X-ray characterization of mesophases of human telomeric G-quadruplexes and other DNA analogues

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Observed in the folds of guanine-rich oligonucleotides, non-canonical G-quadruplex structures are based on G-quartets formed by hydrogen bonding and cation-coordination of guanosines. In dilute 5′-guanosine monophosphate (GMP) solutions, G-quartets form by the self-assembly of four GMP nucleotides. We use x-ray diffraction to characterize the columnar liquid-crystalline mesophases in concentrated solutions of various model G-quadruplexes. We then probe the transitions between mesophases by varying the PEG solution osmotic pressure, thus mimicking in vivo molecular crowding conditions. Using the GMP-quadruplex, built by the stacking of G-quartets with no covalent linking between them, as the baseline, we report the liquid-crystalline phase behaviors of two other related G-quadruplexes: (i) the intramolecular parallel-stranded G-quadruplex formed by the 22-mer four-repeat human telomeric sequence AG3(TTAG3)n and (ii) the intermolecular parallel-stranded G-quadruplex formed by the TG4T oligonucleotides. Finally, we compare the mesophases of the G-quadruplexes, under PEG-induced crowding conditions, with the corresponding mesophases of the canonical duplex and triplex DNA analogues.

Human telomeric sequences AG3(TTAG3)n can form four-stranded G-quadruplexes1 by folding on themselves and matching the G3 segments, enabling the formation of G-quartets2. Polymorphic G-quadruplex structures have been implicated in several biological processes, such as telomere formation in aging and in disease development3. In particular, G-quadruplex conformations of the four-repeat human telomere AG3(TTAG3)n, in the presence of K+ ions have been an important research focus4-6. The parallel-stranded conformation is observed in the crystalline state3 and in K+ solutions6,7 in the presence of polyethylene glycol (PEG). G-quadruplexes formed by the four-repeat human telomere have been shown4 to be thermally more stable than the structures formed by the longer telomeric sequences, implying that the four-repeat telomere is the likely candidate for G-quadruplex formation in human cells8. Here, the parallel-stranded intramolecular G-quadruplex formed by AG3(TTAG3)n will be referred to as the 22-mer HT-quadruplex. This quadruplex9 (PDB: 1KF1) contains a central core formed by the stacking of three G-quartets supported by four parallel sugar-phosphate strands and TTA linkers that connect the adjacent strands by forming side-loops (Fig. 1a,b). Mimicking molecular crowding conditions in vivo, that critically affect the structure of G-quadruplexes10, by systematically varying the osmotic pressure exerted by the

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bathing PEG solutions and combining it with the x-ray structural probe in an osmotic stress experiment\textsuperscript{11}, we examine the liquid-crystalline phase behavior of the 22-mer HT-quadruplex in order to assess its likely state under similar conditions in the cellular environment.

The G-quartet is a planar structure formed by the cyclic arrangement of four hydrogen-bonded guanine residues. We first examine the liquid-crystalline phases of the G-quartets that are made of four GMP monomers (Fig. 1c). The columnar structure built by the stacking of such G-quartets (abbreviated GMP-quadruplex) is a simple model of biologically relevant G-quadruplexes. Helical stacking of G-quartets (Fig. 1d) in the crystal-line state was proposed based on fiber diffraction\textsuperscript{13}. Formation of columnar structures was detected also in the non-crystalline GMP solutions in the presence of K\textsuperscript{+} ions\textsuperscript{14}. By varying the osmotic pressure of the solution set by the concentration of PEG, we investigate the first-order transition from a loosely linked and disordered G-quartet column formed in K\textsuperscript{+} solution to the highly-ordered GMP-quadruplex driven by the osmotic pressure changes in the regime mimicking the biologically relevant molecular crowding conditions. This osmotic-pressure-induced change in order along the columnar axis consequently permits tighter packing of the GMP-quadruplex array between the columns, with progressively longer-ranged hexagonal order in the plane perpendicular to the columnar axis.

