Quadruplex folding of DNA promotes the condensation of linker histones via liquid-liquid phase separation

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Abstract

Liquid-liquid phase separation (LLPS) of proteins and DNA has recently emerged as a possible mechanism underlying the dynamic organization of chromatin. We herein report the role of DNA quadruplex folding in liquid droplet formation via LLPS induced by interactions between DNA and linker histone H1 (H1), a key regulator of chromatin organization. Fluidity measurements inside the droplets and binding assays using G-quadruplex-selective probes demonstrated that quadruplex DNA structures, such as the G-quadruplex and i-motif, promote droplet formation with H1 and decrease molecular motility within droplets. The dissolution of the droplets in the presence of additives indicated that in addition to electrostatic interactions between the DNA and the intrinsically disordered region of H1, π-π stacking between quadruplex DNAs could potentially drive droplet formation. Given that DNA quadruplex structures are well documented in heterochromatin regions, it is imperative to understand the role of DNA quadruplex folding in the context of intranuclear LLPS.

Introduction

Genomic DNA in eukaryotic cells wraps around histone protein cores to form nucleosomes, which are further compacted into chromatin.[1] The level of chromatin condensation is closely related to gene transcription;[2] heterochromatin is a tightly packed form that is inaccessible to polymerases and thus inactivates gene transcription, whereas gene transcription is activated in euchromatin, in which the nucleosomes are loosely packed.[3] Chromatin undergoes highly dynamic changes in its condensed structure during a cell cycle. However, the mechanisms that govern the organization of chromatin remain largely unknown.

Liquid-liquid phase separation (LLPS) has emerged as a possible mechanism for the control of chromatin organization through the promotion of nucleosome packing.[4] Biological LLPS is a process in which mixed solutions of biomacromolecules spontaneously separate into two phases.[5,6] In such events, one phase is usually a small-volume droplet-like phase in which the biomacromolecules are concentrated in aqueous media, while the other is the surrounding phase, which is depleted of biomacromolecules.[7] Multivalent weak intermolecular interactions involving intrinsically disordered regions (IDRs) and/or low-complexity sequences (LCS) of proteins, such as electrostatic, cation-π, and π-π interactions, play crucial roles in LLPS.[5,6] For instance, binding of the IDR-containing heterochromatin protein HP1α to the histone H3K9 methylation site induces LLPS in specific domains of heterochromatin.[8–11] LLPS also occurs in euchromatin regions that are rich in acetylated histone tails when the transcriptional regulator protein BRD4, which contains a long IDR, is co-localized.[12,13] Thus, the nature of the relationship between LLPS-mediated chromatin-condensation and proteins is gradually determined.

Several reports have indicated that DNA is also involved in the LLPS associated with chromatin condensation. The length of the inter-nucleosome linker DNA strongly affects the LLPS of nucleosome arrays.[12] Double-stranded DNA induces LLPS in the presence of histone H1,[14] which is capable of regulating chromatin organization via binding to inter-nucleosome linker DNA.

However, knowledge regarding the structure of the DNA involved in LLPS-mediated chromatin-condensation is still very limited. Therefore, in this work, we have focused on the secondary structures of DNA, especially the most common G-quadruplex structure.[15,16]

G-quadruplex is a stacking planar structure formed through Hoogsteen hydrogen bonds between four guanine residues (Figure 1A).[17] Guanine-rich sequences with the potential to fold into the G-quadruplex structure are frequently observed in oncogene promoter sequences and telomere regions, where they may promote or inhibit the access of transcriptional factors or telomere binding proteins.[18,19] Recently, it has been revealed that G-quadruplex sequences are also abundant in heterochromatin region;[20] however, the role of these sequences on chromatin condensation has not been clarified. Thus, we attempted to elucidate the relationship between the G-quadruplex DNA structure and the LLPS of nucleosome constituents using a variety of sequences that are capable of forming quadruplex structures. The presented findings will facilitate the understanding of the role of G-quadruplex structures in the cell nucleus and chromatin condensation.

