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Duplex/quadruplex oligonucleotides: Role of the duplex domain in the stabilization of a new generation of highly effective anti-thrombin aptamers

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ABSTRACT

Recently, mixed duplex/quadruplex oligonucleotides have attracted great interest for use as biomedical aptamers. In the case of anti-thrombin aptamers, the addition of duplex-forming sequences to a G-quadruplex module identical or very similar to the best-known G-quadruplex of the Thrombin Binding Aptamer (HD1) results in new or improved biological properties, such as higher activity or different recognition properties with respect to HD1. Remarkably, this bimodular fold was hypothesized, based on its sequence, for the only anti-thrombin aptamer in advanced clinical trial, NU172. Whereas cation modulation of G-quadruplex conformation and stability is well characterized, only few data from similar analysis on duplex/quadruplex oligonucleotides exist. Here we have performed a characterization of structure and stability of four different duplex/quadruplex anti-thrombin aptamers, including NU172, in the presence of different cations and in physiological-mimicking conditions in comparison to HD1, by means of spectroscopic techniques (UV and circular dichroism) and differential scanning calorimetry. Our data show a strong reciprocal influence of each domain on the stability of the other and in particular suggest a stabilizing effect of the duplex region in the presence of solutions mimicking the physiological conditions, strengthening the idea that bimodular aptamers present better therapeutic potentialities than those containing a single G-quadruplex domain.

1. Introduction

Aptamers are short DNA or RNA molecules characterized by the ability to bind a molecular target with strong affinity and high selectivity [1]. Aptamers targeting biomacromolecules (proteins, DNA, etc.) that play a biological activity in important physiological or pathological processes have a great potential as drugs [2]. Indeed, they can inhibit or control activity of their targets through recognition of specific binding sites. This mechanism is strictly dependent on the aptamer three-dimensional structure, because the interaction is determined by shape and chemical complementarity between aptamer and target surfaces. A particularly exemplary case is that of anti-thrombin aptamers. Thrombin is a trypsin-like serine protease that plays a pivotal role in haemostasis: it has procoagulant activity, being the only enzyme capable of catalysing the conversion of soluble fibrinogen in insoluble fibrin strands and the most potent platelet activator, as well as anticoagulant and antifibrinolytic activity in the presence of thrombomodulin [3]. Apart from the active site, which is common among all serine proteases, thrombin possesses two electropositive patches on its surface, exosites I and II, that through binding of substrates and cofactors determine the biological properties of the enzyme [4]. These exosites are also targeted by several DNA and RNA aptamers whose potential as anti-thrombotic drugs has been long investigated. The first anti-thrombin aptamer to be selected and deeply studied is the so-called thrombin binding aptamer, HD1 [5], a DNA 15mer that adopts a chair-like G-quadruplex structure and binds thrombin exosite I [6], the fibrinogen recognition site, thus inhibiting thrombin activity. Due to the inherent properties of aptamers, including lack of toxicity and immunogenicity in addition to specificity and controllable

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activity [7–10], HD1 has been considered as a promising anticoagulant drug and reached Phase I clinical trials [11,12]. With the aim to improve HD1 properties and overcome its drawbacks, such as the very short lifetime *in vivo*, many different modified HD1s were produced in the past [13], including sequences employing not canonical residues [14,15], polarity inversion sites [16–18], additional moieties [19]. Despite a great effort in this direction, none of these molecules passed a complete clinical trial. Recently a great scientific interest was focused on bimodular oligonucleotides encompassing both a duplex and a quadruplex module, oligonucleotides that adopt a so-called duplex/quadruplex conformation. These molecules can either represent valuable models for telomeric regions thus helping the design of specific ligands [20–23] or act as aptamers that may be more specific and effective than those presenting a single module (either a duplex or a quadruplex one) [24,25]. Several example of bimodular duplex/quadruplex aptamers (DQ-aptamers) directed against a variety of biological targets, also including thrombin, are reported in the literature [24,26–33]. It has to be noticed that a bimodular structure was hypothesized on the basis of the sequence for the only anti-thrombin aptamer in Phase II clinical trials, a 26mer named NU172 [34,35]. The addition of a duplex forming sequence in a HD1-like quadruplex domain may result in i) improved anti-thrombotic activities, such as the case of NU172 [34], ii) higher thrombin affinity, as happens for a 31mer aptamer named RE31 [36,37], and iii) even completely different binding properties with respect to HD1, such as the case of HD22 aptamers that recognize exosite II [33,38], the heparin binding site. The recognition properties of DQ-aptamers are strictly dependent on the relative orientation of the duplex and quadruplex domains. It has been shown that the recognition of the larger exosite II in the place of exosite I by the duplex/quadruplex anti-thrombin aptamer HD22_27mer depends on the peculiar perpendicular arrangement of the two aptamer domains [38], while in RE31 the coaxial disposition of duplex and quadruplex regions stabilizes the binding at exosite I [39]. It is worth to underline that the peculiar recognition mode of HD22 aptamers assures a high binding specificity. Indeed, these aptamers represent the best alternative to design efficient aptasensor for detection of thrombin in real samples [40].

