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Review

Effects of Molecular Crowding on the Structure, Stability, and Interaction with Ligands of G-quadruplexes

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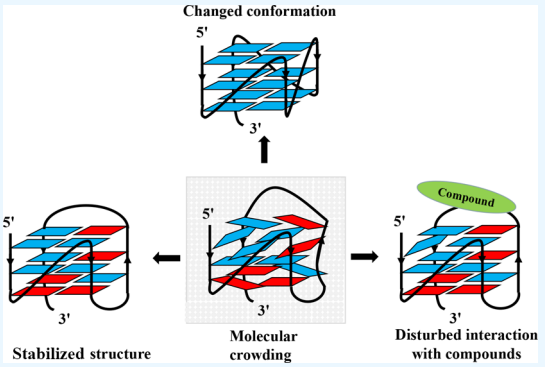
ABSTRACT: G-quadruplexes (G4s) are widely found in cells and have significant biological functions, which makes them a target for screening antitumor and antiviral drugs. Most of the previous research on G4s has been conducted mainly in diluted solutions. However, cells are filled with organelles and many biomolecules, resulting in a constant state of a crowded molecular environment. The conformation and stability of some G4s were found to change significantly in the molecularly crowded environment, and interactions with ligands were disturbed to some extent. The structure of the G4s and their biological functions are correlated, and the effect of the molecularly crowded environment on G4 conformational transitions and interactions with ligands should be considered in drug design targeting G4s. This review discusses the changes in the conformation and stability of G4s in a physiological environment. Moreover, the mechanism of action of the molecularly crowded environment affecting the G4 has been further reviewed based on previous studies. Furthermore, current challenges and future research directions are put forward. This review has implications for the design of drugs targeting G4s.



1. INTRODUCTION

G-quadruplexes (G4s) are formed by guanine-rich nucleic acid sequences and are comprised of two or more stacks of planar G-quartets. Hoogsteen hydrogen bonds stabilize the G4 structures in the presence of monovalent cations such as K^+ and Na^+.[1−4] G4-forming sequences are widely distributed in the genome, and G-rich DNA sequences always exist in eukaryotic telomeres and promoter gene regions such as *c-MYC*, *c-KIT*, *KRAS*, *BCl2*, *VEGF*, etc. The formation and unwinding of G4 structures significantly impact several biological events, including gene replication, recombination, and translation.[5−9] Small molecules targeting the telomeric G4 can inhibit telomere lengthening by affecting telomerase activity, leading to the inhibition of tumor cell growth.[11,12] In addition, small molecules can act on the promoter region of proto-oncogenes, inhibiting the binding of proteins involved in regulating transcription to the promoter region and thereby suppressing gene expression. Therefore, G4s on proto-oncogenes can also be used as targets for screening antitumor drugs.[10−14]

G4s have been discovered in the viral genome, which can be used as targets for antiviral drug screening.[15−17] G4s play a crucial role in regulating the replication, maintenance, and recombination of the virus genome[18−21] and even affect the entire life cycle of the virus.[22−24] The terminal repeat region of Kaposi sarcoma-associated herpesvirus is guanine-rich and can form a G4 structure that regulates viral latency in host cells. The PhenDC3 and TMPyP4 (G4 ligands) were found to slow down the replication fork and disrupt the DNA replication of Kaposi sarcoma-associated herpesvirus.[25] The G4's structure was also identified in the COVID-19 genome and could be an essential drug target for the inhibition of this virus.[26,27]

Current studies on G4s have mainly been carried out under diluted solution conditions. However, in reality, the cellular milieu is highly crowded, with a diverse range of molecules, including proteins, polysaccharides, nucleic acids, and other soluble or insoluble substances, occupying a limited amount of available space.[28] The diluted solution environment of the cell differs from the molecularly crowded environment in multiple ways, such as water activity, osmolarity, pH, ion species, and concentrations. Macromolecules in the cell have a total concentration of approximately 400 mg/mL, representing 30%−40% of the cell volume.[29,30] Such macromolecular restrictions may affect biomacromolecules' structural and physiological conformations and their interactions with

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ligands.[30] Molecular crowding has been widely reported to affect the conformation and stability of nucleic acids.[31,32] G4s are noncanonical DNA structures composed of stacks of stabilized G-quartets. The molecularly crowded environments can dramatically impact the G4's structure, stability, and interactions with ligands.[33] Studying the biochemical responses of G4s in a molecularly crowded environment is essential.