The columnar liquid-crystalline mesophases of planar disc-shaped structures are known and understood\textsuperscript{15}. In these phases, disc-shaped structures are stacked on top of each other and form columns, which in turn self-assemble into arrays. As with other columnar assemblies\textsuperscript{16}, the nature of intra- and intercolumnar ordering can vary, depending on solution conditions. The disordered columnar phases (\(\Phi_{dc}\)) exhibit fluid-like positional intracolumnar order, while in the ordered columnar phases (\(\Phi_{oc}\)) there is long-range positional order within each column. This leads to column–column positional and orientational correlations and consequently to long-range intercolumnar order\textsuperscript{16,17}. In this respect, the mesophase transition of GMP-quadruplex is similar to the \(\Phi_{dc} \rightarrow \Phi_{oc}\) transitions observed in the columnar aggregates of other disc-shaped structures built from the molecules with aromatic rings\textsuperscript{13,17,18}.

Recent experiments demonstrated that the formation of higher-order G-quadruplex motifs in the human telomere is sensitive to the phase in the cell-cycle\textsuperscript{9}, which, on the other hand, is related to macromolecular crowding, that can fine-tune the gene circuit response\textsuperscript{19}. Thus, the \(\Phi_{dc} \rightarrow \Phi_{oc}\) transition of the GMP-quadruplex induced by changing molecular-crowding conditions is relevant for the higher-order G-quadruplex structure formation built from the 22-mer HT-quadruplex repeats\textsuperscript{5,8}. In this view, we also examined the columnar assemblies and the mesophase behaviors of (i) the 22-mer HT-quadruplex and (ii) the intermolecular parallel-stranded G-quadruplex

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**Figure 1. Schematic representations of the structures.** (a) Top view of the parallel-stranded 22-mer HT-quadruplex (PDB: 1KF1) formed by the sequence AG\textsubscript{3}(TTAG\textsubscript{3})\textsubscript{3}. In this conformation (observed in the crystalline state\textsuperscript{5} as well as in the non-crystalline state under molecular crowding conditions\textsuperscript{6,7}), four parallel GGG runs form a stack of three planar G-quartets in the central core and TTA segments fold into loops projecting outwards. (b) Side view of the 22-mer HT-quadruplex. K\textsuperscript{+} ions (purple) reside inside the G-quadruplexes and they are positioned between the adjacent G-quartets. (c) G-quartet made of four GMP monomers. Thin black lines represent the hydrogen bonds holding nucleotides together in a planar cyclic arrangement. (d) Top view of the GMP-quadruplex in ordered columnar phase, where stacking is helical with an azimuthal rotation of 30° between adjacent G-quartets\textsuperscript{13}. G-quartets in c & d are modeled from 22-mer HT-quadruplex (PDB: 1KF1) shown in a & b.
formed by four TG₄T oligonucleotides²⁰ (PDB:244D). The latter (abbreviated TG₄T-quadruplex) is shown in Fig. 2a. This quadruplex was selected because of its resemblance to the 22-mer HT-quadruplex. In both structures, parallel sugar-phosphate backbones interconnect the stacked G-quartets. Contrary to the 22-mer HT-quadruplex, the TG₄T-quadruplex is also missing the flexible side loops.

The Φₑ − Φₑ transition of the GMP-quadruplex is also analogous to the mesophase transitions in duplex (Fig. 2b) and triplex (Fig. 2c) DNA arrays under similar crowding conditions. For duplexes, this transition is described as the cholesteric–columnar hexagonal transition for short DNA²¹ and as the cholesteric–hexatic transition for long DNA²²,²³. While the columnar hexagonal phase of the DNA triplexes has been observed before²⁴, the triplex mesophase transition was not measured. The changes observed in the x-ray diffraction patterns at the mesophase transitions of the duplex, triplex, and G-quadruplex are essentially similar. At these transitions, both the intra- and the intercolumnar order change significantly and abruptly. We discuss the mesophase transitions of the analogue DNA structures with or without sugar-phosphate backbones and with different numbers of bases contributing to the stacking units (i.e., base-pair for duplex, base-triplet for triplex, and G-quartet for G-quadruplex). We then assess the relevance of these transitions for the observed mesophase behaviors.