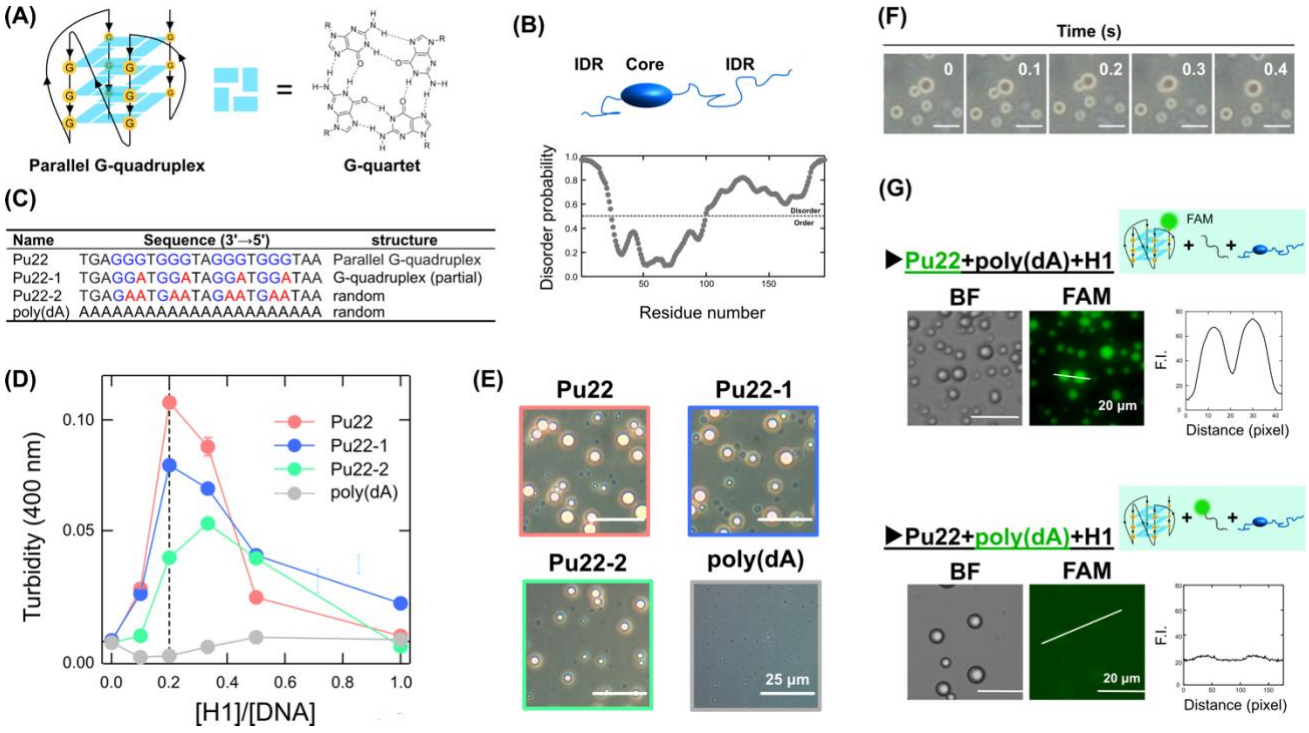

Figure 1. Liquid-like droplets of G-quadruplex-forming ssDNA with H1. (A) Schematic illustration of the parallel G-quadruplex DNA structure (left), which consists of stacked G-quartets (right). (B) Disorder probability of the H1 structure as predicted using the Protein DisOrder prediction System (PrDOS).[21] Regions of the sequence that exhibit a score bigger than 0.5 are defined as intrinsically disordered regions. (C) Sequences and structures of the ssDNA used in this study. (D) Turbidity of solutions that contain various ssDNA sequences (10 μM) and H1 (0-10 μM). (E) Phase-contrast-microscopy images of solutions that contain ssDNA (10 μM) and H1 (2 μM); scale bar = 25 μm. (F) Fusion process of the Pu22/H1 droplets; scale bar = 10 μm. (G) Selectivity of the ssDNA sequences for the droplet formation with H1. Either Pu22 or poly(dA) was modified with FAM, and both (10 μM) were mixed with H1 (2 μM). The fluorescence intensity along the white line was quantified from the brightness of each pixel; scale bar = 20 μm. All experiments were carried out in 10 mM Tris-EDTA buffer (pH = 7.4).

