Mini-Reviews in Medicinal Chemistry, 2016, *16*, 1321-1329

REVIEW ARTICLE

G4 Aptamers: Trends in Structural Design

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ARTICLE HISTORY

Received: July 15, 2015
Revised: January 07, 2016
Accepted: March 21, 2016

DOI:
10.2174/1389557516666160321114715

Abstract: Many potent DNA aptamers are known to contain a G-quadruplex (G4) core. Structures and applications of the majority of such aptamers have been reviewed previously. The present review focuses on the design and optimization of G4 aptamers. General features of bioactive G4s are analyzed, and the main strategies for construction of aptamers with desired properties and topologies, including modular assembly, control of an aptamer folding and some others, are outlined. Chemical modification as a method for post-SELEX G4 aptamer optimization is also discussed, and the effects of loop and core modifications are compared. Particular attention is paid to the emerging trends, such as the development of genomic G4-inspired aptamers and the combinatorial approaches which aim to find a balance between rational design and selection.

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Keywords: Aptamers, chemical modification, combinatorial approaches, drug design, G-quadruplexes, structure-activity relationship.

1. INTRODUCTION

Aptamers are relatively short native or chemically modified nucleic acid fragments that bind with high affinity and specificity to targets such as peptides, proteins, small molecules, viruses, *etc*. Traditionally, aptamers are selected from randomized oligonucleotide (ON) libraries via Systematic evolution of ligands by exponential enrichment (SELEX) [1]. However, the term 'aptamer' is also used to denote target-recognizing oligonucleotides that were obtained by other methods (random screening; functional analysis of genomic nucleic acid structures or rational design based on *in silico* modeling). Aptamers usually fold into well-defined secondary structures that are stabilized by Watson-Crick or noncanonical heterocycle interactions.

G-quadruplexes (G4s) are noncanonical nucleic acid structures composed of stacked planar guanine tetrads. They are present in many potent aptamers (DNA in particular) that have been selected against a broad range of proteins. The specific affinities of G4 structures for various unrelated classes of targets on one hand and the general predominance of such structures in the DNA aptamer pool on the other are conditioned by the polymorphism of quadruplex topologies. G4s differ in the number of tetrads; glycosyl conformations (syn *vs.* anti) and relative strand directions in the G4 core; composition and orientation of loops (fragments that connect G-runs) and inter- *vs.* intra-molecular nature [2].

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Importantly, the definition of G4 structures is being constantly broadened, and our current view of G4 diversity may turn out to be an underestimation. New types of quadruplex architectures, such as G4s with bulges [3], mixed G:A:G:A, G:T:G:T and G:C:G:C tetrads [4, 5] or G-vacancies [6], as well as 'interlocked' or 'intertwined' G4 dimers [7] have been recently reported. In some G4 aptamers, unusual structural elements (A-tetrads sandwiched between G-tetrads [8], A:G4 pentads [9] and others) have been observed. G4 interlocking has been considered as a basis for the assembly of higher-order aptamer structures [10].

Structures and applications of the majority of known G4 aptamers are reviewed in [11] and [12]. Several more bioactive G4s and G4-aptamer-based sensing platforms have been reported in the last several years [13-18]. The present review focuses on the rational design and optimization of G4 aptamers. The conventional SELEX procedure and relating methods, such as Cell-SELEX, CE-SELEX, Counter-SELEX, *etc*., are regarded as a general platform and are not discussed. We analyze here how structure-activity relationships, combinatorial approaches and the use of various chemical modifications enable the construction of potent aptamers on the basis of active G4s selected initially from randomized ON libraries.

2. GENERAL FEATURES OF BIOACTIVE G4s AND THE IMPLICATIONS FOR RATIONAL DESIGN

G4 aptamers are conformationally diverse and include all basic types of quadruplex structures: parallel, antiparallel

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and mixed. In some cases the exact topology has yet to be clarified [19].

The thrombin inhibitor TBA15 [20], one of the most celebrated DNA aptamers that spurred interest in G4s in the 90s and launched an ongoing series of studies, is a typical example of an intramolecular antiparallel quadruplex, characterized by the opposite polarities of G-tetrads, lateral loops and opposite strand directions in the G4 core. Although TBA did not find it clinically useful as an anticoagulant, it is still extensively used as a model antiparallel G4 in fundamental, methodological and 'proof-of-concept' works [21-23].