Finally, 22-mer HT-quadruplex has a propeller-like shape³ though not as pronounced as when 22-mer HT-quadruplex blocks are stacked in a column with TTA linkers between them¹⁵. In this particular model for the columnar stacking of G-quadruplex blocks (that are formed by four-repeat telomeric sequences), TTA segments connect the consecutive blocks that in turn would form an ordered G-quadruplex column with quasi-continuous helical characteristics. Here, we investigate the columnar mesophase behavior of 22-mer HT-quadruplex blocks when they are unlinked. These are similar to the unlinked very-short duplex DNA fragments²⁵, except that the strong stacking interactions between the exposed hydrophobic cores of the duplex DNA fragments²⁶ are missing in the 22-mer HT-quadruplex case. If anything, because of the relatively weak end-to-end stacking interactions, the 22-mer HT-quadruplex blocks could be more prone to repel each other then to attract. This would force the columns of 22-mer HT-quadruplex blocks to be highly disordered.

Results

Under sufficient molecular crowding, as mimicked by the PEG-induced osmotic pressure (see Methods), duplex and quadruplex (GMP-quadruplex, TG₄T-quadruplex, and 22-mer HT-quadruplex) DNA structures self-assemble into stable aggregates in the presence of K⁺ ions without any other ions being added. These aggregates are transferred into PEG solutions of various concentrations for equilibration against known external osmotic pressures. PEG (molecular weight 8000 Daltons) is excluded from the DNA arrays during equilibration. Temperature-dependent osmotic pressures produced by the solutions of PEG at various concentrations are from ref. 11. Unless otherwise stated, all the measurements are at [KCl] = 0.3 M.

The osmotic pressures required for inducing DNA-analogue mesophase transitions strongly depend on solution ionic conditions. At [K⁺] = 0.3 M, in the absence of any multivalent salts, the transition osmotic pressures for duplex and GMP-quadruplex are about the same, varying nearly from 6 to 8 atm (corresponding to from ≈19 to ≈22 wt% PEG 8000 concentration at 20°C). Increasing K⁺ concentration (under fixed external pressure) results in compression in the arrays of duplexes and G-quadruplexes. This observation can be explained as the screening of the electrostatic interactions between intercolumnar phosphate charges.
In this section, we describe and present the experimental data systematically, by giving the priority to G-quadruplex DNA structures (GMP-quadruplex, TG₄₄₄T-quadruplex, and 22-mer HT-quadruplex) and by using the duplex and triplex DNA data as additional information. The osmotic-pressure induced mesophase transitions for duplex DNA in the presence of monovalent NaCl has been observed recently²², while in this paper we additionally present the duplex DNA mesophase transition data in the presence of monovalent KCl. Furthermore, the triplex mesophase transition was not measured elsewhere. Although the main focus of this work is to present and discuss the mesophase behavior of the G-quadruplex structures, we also present duplex DNA data (in the presence of KCl) and the triplex mesophase transition that was measured the for the first time. We then compare the observed G-quadruplex DNA mesophases with duplex and triplex DNA mesophases. We used “long” wild-type DNA (~1 micron long) in the duplex DNA measurements. See below for details on the triplexes.

GMP-quadruplex. Increasing order continuously with increasing osmotic compression in the disordered columnar mesophase (Φₐc) is followed by a sudden collapse into the ordered columnar mesophase (Φₒc) with remarkable changes in the intercolumnar distance (Δdₒc) and the radial order (Fig. 3b). At the Φₐc → Φₒc transition of the GMP-quadruplex, in particular, the change in the packing density corresponds to about 0.3–0.4 nm³ volume change per GMP nucleotide. It occurs concurrently with a significant lowering of the packing disorder. The radial disorder in the Φₐc phase, due to lateral displacements of loosely stacked G-quartets around the mean columnar axis, is evident in the Gaussian-shaped broad diffraction radial intensity peaks. By comparison, the Lorentzian peak shape in the Φₒc phase attests to the long-range nature of positional order. The correlation length in the ordered phase (equal to the inverse of the full-width-at-half-maximum of a Lorentzian function fitted to the x-ray diffraction radial intensity peak) is 5-to-6 neighbor separations for duplex DNA and 9-to-10 neighbor separations for GMP-quadruplex.

