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Kinetics, conformation, stability, and targeting of G-quadruplexes from a physiological perspective

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The particular enrichment of G-quadruplex-forming sequences near transcription start sites signifies the involvement of G-quadruplexes in the regulation of transcription. The characterization of G-quadruplex formation, which holds the key to understand the function it plays in physiological and pathological processes, is mostly performed under simplified *in vitro* experimental conditions. Formation of G-quadruplexes in cells, however, occurs in an environment far different from the ones in which the *in vitro* studies on G-quadruplexes are normally carried out. Therefore, the characteristics of G-quadruplex structures obtained under the *in vitro* conditions may not faithfully reveal how the G-quadruplexes would behave in a physiologically relevant situation. In this mini-review, we attempt to briefly summarize the differences in a few important characteristics, including kinetics, conformation, and stability of G-quadruplex formation observed under the two conditions to illustrate how the intracellular environment might affect the behavior of G-quadruplexes largely based on the previous work carried out in the authors' laboratory. We also propose that unstable G-quadruplex variants may be better drug target candidates to improve selectivity and potency.

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1. Introduction

G-quadruplexes are four-stranded secondary structures formed in guanine-rich (G-rich) nucleic acids that carry multiple runs of guanine tracts (G-tracts) [1]. Putative G-quadruplex-forming sequences (PQSs) are widely present in cells of both prokaryotic and eukaryotic species [2,3]. Because DNA PQSs are mostly enriched in the promoter regions of animal genes, it was strongly believed and now proven that G-quadruplexes commit a major role in gene regulation [4]. Besides, G-quadruplexes are also regarded to participate in other DNA metabolic activities, such as replication, DNA repair, and recombination [5]. Therefore, information on the kinetics, conformation, and stability of G-quadruplex formation in a physiological environment is crucial for understanding the role they play and drug-designing for therapeutic applications.

Experimental investigations on the characteristics of G-quadruplexes can be conveniently performed *in vitro* with several biochemical and biophysical techniques [6]. Due to technical limitations or for convenience, G-quadruplexes are, in most cases, studied using guanine-rich (G-rich) PQS motifs in a free single-stranded form at folding/unfolding equilibrium. However, PQSs in cells are present in a completely different environment. They are by no means free as in the many *in vitro* experiments. Take genomic DNA as an example, a PQS is annealed with a complementary DNA strand, except those in the single-stranded telomere G-rich tail. Besides, a PQS is further constrained in a long duplex DNA of a chromosome. Moreover, DNAs in cells are bound by proteins and are only liberated in a small time window. All these characteristics are connected to a highly dynamic metabolic network in cells that is not present under a non-physiological condition. How these confinements and dynamics impact the behavior of G-quadruplexes is important for understanding the physiological role of and designing drugs targeting G-quadruplexes. Since studies on G-quadruplexes are much more difficult to carry out in cells, one will have to consider conditions that mimic the physiological reality. In this mini-review, we attempt to draw a few concise comparisons between the characteristics of G-quadruplexes revealed under the two different conditions to demonstrate how G-quadruplexes may behave differently under the two conditions and how we may benefit from these characteristics when choosing G-quadruplexes as drug targets.

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2. Kinetics of G-quadruplex folding in single-stranded DNA

The folding kinetics of a G-quadruplex is of great physiological relevance because it determines how fast a PQS responds to cellular activities to make a structural switching to affect an ongoing process, or to mediate the relevant reactions and feedbacks. Many years ago, we measured the folding and unfolding kinetics of the well-known telomere sequence $(TTAGGG)_4$ using the surface plasmon resonance (SPR) technique [7]. In this work, the $(TTAGGG)_4$ oligonucleotide was attached at one end to the surface of a sensor chip and a complementary C-rich oligomer was injected through the chip surface to anneal with the unfolded G-rich partner that underwent folding/unfolding cycles. Through a coupled-hybridization model, the folding and unfolding rate constant of the G-quadruplex formation were respectively derived according to the real-time signal of duplex formation at the chip surface. The folding rate constant obtained at 37 °C in a 150 mM K^+ solution was 0.016 s^{-1}. In a similar work from another lab, a telomeric DNA was immobilized on a solid surface at one end and attached with a magnetic bead at another to study the folding and unfolding kinetics, a smaller folding rate of 0.0082 s^{-1} was obtained [8]. This result suggested that the additional constraint imposed by the magnetic bead reduced the folding rate of the G-quadruplex.