A significant number of cancer-selective cell-binding aptamers - potential drug carriers and tumor-imaging tools - adopt the parallel G4 topology with co-directed strands in the core and side-reversal (propeller) loops. One vivid example is the DNA aptamer to nucleolin - AS1411, which has reached phase II of renal cell carcinoma clinical trials [24]. Interestingly, the G4-forming potential appears to be essential for cancer-selective antiproliferative activity of the ONs. Bates *et al*. [25] investigated 11 combinatorial ON libraries with different nucleobase compositions and showed that G-rich libraries with structural and spectral (circular dichroism) features consistent with G4 formation strongly inhibit cancer cell growth while sparing non-malignant cells. The authors attribute this to the nuclease resistance, efficient cellular uptake and protein binding of G4s.

In another recent study, Shangguan et al. [26] confirmed the antiproliferative activities of DNA quadruplex aptamers and showed that the intramolecular parallel G4 structure may be a key feature that allows an ON to bind to cellular proteins and enhances cellular uptake through the endosome/lysosome pathway. This study was performed on a number of parallel G4 structures, including putative G4 sequences from the human genome.

One vivid example of an RNA aptamer family sharing a common G4 motif is Spinach and its analogs [27]. These RNA mimics of GFP have been adapted for use as imaging tools, live cell labeling tools and sensors [27, 28]. The unusual G4 structure in the Spinach core, comprised of two G-quartets stacked above a mixed-sequence tetrad, has recently been shown to serve as a platform for binding a GFP-like fluorophore and fluorescence activation [29]. This provided foundation for further progress in engineering Spinach-like light-up probes.

The above findings support the idea that structure-activity relationship analysis can be used to identify general G4 scaffolds for the design of specific anti-cancer aptamers, fluorophorogenic RNAs and other aptamers.

These are some of the common characteristics of potent G4 aptamers:

- T-rich quadruplex loops are rather frequent [20, 29-34], presumably because thymine is more thermodynamically favourable than bulky adenine, and its exposure to solution results in lower solvation entropy. C-rich fragments would induce the formation of Watson-Crick hairpins.
- The majority of aptamers are (3+)-tetrad G4s. Because structural stability is related to the biological activity of the aptamer, and the thermal stability of G4 is usually roughly proportional to the number of tetrads, extra tetrads are expected to be beneficial. However, this correlation is not strict, and the longer G-runs required for extra tetrads may alter the loop orientation, which often determines epitope exposure. For instance, the attempt to improve TBA15 by introducing an additional tetrad [35] resulted in a completely different G4 topology.
- Intramolecular quadruplexes are abundant and include aptamers to human thrombin, insulin and vascular endothelial growth factor [20, 36, 37]; HIV-1 nucleocapsid protein and reverse transcriptase [38, 39]; porphyrins, ATP and some other small molecules [40, 41]. Bimolecular structures composed of two DNA/RNA hairpins (for instance, aptamers to nucleolin and HIV-1 integrase [9, 42]) have also been reported; and the aptamers to HIV-1 glycoprotein gp120 are mostly tetramolecular [30, 43]. The intermolecular G4 arrangement is generally prone to conformational polymorphism and concentration-dependent rearrangements, which complicates the handling of such structures and limits their practical application.

3. DESIGN STRATEGIES

3.1. Controlling Aptamer Folding

To overcome the problems associated with intermolecular G4s, Piccialli *et al*. used the TEL-ODN strategy ('tetra-end-linked oligodeoxynucleotides') to obtain pseudo-monomolecular analogues of the previously reported tetrameric aptamers to gp120 [44, 45]. They joined the 3'-termini of the four 5'-end-substituted hexanucleotides, known as Hotoda's sequences [43], through a tetra-branched linker.

A somewhat similar strategy was proposed by Defrancq *et al*. [46]. Topologically constrained 'template-assembled synthetic quadruplexes' (TASQ) were obtained by using a functionalized cyclic peptide. Alternative templates, such as pyrene derivatives [47], have also been reported. Although the concept of template-assisted assembly has so far been considered only for constructing G4-based probes and systems for G4-ligand interaction assessment, it appears promising for broader applications.