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The slight temperature sensitivity of the Φₐc → Φₒc transition is shown for GMP-quadruplex (open circles in Fig. 4a). Temperature has no detectable effect on the packing density in the Φₒc phase. The transition osmotic
pressure ($\Pi_\ell$) increases by about 1 atm upon increasing temperature from 20 to 40 °C. The effects of temperature on $\Pi_\ell$ (as well as the effect of temperature on the DNA density at a fixed osmotic pressure $\Pi_\ell$) are appreciably smaller for other DNA structures and not shown. The transition for the GMP-quadruplex system is fairly sharp, and the integrated diffraction intensity (area under the diffraction peak) does not change through the transition. The integrated diffraction intensities from the same sample in the phase-coexistence region (Fig. 3) are nearly the same ($\Phi_{dc}$ and $\Phi_{oc}$ phases are nearly the same (Fig. 3b)). Phase-coexistence is pronounced over a significantly narrower range of osmotic pressures in the transitions of the duplex and GMP-quadruplex than in the transitions of the other structures. In particular, for GMP-quadruplex, the width of the phase-coexistence region is about 0.5 atm osmotic pressure.

**TG$_a$T-quadruplex.** When we followed the same procedure with the intermolecular TG$_a$T-quadruplex, we observed first-order transitions similar to the $\Phi_{dc} \rightarrow \Phi_{oc}$ transition of the GMP-quadruplex. This suggests an abrupt change in the columnar organization of the short intermolecular TG$_a$T-quadruplex, similar to the change in the columnar organization of the G-quartets at the $\Phi_{dc} \rightarrow \Phi_{oc}$ transition of the GMP-quadruplex. When this change in order is tuned by varying the osmotic pressure, we observe a much broader coexistence-region for the TG$_a$T-quadruplex than for the GMP-quadruplex. For clarity, in Fig. 4a we show the intercolumnar spacings for TG$_a$T-quadruplex only at the upper and lower boundaries of the coexistence-region (inverted triangles). Intercolumnar spacings are about the same in the $\Phi_{oc}$ phases of the TG$_a$T-quadruplex and GMP-quadruplex arrays under the same pressure.

**22-mer HT-quadruplex.** With the aim of obtaining ordered columnar phases of the 22-mer HT-quadruplex arrays (similar to the $\Phi_{oc}$ phase of simpler model GMP-quadruplex), we increased the osmotic pressure compressing the disordered arrays of 22-mer HT-quadruplex and measured their x-ray diffraction patterns. The radial packing order increases with increasing osmotic compression. However, the very sharp changes observed in the radial intensity profiles at the first-order $\Phi_{dc} \rightarrow \Phi_{oc}$ transition of the GMP-quadruplex are not seen in the radial intensity profiles of 22-mer HT-quadruplex arrays (Fig. 3e).

When equilibrated under osmotic pressures less than the $\Phi_{dc} \rightarrow \Phi_{oc}$ transition osmotic pressure of the GMP-quadruplex, the measured positional disorder in 22-mer HT-quadruplex array is smaller than the disorder in GMP-quadruplex array at the same pressure (Fig. 5), which can be argued to be a consequence of the constrained lateral motion of the G-quartets, connected by the sugar-phosphate backbone. This connectivity increases the stability of 22-mer HT-quadruplex relative to the GMP-quadruplex and decreases its positional as well as stacking disorder. This is obviously true despite the TTA loops of the 22-mer HT-quadruplex that extend laterally from the core G-quartets, and could be conceived as promoting and not suppressing the disorder of the columns. However, when equilibrated under osmotic pressures greater than that of the $\Phi_{dc} \rightarrow \Phi_{oc}$ transition of GMP-quadruplex, $d_{int}$ is 2–3 Å bigger for 22-mer HT-quadruplex than for GMP-quadruplex at the same pressure (Fig. 4a). Under these conditions, the radial disorder in the 22-mer HT-quadruplex array remains almost the same as in the disordered phase, while that of the GMP-quadruplex array drops significantly (Fig. 5, blue circles), possibly signaling the reverse action of laterally extended TTA loops in this case, amounting to a simple increase of the effective diameter of the columns.
changes in the length (\(P\)), are from refs 13, 26 and 32. The distances \(d_{\text{int}}\) are measured in 0.3 M K\(^+\) Poly DNA once the osmotic pressure. Thus, the reduction in the packing order in duplex and GMP-quadruplex for duplex and GMP-quadruplex compared with the TG\(_2\)T-quadruplex. The error in the determination of FWHM is as big as \(\approx 0.1\) nm\(^{-1}\) in the case of broad Gaussian peaks (in the disordered phases). The errors in FWHM are smaller for the sharp Lorentzian peaks in the ordered phases (see SI Appendix).

