Kinetic ESI-MS Studies of Potent Anti-HIV Aptamers Based on the G-Quadruplex Forming Sequence d(TGGGAG)

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S Supporting Information

ABSTRACT: To investigate what properties make tetramolecular G-quadruplex ODNs good anti-HIV aptamers, we studied the stoichiometry and the self-assembly kinetics of the highly active 5'-end modified G-quadruplexes based on the d(TGGGAG) sequence. Our results demonstrate that the 5'-end conjugation does not necessarily increase the folding rate of the G-quadruplex; indeed, it ascribes anti-HIV activity. Unexpectedly, the G4-folding kinetics of the inactive G4 is similar to that of the 5'-end modified sequences. ESI-MS studies also revealed the formation of higher order G4 structures identified as octameric complexes along with tetramolecular G-quadruplexes.

KEYWORDS: Anti-HIV aptamers, tetramolecular G-quadruplexes, mass spectrometry, kinetic studies, modified oligonucleotides

In the last 30 years, there has been growing interest in the potential G-quadruplexes as novel aptamers.¹ G-quadruplexes are noncanonical DNA structures that, due to their peculiar three-dimensional arrangement, are able to recognize specific targets playing important roles in controlling gene expression.² These structures are known to act as potent inhibitors of HIV-infection at different stages of virus life cycle.³ The potential targets of HIV infection are HIV-1 reverse transcriptase, HIV RNase H, HIV-1 integrase (IN), and the viral surface glycoprotein known as gp120.⁴ Relevant G4 aptamers reported as potent inhibitors of integrase and RT RNase H domain are 93del and derivatives thereof (T30177, T30923, etc.).^{5,6} All these anti-HIV aptamers present parallel G4 structures with a variety of topologies ranging from intramolecular parallel G4 structure for T30177 to a peculiar interlocked bimolecular parallel G4 structure for 93del. The particular arrangement of the 93del highlights the marked polymorphism of G-quadruplex complexes, exhibiting the potentialities of G4 structures also to form higher-order structures by noncovalent stacking of the external quartets. The first aptamer able to target glycoprotein gp120 of HIV virus has been the phosphorothioate ISIS-5320 d-(TTGGGGTT) formed by a tetramolecular G-quadruplex structure.⁷ Subsequently, other short G-rich sequences have been identified as anti-HIV agents, among these the “Hotoda’s sequence”. These short d(TGGGAG) sequences formed very stable parallel tetramolecular G4 and showed high anti-HIV activity (EC₅₀).^{8,9} Surprisingly, activity was observed only when

the sequence was conjugated at the 5'-end with aromatic groups. In this regard, while for ISIS-5320 the active form has been reported to be the G-quadruplex structure,¹⁰ the recognition mechanism between Hotoda’s sequences and the glycoprotein gp120 is not completely clear and herewith also the role of the G-quadruplex.³ In this context, our efforts have been focused on the synthesis and characterization of new and highly active anti-HIV aptamers based on “Hotoda’s sequence”. Recently we have reported the synthesis of a mini-library of 5'-end modified d(TGGGAG) derivatives, carrying a variety of aromatic and hydrophobic groups at the 5'-end through a phosphodiester bridge^{11,12} (some of these are reported in Figure 1). All 5'-end modified d(TGGGAG) sequences (Figure 1) formed very stable parallel tetramolecular G-quadruplexes as confirmed by CD studies in potassium buffer ($T_{1/2} > 57\text{ }^{\circ}\text{C}$), and some of these show also a strong anti-HIV activity (EC₅₀ = 0.061–0.13 μM).¹¹ Most melting curves recorded by heating a preformed quadruplex do not correspond to equilibrium melting curves; the apparent melting temperatures T_m or $T_{1/2}$ determined by CD, strongly depend on the rate of heating.¹³ By examining the results obtained on 5'-end modified d(TGGGAG), we can see that, although the stability of G4 structures should be important for the activity, there is no relationship between the higher thermal stability of 5'-end

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Figure 1. Unmodified d(TGGGAG) sequence and its modified d(TGGGAG) ODNs carrying aromatic groups at 5' end by a phosphodiester bridge.¹¹

modified G-quadruplexes and their anti-HIV potency. We therefore reasoned that the kinetics of G4s formation could be the key factor, given that the folding of tetramolecular G4s is typically very slow.¹³

Along this line, Piccialli et al.¹⁴ reported the synthesis of TEL-ODNs, monomolecular G4s based on d(TGGGAG) with prominent anti-HIV activity that have faster formation kinetics due to tethering at the 3'-end.

