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RNA G-Quadruplexes as Key Motifs of the Transcriptome

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Abstract G-Quadruplexes are non-canonical secondary structures that can be adopted under physiological conditions by guanine-rich DNA and RNA molecules. They have been reported to occur, and to perform multiple biological functions, in the genomes and transcriptomes of many species, including humans. This chapter focuses specifically on RNA G-quadruplexes and reviews the most recent discoveries in the field, as well as addresses the upcoming challenges researchers studying these structures face.

Keywords Gene expression regulation, RNA G-quadruplex, RNA structure

Contents

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Abbreviations

ASO	Antisense Oligonucleotides
EBV	Epstein–Barr virus
HCV	Hepatitis C virus
HIV	Human immuno-deficiency virus
HPV	Human papilloma virus
HSV	Herpes simplex virus
hTelo	Human telomeric sequence
hTERC or hTR	Human telomerase RNA component
hTERT	Human telomerase reverse transcriptase
IRES	Internal ribosome entry site
lncRNA	Long non-coding RNA
miRNA	MicroRNA
mRNA	Messenger RNA
pre-miRNA	MicroRNA precursor
RBPs	RNA binding proteins
siRNA	Small interfering RNA
TERRA	Telomeric repeat containing RNA
tiRNA	tRNA-derived, stress-induced RNA
tRNA	Transfer RNA
UTR	Untranslated region

1 Introduction

The "Big Bang" moment of the G-quadruplex field happened all the way back in 1900, when it was reported that concentrated guanylic acid solutions tend to form a gel [1]. Much later, Gellert et al. were the first to show that the guanines in these gels adopt a helical structure that is now known to be the G-quadruplex [2]. This peculiar structure, first thought to be only a laboratory curiosity, is now regarded as a crucial regulatory motif that is implicated in multiple biological functions.

Guanine-rich DNA and RNA sequences can fold into the G-quadruplex structure. The former were the first discovered and they have been more extensively studied. The latter are the subject of this chapter. RNA quadruplexes tend to be more stable than their DNA counterparts and have less topological diversity [3]. Indeed, RNA G-quadruplexes almost exclusively adopt a parallel conformation in which the four strands all have the same directionality. This observation is explained by the fact that the 2′-hydroxyl group of the ribose locks the RNA in an *anti* conformation, which favors this parallel topology [4].

Balasubramanian's group was the first to report a biological role for an RNA G-quadruplex. They showed that a guanine-rich sequence located in the 5′UTR of the NRAS oncogene's mRNA can fold into a G-quadruplex structure and repress the mRNA's translation [5]. Subsequently, many biological functions have been attributed to RNA G-quadruplexes [6]. Furthermore, many G-quadruplex-forming

sequences have been found to be conserved over a large number of species [7, 8]. Recently, compelling evidence of the in vivo folding of RNA G-quadruplexes was provided using a structure-specific antibody [9]. This chapter aims to review both the current knowledge and the recent discoveries in the field of RNA G-quadruplexes. It also addresses the perspectives and challenges involved in the study of these structures.

2 Definition of a G-Quadruplex

The guanine base possesses two proton acceptor groups located on its Hoogsteen face, and two proton donor groups located on its Watson–Crick face (Fig. 1a) [10]. These features enable two guanines to interact in a Hoogsteen base pair through the formation of two hydrogen bonds. As the two guanines are involved in the base pair through only one of the two faces, they can still interact with another guanine to complete a planar cycle composed of four guanines linked through a total of eight hydrogen bonds [2]. This planar interaction is called a G-quartet and is usually not observable in solution because the partial negative charges of the oxygen atoms located in its center destabilize the whole structure. The formation of a G-quartet in solution requires the presence of a monovalent cation in its center to offset this partial negative charge (Fig. 1b). A cation of the appropriate radius forms an electrostatic interaction with the oxygens, increasing the stability of the structure. Among the cations found naturally in cells, potassium possesses the most appropriate radius in addition to being the most abundant intracellular cation.