In the present work, we reviewed the changes in conformation and stability of G4s and the effects of G4−ligand interactions in molecularly crowded environments simulated *in vitro*. Additionally, we have summarized the mechanisms by which molecularly crowded environments affect G4s based on previous studies. This review is important for understanding the physiological functions of G4s inside the organisms' genomes and targeted drug design.

2. REAGENTS SIMULATING THE MOLECULARLY CROWDED ENVIRONMENT IN CELLS

Studies on G4s have not yet been carried out purely in an intracellular setting due to the complexity of the intracellular environment. However, some research on G4s under physiological conditions has been conducted in environments that simulate molecular crowding.[34−36] As a molecular crowding reagent for simulating the intracellular environment, it is required to increase the rejection volume, alter the water activity, and meet the requirement of not interacting directly with nucleic acids and the ligand. To achieve a purposeful study on the conformation of G4s and ligands, many reagents are used to simulate the molecularly crowded environment, such as poly(ethylene glycol) (PEG), acetonitrile, glycerol, dextran, glucose, and dimethyl sulfoxide (DMSO), etc.[35,37−39] PEG with different molecular weights (PEG 200, PEG 400, PEG 2000, and PEG 8000) is commonly adopted to mimic the crowded cellular environment because it is water-soluble, is chemically inert, and does not interact efficiently with biological macromolecules. Moreover, different molecular weights of PEG can be obtained by changing the degree of polymerization, thus allowing the simulation of molecules of different sizes in living organisms and better representing the intracellular environment.[40,41] Changing the external environment and the metabolic activities of cells will inevitably affect the physicochemical properties of the internal environment, such as pH, osmolarity, and temperature, at all times. Thus, there are differences between the reagent-simulated cellular environment and the natural intracellular environment. However, it has contributed to a certain extent to the study of intracellular G4 conformation, stability, and interactions with small molecules.

3. MOLECULARLY CROWDED ENVIRONMENT CHANGES THE CONFORMATION OF THE G4

The conformation of nucleic acids can be affected by a molecularly crowded environment.[42,43] As a higher-order nucleic acid structure, the conformation of the G4s is even more influenced by the molecularly crowded environment. Telomeric G4 can fold into more than four conformations, including parallel, antiparallel, and (3 + 1) hybrid-1/-2 structures, and the conformational changes of telomeric G4s are most extensively studied in a molecularly crowded environment (Figure 1).[44] The conformation of the telomeric G4 in K^+ solutions was significantly affected by PEG 200, switching from a hybrid to a parallel structure, while no

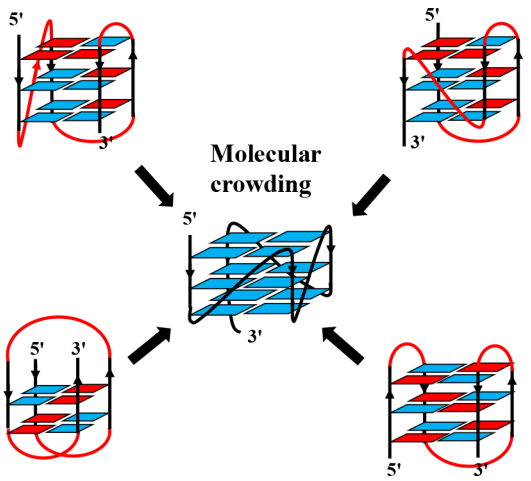

Figure 1. Conformation of the telomeric G4 was significantly affected by molecular crowding.

changes were found in the presence of Na^+.[45] When ethanol is used as a molecular crowding reagent, 20% (w/v) ethanol stabilizes the antiparallel conformation of human telomeric G4 ($G_3(TTAG_3)n$) in the absence of K^+ solutions, and the molecularly crowded environment induces the human telomeric G4's conformation to pass through an intermediate hybrid structure from an antiparallel to a parallel structure upon adding a certain amount of K^+.[46] However, in the K^+ solution containing 40% (w/v) of PEG 200, the telomeric G4 structure could not completely transition from a hybrid to a parallel structure; instead, a conformational complex consisting of both hybrid and parallel structures were found,[34,47] which suggested that there may be multiple conformations of human telomeric G4s present in the natural intracellular environment. Li *et al.*'s results showed that the structure of the human telomeric G4 was transformed with increasing concentrations of PEG, and this change was accompanied by structural compaction and increased stability,[48] which indicated that the molecularly crowded environment induces both structural changes and size modifications in the human telomeric G4.

