Electronic Supplementary Information (ESI) for:

**π-π Stacked DNA G-Wires Nanostructures Formed by a Short G-Rich Oligonucleotide Containing a 3'-3' Inversion of Polarity Site**

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**ODN Synthesis and Purifications.** The G4-forming ODN sequences d(TGGGGT) and d(CGGTGTT), used for the obtainment of the G4 length markers, were chemically synthesized on an Expedite 8909 DNA synthesizer (PerSeptive Biosystems, Framingham MA, US) using a universal CPG support UnySupport 500, purchased from Glen Research (Sterling VA, US). The syntheses were performed adopting the standard β-cyanoethyl phosphoramidite chemistry at 1-2 µM scale, and the products were purified as described below. The synthesis of the ODN 2 d(5'-CGG-3'-3'-GGC-5') was also performed by standard procedure. The inversion of polarity site within the sequence was obtained by initially assembling the first 5'-CGG-3' tract using 5'-phosphoramidites and then the 3'-GGC-5' tract using standard 3'-phosphoramidites. The combined filtrates and washings were concentrated under reduced pressure and purified on a Jasco HPLC system equipped with a PU2089 Plus quaternary pump and a 2075 Plus UV detector (Jasco Europe S.r.l., Cremella, Italy) using an anion exchange column (1000-8/46, 4.4×50 mm, 5 µm; Macherey-Nagel, Düren, Germany) eluted with a linear gradient from 0 to 100% B in 30 min, flow rate = 1 mL/min and detected at 260 nm (buffer A: 20 mM NaH₂PO₄ aq. solution pH 7.0, containing 20% (v/v) CH₃CN; buffer B: 20 mM NaH₂PO₄ aq. solution pH 7.0, containing 1 M NaCl and 20% (v/v) CH₃CN). The purified ODNs were desalted by molecular exclusion chromatography on Biogel P2 fine (Biorad, Milano, Italy) eluted with 20% aq. ethanol and then lyophilized. The chemical identity of 2 was confirmed by ESI-MS and ¹H NMR analyses. ESI-MS (m/z) calcd. for [M – 2H]²⁻ 915.2, found 915.0 (Figure S8). ¹H NMR (700 MHz, D₂O, 50 °C, Figure S9), selected signals in the aromatic and anomeric regions at δ: 8.00 (s, 2H, 2×G H8), 7.81 (s, 2H, 2×G H8), 7.52 (d, ²J = 7.2 Hz, 2H, 2×C H6), 6.06 (m, 4H, 4×H1'), 5.92 (d, ²J = 7.2 Hz, 2H, 2×C H5), 5.87 (m, 2H, 2×H1').

**Annealing Procedure.** The ODNs concentrations were determined spectrophotometrically at λ = 260 nm at 90 °C, using molar extinction coefficient ε = 54,400 M⁻¹ cm⁻¹ for 5'-CGGGGCG-3', as determined using the Sigma-Aldrich OligoEvaluator™ web tool.
(www.oligoevaluator.com). The 0.05, 1.0 and 3.6 mM solutions of 2 were obtained by dissolving the lyophilized sample in ultrapure water containing 90 mM KCl and 10 mM KH$_2$PO$_4$ (100 mM K$^+$ buffer) or 900 mM KCl and 100 mM KH$_2$PO$_4$ (1.0 M K$^+$ buffer). The pH was adjusted at 7.0 using HCl/KOH. For ESI-MS experiments, the lyophilized samples were redissolved in 150 mM NH$_4$OAc, and the pH was adjusted to 7.0 using AcOH/NH$_4$OH. Samples were annealed by heating at 90 °C for 10 minutes and then rapid cooling and storage at 4 °C for at least 24 h before further studies.

**Non-denaturing Polyacrylamide Gel Electrophoresis (PAGE).** Native gel electrophoresis experiments were performed on 15% polyacrylamide gels containing TBE 1x (8.9 mM Tris, 8.9 mM borate, 0.2 mM EDTA; Bio-Rad Laboratories, Hercules CA, US) and 30 mM KCl, pH 7.0 at a constant voltage of 130 V for 2 h at 5 °C. The ODN samples, annealed at 1.0 and 0.05 mM ss concentration in 1.0 M K$^+$ buffer, were diluted at 0.6 mM loading concentration just before PAGE runs. Glycerol was added (10% final) to facilitate sample loading in the wells. The gels were finally stained with SYBR Green (Sigma-Aldrich, St. Louis MO, US) and visualized using the Bio-Rad Laboratories Gel Doc™ XR apparatus.

**HPLC-Size Exclusion Chromatography (SEC) analyses.** HPLC-SEC analyses and purifications were performed on a Phenomenex Yarra SEC-2000 column (Phenomenex, Torrance CA, US) operating in the MW range of 1,000-300,000 Dalton (300 × 7.8 mm, 3 µm) eluted with 90 mM KCl and 10 mM KH$_2$PO$_4$/CH$_3$CN (80:20, v/v), flow rate 0.6 mL min$^{-1}$, detector at λ = 260 nm. The analyses were performed at room temperature using a Jasco PU-2089 Plus quaternary gradient pump equipped with a Jasco UV-2075 Plus UV/VIS detector.