Studies on the kinetics of G-quadruplex folding have been carried out using spectroscopic methods using free-floating telomere oligonucleotides [9]. In comparison with the results in such studies (4.6–26.7 s^{-1}), the folding kinetic rate constant obtained with the immobilized oligonucleotide was one to two orders of magnitude smaller. The immobilization in our experiments imposed a constraint on the telomere oligonucleotide which, to some extent, resembled the *in vivo* situation where the single-stranded telomere DNA tail is attached to the end of a chromosome. The difference in the kinetic rates observed under the two conditions indicates that G-quadruplex formation in a confined condition would be much slower than what was observed with free oligonucleotides.

The impact of confinement on the kinetics was also observed in the folding/unfolding equilibrium of G-quadruplexes. In another one of our studies, we attached a 46 kDa protein to one or two ends of a telomere oligonucleotide to mimic the association of proteins in cells. We measured the equilibrium constant of these two oligonucleotides along with the free one [10] and found that attaching the protein to one end of the oligonucleotide reduced the folding equilibrium constant K_F by 5-fold and attaching the protein to both ends reduced the K_F by 50-fold. Collectively, these results show that the folding/unfolding rate and the equilibrium between the two structural states of a G-quadruplex in a constrained condition are also significantly affected and differ from those of the corresponding free oligonucleotides.

3. Kinetics of G-quadruplex formation in duplex DNA

Except for the telomere DNA, all PQSs in a genome are accommodated in a long stretch of duplex DNA in which G-quadruplex formation occurs along with a variety of DNA metabolic activities catalyzed by DNA-protein interactions. In this situation, a PQS is even more severely confined and the formation G-quadruplex is further modulated by DNA metabolism. To investigate in a more physiologically relevant condition, we studied G-quadruplex formation in DNA duplexes under two distinctive conditions with respect to the position of PQS relative to the transcription start site (TSS) using the well defined T7 transcription model. When a PQS is positioned upstream of a TSS, the formation of G-quadruplex is triggered by the upward transmission of negative supercoiling waves generated by the downwards translocation of RNA polymerase (RNAP) [11]. When a PQS is located at the downstream side of a TSS, the DNA duplex is denatured in front of the RNAP to form a G-quadruplex [12].

Using a real-time fluorescence technique, we were able to monitor the kinetics of G-quadruplex formation in duplex DNA driven by transcription with T7 RNAP in both cases [13,14]. At the upstream side of TSS, we observed a maximal rate constant of >0.03 s^{-1} for the formation of G-quadruplex in the $(G_3T)_3G_3$ motif at the saturating concentration of T7 RNAP [13]. At the downstream side, a maximum rate constant of 0.0045 s^{-1} was obtained in a $(G_3A)_3G_3$ motif [14]. The rate constant is far smaller in the latter than in the former case, which is most likely caused by the formation of the R-loop, an RNA:DNA heteroduplex as a by-product of transcription [15], at the downstream side of the TSS [16] that may suppress the contraction of the DNA duplex during the folding of the PQS. The presence of an R-loop also makes a G-quadruplex more persistent by resisting the annealing of the DNA duplex [14]. This observation indicates a spatially distinctive feature of G-quadruplex formation in transcription. It also suggests that the formation of G-quadruplexes is more sensitive in response to transcription at the upstream side than at the downstream side of a TSS.