Results and Discussion

LLPS of G-quadruplex-forming ssDNA with H1

To investigate the effect of G-quadruplex formation on LLPS in the context of chromatin condensation, we used histone H1 (H1) and various single-stranded DNA (ssDNA) sequences. H1 controls the packing density of nucleosomes via non-specific electrostatic interactions between its positively charged lysine-rich IDR at its C-terminal and negatively charged DNA (Figure 1B).[22] Four 22 nt ssDNA sequences were initially prepared (Figure 1C): an oncogene *c-myc* promoter sequence that can fold into a parallel G-quadruplex structure (Pu22)[23]; sequences in which one or

two of the successive guanines of Pu22 were replaced with adenine (Pu22-1 and Pu22-2, respectively); and a simple repeat of deoxyadenylic acid (poly(dA)) with a random coil structure. The nucleotides are arranged an all-*anti* configuration in the parallel forms of G-quadruplexes, while the antiparallel forms contain nucleotides in both *syn* and *anti* configurations (Figures 1A and S2A). The secondary structure of each ssDNA was examined by circular dichroism (CD) measurements, which revealed that with decreasing number of guanines, the content of G-quadruplex structures decreased (Pu22 to Pu22-1), whereby Pu22-2 exhibits a random coil-like structure (for details, see section 3 of the Supporting Information).

The turbidity of the aqueous solutions of the guanine-containing sequences (Pu22, Pu22-1, and Pu22-2) increased upon addition of H1 up to a certain concentration ([ssDNA]/[H1] = 0.2-0.4) (Figure 1D), indicating that interactions between the ssDNA sequences and H1 resulted in the formation of large assemblies. Interestingly, the maximum turbidity values of the solutions increased with the number of continuous guanines in the ssDNA sequence (Pu22 > Pu22-1 > Pu22-2). The decreased turbidity at high concentrations of H1 probably results from repulsive forces that arise from the excess of positive charge;[14,24] thus, electrostatic interactions between the cationic H1 and anionic ssDNA are presumably a dominant force in the formation of the assemblies. However, although poly(dA), like the other ssDNA sequences, is anionic, the poly(dA) solution did not exhibit an apparent increase in turbidity upon addition of H1.

Spherical assemblies were observed via phase-contrast microscopy for all turbid ssDNA solutions ([ssDNA]/[H1] = 0.2; Figure 1E), similarly to our recent studies of cationic protein/anionic polymer pairs.[25,26] Time-lapse images showed rapid, sub-millisecond fusion of the assemblies (Figure 1F). This behavior indicates that these assemblies are not gel-like aggregates, but instead liquid-like droplets with highly fluid properties, as have been observed for other phase-separating proteins.[27,28] Consistent with the turbidity measurements (Figure 1D), the size of the observed droplets decreased with decreasing number of continuous guanines in the ssDNA sequence. In the

case of poly(dA), only small droplets (diameter < 1.0 μm) were formed. Similar behavior was observed for other ssDNA sequences, including an anti-parallel G-quadruplex sequence present in telomeric regions (22AG), its derivatives, and a simple repeat of deoxythymidylic acid (poly(dT)) (Figure S2). Thus, the relationship between the G-quadruplex-forming sequence and the formation of droplets may be key to the assembly of ssDNA with H1.

To study the sequence selectivity of the droplet formation, either Pu22 or poly(dA) was labeled with carboxyfluorescein (FAM), and then both were mixed with H1. When Pu22 was labeled, the inside of the droplet emitted strong fluorescence, whereas the fluorescence inside and outside of the droplet was comparable for labeled poly(dA) (Figure 1G). This result suggests sequence selectivity not only in the formation of droplets with H1, but also in the incorporation of ssDNA into the resulting droplets.

Based on these results, it seems feasible to conclude that the interaction between H1 and ssDNA sequences that can fold into G-quadruplex structures plays a significant role in both the generation of LLPS and the ability of the formed droplets to incorporate other ssDNA. This assumption is supported by the linear relationship between the turbidity of the solutions that contain the droplets and the fluorescence intensity of a G-quadruplex structure-specific probe (Figure S3).