In 2014, following the same aim, we reported the synthesis and characterization of new bimolecular G-quadruplexes based on the d(TGGGAG) sequence.¹⁵ Biological studies reported in that paper confirmed that only bimolecular G-quadruplexes containing 5'-end moieties exhibited significant anti-HIV activity, highlighting again the crucial role of the group at 5'-end. Overall, the data do not explain the strong activity of some 5'-end modified d(TGGGAG) sequences, but they clarify that the anti-HIV activity of these G4s is dependent on the presence of hydrophobic groups at 5'-end of d(TGGGAG) (Figure 1; Table 1). This result is in agreement with a recent paper by

Table 1. Equilibrium Constants for G-Quadruplex and Octamer Folding; $t_{50\%}$ of Sequences I–IV and Relative EC_{50}

ODNs	$t_{50\%}$ (hours)	K_{eq1} (mM^{-3})	K_{eq2} (mM^{-1})	EC_{50} (HIV-1) (μM)
I	9.4	161	1.54	>40 ^a
II	7.6	60	0.88	0.061 ± 0.04 ^b
III	8.2	214	1.94	0.51 ± 0.25 ^b
IV	49	25	1.04	0.50 ± 0.33 ^b

^aSee ref 8. ^bSee ref 11.

D'Onofrio et al.¹⁶ in which it is revealed that the conjugation with polar moieties like sugars at 5'-end of d(TGGGAG) does not confer anti-HIV activity and thermal stability to d(TGGGAG) sequence.

In an attempt to achieve a more complete picture of structure–activity relationship depending on the modifications at 5'-end of d(TGGGAG), the main goal of the present study is the evaluation of kinetic aspects involved in the tetramolecular G-quadruplex folding for the most active 5'-end modified d(TGGGAG) (II–VI, Figure 1).

A kinetic investigation of G-quadruplex folding mechanism of d(TGGGAG) could reveal important aspects that may be useful for the understanding of the biological activity. To this

end, the G-quadruplex folding kinetics were studied by electrospray mass spectrometry (ESI-MS), using soft conditions of electrospray ionization (ESI) and of ion transfer that allow preserving noncovalent interactions throughout the MS analysis.¹⁷ Furthermore, ESI-MS enables unambiguous and accurate detection of all noncovalent complexes formed.¹⁸ By ESI-MS, it is possible to determine the number of strands, the inner cations for each complex, as well as their equilibrium binding constants.¹⁹ The time-scale selected for our tetramolecular G4 kinetic studies was a day because differently from a monomolecular G4, the kinetics of tetramolecular G4-folding are rather slow. We followed the kinetics of G-quadruplex formation during 14 days in the presence of an internal reference (dT₆) and in ammonium acetate buffer (150 mM NH₄OAc). During 14 days the samples were stored at 20 °C and at each time point an aliquot was diluted to 20 μM , final single-strand concentration, and immediately injected in the ESI-MS. Before G-quadruplex formation, aqueous stock solutions of each sequence were heated at 80 °C for 5 min. Heating was found crucial to remove any preformed G-quadruplex that could come from the synthesis and purification steps.

The folding kinetics was carried out at 600 μM of ODNs, fixing as the reaction starting time (t_0) the time of buffer addition (150 mM NH₄OAc). We chose to study the G4-folding kinetics in these experimental conditions because they resemble those used in the anti-HIV assays in terms of both ionic strength and concentration-scale. The formation of G4 structures has been verified also in NH₄OAc buffer qualitatively by CD, showing a typical profile of a parallel tetramolecular G4 (see Supporting Information; S9). Signal intensity of d(T₆) was used for the response factor determinations of each signals.²⁰ In general, MS data show a rapid conversion of the single strand (M) into two intermediates, the dimer (D) and trimer (T), followed by the slower formation of tetramolecular G-quadruplex structure (Q). Tetramolecular G-quadruplex folding pathways that includes the formation of dimer and trimer intermediates was recently reported by us¹⁹ and previously predicted by Stefl.²¹ An unexpected finding of the present study was the detection of a higher-order G4 structure, identified as an octameric complex (O = 2Q). The formation of a supramolecular complex for d(TGGGAG) was recently hypothesized by NMR studies on Hotoda's sequence by Galeone and co-workers.²² They observed the coexistence of multiple but not well-defined species of G-quadruplexes, hypothesizing the formation of higher-order aggregates. For the first time, our present ESI-MS experiments have unambiguously uncovered the formation of octameric complexes (O) for the unmodified d(TGGGAG) sequence and its 5'-end modified congeners. Similar octameric species were observed by MS previously for an unrelated sequence, d(CG GTGGT).²³ A representative ESI-MS spectrum of d(TGGGAG) sequences is reported in Figure 2. In the spectrum are shown the peaks related to all complexes observed after 14 days (monomer, dimer, trimer, quadruplex, and octamer). The monomer was detected at charges state 2[−] (m/z = 934.93) only with some sodium ions nonspecifically bound. Dimer and trimer were also detected with nonspecific sodium adducts, issuing respectively at m/z = 1246.99 and m/z = 1402.83. A tetrameric species were detected at two different charge states, 5[−] (m/z = 1503.69) and 4[−] (m/z = 1884.16). The octamer was observed unambiguously at charge state 7[−] (m/z = 2153.54). The absolute concentration of the species

Figure 2. Electrospray mass spectrum of d(TGGGAG) (20 μ M in 150 mM NH_4OAc) recorded after 10 days from ammonium acetate addition. **M** (single stranded, ss) for monomer, **D** for dimer, **T** for trimer, **Q** for tetramer, and **O** for octamer.