The most recent strategy for precise control of G4 folding is based on 'hairpin' ONs [48]. Di Fabio et al. used Hotoda's hexanucleotides to design hairpin mimics that formed a bimolecular analogue of the tetrameric anti-HIV aptamer. The original idea behind the 'hairpin ON' strategy was that polarity inversion, which is required for the desired parallel G4 topology, can be achieved by linking the 3'-3' or 5'-5' termini of the hexanucleotides via a non-natural fragment (loop of the hairpin mimic), *e.g.*, a hexaethylene glycol (HEG) moiety.

In [45] and [48], mini-libraries of TEL-ODN-based or 'hairpin'-based G4s, respectively, differing in linker position

or type and terminal substituents, were synthesized and tested for the ability to inhibit viral entry. Several pseudo-mono/bi-molecular G4s demonstrated high stability and substantial anti-HIV activity. These studies can be regarded as examples of successful post-SELEX aptamer design using controlled G4 folding. The folding control strategies are illustrated in (Fig. **1**).

All of the above-mentioned strategies are aimed at ensuring unambiguous G4 folding, which is reasonable for many therapeutic applications. On the contrary, some nanotechnology-related applications, such as nanomotor engineering, require reversible G4 folding and unfolding. In this regard, G4-to-duplex transitions are in the spotlight. Known strategies for controlling G4-duplex equilibrium rely on the use of cations, ligands, temperature, electrical-field strength, *etc*. [49]. Photoregulation of conformational switches is gaining particular attention due to its usability and compatibility with physiological conditions [50]. The strategy is based on light-mediated isomerization of photochromic ligands (*e.g.*, azobenzene or its derivatives [51, 52]) or modified nucleobases in G4s [53] and guarantees high conversion efficiency even in the case of thermodynamically extra-stable G4s.

3.2. Modular Assembly and the Combinatorial Approach

Preliminary knowledge about structure-activity relationship for a series of ligands to similar targets enables researchers to use a combinatorial approach for aptamer design, *i.e.*, the combination of SELEX with various rational design methods. A promising strategy within the framework of the combinatorial approach is modular assembly. So far, modular assembly has been implemented mainly for thrombin-binding aptamers. The active G4 module can be coupled to an additional duplex module to improve the thermodynamic stability of the structure [54, 55]. Alternatively, polyvalent aptamers [56] can be obtained by conjugating similar G4 modules (homo-polyvalent aptamers) or different modules (hetero-polyvalent aptamers), that recognize distinct sites on a particular target protein [57-59] or distinct targets [60]. Rational design of a linker between the modules represents a separate challenge. Therefore, selection from combinatorial libraries with randomized linkers and constrained (intact) active modules can be used instead [61].

Aptamer selection from partially randomized G-rich libraries may also be advantageous if a particular G4 scaffold is associated with a certain activity (for instance, inhibition of viral entry for tetrameric parallel G4s [30, 43], or cancer-selective cell binding for monomeric parallel G4s [26]). However, in some cases, even minor variations in G4 sequences may have dramatic effects on aptamer stability and/or activity [14, 62-64], possibly due to altering G4 topology (*e.g.*, an intramolecular antiparallel G4 can be converted to the intermolecular parallel one [65]) or causing the aptamer to target a different epitope of the protein [66]. Thus, the extent of randomization is always a compromise between sequence variant coverage and G4-folding maintenance [67]. In the case of an intermolecular scaffold, the combinatorial libraries usually contain several consecutive guanosines (G-runs) with randomized flanks [30]. In the case of an intramolecular scaffold, the G4 core is constrained (kept intact), and loops are randomized.

The combinatorial approach is summarized in (Fig. **2**).