Table 1. Quantitative information regarding the structure and the \(\Phi_{\text{dc}} \rightarrow \Phi_{\text{oc}}\) transitions of duplex, Poly(AT\(^+\)T)-triplax, and GMP-quadruplex DNA. The structural parameters, radius (\(a\)) and helical pitch length (\(P\)), are from refs 13, 26 and 32. The distances \(d_{\text{int}}\) are measured in 0.3 M K\(^+\) solutions for duplex and GMP-quadruplex. These distances for duplex and GMP-quadruplex decrease without a significant change in \(d_{\text{int}}\) with increasing K\(^+\) concentration. The Poly(AT\(^+\)T)-triplax measurements are carried out in the presence of 0.3 M K\(^+\) and 5 mM Mg\(^{2+}\). Lowering Mg\(^{2+}\) concentration any further (less than 5 mM), while keeping the K\(^+\) concentration fixed at 0.3 M, results in the disassociation of the triplexes (see SI Appendix). \(A_{\text{cell}}\) is the hexagonal cross-sectional area surrounding the duplex, triplex, or GMP-quadruplex. \(A_{\text{cell}}\) is the change in the Wigner-Seitz cell area at the transition. \(\Delta V_{\text{pn}}\) is the change in the volume per nucleotide at the transition. The change in the volume per stacking unit (i.e., base-pair for duplex, base-pair for triplex, and G-quartet for GMP-quadruplex) is equal to \(\Delta A_{\text{cell}}\) multiplied by the base-stacking height (see SI Appendix). The overall uncertainty in the determination of \(\Delta V_{\text{pn}}\) is about 10%.

### Poly(AT\(^+\)T)-triplax

Poly(AT\(^+\)T)-triplax samples are prepared in the presence of 5 mM Mg\(^{2+}\). The role of Mg\(^{2+}\) in the stability of DNA triplexes has been investigated\(^{24}\), and stable Poly(AT\(^+\)T)-triplax at [Mg\(^{2+}\)] = 5 mM is reported. Under sufficient osmotic pressures in presence of 5 mM Mg\(^{2+}\), Poly(AT\(^+\)T)-triplax self-assemble into columnar aggregates (see Methods). Addition of 0.3 M K\(^+\), while keeping [Mg\(^{2+}\)] = 5 mM, causes the following changes in the Poly(AT\(^+\)T)-triplax arrays (under fixed pressure): (i) in the \(\Phi_{\text{dc}}\) phase, expansion in the lateral direction (see SI Appendix) and (ii) in the \(\Phi_{\text{oc}}\) phase, destabilization of the Poly(AT\(^+\)T)-triplax. The osmotic pressure required to prevent the destabilization of the Poly(AT\(^+\)T)-triplax depends on the concentrations of K\(^+\) and Mg\(^{2+}\). At the \(\Phi_{\text{dc}} \rightarrow \Phi_{\text{oc}}\) mesophase transition of the Poly(AT\(^+\)T)-triplax (in the presence of 0.3 M K\(^+\) and 5 mM Mg\(^{2+}\)) \(d_{\text{int}}\) changes from about 37 Å to 33 Å (Fig. 4a). The changes were reversible for \(d_{\text{int}}\) less than \(\approx 38\) Å. At larger spacings, once the Poly(AT\(^+\)T)-triplax dissociate, the triplex formation cannot be reestablished by simply increasing the osmotic pressure. Thus, the \(d_{\text{int}}\) values for the Poly(AT\(^+\)T)-triplax that we report in Table 1 are near the biggest distances where the transition can be observed at [K\(^+\)] = 0.3 M.