**Circular Dichroism (CD).** CD spectra and CD melting profiles were recorded on samples annealed in 100 mM and 1.0 M K$^+$ buffers, prepared as described above, at the 20 µM final single strand concentration using a Jasco 1500 spectropolarimeter equipped with a Jasco PTC-348-WI temperature controller (λ = 200–320 nm range, 0.1 cm path-length cuvette).
The CD data obtained from samples annealed in 1.0 M K+ were almost superimposable to those obtained in the weaker buffer and are not shown here. CD spectra were averaged over three scans recorded at 100 nm min⁻¹ scanning speed, 4 s response time, 2 nm bandwidth at 5 °C. The buffer baseline was subtracted from each spectrum, and the spectra were normalized to have zero ellipticity at 320 nm. The CD spectra reported in Figure S4 were recorded by collecting the eluate of peaks corresponding to Q₁, Q₂, Q₃ and Q₄–n (Figure 4B) obtained from a single injection of 1.0 mM 2 annealed in 1.0 M K⁺ buffer. CD melting curves were registered at λ = 253 nm at the 0.5 °C min⁻¹ heating rate in the temperature range 5–85 °C.

**Nuclear Magnetic Resonance (NMR).** 1H NMR spectra were recorded on Bruker Avance Neo 400/700 MHz instruments (Bruker-Biospin, Billerica, US) and processed using the Mnova NMR software package (Mestrelab Research, Santiago de Compostela, Spain). The spectra were acquired as 32,768 data points with a recycle delay of 1.0 s at 25, 45, 65 and 85 °C and were apodized with a shifted sine bell squared window function. Water suppression was achieved by excitation sculpting using the Bruker ZGESGP pulse sequence. 1 NMR samples were prepared at the concentration of 1.6 mM single strand in 200 μL of H₂O/D₂O 9:1 containing 900 mM KCl and 100 mM KH₂PO₄.

**Atomic Force Microscopy (AFM).** The AFM imaging of Qₙ G-wires was performed by using a Park Systems XE-100 instrument (Park Systems, Suwon, South Korea). NCM amplitude and phase were obtained using 125 μm long silicon/aluminium-coated cantilevers (PPP-NCHR 10M; Park Systems; tip radius lower than 10 nm), with a nominal force constant of 42 N/m and a resonance frequency of 200 to 400 kHz. The scan frequency of the 512×512 pixels images was typically 0.5 Hz per line. When necessary, the AFM images were processed by flattening to remove the background slope, and the contrast and brightness were adjusted. As the AFM substrate (about 1 cm² surface), we used the muscovite mica because of its large atomically flat areas obtained by the perfect cleavage along a <001>
plan. Moreover, after cleavage, the K$^+$ ions bonding the mica layers are highly mobile resulting in a positive overcharging of the mica surface, which enables the deposition of molecules that hold a net negative charge, such as DNA$_2$ and a super-hydrophilicity that provides a low interaction between suspended biomolecules during the evaporation of the solvent. Mica was freshly cleaved using adhesive tape before each deposition to establish its cleanliness. 2 µl aliquots of the DNA/imaging buffer were directly cast onto early-cleaved muscovite mica, gently washed with deionized water and then dried at room temperature under a ventilated fume hood.

**Electrospray Mass Spectrometry (ESI-MS).** An API4000 triple quadrupole LC/MS-MS mass spectrometer (AB-SCIEX, Concord, ON, Canada) was used to confirm the structure of the single-stranded 2, as well as the strand molecularity of the shorter members of the G4 multimers. The analysis of the single-stranded 2 was performed by dissolving the desalted lyophilized sample in ultrapure water at 1.0 mM single strand concentration. To analyse the G4 species, the lyophilized sample was dissolved in 150 mM NH$_4$OAc, and 60 % methanol was added after the annealing. All samples were infused at 15 µL/min in the electrospray (ESI) source that was operated in the negative ions Enhanced Resolution (ER) mode (voltage –3,3 kV, 80 °C, GS1 20 psi, GS2 30 psi, scan time 0.5 sec).
References


Figure S1. Formation and structures of the size markers $Q_1^*$ and $Q_2^*$ formed respectively by the single strands $d(TGGGGT)$ and $d(CGTTGGT)$. 
Figure S2. (top) Enhanced resolution ESI-MS spectrum of ODN 2 recorded in the negative ion mode. (bottom) Closeup showing the isotopic distribution of peaks belonging to the [M – 2H]²⁻ pseudomolecular ion. Calculated 915.2, found 915.0.
**Figure S3.** Aromatic and anomeric protons region of the 1H NMR spectra recorded at 25 and 50 °C of ODN 2 dissolved in D₂O.

**Figure S4.** Enhanced resolution ESI-MS spectrum of ODN 2 (negative mode) annealed in 150 mM ammonium acetate buffer (1.0 mM ss, pH 7.0).
Figure S5: Purification by HPLC-SEC of Q₁–₃ isolated from Qₙ multimers formed by 2 (panel A) and control of their stability/purity after 30 min (left panels) and after 24 h (right panels) at room temperature.
Figure S6. HPLC-SEC profiles of Qₙ multimers from ODN 1 (A, left panels) and Qₙ multimers from ODN 2 (B, right panels) after heating at 25, 45, 65 and 85 °C.
Figure S7. CD denaturation profile of the G-wires distribution $Q_n$ obtained by annealing 2 in 100 mM K$^+$ buffer.
Figure S8. Imino, aromatic and anomeric protons region of 1H NMR spectra recorded at 25, 45, 65 and 85 °C of the Qn distribution obtained by annealing 2 in 1.0 M K+ buffer (1.5 mM ss concentration).
Figure S9. AFM Statistical analysis of Qₙ multimers formed by ODN 2. The distribution of height, width and length is reported in the table.

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<th>Length(nm)</th>
<th>wideness(nm)</th>
<th>highness(nm)</th>
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<td>Mean</td>
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<td>21,21</td>
<td>1,76</td>
</tr>
<tr>
<td>Std.</td>
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