was determined by peak integration (not peak height) using the internal standard method.²⁰ Native gel electrophoresis experiments (PAGE) confirmed the formation in solution of higher order G-quadruplex structures for these sequences. Aiming at observing the mobility of all complexes, all sequences (I–IV) were loaded on the gel in single stranded (ss) conditions (no potassium added, indicated by “–”) and G4 conditions (100 mM KCl, indicated by “+”) (more details in [Supporting Information](#); S8). For all ODNs loading under “salt-free” conditions (“–”), the more intense band is that related to the ss in agreement with the fastest migration. Sequences I, II, and III (“+”) exhibit a retarded migration with respect to the G4 reference, $[\text{d}(\text{TG}_4\text{T})]_4$. We therefore assigned the slower migrating PAGE bands to octameric self-assembly structures. According to ESI-MS data (orange lines, [Figure 4](#)), I and III are able to form detectable amounts of octamer complexes also in “salt-free” conditions of PAGE ([Figure 3](#), “–”). This behavior displays their major tendency to aggregate.

Lower mobility is also observed for the G4 of I compared to the others (II–IV). These data are explained from the major compactness of $[\text{d}(\text{TG}_4\text{T})]_4$ because of its four consecutive G-

planes. Concurrently, also G4 of modified sequences (II–IV) thank to hydrophobic interactions by lipophilic 5'-end moieties could display more compactness with respect to G4 of I. Finally, the sequence IV showed an evident smearing in terms of intensity of band in agreement with the slow kinetics observed by ESI-MS ([Figure 4](#)).

The time evolution of the concentration (mM) of the more abundant species (monomer, tetramer, and octamer) for the sequences I–IV, are reported in [Figure 4](#). Analyzing ESI-MS data, we observed that the single strand (**M**), in rapid equilibrium with dimer (**D**), was converted into tetramer (**Q**) and octamer (**O**) complexes within 1 day (except for julolidine derivative IV) and continued to proceed during the following 14 days, until reaching a plateau ([Figure 4](#)). Formation of the octamer began simultaneously with that of the tetramer, but octamer abundance remained lower than that of tetramer. Dimer and trimer were formed in very small amounts and were likely in rapid equilibrium with the monomer. Unfortunately, two of these modified ODNs (V–VI), likely owing to their more lipophilic nature, aggregated at 600 μ M (as indicated by a loss of total intensity depending on time; see [Supporting Information](#); S7), preventing the observation of their association kinetics.

Consequently, they were not taken into account in the reaction equilibria, which were defined as follows:

The equilibrium constants for both equations (eqs 1 and 2) and the kinetic parameter $t_{50\%}$, i.e., the time needed to form half of the final amount of G-quadruplex, are reported in the [Table 1](#).

Strikingly, the $t_{50\%}$ of the unmodified sequence I (9.4 h) was in the same order of magnitude as the $t_{50\%}$ of II and III (respectively, 7.6 and 8.2 h; [Table 1](#)) suggesting that conjugation of 5'-end moieties does not necessarily accelerate the G4 assembly process. Only sequence IV presented a kinetics of G-quadruplex and octamer folding slower than other sequences. In this last case, a role of julolidine group at 5'-end can be hypothesized, likely due to the presence of a protonated intracyclic nitrogen atom that could slow down the folding process. In summary, ESI-MS studies on 5'-end modified d(TGGGAG) derivatives confirm the formation of tetramo-

Figure 3. Native gel electrophoresis of I–IV sequences in two conditions (“–”, no potassium added; “+” in 100 mM KCl); 15% polyacrylamide gel supplemented with 100 mM KCl. The gel was run at 26 $^{\circ}\text{C}$ at constant voltage (110 V) for 2.5 h.

Figure 4. Time-evolution of the concentration (mM) of single strands engaged in monomeric form (M) (green points), tetramolecular (Q), and octameric G-quadruplexes (O) (respectively, red and orange points). The lines represent the data fittings of each species fitted independently using a single exponential function.

lecular G-quadruplexes and show up the formation of unexpected octameric complexes. Native PAGE experiments reveal the simultaneous formation of G-quadruplex and octameric complexes also in 100 mM KCl solution. Kinetic ESI-MS experiments have shown that the rate of G-quadruplex-folding does not increase with the conjugation at 5'-end of hydrophobic groups. Surprisingly, the folding rate of the inactive G4 of d(TGGGAG) is very similar to that of high-active ones formed by 5'-end modified sequences II and III. However, the slower kinetics of G4 formed by sequence IV is far from its moderate anti-HIV activity. The present work extends the current knowledge on the G-quadruplex folding mechanism of d(TGGGAG) sequences and reveals interesting features useful to get a more complete picture of structure–activity relationships.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.5b00408.

Description of synthesis procedures and identification of oligomers (I–VI) and protocol for the ESI-MS experiments (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ODNs, oligodeoxyribonucleotides; TEL, tetra-end-linked; G4, G-quadruplex; ESI-MS, electrospray mass spectrometry; PAGE, poly-acrylamide gel electrophoresis; HIV, human immunodeficiency virus

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