2 μM) before depositing on mica surface. Deposition of 2 μM d(GA4GA4) G-wires could not be accurately calculated since $L/d$ ratio is $>30$, which exceeds the range of theory validity. The number of stacked G-quadruplexes ($n$) was calculated based on average stacking distance of 0.34 nm and our G-quadruplexes folding model. Furthermore, it should be noted that DLS signal favors larger species as the intensity of scattered light scales with the square of the scattering volume.

**Sample preparation for SEM and TEM.** Sample concentration used for electron microscopy imaging was the same as for NMR and DLS experiments. G-wire samples were deposited on graphene oxide on lacey carbon 300 mesh copper TEM grids (Structure Probe, Inc., PA, USA). We deposited 10 μL of the sample (1.0 mM oligonucleotide and 100 mM KCl concentrations) on the TEM grid, waited 1 min for adsorption and removed the excess with lint free lens tissues. We washed the sample by depositing 10 μL miliQ water, waiting 1 min and removing the excess with lint free lens tissues. The washing procedure was repeated five times.

**Electron microscopy imaging.** SEM was carried out using a Zeiss Supra TM 35 VP (Carl Zeiss, Oberkochen, Germany) scanning electron microscope. The operational voltage was set to 0.7 kV with 3.5 nm working distance, 30 μm aperture size and a secondary electron detector.

**Data availability**

All relevant data supporting the findings of this study are available in the Supplementary Information. Any additional data are available from the corresponding author upon request.

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