In the presence of Na^+, PEG could not induce the conformational change of human antiparallel-structured telomeric G4, but it could alter the antiparallel conformation of some nonhuman telomeric G4s. It has been reported that the thrombin-binding aptamer sequence, 5′-GGGTTGGGTGTGGGTTGGG (G3), can be folded into an antiparallel-structured G4 in Na^+ dilute solution. G3 remains in the antiparallel form at low PEG concentrations, but at 30% (w/v), its conformation in solution consists of both antiparallel and parallel structures. When the PEG concentration exceeded 40% (w/v), G3 exhibited a perfectly parallel conformation. In the presence of K^+, the G3 conformation remains parallel even as the PEG concentration increases.[49] Sugimoto and co-researchers investigated the conformational changes of the G4 in the telomeric DNA sequence $dG_4T_4G_4$ of *Trichoderma aculeatus* at 100 mM Na^+, using neutral PEG 300, propanetriol, and positively charged butylenediamine, pentanediamine, and spermidine to simulate the molecularly crowded environment *in vivo*. They found that the molecularly crowded environment caused a conformational change in the G4 from an antiparallel structure to a parallel one.[50]

A molecularly crowded environment can also induce dimer formation from the monomeric G4s, and RNA G4s have been

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reported to form dimeric G4s through the 3′−3′ stacking of two monomeric G4 subunits with an ammonium ion sandwiched between the interfaces at a high concentration of NH_4^+ (100 mM) or in a molecularly crowded environment.[51] By employing the NMR in living cell experiments, the studies confirmed that the higher-order G4 exists in cells and demonstrated that telomere RNA G4s are converted to the higher-order G4 in a molecularly crowded environment. The studies also exhibited that the higher-order G4 has high thermal stability in molecularly crowded solutions.[52]

The conformation of the G4 determines its function, and molecular crowding affects the conformational transition of the G4, promoting the conformation of the intracellular G4 to assume polymorphism. This provides a mechanism for conformational adjustment of its biological function, which should be considered when designing drugs targeting the G4.

4. MOLECULARLY CROWDED ENVIRONMENT ALTERS THE THERMAL STABILITY OF G4S

Molecularly crowded environments induced by high intracellular concentrations of macromolecules provide support for producing and stabilizing higher nucleic acid structures.[32,53] The G4 structure is significantly more stable in a molecularly crowded environment than in dilute solution conditions (Figure 2). The melting temperature (T_m) of human telomeric

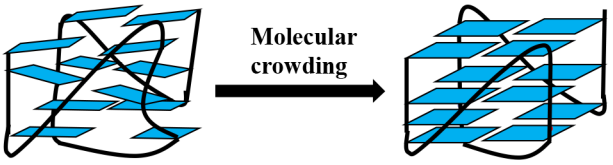

Figure 2. G4 structures are stabilized in a molecularly crowded environment.

G4s in K^+ dilute solutions was reported to be only 68.4 °C. The addition of 40% (w/v) PEG 200 resulted in an unusual stability of telomeric G4 (T_m > 80 °C).[45] Miyoshi's group investigated the changes in the thermal stability of the antiparallel G4 structure formed by thrombin aptamer sequences (TBA) in a molecularly crowded environment created by PEG 200. The results showed that with the increase in concentration (0 to 40%) of PEG 200 (w/v), the T_m value of the TBA sequence gradually increased from 54.1 to 58.7 °C.[54] There is a close correlation between G4's stability and the molecular weight of the molecular crowding imitated reagent. For example, PEG 8000 stabilizes the M2 G4 (dTAGGGACGGGCGGGCAGGG) to a greater extent than ethylene glycols at 20% (w/v). The stability of the M2 G4 in the presence of 20% (w/v) ethylene glycols can be comparable to that of 10% (w/v) PEG 8000.[40]