Further stabilization is possible via π–π stacking of the guanines' aromatic cycles, which results in the formation of a tetrahelical structure [2]. This tetrahelix can be formed by several nucleic acids as an intermolecular G-quadruplex, or within a single nucleic acid string as an intramolecular G-quadruplex (Fig. 1c). The folding of an intramolecular G-quadruplex requires the presence of series of consecutive guanines which build the four strands of the helix. These four guanine series are spaced by three loops of variable lengths and nucleotide compositions. According to the classical definition, the loops lengths vary between 1 and 7 nucleotides (Fig. 1d).

This definition of a G-quadruplex permitted the evaluation of the number of potential G-quadruplexes present in the human genome using Quadparser, software that functions solely by pattern research [11]. This first estimation reported the presence of more than 376,000 potential G-quadruplexes, and had a large impact on the investigation of G-quadruplexes within human genomic sequences. The QGRS mapper software soon followed and provided greater flexibility in the definition of the motif. It also implemented a score which represents the sequence's likelihood of folding into a G-quadruplex [12]. This tool found the number of potential G-quadruplexes within human pre-mRNA sequences to be between 197,000 and 2,391,000 depending on the stringency of the parameters used by the algorithm [13].

Recently, a novel high throughput method of DNA G-quadruplexes detection revealed that the first estimation of the number of potential genomic G-quadruplexes was underestimated by about twofold. Precisely 716,310 distinct

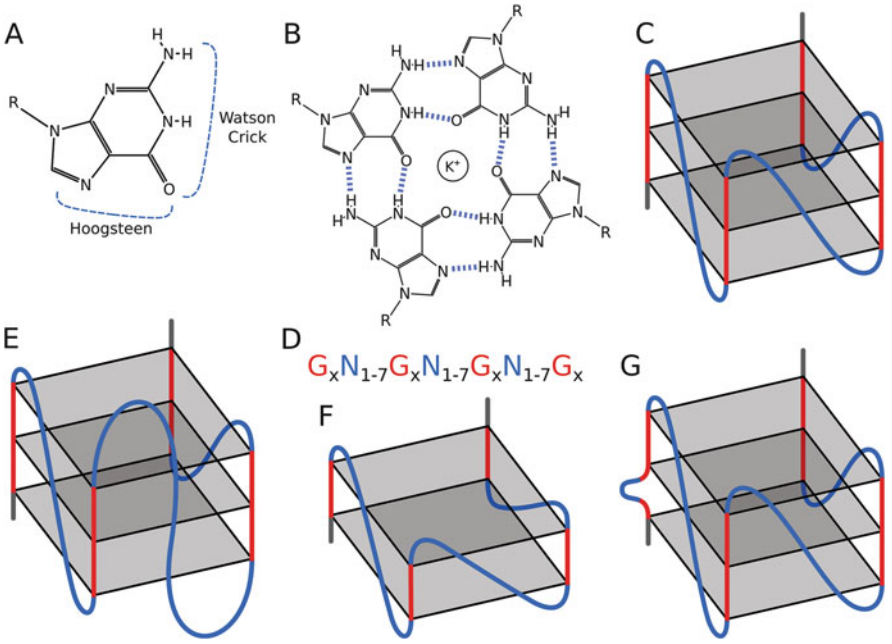

Fig. 1 The G-quadruplex structure. (**a**) Guanine base and its pairing faces. (**b**) The G-quartet. (**c**) Classical G-quadruplex. (**d**) Description of the motif originally used to find G-quadruplexes where $x \geq 3$. (**e**) G-quadruplex with a long central loop. (**f**) Two-tiered G-quadruplex. (**g**) Bulged G-quadruplex

G-Quadruplex structures were detected [14]. This discrepancy is mostly explained by G-quadruplex structures that do not respect the motif searched for by a variety of prediction software. Because such unusual structures have also been observed in RNA, the RNA G-quadruplexes estimation is thought to be an underestimation as well.