4. Conformations of freshly formed G-quadruplexes

G-quadruplexes are considered promising drug targets for a variety of diseases, including cancer [17–19]. The folding topology of G-quadruplexes is a key characteristics in rational drug designing and targeting. So far, the *in vitro* studies on G-quadruplexes have mostly been carried out in a thermodynamically equilibrated condition that is certainly not a physiologically relevant situation. DNA metabolism is highly dynamic and takes place at a pace that should be much faster than the time required for a G-quadruplex to reach equilibrium. For example, the human genome, which accommodates >370,000 PQSs [3], is replicated in roughly 8 h [20]. The *E. coli* genome, carrying nearly 3000 PQSs, is replicated in ~40 min [21]. Therefore, the conformation of biological and pathological importance would be the one adopted shortly after a PQS is liberated to permit a formation of G-quadruplex. In this case, the kinetics matters.

In an attempt to address the question of what conformation a telomere DNA would adopt when it was given a chance to fold into a G-quadruplex [22], we annealed a $G_3(TTAG_3)_3$ motif to a C-rich DNA strand, yielding a heteroduplex with a single-stranded protruding overhang at the 3' end of the C-rich strand. This duplex DNA was then loaded with a BLM helicase at the overhang and the $G_3(TTAG_3)_3$ oligonucleotide was then released by adding ATP to the solution. We found that the conformation of the G-quadruplex freshly formed after the release was a parallel/antiparallel hybrid but not the parallel type that was adopted at equilibrium in the same solution. After hours of incubation, the fresh conformation finally converted to the one adopted at equilibrium. In cells, there would be not enough time for such a structural conversion to occur. Rather, a fresh G-quadruplex would, in reality, interact shortly with other cellular components such as G-quadruplex-interacting proteins [23,24]. Therefore, the fresh conformation of a G-quadruplex should be the preferred candidate for drug targeting.

5. Effect of molecular crowding on G-quadruplex conformation

Molecular crowding is one of the intracellular features that can make a difference in the conformations of G-quadruplexes. A cytoplasm volume is crowded with a high concentration of macromolecules [25], which has long been demonstrated to have an impact on the structure of nucleic acids [26], including the folding topology of G-quadruplexes [27]. Take the telomeric motif $G_3(TTA\ G_3)_3$ as an

example, this DNA forms a parallel-stranded G-quadruplex in K^+ solution containing PEG as a crowding agent [22]. In contrast, the DNA adopts a different hybrid-type mixed parallel/antiparallel quadruplex in a dilute solution. Such differences in conformation have also been found in other PQS motifs. For instance, crowding has shown to cause a transition from antiparallel to parallel G-quadruplex in *Oxytricha nova* telomeric DNA in Na^+ solution [27], and transition from intra-molecular G-quadruplex to long multi-stranded G-wire in *Tetrahymena* $(T_2G_4)_3T_2G_2$ [28].

6. Effect of molecular crowding on the stability of G-quadruplexes

Besides the influence on the folding conformation, molecular crowding also has a dramatic influence on the stability of G-quadruplexes. For a single-stranded DNA (ssDNA), molecular crowding can promote G-quadruplex formation in a solution deficient in salt [29] that was previously thought a prerequisite for G-quadruplex formation. For a duplex DNA, the effect of molecular crowding inserts two opposite effects on the G-quadruplex and duplex by stabilizing the G-quadruplex and, at the same time, destabilizing duplex hybridization [30]. This combination seemed to be a key factor for the G-quadruplex formation in a duplex DNA because we could hardly detect G-quadruplex formation in a duplex DNA in dilute solutions. To seek the biological implication, we found that the parallel and more stable G-quadruplex formed in a molecularly crowded condition led to a dramatically enhanced inhibition on the processivity of telomere extension by telomerase [22]. Besides, our study also shows that molecular crowding might contribute to the reduced or diminished stabilization of the telomere G-quadruplex by several small molecule ligands [31].