3.3. Chemical Modification

Unmodified nucleic acid aptamers *per se* are rather unstable in biological fluids due to enzymatic degradation. Chemical modifications that improve the biostability of nucleic acid aptamers in general are reviewed in [68] and [69]. Modifications in G4s are reviewed in [70] and [71]. Aptamers with non-natural fragments can be either selected from chemically modified libraries [72-73] or obtained by post-SELEX optimization. In this section, we focus on the

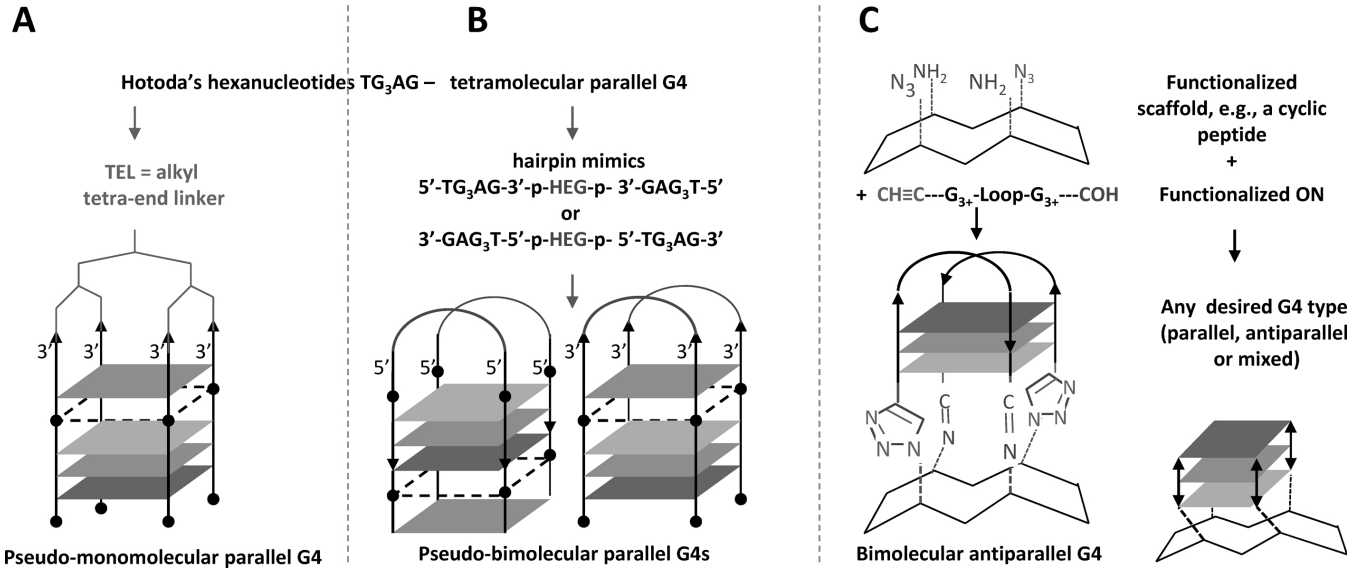

Fig. (1). Control of G4-folding. A: The 'TEL-ODN' strategy, based on the use of tetra-end linked ONs. **B**: The 'Hairpin ON' strategy, based on the use of hairpin mimics – ONs with inverted polarity. HEG is a hexaethylene glycol linker. **C**: The 'TASQ' strategy, based on template-assisted quadruplex assembly.