Numerous RNA G-quadruplex topologies have been reported with various combinations of guanine series and loop lengths that do not fit the previously described motif. The maximum limit of seven nucleotides per loop was shown to be incorrect, as very large second loops combined with loops one and three (which are limited to just one nucleotide each) have been observed (Fig. 1e) [15, 16]. These large loops were shown neither to be specific to the second loop nor to hinder the presence of another large loop, at least in vitro [16]. The minimum of three G-quartets implied in the motif is also erroneous. A two-quartet G-quadruplex (Fig. 1f) was shown to fold in the 5′UTR of the KRAS mRNA [17]. Additionally, G-quadruplexes that exhibit a nucleotide bulging out of a strand, which requires this nucleotide to be located within a guanine track, have been reported (Fig. 1g) [18]. An unexpected artificial G-quadruplex was observed with non-guanine bases involved in the formation of a quartet, thus widening the number of sequences that can potentially fold into G-quadruplexes [19, 20]. These multiple topologies,

and the related work on DNA suggesting more structures, such as those involving triads [21], harden the challenge of identifying putative RNA G-quadruplexes.

Efforts have been made to accommodate the changing definition of what constitute a G-quadruplex in the predictions of the structure in various nucleic acids. The QGRS mapper has options to search for two quartet G-quadruplexes, as well as sequences possessing a bulge in one of the guanine series, to reduce the number of false negatives [12]. However, being more permissive increases the rate of false positive predictions and few solutions have been developed to address this problem. To provide greater confidence in its predictions, a semi-global aligner was included with the QGRS mapper in the QGRS-H predictor [22]. A potential G-quadruplex is likely to be observed if it possesses a function which should be conserved across species. The consecutive guanosine/consecutive cytosine (cG/cC) scoring system uses a different strategy by offering the user a value that reflects the presence of consecutive guanines as compared to that of consecutive cytosines. A guanine-rich environment with a low presence of cytosine is favorable to G-quadruplex formation although a balance between the two might favor Watson–Crick structures [23]. A similar strategy was used to develop G4Hunter, a program which was tested on both RNA and DNA sequences. It estimated that the number of potential G-quadruplexes in the human genome should be revised up by two- to tenfold [24]. These two tools improved G-quadruplex prediction by considering the regions flanking the potential structure.

The best strategy for identifying potential G-quadruplexes is still up for debate. The motif search provides good control of what should be considered a hit to the user. However, it is dependent on a constantly changing definition, and is therefore not appropriate for identifying new topologies of G-quadruplexes. Scoring systems favoring guanine richness correlate well with the presence of G-quadruplexes and permit the users to set their own threshold to fit their needs. However, they do not evaluate the sequence for the presence of the minimal requirements that would support intramolecular folding, and thus they promote the generation of false positives. All strategies were based on experts' observations which seem to contain a bias toward a sub-group of all possible G-quadruplexes. Fortunately, this challenge is recognized by the research community. Both the large amount of work being done and the increasing interest the field is generating should provide the volume of data (experimentally tested sequences) required to infer rules and/or patterns in the coming years. As soon as a sufficient amount of data is available, various technologies should be considered in order to learn from it. Motif detection software should obviously be used, but more advanced tools, such as computationally automated techniques, should also be considered. The latter are known to retrieve subtleties and interrelations between features that are barely noticeable by manual analysis, and are suited for use with both genomic and transcriptomic data [25].

Even though the core structure of a G-quadruplex has been thoroughly described, sequences folding into a G-quadruplex are still in need of an inclusive definition. Such a definition is almost within our grasp for intramolecular G-quadruplexes, but they represent only a fraction of all of the G-quadruplexes

present in a cell. There have been reports of intermolecular G-quadruplexes involving multiple RNA molecules [26, 27]. Additionally, there have been observations of hybrid G-quadruplexes resulting from the interactions of the RNA and DNA molecules located in both telomeres and transcription forks [28, 29]. These intermolecular G-quadruplex structures force investigators to broaden the definition of what constitutes a G-quadruplex, adding another layer of complexity to the structure prediction problem. Another key challenge is to integrate G-quadruplex structure into RNA secondary structure folding software. To our knowledge, the ViennaRNA package is the only one to date that offers this option [30]. However, it relies on pattern research similar to that of Quadparser which was found to be too restrictive. It supplies a calculated evaluation of the minimal free energy of the structure based on experimental observations [31]. This evaluation allows comparison with potential Watson–Crick structures. Double-stranded structures are favored according to its predictions, but its authors are aware that the energy function of the minimal free energy was simplified in the writing of the program. It should be revised when more thermodynamics data are available, especially when considering the G-quadruplexes that do not possess three quartets, or those possessing asymmetrical loops.