G-quadruplex folding within liquid-like droplets

The stability of the G-quadruplex structure is affected by protein binding.[29,30] Since the components of the droplets formed through LLPS are generally concentrated within the droplets by a factor of several to several hundred compared to the surrounding phase,[12,31] the G-quadruplex content might fluctuate due to the presumably high concentration of H1 inside the droplets. Therefore, we investigated the folding state of the ssDNA within the droplet using two fluorogenic probes that selectively bind to G-quadruplex structures, thioflavin T (ThT) and *N*-methylmesoporphyrin IX (NMM) (Figure 2A). ThT binds to G-quadruplex structures regardless of

their conformation,[32] while NMM recognizes only parallel-folded G-quadruplexes.[33] These probes are almost nonfluorescent in aqueous solution, but exhibit strong emission when bound to G-quadruplex structures.

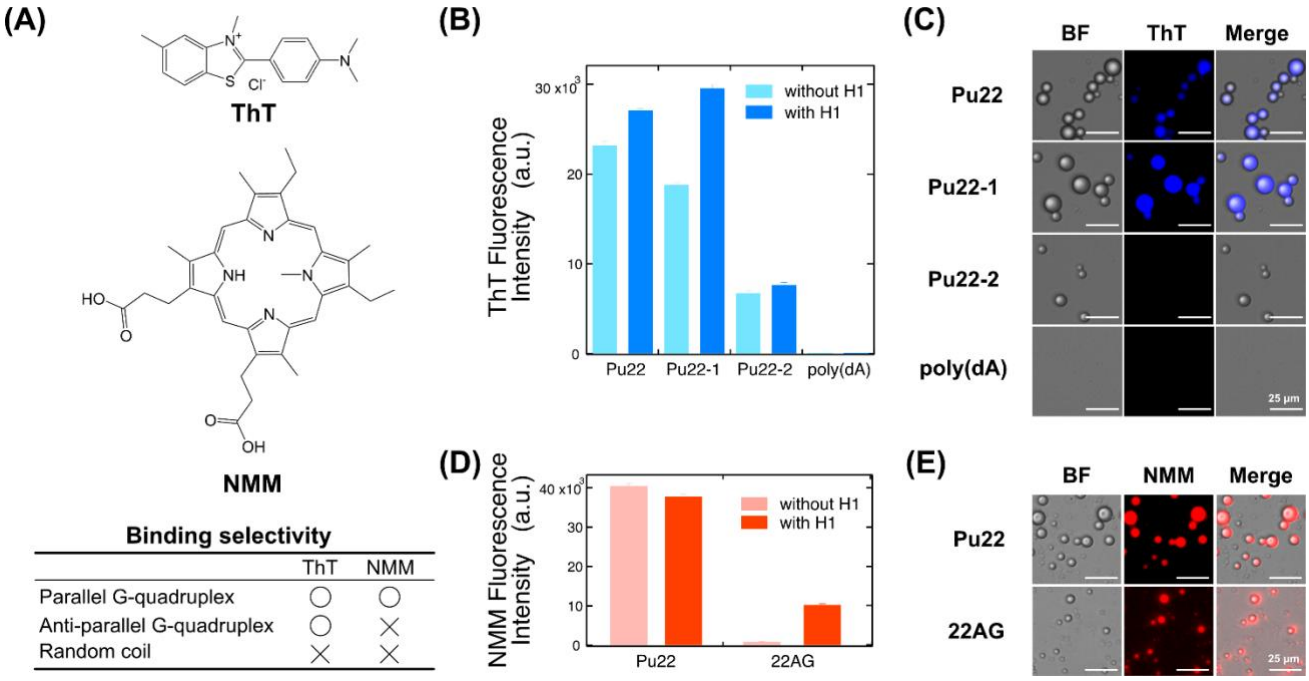

Figure 2. Analysis of G-quadruplex structures inside the liquid droplets using fluorescent molecular probes. (A) Chemical structures of the fluorescent probes ThT (left) and NMM (right), which exhibit greatly enhanced fluorescence upon their selective binding to G-quadruplex structures. The binding selectivity of ThT and NMM is taken from refs 20 and 30, respectively. Fluorescence intensity of (B) ThT and (D) NMM in solutions that contain ssDNA (10 nM) with or without H1 (2 nM). Fluorescence microscopy images of ssDNA/H1 liquid droplets after the addition of (C) ThT and (E) NMM; scale bar = 25 μm.