### Discussion

Fiber diffraction data provide substantive evidence of helical stacking of the G-quartet\(^{13}\) in GMP-quadruplex, as well as helical stacking of the base-pairs\(^{8}\) in duplex DNA, at relative humidities corresponding to the osmotic pressures produced by the bathing PEG solutions that induced \(\Phi_{\text{oc}}\) phases of these structures. These helical details are expected to be pronounced only in the presence of strong correlations between repeating units (base-pair for duplex and G-quartet for GMP-quadruplex) along the columnar axes. Based on this, one can argue that the helical nature of the base-stacking in DNA structures plays a key role in the \(\Phi_{\text{dc}} \rightarrow \Phi_{\text{oc}}\) transitions of the DNA arrays. Transitions occur when the intercolumnar spacings (\(d_{\text{int}}\)) are comparable to the helical pitch length (\(P\)), i.e., the axial distance per helical turn along the columnar axis (see Table 1). This implies that the formation of long-range translational and helical order along the columnar axis leads to long-range intercolumnar order in the structures.
the $\Phi_n\rightarrow\Phi_\infty$ phase. Additionally, the strength of the attraction, as seen in the volume change per nucleotide ($\Delta V_{nt}$) in Table 1, increases with increasing number of strands in the structure. Counterintuitively, the attraction induced at the transition increases with increasing linear or surface charge density ($\lambda$ and $\sigma$, respectively). This scenario has been elaborated$^{22,24}$ theoretically but is difficult to corroborate experimentally.

We examined the $\Phi_n\rightarrow\Phi_\infty$ mesophase transitions of duplex, triplex, and quadruplex DNA structures. In particular, the spontaneous formations of highly-ordered G-quadruplex columns (GMP-quadruplex and TG$_4$T-quadruplex) under biologically relevant molecular crowding conditions are significant for their analogies with the stacking organization of G-quadruplex structures in the human telomere. A quasi-continuous helix that runs along the columnar axis by the stacking of G-quartets within the TG$_4$T-quadruplex columns$^{26}$ (similar to the helical stacking of the G-quartets in GMP-quadruplex) is possible with azimuthal rotations and arrangements of the TG$_4$T-quadruplex blocks relative to the adjacent blocks.

The formation of uniaxially ordered columnar liquid crystals has been observed also in the case of the stacking of very-short-fragment (6 base-pairs long) DNA duplexes$^{25}$. This stacking behavior was explained by the end-to-end adhesion of the exposed hydrophobic cores of the short DNA segments. From the observed $\Phi_n\rightarrow\Phi_\infty$ first-order mesophase transition of the TG$_4$T-quadruplex arrays, as seen in the discontinuous change in the x-ray diffraction patterns tuned by varying molecular crowding conditions, it is possible to argue that the high-planarity of the G-quartets in the TG$_4$T-quadruplex blocks makes the end-to-end stacking favorable. However, one could claim that the presence of the thymine bases at the ends of TG$_4$T-quadruplex blocks would likely weaken the stacking interactions and in turn make the uniaxial helical ordering unfavorable. The broad range of osmotic pressures where the phase coexistence is observed in TG$_4$T-quadruplex arrays (Fig. 5) might be attributed to the increased disorder due to the thymine bases at the ends.

In order to investigate the formation of ordered G-quadruplex columns in the telomere, the 22-mer HT-quadruplex was specifically chosen for two reasons: (i) the four-repeat sequence AG$_4$(TTAG)$_4$ is the likely candidate for the G-quadruplex formation in $\textit{in vivo}$ and (ii) G-quadruplex conformation of this sequence under molecular crowding conditions is known. However, to the best of our knowledge, there is no direct evidence that the 22-mer HT-quadruplex blocks would be organized into a columnar assembly in the non-crystalline state. Our osmotic stress experiments were designed to detect any sudden changes in the packing density and order, similar to the observed first-order mesophase transitions of the other model G-quadruplexes (GMP-quadruplex and TG$_4$T-quadruplex). However, at all osmotic pressures 22-mer HT-quadruplex blocks (when they are unlinked) were observed to make only disordered columns, characterized by almost unchanged radial disorder (Fig. 5), implying also a pronounced stacking disorder. At large osmotic pressures it appears as if the disordered columns would have an effective diameter augmented by the contribution of the dangling TTA loops, while at small osmotic pressures the phosphate backbone connectivity of the 22-mer HT-quadruplex blocks enhances their stability. The robust disordered columnar assembly would possibly be the outcome of the attenuated stacking interactions between the G-quartet cores of the 22-mer HT-quadruplex blocks, compared with the very pronounced stacking interactions within the highly-ordered columnar phases of the GMP-quadruplex and the TG$_4$T-quadruplex. This preserves the fluid-like order in the 22-mer HT-quadruplex columns at all crowding conditions. In fact, this could well be important in the $\textit{in vivo}$ context.