3 Regulatory Roles of RNA G-Quadruplexes

RNA G-quadruplex functions have been identified at many levels of the post-transcriptional regulation of mRNA, from its transcription to its translation. Furthermore, the prediction and the identification of this structure in other families of RNA, such as miRNA, long non-coding RNA and TERRA, to name just a few, suggests that G-quadruplexes could be involved in all RNA-associated processes. This section presents an overview of the demonstrated roles of RNA G-quadruplexes, with emphasis on the most recent discoveries and on some perspectives on the global role of this regulatory motif. For more details about each G-quadruplex's functions, readers are invited to consult two recently published reviews on the subject [6, 32].

DNA G-quadruplexes located in promoters have been shown to act as transcriptional repressors [33]. Recently, Zheng et al. demonstrated not only the co-transcriptional formation of hybrid intermolecular RNA:DNA G-quadruplexes between the nascent transcript and the template DNA strand but also that these hybrid G-quadruplexes repress transcription [29]. A subsequent bioinformatic survey by the same group showed that these motifs are both conserved and highly abundant [34]. However, the best characterized roles of RNA G-quadruplexes are described in the last steps of the mRNA transcription, more specifically the termination [35] and the 3′ end-processing of the pre-mRNA [36–38]. RNA:DNA hybrids were also found to be involved in the mitochondrial transcription termination steps [39]. Overall, both RNA G-quadruplexes and RNA:DNA hybrid G-quadruplexes can be considered as *cis* regulatory elements that affect both the

transcription termination and the 3′ end processing steps of polyadenylation site recognition and cleavage.

It is estimated that 92–94% of all mRNA transcripts undergo alternative splicing, creating a diversity of transcript isoforms with different roles, locations, and degrees of expression [40]. The first example of a G-quadruplex affecting alternative splicing was found in the human telomerase RNA hTERT [41]. Subsequently, RNA G-quadruplex formation and stabilization with ligands has been shown to affect the splicing of many different mRNAs, some of which are important in certain cancers [42–48]. A recent high throughput study of G-quadruplex formation in the human genome showed a high density of G-quadruplexes located near splicing sites [14]. This suggests that the splicing of many more mRNAs could be regulated by this structure. It has also been demonstrated in vitro that RNA oligonucleotides corresponding to the excised intron 1 of VEGF-A, as well as those from the introns of both PDGF A and PDGF B, could not only form G-quadruplexes but also bind to the proteins they encode. This represents yet another example of the G-quadruplex regulation of RNA splicing [49].

The specific localization of mRNA in a particular cell compartment is essential prior to its translation. G-quadruplex structures located in the 3′UTRs of both the PSD-95 and the CaMKIIa mRNAs were shown to be recognized by protein cofactors, and to be used as a signal to direct them to the neurite [50]. This aspect has been reviewed elsewhere, and lately the protein TDP-43 has been recognized as being important for the G-quadruplex mediated mRNA targeting in neurons [51, 52].