In the absence of H1, the overall fluorescence intensity of the solutions in the presence of ThT follows the order Pu22 > Pu22-1 > Pu22-2 >> Poly(dA), i.e., the abundance of G-quadruplex structures increases with increasing number of successive guanines in the ssDNA sequence (Figure 2B; for the fluorescence spectra, see Figure S4A). The fluorescence intensity of ThT after the droplet formation is comparable for the Pu22-2/H1 or poly(dA)/H1 solutions, while that of the Pu22/H1 and Pu22-1/H1 solutions increases 1.2- and 1.6-fold, respectively (Figure 2B). The interaction with H1 to induce LLPS might have facilitated the folding of the ssDNA into G-quadruplex structures, especially in the case of Pu22-1. Molecular crowding causes dehydration of G-quadruplex structures,

which stabilizes this conformation;[29,34] this phenomenon is likely responsible for the enhanced folding inside the droplets. Fluorescence microscopy images of the Pu22 and Pu22-1 solutions showed significant fluorescence inside the droplets, while Pu22-2 solutions exhibited very weak fluorescence (Figure 2C). In light of the finding that other fluorescent dyes with no specificity for the G-quadruplex were concentrated in the droplets regardless of the ssDNA sequence (Figure S5), we concluded that the G-quadruplexes formed by Pu22 and Pu22-1 were also concentrated inside the droplet, and that ThT was bound to these internal G-quadruplex structures. Similar results were obtained for a telomere-derived sequence (22AG) that is capable of folding into an anti-parallel G-quadruplex structure (Figure S4B).

Aqueous solutions of Pu22 both with and without H1 showed strong fluorescence when the probe NMM, which is specific to parallel-type G-quadruplexes, was added (Figure 2D). The intensity of the NMM fluorescence decreased only slightly due to the formation of droplets in the presence of H1, indicating that the parallel G-quadruplex structure of Pu22 is not significantly perturbed by LLPS. On the other hand, substantial NMM emission was observed from the entire solution of anti-parallel 22AG only after droplets were formed by the addition of H1. Similar to the emission of ThT, that of NMM was concentrated inside the droplets for both ssDNA sequences (Figure 2E). The enhancement in the fluorescence after droplet formation for 22AG suggests that either the G-quadruplex structures of 22AG transitioned from the anti-parallel to the parallel form due to droplet formation, or the affinity of NMM towards the anti-parallel structures was enhanced in the concentrated droplet environment. Molecularly crowded environments have been reported to induce anti-parallel-to-parallel transition in telomere-derived ssDNA, which inhibits telomerase processability.[35] Thus, if transition of the G-quadruplex structures of 22AG is coupled to the droplet formation, it might be involved in the telomere activity switching mechanism in the cell nucleus.

Generality of the promotion of LLPS by the quadruplex conformation

To clarify the generality of the promotion of LLPS by quadruplex structures in ssDNA, we tested another quadruplex structure formed by successive cytosine bases, the so-called i-motif (Figure 3A).[36] As expected, the increase in turbidity upon addition of H1 was greater for an i-motif-forming sequence (22CT) than for a shuffled variant of this sequence in which the cytosines are not successive ($22CT_{shuffle}$) (Figure 3B).