7. Non-canonical G-quadruplexes as drug targets

The significant enrichment of PQS motifs near TSS implies their regulatory role in transcription. Their existence in genomic DNA is anticipated to affect several essential cellular processes, for instance, the translocation of motor proteins tracking along a DNA molecule [32] and binding of transcription factors [4]. For this reason, there has been extraordinary enthusiasm in searching for small molecules to target G-quadruplexes for therapeutic applications in the past two decades [33–37]. The total number of such small molecule ligands able to specifically bind G-quadruplexes reported so far is approaching 1000 [38] and more is still emerging. Previously, these small ligands have been almost exclusively intended to target the canonical perfect G-quadruplexes.

When such a small ligand binds a G-quadruplex, the G-quadruplex is stabilized and the enhancement in the stability of the ligand-G-quadruplex complex is a major factor in determining the impact of the ligand on the physiological activity in which the G-quadruplex is involved. A larger gain in the stability results in a more persistent structure, making the G-quadruplex more difficult to be resolved or prolonging the blocking of native G-quadruplex-interacting activities [39]. The canonical perfect G-quadruplexes are quite stable structures, such that the room for their further stabilization by small ligands will be limited. This may be particularly true in cells due to the stabilization with molecular crowding [40] since our study shows that small-molecule ligands that were effective in stabilizing G-quadruplexes in dilute solutions were not so in a molecularly crowded environment [31]. In this regard, less stable G-quadruplexes may be more appropriate to serve as drug targets.

Except for the canonical perfect G-quadruplexes, there exist a large number of non-perfect G-quadruplex-forming motifs that are able to form G-quadruplexes with additional features such as a G-vacancy [41–43] or bulge [44]. These sequence motifs are as abundant as the canonical G-quadruplexes in the human genome [41]. In theory, there are benefits in at least two aspects in targeting such structures. First, these imperfect G-quadruplexes are, in general, much less stable than the corresponding canonical ones, such that they offer more room for further stabilization to gain a larger stability increment upon ligand binding. Second, the vacancy and bulge provide additional features to improve recognition selectivity. Small ligands are mostly cationic to enhance the binding to the intended G-quadruplexes containing negatively charged phosphate backbone. However, this also causes non-specific binding to other forms of nucleic acids due to the electrostatic attraction. As a result, small ligands often have to compromise between selectivity and affinity. A G-vacancy and a bulge may serve as an additional and distinct recognition site to improve specificity because they are absent in other forms of DNA structures.

8. Summary

The intracellular environment differs in many aspects, to mention a few, flexibility confinement, viscosity, excluded volume, dielectric constant, from the *in vitro* conditions under which many G-quadruplex assays are carried out. Moreover, the dynamic nature of cellular metabolism imposes a further impact on the formation of G-quadruplexes. How these environmental parameters and metabolism affect the properties and behavior of G-quadruplexes is of great importance for understanding the consequences of G-quadruplex formation in a physiological environment. From the limited sources of information, we can see that some factors have to be considered when extrapolating from the *in vitro* knowledge to the *in vivo* anticipation. Firstly, the physical constraint has a great impact on the kinetics of G-quadruplex formation, which normally reduces the folding/unfolding kinetics of G-quadruplexes that determines how fast G-quadruplex formation reacts to cellular metabolism. Secondly, the folding topology of G-quadruplexes in cells may be different from what we see *in vitro* under an equilibrated condition and is determined by the folding kinetics and environmental parameters. Thirdly, the targeting of G-quadruplexes should also take into account the possibility that the G-quadruplexes in cells may differ from what we observed in the *in vitro* experiments in order to design more effective drugs. Awareness of the differences between the *in vivo* and *in vitro* conditions regarding G-quadruplex formation will help us better understand the biological function of G-quadruplexes and design drugs towards G-quadruplexes.

Declaration of competing interest

The authors declare no conflict of interests.

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