The regulation of translation is probably the most studied role of RNA G-quadruplexes. In a general way, RNA G-quadruplexes located in the 5′UTR are known to repress cap-dependent translation [15, 32, 53–57]. Conversely, there are some exceptions in which the presence of a G-quadruplex actually enhances the translation, either by being part of an IRES structure [58, 59] or by an as yet unknown mechanism [60, 61]. G-quadruplexes located in the 3′UTR have also been shown to reduce translation [62, 63]. The mechanism of the 5′UTR G-quadruplex repression of translation has not been fully determined, but two major possible mechanisms have been proposed. First, because of their high stability, they may either stall or impair the ribosome scanning of the 5′UTR. Second, they may impair the recognition of the cap structure by the eIF4e initiation factor. Further studies are also needed to decipher the exact molecular mechanism of the translational repression by G-quadruplexes located in the 3′UTR. They could recruit translation protein co-factors, affect the miRNA mediated regulation, or even influence the location of the mRNA transcript. G-quadruplexes present in the coding regions of mRNA may also impair translational elongation. Because of their high stability, they were shown to repress translation by acting as road-blocks to elongation [64, 65] and also to trigger ribosomal frame-shifting [66, 67]. The translational halt during elongation was also shown to affect the folding of the resulting protein and thus to affect its proteolytic cleavage [68]. Moreover, it has been shown that the halting effect in elongation is dependent on the G-quadruplex's exact position with respect to the open reading frame [69]. G-quadruplexes seem to be a common motif in

global translational regulation. It was shown that the inhibition of eIF4A, the helicase component of the translational initiation complex, leads to the translational repression of a subset of genes, all of which are enriched in G-quadruplex motifs [70]. Interestingly, a lot of these genes were already known for their implications in various cancers. Understanding of the exact molecular mechanisms of G-quadruplex translational regulation is an essential problem to solve as more and more G-quadruplex motifs are identified and selected as potential regulatory targets. Interestingly, the regulation of translation by G-quadruplexes is well-conserved among many species, as even prokaryotes use a similar mechanism. A method has been developed to study the impact of RNA G-quadruplex motifs on mRNAs translation in *Escherichia coli* [71]. It showed that naturally occurring *E. coli* G-quadruplex motifs proximal to the ribosome binding site could affect translation [72]. Undoubtedly, RNA G-quadruplex formation could also have a tremendous impact on bacterial gene regulation and requires further investigation.

Telomere regions are highly enriched in DNA G-quadruplexes. Nevertheless, these chromosomal extremities are transcribed into the heterogeneous non-coding RNA called TERRA (telomeric repeat-containing RNA). They play a crucial role in the regulation of telomerase activity. Their sequences, multiple repeats of UUAGGG, have been shown to fold into intramolecular G-quadruplexes [73] and to form dimeric DNA/RNA hybrid G-quadruplexes [28]. Many proteins important for telomere maintenance bind to TERRA via the G-quadruplexes [74, 75]. TERRA can cause genome-wide alterations of gene expression in cancer cells [76]. Furthermore, the telomerase, the enzyme responsible for the elongation of these terminal sequences, uses an RNA template called human telomerase RNA (TERC or hTR), which was also shown to possess a G-quadruplex structure [77]. The latter was found to be unwound by the RHAU helicase (also known as DHX36) [78]. In summary, RNA G-quadruplexes play a central role in telomere homeostasis, thus making them a good potential therapeutic target as the telomerase is active in most cancers [79].

Putative G-quadruplex sequences have been found within long non-coding RNAs (lncRNA). Most of these were RNAs 200–300 nucleotides long and harbored G-quadruplex motifs possessing short loops of only 1 or 2 nucleotides, which are known to be very stable [80]. In addition, co-immunopurification and RNA binding assays revealed that RHAU helicase interacts with lncRNA BC200 [81]. By itself, BC200 does not form a G-quadruplex, but it has been shown to bind the unwound G-quadruplexes of the human telomerase RNA (hTR). These studies show that some lncRNAs are predicted to adopt G-quadruplex structures that are stable enough potentially to fold in vivo, and that different lncRNAs and helicases could regulate the folding of G-quadruplexes in other RNAs.