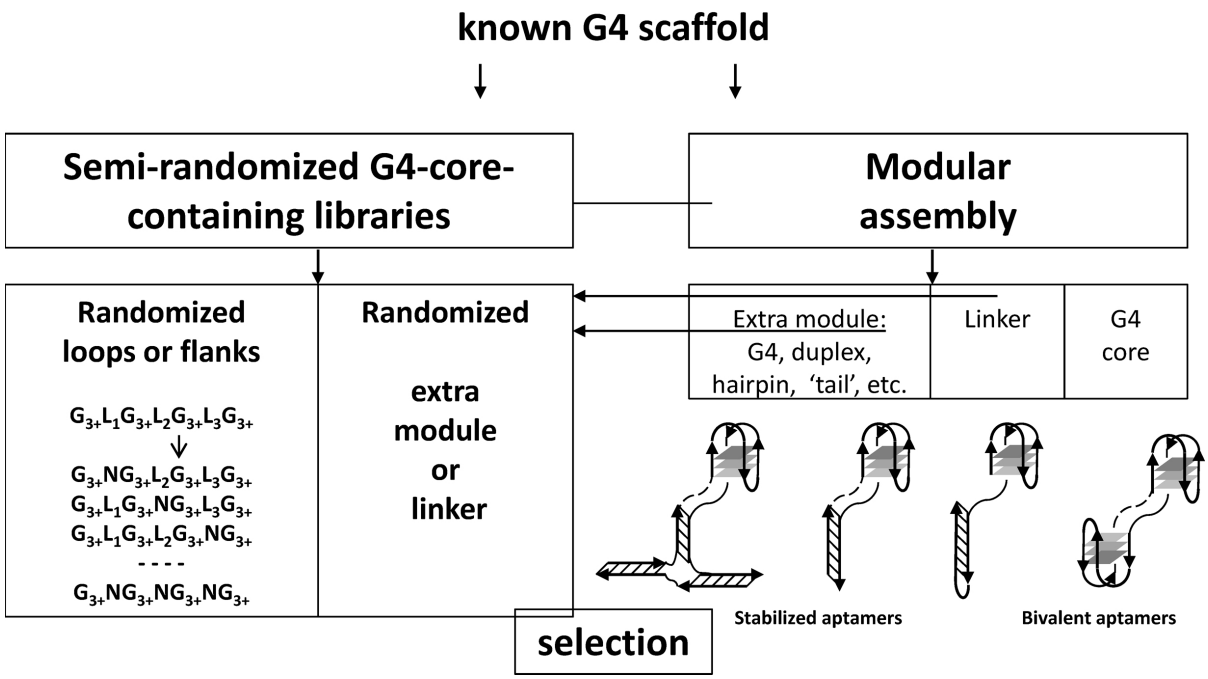

Fig. (2). Modular assembly of G4 aptamers and the 'partial randomization' design strategy. (Loop randomization is presented schematically. Primer binding sites are not shown).

latter method.

The G4 core provides protection from endonuclease digestion due to compact folding. Loops and strand termini are the primary positions for nuclease attack and, therefore, are commonly subjected to modification. In addition to nuclease resistance, chemical modifications may also endow such favourable properties as increased thermal stability [74], enhanced target affinity and binding specificity. We chose two representative examples (thrombin inhibitor TBA as a model intramolecular G4 and Hotoda's anti-HIV aptamer as a model intermolecular G4, Fig. **3**) to illustrate how terminal substitutions, loop modifications and certain core modifications are typically used to improve aptamer resistance to nucleases and general efficiency.

Hotoda's 5'-dimethoxytrityl-substituted hexamer TG_3AG, which forms a tetramolecular parallel G4 with four G-tetrads and one internal A-tetrad [8], was shown to inhibit HIV-1 entry through gp120 binding [43]. The 5'-terminal hydrophobic moiety contributed significantly to aptamer activity, presumably because it facilitates intermolecular G4 folding and participates in aptamer-protein interactions. Hotoda's findings inspired several research groups to further optimize the TG_3AG aptamer, and a large number of modifications have been described. 3'-Terminal 2-hydroxy-ethylphosphate groups [75, 76] and monosaccharide (glucose or mannose) residues [77], as well as 5'-terminal 3,4-dibenzyloxybenzyl [75, 76], tert-butyldiphenylsilyl [78] groups and some other aromatic groups [79] were shown to enhance aptamer activity. Bulky pyrene derivatives at the 5'-terminus altered G4 folding and led to activity loss [80]. Modifications of the G4 core were also reported [80-81]. Such modifications are usually meant to improve thermal stability and/or impart additional structural rigidity by constraining the aptamer in a particular conformation associated with activity. In this regard, the introduction of LNA residues ('locked' nucleic acid fragments) appears reasonable. Substitution of all guanosines except for the 5'-terminal one and substitution of dA and the next-neighbouring dG with LNA analogues in Hotoda's sequence resulted in highly efficient HIV inhibition [80].

The other aptamer for which the effects of chemical modifications have been studied over last twenty years is the antiparallel 2-tetrad G4 TBA15 (GGTTGGTGTGGTTGG), which has two TT loops and the central TGT loop. Most of the TBA derivatives are reviewed in [82], and some additional modifications have been reported in the last two years [83-87].

