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Quantitative characterization of oligomeric state of G-quadruplex antithrombin aptamers by size exclusion HPLC

Rugiya R. Alieva, Elena G. Zavyalova,* Vadim N. Tashlitsky and Alexey M. Kopylov

Department of Chemistry, M. V. Lomonosov Moscow State University, 119991 Moscow, Russian Federation. Fax: +7 495 939 3149; e-mail: zlenka2006@gmail.com

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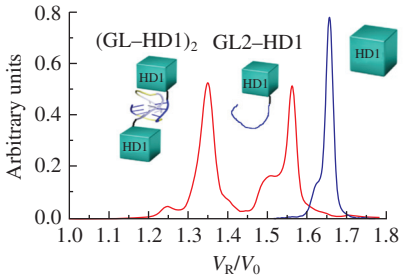
A quantitative technique for estimation of the oligomeric state of G-quadruplexes has been developed for the first time. The simplest 15-mer G-quadruplex antithrombin aptamer HD1 and its analogues, namely RA36 as a covalent dimer of HD1 as well as GL2–HD1 as a non-covalent dimer with G-quadruplex lock, were used as model objects. HD1 and RA36 have been shown to be single-stranded G-quadruplexes, whereas GL2–HD1 forms non-covalent dimers $(GL2–HD1)_2$ in a concentration-dependent manner, affording ~50% of the dimers at 1 mmol dm^{-3} concentration.



Nucleic acid G-quadruplexes have an intrinsic conformational polymorphism and are capable of forming several oligomeric states. These properties are affected by a buffer medium composition and the nucleic acid sequence, in particular a loop length, a number of guanine residues in the G-block and the presence of terminal extensions.[1–5] Therefore, under different conditions G-quadruplexes can reveal various properties, and the investigation of G-quadruplex polymorphism is of importance for both fundamental and applied research.[6–11]

HD1 or TBA is a well-known 15-mer G-quadruplex aptamer, which targets thrombin as a coagulation factor protein.[12–14] A number of aptamer analogues of HD1 have been synthesized, including duplex–G-quadruplex bimodular structures,[15,16] covalent dimer RA36[15] as well as non-covalent dimers linked by locks of double helix and G-quadruplex types.[17] The oligomerization of aptamer HD1 affects its pharmacokinetics and therefore requires a thorough consideration during the development of new aptamer-based antithrombotic drugs.[12]

In general, a simple and robust technique is necessary for the quantification of various oligomeric and conformational states of G-quadruplexes. The currently used approach is the size exclusion high performance liquid chromatography (SE HPLC),[18] however, its common protocols lack a linear correlation between the chromatographic retention volume and the logarithm of molecular weight for monomeric G-quadruplexes, the reason being probably in suboptimal separation conditions.[19] As a result of such complications, the molecular mass of aptamer HD1 was twice overestimated.[18] Analytical ultracentrifugation is used as an additional method for the estimation of G-quadruplexes oligomeric state for the HPLC fractions obtained.[19]

In this work, SE HPLC was applied to the investigation of the oligomeric composition for antithrombin G-quadruplex aptamers. Optimization of the chromatographic technique allowed us to achieve the required linear dependence between the retention volume and the logarithm of molecular weight for the range of duplex weight of 6.5–65 kDa. The HPLC column was calibrated with duplexes for the subsequent determination of G-quadruplex oligomeric state.[†]

For the column calibration, the separation of duplexes with lengths 10–100 bp was carried out. The linear calibration curve as the logarithm of molecular weight *vs.* relative retention volume had R^2 value of 0.995. The following resulting equation was used for the estimation of the oligomeric state of G-quadruplexes: $\lg M = -1.541 V_R/V_0 + 6.337$.

The chromatogram for aptamer HD1 has a single peak with relative retention volume V_R/V_0 of 1.66 [Figure 1(*a*)], which is out of the calibration range. The formal calculation gave the number of strands of 1.29 (Table 1). Aptamer RA36 has a major peak at V_R/V_0 of 1.52 (94%) and a minor peak at V_R/V_0 of 1.33 (6%) [Figure 1(*b*)], both values being within the calibration range. The calculated number of strands per G-quadruplex is 1.04 for the major peak and 2.00 for the minor peak (Table 1). Thus,

† *Sequences of oligonucleotides*. HD1: ggttggtgtggttgg. Complementary sequence to HD1: ccaaccacaccaacc. RA36: ggttggtgtggttggtggttggtgtggttgg. Complementary sequence to RA36: ccaaccacaccaaccaccaaccacaccaacc. GL2–HD1: ggttggtgtggttggttttggggttttgggg. All solutions were prepared in ultrapure water with resistivity 18.2 MΩ. Folding of the aptamers was carried out in 20 mM PBS buffer pH 7 containing 10 mM KCl and 140 mM NaCl by heating at 95 °C for 5 min for a denaturation and then cooling to room temperature for the folding itself.