Both the RNA silencing mechanism and small non-coding RNAs have been shown to be crucial in the biology of the cell. MicroRNA (miRNA; 20–22 nucleotides) silence mRNA expression, and their biogenesis involves the transcription of a primary miRNA which is then cleaved in the nucleus by Drosha, creating a precursor miRNA (pre-miRNA). The pre-miRNA is subsequently exported to the cytoplasm and is further processed by the ribonuclease Dicer, at the extremities of

its characteristic stem-loop, to create the mature miRNA. G-quadruplexes have been found in pre-miRNAs [82, 83]. It was shown that the G-quadruplex structure is in equilibrium with the stem-loop, and that the G-quadruplex impedes the Dicer cleavage, thus reducing the amount of mature miRNA. Moreover, it was demonstrated that the G-quadruplex present in close proximity to the miR-125a binding region in the 3′UTR of the target mRNA PSD-95 modifies its accessibility [84]. In addition, the proteins Lin28 and Nucleolin were shown to bind RNA G-quadruplexes in miRNA and mRNAs and to modulate their folding [85, 86]. It was recently shown that a mature miRNA sequence can fold into a G-quadruplex and that addition of stabilizing ligand prevents the miRNA from binding to its target [87]. G-quadruplexes were also reported in Piwi-RNA, a class of 23–30 nucleotides small non-coding RNAs involved in the silencing of retrotransposon elements in germline cells. In this case, with their protein partner, they appear to be both important landmarks and binding sites for a helicase involve in Piwi-RNA processing [88]. Finally, G-quadruplexes were also found in tiRNAs, which are produced by the cleavage of mature tRNA in the anticodon loop by the ribonuclease angiogenin [89]. The resulting 5′ fragments exert cytoprotective functions in cells by repressing translation and by triggering the formation of stress granules. In brief, G-quadruplex formation affects both the biogenesis and the functions of many kinds of small non-coding RNAs. Complementary information can be found in this review [90]. It could be interesting in the future to be able to predict their formation and to study their diverse roles in all processing intermediates, as well as in other RNA families such as small nuclear, small nucleolar, and ribosomal RNA.

G-quadruplex-forming sequences have been found in the genomes of the human immunodeficiency virus (HIV-1), the Epstein–Barr virus (EBV), papillomaviruses (HPV) [91], herpes simplex viruses (HSV) [92], and, more recently, the hepatitis C virus (HCV) [93]. In the case of HIV, G-quadruplexes have been identified at both the DNA and RNA levels. The formation of dimeric RNA G-quadruplexes from two G-rich regions of the viral genome is important in its replication [94]. In EBV, the level of EBNA1 protein expression is regulated by the presence of a G-quadruplex in its mRNA [95]. In HPV, whose proteins are coded in an overlapped fashion, the formation of RNA G-quadruplexes could affect the alternative splicing essential to their expression [91]. HSV possesses a highly-G-rich genome and was shown to fold into DNA G-quadruplexes [92]. Even if RNA G-quadruplexes have not yet been reported for this virus, unusual G-rich RNA structures have been observed, and the presence of RNA G-quadruplexes important for virus regulation is plausible [96]. Recently, Wang et al. identified a conserved G-rich sequence located in the core gene of HCV [93]. This sequence could fold into a G-quadruplex and be targeted by G-quadruplex ligands, which results in the repression of the viral replication at both the RNA and the protein levels. Overall, G-quadruplex formation in viruses seems to be very important for both their replication and their gene expression, and is considered a promising therapeutic target going forward [97].

In light of these findings, it seems that most if not all aspects of RNA biology can be regulated by G-quadruplexes. As new classes of RNA, or new roles for known

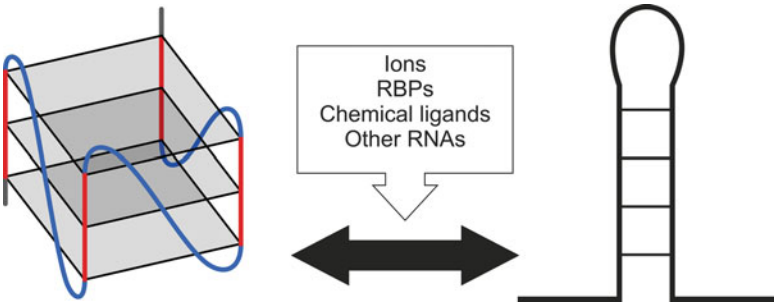

Fig. 2 Modulation of G-quadruplex folding. Different modulators used by either the cell or researchers in order to influence the folding balance between G-quadruplex and canonical structures

RNAs, are discovered, additional layers of G-quadruplex mediated regulation are sure to follow.