Much like Hotoda's aptamer, TBA and its complex with thrombin have been characterized extensively. However, some aspects, including the stoichiometry of binding, are still a subject of debate. According to the most recent X-ray diffraction studies of the TBA-thrombin complex, the TT loops are the primary binding epitopes, and they have polar contacts (T4 and T13) and hydrophobic contacts (T3 and T12) with amino acid residues in the fibrinogen binding site (exosite 1) of thrombin [88-89], whereas the TGT loop may interact with the heparin binding site (exosite 2) [90]. Exosite 1 is the major target of TBA, and in the case of 1:1 binding, the TT loops are shielded by the protein, while the central loop is exposed and prone to nuclease degradation. For this reason, the central loop is commonly modified to improve TBA biostability. TBA analogues with enhanced stability, activity or both, containing 2′-deoxyisoguanosine, isothymidine, 2′-deoxy-2'-fluoroarabinonucleotides, 4-thio-2′-deoxyuridine, 5-hydroxymethyl-2'-deoxyuridine, and

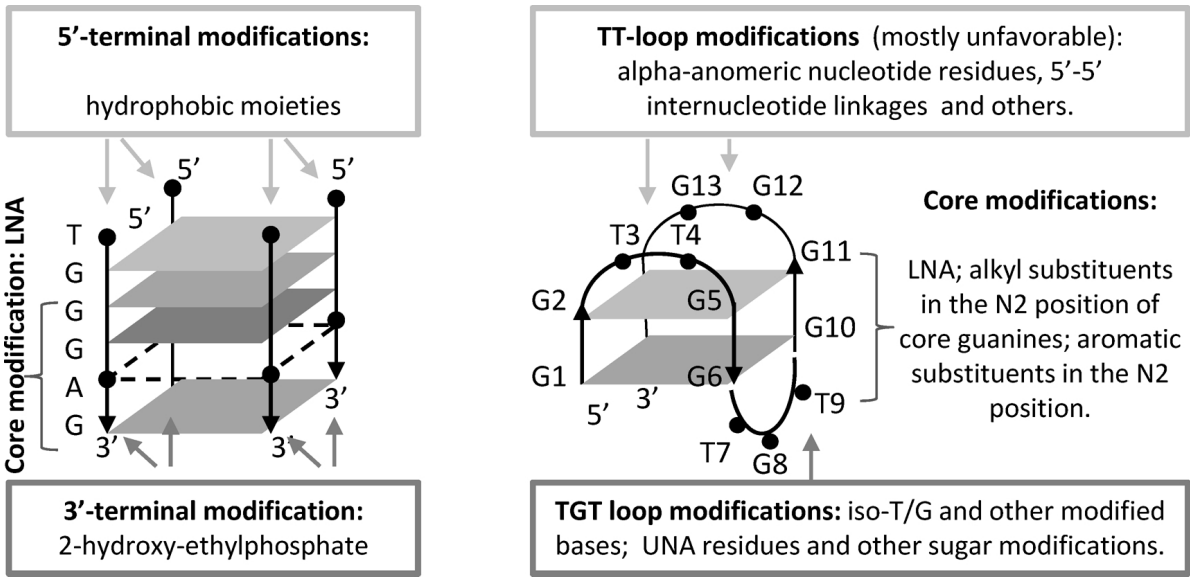

Fig. (3). Post-SELEX chemical modification of the G4 inhibitors of HIV-1 (left) and human thrombin (right). LNA = locked nucleic acid monomer (2′-*O*, 4′-*C*-methylene-linked bicyclic ribonucleotide); UNA = unlocked nucleic acid monomer (2'-3'-seco-nucleotide).

UNA (unlocked nucleic acid) residues in the TGT loop have been described [83, 87, 91-94].

The G4 core can also be optimized to improve thermal stability, which supposedly correlates with the inhibitory activity of the aptamer. Beneficial modifications include the introduction of a benzyl group into N2 of G6 and G11; naphthylmethyl groups into N2 of G6 and methyl or propynyl groups in the C8 positions of G1, G5, G10 and G14 [95]. LNA modification of G2 or G15 is also advantageous. Surprisingly, the thermal stabilities of LNA-bearing TBA analogues do not correlate with the inhibitory activity [96, 97].