Several studies have dealt with the high resolution structural characterization of bimodular oligonucleotides as well as their stability [20,21,23,41], however, designing and optimizing better aptamers should take into consideration not only the interactions between the two structural elements, but also the role of metal ions in stabilizing them [26]. Indeed it is well-known that cations play a key role in determining quadruplex secondary structure [42–45] and affect the aptamer stability and activity, as shown in the case of HD1 properties [6,33,45], but only very few studies analysed their effect on mixed duplex/quadruplex oligonucleotides [35].

Here we present a thorough investigation of conformation and stability of four anti-thrombin aptamers RE31 [36,37,39], NU172, HD22_27mer and HD22_29mer [33,38], for which a mixed duplex/quadruplex fold was hypothesized on the basis of the sequences or proved by X-ray crystallography, in the presence of different cations as well as in Phosphate Buffered Saline (PBS), which mimics physiological conditions. The study was carried out by means of UV and circular dichroism (CD) spectroscopy and differential scanning calorimetry (DSC). Our results confirm the presence of both domains within these oligonucleotides in different ionic conditions and indicate that DQ-aptamers are likely to change conformation depending on the cations employed, with the only exception of RE31. Duplex and quadruplex domains strongly affect each other. Finally, comparison with HD1 well highlights that duplex domain has a stabilizing effect on G-quadruplex in sodium-containing buffer and, remarkably, in PBS.

Table 1
Sequences of the different oligonucleotides used. Guanines that were hypothesized to form G-tetrads on the basis of the sequence are underlined, those that were seen to belong to G-tetrads in the high resolution crystal structures of HD1 [6], RE31 [39] and HD22_27mer [38] are in red. (For interpretation of the references to colour in this table legend, the reader is referred to the web version of this article.).

Name	Sequence (5'-3')
HD1	GGTTGGTGTGGTTGG
RE31	GTGACGTAGGTTGGTGTGGTTGGGGCGTCAC
NU172	CGCCTAGGTTGGGTAGGGTGGTGGCG
HD22_27mer	GTCCGTGGTAGGGCAGGTTGGGGTGAC
HD22_29mer	AGTCCGTGGTAGGGCAGGTTGGGGTGACT
Rand31	GTACGGTTGATCCGTGTTAGGTTGTGTGCAC

2. Material and methods

2.1. Materials

HD1 and the duplex/quadruplex aptamers (DQ-aptamers) NU172, HD22_27mer, HD22_29mer, were purchased from Sigma-Genosys. RE31 was synthesized as previously reported [46]. A random 31mer sequence (Rand31) to be used as control was synthesized by Syntol (Russia). Sequences of all the oligonucleotides are reported in Table 1.

2.2. Sample preparation

Stock solutions of each oligonucleotide were prepared by dissolving the lyophilized oligonucleotide in different buffers: A) 10 mM potassium phosphate buffer pH 7.2 and 100 mM potassium chloride (potassium buffer); B) 10 mM sodium phosphate buffer pH 7.2 and 100 mM sodium chloride (sodium buffer); C) 10 mM sodium phosphate pH 7.4, 137 mM sodium chloride and 3 mM potassium chloride (PBS); D) 10 mM Tris/HCl pH 7.5 and 100 mM lithium chloride (lithium buffer). Buffers A and B were chosen in order to highlight the effects of the most common cations able to stabilize G-quadruplexes, buffer D was chosen since lithium is a monovalent cation that is not particularly able to stabilize G-quadruplexes. Finally, PBS was chosen since it more closely resembles physiological conditions. The concentration of each sample was calculated at 90 °C by means of UV spectroscopy at 260 nm, using molar extinction coefficients calculated from the primary sequence. Each sample was annealed, in order to induce folding: it was heated for 5 min at 90 °C, then slowly cooled down to room temperature, and finally stored at 20 °C over-night.

2.3. CD spectroscopy

Circular dichroism (CD) spectra were recorded at 10 °C using a Jasco J-710 spectropolarimeter equipped with a Peltier thermostatic cell holder (Model PTC-348WI). CD measurements were carried out in the 220–320 nm range, using a 0.1 cm path length cell and 40 μM oligonucleotide solutions.

Thermal unfolding curves were obtained by following the CD signal at 295 nm and by recording 220–320 nm spectra in 2 °C steps with 5 s equilibration time between readings, in the 10–90 °C range, at heating rate 1.0 °C min^{-1}. Fraction of folded oligonucleotide was calculated as

$$F_{folded} = (I_{obs} - I_u)/(I_f - I_u)$$