Another feature of the ordered phases of the DNA structures is revealed by comparison of the characteristic decay lengths in the II vs. $d_{oc}$ curves (Fig. 4a) of duplex, triplex, and GMP-quadruplex DNA. On theoretical grounds one can quantify the intercolumnar distance dependence of the osmotic pressure of the array in terms of a short-range hydration characteristic length and a longer-ranged Debye screening length$^{29}$. This approach describes the hydration and the electrostatic components of the total interaction between the columnar structures in an ordered array. In the case of duplex DNA in monovalent salt solutions, at large separations in the ordered phase, the apparent decay length from the II vs. $d_{oc}$ curve is close to the Debye length, suggesting that the electrostatic interactions dominate at these separations$^{22}$. When the surface-to-surface separation is smaller than about 7–8 Å, the II vs. $d_{oc}$ curves for all ionic concentrations converge to a single curve, suggesting also a universal short-range hydration repulsion that is independent of ionic strength (for details see ref. 22). Similarly, triplex DNA exhibits the same interaction regimes, with comparable Debye lengths at large interaxial separations, except that the hydration decay length in the high density regime is smaller than in the case of duplex DNA. In this respect the triplex DNA lies in between the behavior exhibited by the duplex DNA and the G-quadruplexes. In fact, in the case of G-quadruplexes, neither the Debye length nor the expected characteristic length for the short-range hydration interactions (on the order of the size of a water molecule) are anomalously small as the apparent lengths reported here (~ 1 Å). The nature of these extremely short range interactions observed in G-quadruplexes thus remains to be elucidated.

As a side note, the radial disorder in the $\Phi_n\rightarrow\Phi_\infty$ phase is more pronounced in the GMP-quadruplex arrays than in the duplex, 22-mer HT-quadruplex, and TG$_4$T-quadruplex arrays (Fig. 5). In the disordered phases, the repeating units along the columnar (or molecular) axes are constrained by (i) the relatively weak base-stacking interactions and (ii) the sugar-phosphate links between the adjacent units. The data in Fig. 5 point to the increased molecular stability by the sugar-phosphate backbone in duplex, 22-mer HT-quadruplex, and TG$_4$T-quadruplex relative to the GMP-quadruplex. Nonetheless, the base-stacking interactions between the G-quartets are obviously strong enough and lead to the formation of disordered GMP-quadruplex columns at osmotic pressures as low as $\approx$3–4 atm (corresponding to $\approx$15 wt% PEG 8000 concentration).

Molecular crowding - as quantified by its proxy, the PEG solution osmotic pressure - certainly plays an important role in the stabilization of different DNA structures and regulation of their respective functions$^{10}$. By explicitly showing and analyzing how the solution PEG osmotic pressure controls the DNA density, the related molecular order, and the phase transitions between differently ordered dense DNA-analogue arrays, we can take an additional step in understanding how the complicated $\textit{in vivo}$ environment regulates different functionalities of nucleic acids.
Methods

Sample preparation. DNA oligonucleotide samples were ordered from Integrated DNA Technologies and were stored in a freezer until the time of measurements. Below we describe the methods of preparations of each DNA structure considered in this manuscript.

GMP-quadruplex. We prepared the GMP solutions (1 mg/ml) at [KCl] = 0.3 M with stirring at room temperature for about 2–3 hours. We then mixed 1 ml samples of the prepared GMP solution with 4 ml 25 wt% PEG 8000 solutions (containing 0.3 M K+), i.e., the final solution contains ~1 mg of GMP under 20 wt% PEG 8000 and 0.3 M K+. Under these conditions, GMP precipitates and pellets are formed by centrifugation. We transferred the collected pellets into new PEG 8000 solutions (at various wt% concentrations) for the x-ray diffraction experiments.