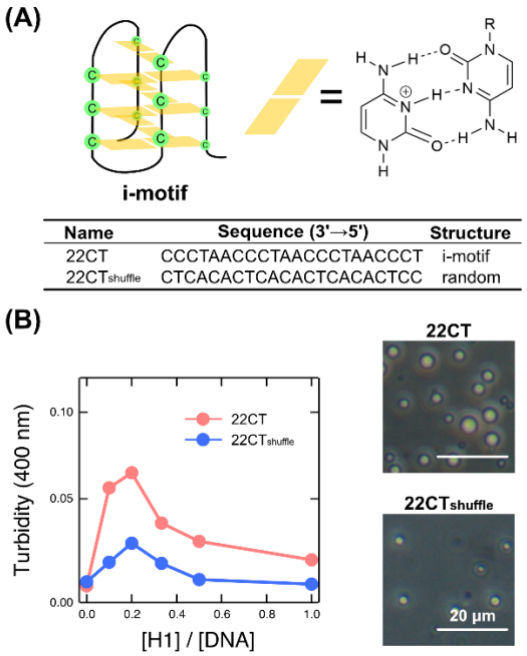

Figure 3. Droplet formation in a solution that contains cytosine-based quadruplex structures and H1. (A) Schematic illustration of the i-motif structure and the DNA sequences used in this experiment. (B) Formation of liquid droplets in solutions of i-motif DNA (22CT) or a shuffled sequence ($22CT_{shuffle}$) with H1 (2 nM); scale bar = 20 μm.

Consistent with the turbidity results, the droplets formed by 22CT were significantly larger than those formed by $22CT_{shuffle}$ (Figure 3B). The dependence of the phase separation behavior on the arrangement of the ssDNA suggests that the quadruplex structure is important in promoting LLPS with H1. This result is somewhat unexpected, as nucleic acids form gel-like aggregates rather

than liquid-like droplets in the presence of cationic macromolecules,[37] or are excluded from protein droplets[38] when stiffened by the formation of secondary structures such as double strands or loops. This unprecedented result might be due to the tendency of DNA with quadruplex structures to stack intermolecularly in longitudinal direction at high concentrations.[39,40] In the case of G-quadruplexes, π-π interactions between planar guanine bases is thought to be the main driving force for intermolecular stacking, as well as the G-quartet stacking that occurs during the formation of intramolecular G-quadruplex structures.[17,41] In protein phase separation, π-π interactions are significant in directing the state of the assembly towards liquid-like droplets rather than gel-like aggregates.[42,43] Therefore, stacking between the quadruplex structures via π-π interactions may have promoted droplet formation.

It should be noted that the guanine bases themselves may also contribute to the promotion of LLPS, as demonstrated in studies using ssDNA with shuffled variants of G-quadruplex-forming sequences and nucleotide monophosphates (for details, see Section 4 of the Supporting Information). Because H1 contains few aromatic amino acids (only one Phe and one Tyr in 214 aa), hydrogen-bonding interactions between partially exposed guanine bases and lysine residues[44,45] of H1 may be involved.

Characteristics of liquid-like droplets containing G-quadruplex structures

To gain further insight into the driving forces of the droplet formation, we added NaCl and 1,6-hexanediol, which inhibit intermolecular electrostatic and hydrophobic interactions, respectively,[46] to solutions that contain the liquid droplets. NaCl markedly reduced both the turbidity of the solution and the droplet size (Figures 4A and S8); the droplets disappeared completely at higher-than-physiological NaCl concentrations (~300 mM). This result demonstrates that the electrostatic interactions between the cationic C-terminus of H1 and the anionic ssDNA are the dominant driving force in the generation of LLPS. The droplets gradually dissolved upon

increasing the 1,6-hexanediol concentration (Figures 4B and S9), which suggests that π-π stacking between quadruplex structures and, possibly, hydrophobic interactions between the nucleobases of the ssDNA and H1 also stabilize the droplets.