A few modifications of the TT loops have been reported [87, 94, 98-100]. One particularly interesting example is the TBA analogue containing a 5'-5' inversion of polarity site (3'GGT5'-5′TGGTGTGGTTGG3') [99]. The analogue displayed somewhat reduced activity. However, the introduction of the non-hydrolysed unnatural 5'-5' or 3'-3' [101] linkages appears a promising approach to aptamer optimization in general. It can be used for constructing G4s with predetermined strand orientation (see subsection 'Controlling aptamer folding'). Another strategy for polarity control with simultaneous protection from nucleases is introduction of anomeric monomers, which also mimic strand reversal. For instance, anomeric phosphorothioate ONs d(T2G4T2) were reported to form anti-HIV-active parallel G4s [30]. In the case of TBA, local anomeric modifications in loops resulted in enhanced biostability but reduced activity. Core modifications in most instances hampered G4 formation [86].

In summary, core modifications of intermolecular and intramolecular quadruplexes are rarely beneficial. Local LNA modifications are among the most promising modifications, but the optimal positions of LNA residues must be experimentally determined for each aptamer. Optimal positions of intramolecular G4 loop modifications depend on the aptamer-protein binding mode and are better tolerated in loops that do not participate in interactions with protein. Importantly, chemical modifications of G4 aptamers often require significant synthetic effort, but the effects are hardly predictable. If the exact conformation of the aptamer and its complex with the target are not fully characterized, a 'structural' approach to aptamer optimization (e.g., addition of a stabilizing duplex module, see subsection 3.2.) appears more reasonable. The structural and chemical approaches are discussed and compared in [54].

4. FUTURE PROSPECTS

Recently, several G4 motifs from the human genome have been considered as aptamer candidates. The ON representing the quadruplex-forming sequence located in the insulin promoter was reported to recognize insulin and insulin-like growth factor 2 [102-103]. A promoter-derived aptamer selection method (G4PAS) was proposed [104] and was used successfully to identify G4s with affinities to vascular endothelial growth factor, platelet-derived growth factor-AA and retinoblastoma protein RB1. Although the affinities of certain G4s from gene promoters towards respective (coded) proteins may be a special case or a mere coincidence rather than a general tendency (*i.e.*, there may be some 'negative feedback'-type regulation mechanism [103]), known genomic G4s and their functions should undoubtedly be taken into account in aptamer design. In view of the G4 motif abundance in the human genome [105] and the diverse regulatory roles of G4s [106], genomic G4s (particularly those located in promoters) can be regarded as putative aptamer scaffolds. For example, native and modified ON mimics of G4s from RAS proto-oncogenes have been shown to compete with DNA-protein complexes that regulate transcription and therefore can be used as decoys for transcription factors [107, 108]. Rational design of G4 decoys, first described by Xodo's group, appears to be a promising trend in anticancer studies [109].

Thus, two current anticancer strategies – targeting G4s in oncogenes [110] and targeting cancer cells with antiproliferative G4s [25, 26] – are being developed in parallel and may converge at some point. Likewise, rapid progress in the studies of the G4 sites of HIV-1, Epstein-Barr virus and papillomavirus genomes and their participation in viral life cycles facilitates the development of new G4 antiviral agents [111]. One can expect that more genomic G4-inspired aptamers will be reported in the near future.

CONCLUSION

In conclusion, G4s are increasingly harnessed as aptamer scaffolds. Preference is generally given to monomolecular structures, and parallel G4s are particularly promising in the field of cancer-selective aptamers. Several elegant strategies for controlling inter- *vs*. intra-molecular folding and mimicking strand reversal have been proposed recently, which allows for constructing G4s with desired (predetermined) topologies. The traditional approaches to aptamer optimization, such as post-SELEX chemical modification, can also be beneficial in the case of G4s, but extensive G4 core modification is usually avoided. The rapidly developing combinatorial approaches (modular assembly in particular) allow for obtaining multifunctional and/or multivalent aptamers on the basis of known active G4 scaffolds.

CONFLICT OF INTEREST

This work was supported by Russian Science Foundation [14-25-00013] (AV, IS and GP – major funding) and in part by Russian Foundation for Basic Research [14-04-01244] (NI and AV). The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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