4 Modulation of G-Quadruplex Folding and Its Biotechnological Applications

RNA folding is a dynamic process. One given sequence can fold into several distinct structures. Therefore, in a cell, the multiple copies of an RNA molecule can reach equilibrium between alternating structures. Given that these changes can affect the RNA's function, the balance between the alternate structures can be used both to modulate and to fine-tune the function of a given RNA (Fig. 2). Many factors can change RNA folding, including temperature, ionic concentrations, and the binding of a metabolite, a protein, or another RNA molecule, thus adding another layer to RNA regulation (reviewed in [98]).

Competition between canonical Watson–Crick and G-quadruplex folding is a well-documented phenomenon [23, 53, 82, 83, 99]. It has been shown that the presence of consecutive cytosines in the vicinity of the G-runs prevents quadruplex folding [23, 53]. Conversely, in the past year, two groups have independently shown that the presence of G-runs in pre-miRNAs leads to quadruplex folding and prevents processing by Dicer [82, 83]. The competition between a hairpin and a G-quadruplex structure was studied at various Mg^{2+} (which favors the hairpin) and K^+ (which favors the G-quadruplex) concentrations, and showed that, under physiological conditions, the quadruplex was the main form present [99]. However, it should be noted that only one sequence was tested, and that dissimilar hairpins or G-quadruplexes might have different relative stabilities and thus behave oppositely. Furthermore, in some precise situations, such as neuronal polarization/depolarization, K^+ concentrations can vary greatly. As G-quadruplexes are known to be important for the specific localization of some mRNAs to the neurite, some have

speculated that the G-quadruplexes are used as a K^+ concentration sensor and thus regulate both mRNA location and translation according to neural activity [50]. This interesting hypothesis remains to be tested. Another interesting case occurs in plant cells, where K^+ deficiency leads to major changes in the expression levels of multiple genes [100]. As several plant genomes contain putative G-quadruplex sequences [101–104], it is tempting to assume that these G-quadruplexes could play a role in the K^+ homeostasis in plants, but further studies are required to confirm this.

Another manner in which the cell can regulate RNA folding is to use RNA-binding proteins (RBPs). Many RBPs have been shown to bind to G-quadruplex structures [49, 57, 65, 105]. Some were shown to unwind RNA G-quadruplexes, including RHAU [106], lin28 [85], DHX9 [107], and eIF4A [108]. Among these, RHAU is the best characterized [78, 109–111]. Many other RBPs/G-quadruplex interactions are notably less well-studied. Furthermore, given the high number of both RBPs and G-quadruplex-forming sequences, it is highly probable that much of the RBPs/G-quadruplexes interactome remains unknown. With the advent of high throughput screening technologies, more interactions are still to be discovered. Co-immunoprecipitation, followed by RNA deep sequencing, can lead to the discovery of new RNA targets for known G-quadruplex binding proteins [81, 112]. On the other hand, G-quadruplex-forming RNAs can be used to pull down proteins which can then be identified by mass spectrometry [49, 55]. By knowing its binding partners, researchers can gain new insights into either an RBP's or an RNA G-quadruplex's functions. It is then conceivable to affect G-quadruplex-related functions by targeting the desired RBPs. In this way, a subset of G-quadruplexes involved in a particular pathway could be targeted via the right RBP.

Many chemical ligands possessing a high affinity for G-quadruplex structure have been developed. They also possess high selectivity toward the quadruplex over other nucleic acid structures [113]. As G-quadruplexes are enriched in proto-oncogenes, some of these ligands are considered to be good therapeutic candidates with which to treat various cancers [114]. However, the selectivity of a ligand for a given subclass of G-quadruplexes, including the selectivity for RNA over DNA and even for a single sequence, is the greatest challenge in the field of G-quadruplex ligands as off-target effects remain an issue [113]. Another problem is the fact that these ligands could induce G-quadruplex folding in sequences that would not adopt this structure under normal conditions [14, 115].