The selective behavior of molecularly crowded environments was found to depend on both the stability of G4s and the number of G-tetrad layers in the G4. PEG200 has been reported to stabilize RNA G4s with three and four G-quartets but not those with two G-quartets.[55] Additionally, Wu *et al.* showed that molecular crowding promoted the formation of the telomeric G4s with three G-quartets without affecting the conformation of the telomeric G4s with two G-quartets, while also increasing the stability of all telomeric G4s.[56] Molecular crowding does not have the same stabilizing effect on different G4s. In the study of human telomeric G4s (TTAGGG)*n*, Sugimoto *et al.* found that the stability of monomeric G4s under a molecularly crowded environment was much higher than that under diluted conditions, and the effect of molecular crowding on the stability of G4s formed from long-stranded DNA sequences was diminished.[53] Although the molecularly crowded environment, to some extent, prevents the binding of small molecules to G4 structures, it can stabilize human telomeric G4s, which provides favorable conditions for the formation of intracellular human telomeric G4 structures. The human telomeric G4 ($G_3(T_2AG_3)_3$) has been reported to form a hybrid conformation in a cation-free molecularly crowded environment.[57] Similarly, the human telomeric sequence $(T_2AG_3)_4$ was found to fold into antiparallel and (3 + 1) hybrid-2 structures in a molecularly crowded environment with low concentrations of KCl (0−1 mM).[34]

5. A MOLECULARLY CROWDED ENVIRONMENT AFFECTS THE INTERACTION OF G4S WITH LIGANDS

G4s can be used as drug targets; however, the molecularly crowded environment somewhat inhibits the interaction between G4s and small molecules (Figure 3). G4 stabilizing

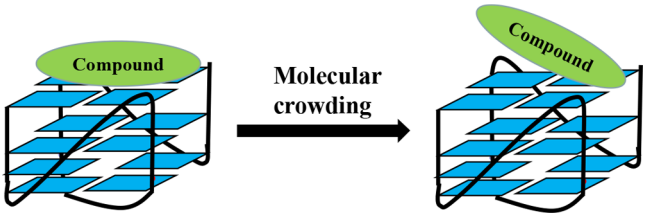

Figure 3. Interaction between the G4 and ligand is disturbed in a molecularly crowded environment.

ligands that work effectively under diluted conditions exhibit reduced or negligible G4 stabilization in a molecularly crowded environment. Therefore, designing ligands that target G4s can be challenging due to the inhibitory effects of the molecularly crowded environment. It was reported that the ability of some ligands to stabilize human telomeric G4s is significantly reduced in a molecularly crowded environment created by PEG 200 (ΔT_m values were reduced by 14 °C), and the binding constants were reduced by a factor of two−threefold.[58,59] The ability of small molecules to stabilize the G4 is reduced in a molecularly crowded environment, mainly caused by the decrease in binding strength between the ligand and the G4. The decrease in water activity in the system, caused by the molecularly crowded environment, is an essential factor inhibiting small molecules' binding to the G4.[60] Although the binding strength of small molecules to the G4 is somewhat influenced in a molecularly crowded environment, it still shows a significant effect on the G4 compared to double-stranded DNA.[59] It suggested that G4s remain a valid target for screening drugs with low side effects. Furthermore, human telomeric sequences can fold into multimeric G4s; they possess a more complex conformation and serve as more meaningful targets for screening selective drugs for antitumoral compounds in a molecularly crowded environment. It has now been found that some small molecules can selectively bind human multimeric G4s even in a molecularly crowded environment.[60]

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6. MECHANISM OF ACTION OF A MOLECULARLY CROWDED ENVIRONMENT AFFECTING THE G4 STRUCTURE AND ITS INTERACTION WITH LIGANDS

Many proteins, nucleic acids, and molecules like polysaccharides create a highly concentrated and restricted molecularly crowded environment within the cell, which has a significant impact on the medium in which the cell's various reactions take place. These effects are mainly due to the excluded volume effect.[61] PEG 400 was reported to induce a faster structural transformation of telomeric G4s than PEG 200 in a molecularly crowded environment without cations.[48]