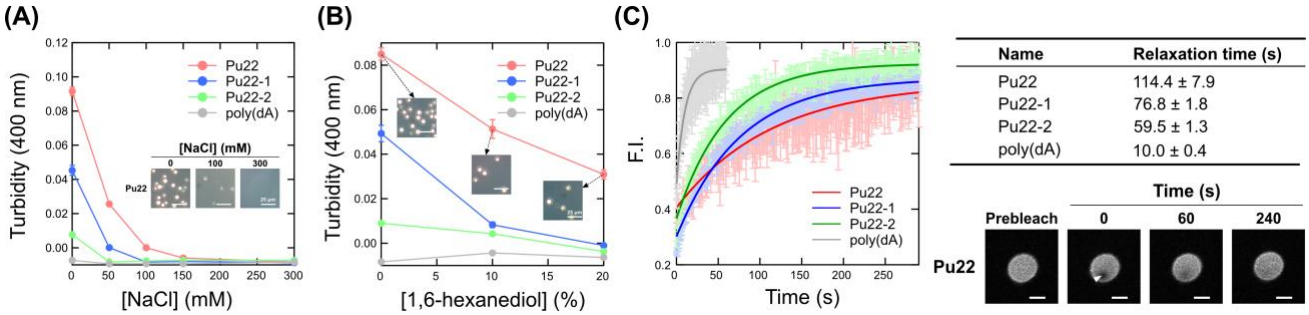

Figure. 4. Physicochemical properties of the DNA/H1 liquid droplets. (A and B) Solution turbidity of the ssDNA/H1 liquid droplets in the presence of (A) 0-300 mM NaCl or (B) 0-20% 1,6-hexandiol. Insets contain phase-contrast images of the G4/H1 liquid droplets in the presence of each additive; scale bar = 25 μm. (C) Left: FRAP recovery curves for the different ssDNA structures. Right: relaxation times of fluorescence recovery calculated by exponential fitting (colored lines; N = 3), and fluorescence images obtained during the FRAP measurement of a Pu22/H1 liquid droplet. The white arrow indicates the bleaching site; scale bar = 5 μm.

Finally, the effect of the ssDNA sequence on the fluidity inside the droplet was compared using fluorescence recovery after photobleaching (FRAP), which is a common method for evaluating the motility of molecules in droplets.[47] The diffusion rate of the FAM-modified ssDNA sequences increased with decreasing number of continuous guanines [poly(dA) > Pu22-2 > Pu22-1 > Pu22], i.e., in the opposite order of the content of quadruplex structures (Figure 4C, D). This result suggests that the formation of G-quadruplexes by the ssDNA controls not only the formation of droplets with H1, but also the motility of the molecules within the resulting droplets, which could potentially affect cellular functions such as the inhibition of gene transcription.

Conclusions

In summary, we have demonstrated that the formation of quadruplex structures in single-strand DNA (ssDNA), including guanine-based parallel and anti-parallel G-quadruplexes and

cytosine-based i-motif structures, promotes the formation of liquid-like droplets with linker histone H1 via liquid-liquid phase separation (LLPS). The quadruplex folding is maintained or, in some cases, promoted inside the droplet, with increases the quadruplex content and decreases the motility of the molecules that comprise the droplet. These droplets are likely formed via not only electrostatic interactions between the anionic ssDNA and the cationic C-terminus of H1, but also via π-π interactions between the quadruplex structures. Thus, DNA quadruplex structures may be capable of regulating LLPS-mediated dynamic chromatin condensation in the nucleus. DNA and RNA with G-quadruplex structures can selectively interact with nuclear proteins such as fused in sarcoma (FUS) and hnRNPA1, which cause amyotrophic lateral sclerosis (ALS) and tend to phase-separate.[48–50] Accordingly, we expect that the quadruplex structure can act as a hub that regulates biological processes such as chromatin condensation in the nucleus via LLPS.

Acknowledgements

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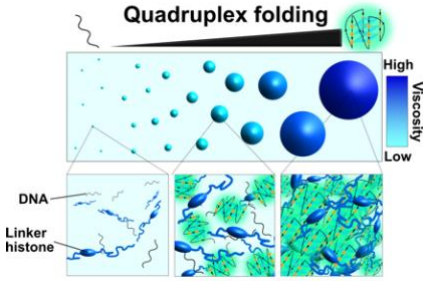
TOC



Quadruplex folding of DNA promotes liquid droplet formation with linker histone H1 via liquid-liquid phase separation (LLPS), and the motility of the molecules in the droplet decreases with increasing quadruplex formation. These findings demonstrate the importance of understanding the function of quadruplex structures in chromatin condensation in the context of LLPS.

Keywords: G-Quadruplex, Histone, Liquid-liquid phase separation, DNA structures, Intrinsically disordered protein