Alternatively, engineered proteins can be used to bind and study G-quadruplexes. A G-quadruplex-specific antibody has been used to visualize these RNA structures in live cells [9]. In another study, the RNA binding domain of the TLS protein was used to create two distinct proteins that were able both to discriminate and to specifically bind to the hTelo and the TERRA G-quadruplexes, the DNA and RNA telomeric sequences, respectively. This enabled the authors to study the distinct effects that these two G-quadruplexes have on telomeric DNA chromatin's status [116]. As more quadruplex binding proteins are found, and the development of protein engineering continues [117], more of these valuable tools should become available for the study and targeting of RNA G-quadruplexes.

Antisense oligonucleotides (ASO) are yet another tool that can be used to target G-quadruplexes. Their main advantage is their specificity, as they recognize their target via Watson–Crick base pairing. Moreover, they have a great plasticity, as many chemical modifications of the ASO backbone and/or sugar moieties can be used to alter the pharmacokinetics properties, stability, specificity, and affinity of the ASO, so as to suit specific needs [118]. Besides, ASO can be used to modulate G-quadruplex folding in both directions. ASO can target guanine stretches and prevent quadruplex folding, thus leading to enhanced translation of specific mRNAs [95, 119]. Alternatively, ASO can target G-quadruplex neighboring sequences, thus interfering with competing canonic structures and enhancing quadruplex folding [119]. Other ASO can invade existing quadruplexes and form hetero G-quadruplex structures, leading to a decrease in mRNA translation [120]. Some can even form a hetero quadruplex with sequences that do not fold into G-quadruplexes on their own, leading to either a decrease in translation [121] or a hindrance of viral replication [122]. Differences in both the length and the chemical composition of the ASO, as well as the targeted sequence, can lead to different outcomes [123]. Further optimization of these parameters should lead to the generation of efficient ASO possessing a wide range of effects. The major hurdle preventing the use of ASO as therapeutic agents is their delivery to specific cells. Fortunately, a lot of research has been done on this problem for siRNAs [124–127], and the lessons learned there can be useful for the delivery of ASO.

It is also possible to use more than one quadruplex binder at a time [128]. Future research should focus on the creation of chimeric ASO/chemical ligands/protein compounds that can combine the different quadruplex binders' respective strengths, compensating for their respective weaknesses. Bifunctional oligonucleotides composed of an antisense portion that determines target specificity, and a non-hybridizing tail that recruits proteins or RNA/protein complexes, have been successfully used to modulate specific mRNA splicing [129]. A similar strategy could be used with a quadruplex folding tail to recruit quadruplex binding proteins to different targets and thus regulate different biological processes. ASO could also be fused to quadruplex ligands in order to enhance their specificity. Given the high prevalence of quadruplex-forming sequences, and their implication in a plethora of biological functions, the imagination and creativity of researchers seem to be the only limits to the potential usefulness of G-quadruplex modulation.

Clearly, there are many ways to manipulate the equilibrium between G-quadruplexes and other secondary structures. One great technical challenge that remains is to find a way to quantify the proportion of a given RNA that folds into each of its various structures in solution, or even within a cell.

Additionally, RNA G-quadruplexes are important in other biotechnological applications. For example, two different RNA aptamers designed for binding and activating ligand fluorescence, namely RNA mango [130] and RNA spinach [19, 20], were both shown to bind to their respective ligand with a G-quadruplex structure located within their binding pockets. Interestingly, many known DNA and RNA aptamers fold into G-quadruplex structures [131, 132]. This exciting field is

sure to develop in the near future as more knowledge on quadruplexes becomes available.

5 Conclusion

The RNA G-quadruplex field has been expanding quickly in the last few years, but these fascinating structures have yet to reveal all their secrets. As more and more tools become available for their study, more functions can be identified. In parallel, the number of possible applications and biotechnological tools utilizing them continue to grow. RNA G-quadruplexes are sure to keep scientists from many fields busy for years to come.

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