The crowded environment can affect a molecule's diffusion rate and collision efficiency. In addition, the various substances in the crowded environment are tightly bound to the water molecules, forming an ordered layer of water molecules, which restricts the free infiltration of water and ultimately significantly affects the equilibrium state and rate of reactions of biological macromolecules within the cell.[62] The stability and conformational changes in the structure of G4s in a molecularly crowded environment may be due to dehydration during their formation under conditions of reduced water activity.[53] A hydration study on an antiparallel-structured G4 discovered that more water molecules are bound in divalent cations than monovalent ones, and crowding agents stabilized parallel-structured G4 but did not affect the stability of antiparallel or hybrid G4 structures, even in the presence of divalent cations.[62] Sugimoto *et al.*'s study revealed that the interior of the human telomeric G4 unit is dehydrated, while the spatial environment of the TTA linkage loop between the two units is hydrated. These linking loops interconnect the G4 units formed by the long telomeric DNA sequences to form a beads-on-a-string multimeric G4, a structure that has important implications for the stability of the overall structure of long telomeric DNA (Figure 4).[53]

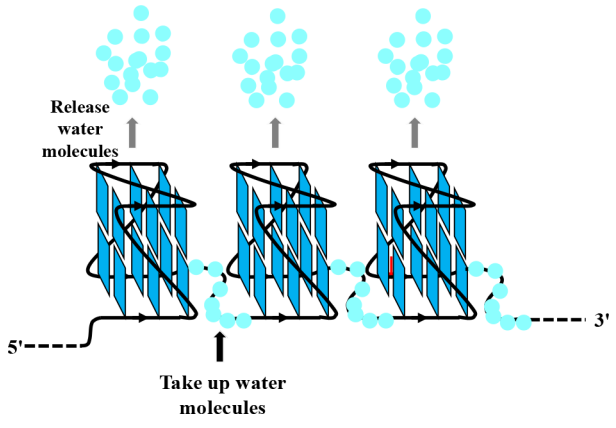

Figure 4. Scheme of hydration of the human multimeric G4 structure. Light blue circles indicate water molecules.

The mechanism of action of a molecularly crowded environment on the G4 also depends on the molecular weight and type of molecular simulation reagent. The dehydration effect is a crucial factor in the ethylene glycol-induced increase in the G4 stability, whereas the macromolecule PEG 8000 stabilizes the G4 mainly through its interaction with the G4.[40] NMR analyses and molecular dynamics calculations suggest that tetraethylene glycol interacts with bases in the G-quartet and loop via CH−π and lone pair−π interactions.[63] In addition to biomolecules such as proteins, nucleic acids, and polysaccharides, cells also contain organelles such as mitochondria, Golgi apparatus, and endoplasmic reticulum, as well as various secondary metabolites and ions of various molecular sizes, creating an extremely complex environment. Therefore, biomolecules within cells may influence the structure and function of G4s through multiple mechanisms of action, which still need more investigation.

7. CONCLUSION AND DISCUSSION

G4s are secondary structures formed by sequences of guanine-rich nucleic acids and are widely found in cells involved in important biological functions. Compounds targeting the G4 frequently exhibit significant biological activity, making it an attractive target for antitumor and antiviral drug design. Current research on G4s is mostly conducted in dilute solutions; however, 30%−40% of the cell's volume consists of organelles and many biomolecules, resulting in a constant state of molecular crowding in the intracellular environment. Molecular crowding increases the excluded volume and alters water activity, with implications for the kinetics and thermodynamics of biomolecules. Therefore, the results obtained under dilute solution conditions are not necessarily reflected in the molecularly crowded environment for G4.

In this review, we discuss the conformational transition of G4s, changes in their stability, and the interaction with small molecules in a crowded environment. The mechanism of action of the molecularly crowded environment affecting the G4 has been further reviewed based on previous studies. It has been shown that the molecularly crowded environment significantly affects the conformation of human telomeric G4s and has a lesser effect on the conformation of non-telomeric G4s with parallel and antiparallel structures. Understanding the effects of a molecularly crowded environment on human telomeric G4s' conformation and biological properties is critical for developing telomeric G4-targeting anticancer drugs. The molecularly crowded environment significantly improves the stability of nucleic acid structures, including G4s, which provides favorable conditions for the development of biological functions of the advanced nucleic acid structures.

However, the molecularly crowded environment induced *in vitro* differs somewhat from the intracellular environment. According to previous studies, different molecular crowding-inducing reagents have different mechanisms of action and have different results on G4s. Therefore, the structure and function of the intracellular G4 remain to be investigated in depth. The intracellular environment is highly complex, and more studies are now finding that the conformation of G4s in cells may be multiple and dynamically switched. Therefore, multiple conformations and dynamic transitions between conformations should be considered while designing drugs targeting the G4 in a molecularly crowded environment.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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