An equilibration with three volumes of the column was carried out before the first injection. A 10–1000 μM aptamer solution in PBS buffer (1 μl) was injected into an Agilent 1200 HPLC system (Agilent Technologies), consisting of a pump with a degasser, a column thermostat, an autosampler, an UV detector and a ChemStation control program. A TSKgel G2000SWXL column, 7.8 × 300 mm, 5 μm spherical silica gel particles, 125 Å pores (Supelco) thermostated at 25.0 °C was used with elution at 0.5 ml min^{-1} by aqueous buffer–acetonitrile, 90 : 10 v/v. The buffer contained 60 mM KH_2PO_4 and 140 mM K_2HPO_4, pH 6.85. Detection was carried out by absorbance in the range of 200–400 nm at bandwidth of 10 nm with a sampling frequency of 150 Hz. Final chromatograms represented the absorbance at 260 nm *vs.* the relative retention volume, V_R/V_0. To calibrate the column, a 10–300 bp mixture of O'GeneRuler Ultra Low Range DNA Ladder duplexes (Thermo Fisher Scientific) was used. The complete dead volume V_0 was determined as the retention volume V_R for the 300 bp DNA duplex with molecular weight > 150 kDa from the DNA Ladder.

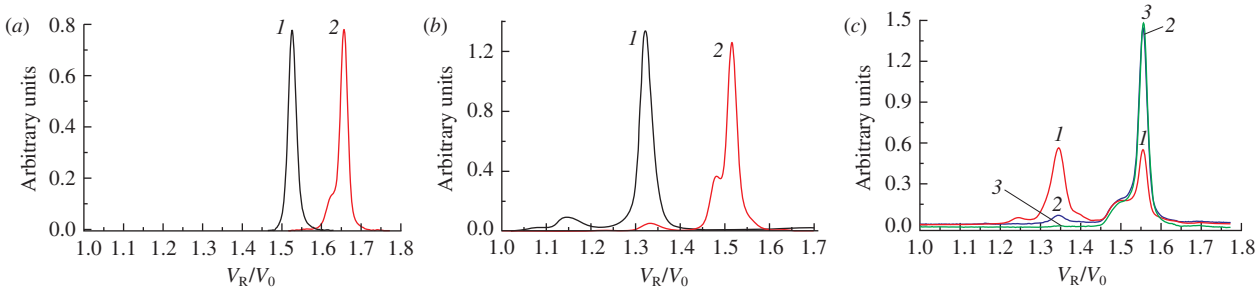


Figure 1 Chromatograms of G-quadruplexes with peaks numbered in the order of elution for aptamers (*a*) HD1, (*b*) RA36 as a duplex (*1*) with and (*2*) without the complementary oligonucleotide and (*c*) GL2–HD1 folded at concentration, μM: (*1*) 1000, (*2*) 100 and (*3*) 10.

Table 1 Oligomeric state of G-quadruplexes and their duplexes (ds) formed after addition of the complementary oligonucleotides.

Aptamer	Concentration/ μmol dm^{-3}	Expected molecular weight for monomer/ Da	V_R/V_0	Relative peak area (%)	Experimental molecular weight/Da	Calc. no. of strands
HD1	1000	4721	1.66	100	6094	1.29
RA36	1000	9611	1.52	94	10038	1.04
			1.33	6	19230	2.00
dsHD1	1000	4564	1.53	100	9707	2.12
dsRA36	1000	9398	1.32	90	19970	2.12
			1.15	10	37227	3.96
GL2–HD1	10	9763	1.56	100	8722	0.89
	100		1.56	96	8722	0.89
			1.35	4	18401	1.88
	1000		1.56	50	8722	0.89
			1.35	50	18401	1.88

aptamer RA36 is predominantly monomeric and contains small amount of a dimer.

Note that for aptamers HD1 and RA36, the peaks of monomeric species were non-symmetric and had shoulders with slightly smaller values of relative retention volumes. Presumably, these shoulders corresponded to conformers that were unfolded and had an increased hydrodynamic radius but the same molecular weight.

To test the nature of these shoulder peaks, both G-quadruplexes HD1 and RA36 were annealed with the corresponding complementary oligonucleotides in 1 : 1 stoichiometric ratio. The resulted duplex structures were symmetric. As a result, peaks of monomeric G-quadruplexes disappeared from the chromatograms completely, together with the shoulders, which supports the existence of two conformers that can be readily converted into identical duplexes by the action of the complementary oligonucleotides.

In general, a non-covalent dimer of aptamer HD1 can be assembled using intermolecular G-quadruplex lock as a joining unit, the first successful example being GL2–HD1.[17] To optimize the conditions for this assembly, the known aptamer GL2–HD1 was folded at different concentrations and analyzed by SE HPLC [Figure 1(*c*) and Table 1].

At 10 μmol dm^{-3} concentration of aptamer GL2–HD1, the dimer was not detected and a single peak with V_R/V_0 of 1.56 corresponded to the monomer. Note that for aptamer GL2–HD1, a shoulder of the monomer peak was observed similarly to G-quadruplexes HD1 and RA36.

When the concentration of aptamer GL2–HD1 increased to 100 μmol dm^{-3}, two peaks were observed. In addition to the major peak that corresponded to the monomer, a minor peak (4%) of the dimer appeared with V_R/V_0 of 1.35. At 1000 μmol dm^{-3} concentration, the amount of the dimer increased to ~50%. Thus, the dimerization of aptamer GL2–HD1 occurred in a concentration-dependent manner.

In summary, SE HPLC turned out to be the appropriate simple and robust technique for the quantitative estimation of both oligomeric composition and conformational polymorphism of G-quadruplexes. The approach developed allowed us to predict the molecular weight of DNA G-quadruplexes from the calibration with a set of DNA duplexes. The single stranded state of aptamers HD1 and RA36 has been confirmed. Aptamer GL2–HD1 with intermolecular G-quadruplex lock has been shown to form dimers in a concentration-dependent manner. At 1000 μmol dm^{-3} concentration the amount of its dimers reaches 50%.

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