TG₄T-quadruplex. Quadruplex formation was induced by heating TG₄T oligonucleotide solution at 80 °C for 5 min and then cooling to room temperature at [KCl] = 0.1 M. The oligonucleotide concentration in the annealing solution was ~0.1 mg/ml. TG₄T-quadruplex is extensively studied in the literature. Their conformation in K⁺ solutions is well-known. We measured the CD spectra of the TG₄T solutions before and after the heat incubation to confirm the parallel-stranded intermolecular TG₄T-quadruplex formation (see SI Appendix).

22-mer HT-quadruplex. Quadruplexes were formed by heating the four-repeat telomeric sequence AG₆(TTAG₃)₃ solution at 95 °C and [KCl] = 50 mM for 5 min and then cooling to room temperature. The oligonucleotide concentration in the annealing solution was ~0.1 mg/ml. We measured the CD spectra of the samples to ensure the formation of the structure (see SI Appendix). We then equilibrated the quadruplexes in PEG 8000 solutions (40 wt%). The structural conversion of the AG₆(TTAG₃)₃ sequence to parallel-stranded 22-mer HT-quadruplex conformation in PEG solutions was seen. 22-mer HT-quadruplex arrays aggregated in the 40 wt% PEG 8000 solutions were collected by centrifugation. The concentrated pellet was then transferred into new solutions of PEG 8000 (at various wt% concentrations) and [KCl] = 0.3 M for equilibration (~48 hours). Before the x-ray diffraction experiments, the solutions were centrifuged for long hours (~20–30 hours) at 4 °C and 30,000 g. The pellets (~1 mg of weight) were held under the same solution conditions (0.3 M K⁺ and the desired PEG wt%) during the diffraction measurements.

Poly(AT*)₃-triplex. Poly(AT*)₃-triplex structures are made of 50 bases long Poly(A) and Poly(T) sequences, by heating the solutions of Poly(A) and Poly(T) (mixed at 1:2 ratio) to 90 °C and slowly cooling to room temperature. The oligonucleotide concentration in the annealing solution (containing 5 mM Mg²⁺) was ~0.1 mg/ml. Following the annealing, we measured the CD spectra of the samples to ensure the Poly(AT*)₃-triplexes were formed (see SI Appendix). We then concentrated the Poly(AT*)₃-triplex solution to about 1 mg/ml oligonucleotide concentration. The Poly(AT*)₃-triplex arrays are formed in PEG 8000 solutions: We mixed 1 ml samples of the 1 mg/ml Poly(AT*)₃-triplex solution with 1 ml 50 wt% PEG 8000 (both containing 5 mM Mg²⁺). Thus the final solution contained ~1 mg of Poly(AT*)₃-triplex under 25 wt% PEG 8000. Self-aggregated Poly(AT*)₃-triplex arrays are equilibrated in the 25 wt% PEG 8000 solutions for about 48 hours and then collected by centrifuging for ~20 hours at 4 °C and 30,000 g. We then transferred the collected pellets into large volumes (~5 ml) of PEG 8000 bathing solutions at various wt% concentrations (also containing the desired ionic conditions, i.e., 5 mM Mg²⁺ and 0.3 M K⁺). We performed the x-ray diffraction measurements after equilibrating the Poly(AT*)₃-triplex arrays in the bathing solutions for about 48 hours.

Data collection. X-ray diffraction measurements are made using the in-house setup at the UMass Amherst Physics Department. Brief explanations of the x-ray diffraction data analysis are given in the caption to Fig. 3. See also SI Appendix for more details. CD spectra measurements are carried out in the School of Medicine at Case Western Reserve University.

Osmotic pressure data. Temperature-dependent osmotic pressure data of PEG (molecular weight 8000 Daltons) solutions are from ref. 11. The osmotic pressure of PEG, as well as the temperature dependence of the osmotic pressure of PEG, are not new. They have been reproduced by a variety of experimental methods (e.g., vapor pressure osmometer, membrane osmometer).

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Author Contributions
S.Y., N.F.S. and R.P. designed research; S.Y., J.B.S. and R.H.F. performed research; S.Y., M.A.A., R.P. and V.A.P. analyzed data; S.Y. prepared figures; and S.Y. and R.P. wrote the paper. All authors reviewed and edited the manuscript.

Additional Information
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