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# Binding modes of thrombin binding aptamers investigated by simulations and experiments

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**ABSTRACT.** Thrombin binding aptamers HD1 and HD22 are the most studied aptamers, both for therapeutic and sensing purposes. Yet there is still no commercialized aptamer-based sensor device for thrombin detection, suggesting that the binding modes of these aptamers remain to be precisely described. Here we investigate thrombin-aptamer interactions with molecular dynamics simulations, and show that the different solved structures of HD1-thrombin complex are energetically similar and consequently possibly co-existing. Conversely HD22 folding is much more stable and its binding energy with thrombin is significantly larger than that of HD1 complexes. These results are confronted to experiments, which consist in monitoring aggregation of aptamer-functionalized gold nanoparticles triggered by thrombin. HD1 alone, but not HD22, can trigger aggregation, meaning that this aptamer has multiple sites of interactions with thrombin. Furthermore, pre-incubation of HD22 with thrombin impedes HD1 aggregation, suggesting that HD1 and HD22 have competing affinities for the same binding site. Altogether this study shows that the characterization of aptamer-thrombin interactions by structural and kinetic experiments joined to simulations is necessary for the development of biosensors.

Aptamers are single-stranded nucleic acids able to bind to a variety of targets with high specificity and affinity<sup>1-3</sup>. Their selection is achieved through the SELEX protocol, which consists in consecutive cycles of selection and amplification, starting from a large library of oligonucleotides ( $10^{13}$ - $10^{18}$  sequences)<sup>4</sup>. Due to their attractive properties in terms of ease of synthesis, low-cost, chemical stability, and high affinity and specificity for a broad range of targets, aptamers have attracted much attention, particularly as potential therapeutic inhibitors and sensing elements for a future generation of biosensors<sup>5</sup>. Thrombin binding aptamers (TBA) HD1 and HD22 have been the most studied aptamers for biosensing, representing ~20% of the 5000 papers published about the use of aptamers for analytical technologies<sup>6</sup>. Surprisingly there is little consensus on their binding properties in the literature. A rapid survey of Surface Plasmon Resonance (SPR) data shows that the reaction constant of HD1 and HD22 spans ~2

decades from 6 nM<sup>7</sup> to 170 nM<sup>8</sup> and from 2 nM<sup>9</sup> to 110 nM<sup>8</sup> for HD1 and HD22, respectively. Efforts to characterize the interaction of aptamers with thrombin are thus needed to improve their performances as sensing layers in a device, and definitively establish aptamer-based technologies.

HD1, the 15-mer DNA (5'-GGTTGGTGTGGTTGG-3') TBA that binds the fibrinogen site, also known as exosite I, was the first to be described in 1992<sup>10</sup> and structurally characterized in 1993<sup>11</sup>. HD1 was shown to adopt a G-quadruplex structure with one TGT and two TT loops (see Fig. 1). The loops have been shown to bind thrombin in different ways, according to X-ray and NMR models respectively<sup>12</sup>, and the determination of multiple crystal structures of the thrombin-HD1 complex has left a doubt on the existence of a unique complex vs. several co-existing states<sup>13</sup>. On the other hand, only one structure of thrombin-HD22 complex has been reported in the literature. This 29-mer (5'-AGTCCGTGGTAGGGCAGGTTGGGGTGACT-3'), which presents a duplex-quadruplex folding, binds to the heparin site, known as exosite II, with a higher affinity<sup>14</sup> than HD1.

In this study, we considered all TBA structures available in the Protein Data Bank (PDB), namely four HD1- and one HD22- thrombin structures (Fig. 1). Our goal was to compare their energy in their equilibrium configuration, as well as their binding energy to thrombin. We first focused on the two HD1 low-resolution structures (2.80Å) solved in 1996<sup>12</sup> with NMR-based and X-ray-based models (PDB code: 1HAO and 1HAP respectively). In 1HAO, G8 and T9 nucleic bases from the TGT loop are stacked on the G-quadruplex while T7 is rejected outside the loop. Interaction with thrombin is established through both TT loops. In 1HAP, the same configuration of the TGT loop can be observed but it is directly interacting with thrombin. Two higher resolution X-ray structures of HD1 (1.80Å) solved more recently<sup>15</sup> in the presence of Na<sup>+</sup> and K<sup>+</sup> (PDB code: 4DIH and 4DII respectively) were also examined. 4DIH and 4DII chains follow the same orientation as 1HAO, and interaction with thrombin occurs through the TT loops. However the TGT loop undergoes a different configuration because T7 and G8 bases are now stacked on the G-quadruplex whereas T9 is rejected outside. The orientation of HD1 on thrombin is rotated by 180° around the helix axis (Fig.1). The other meaningful difference comes from the cation position, which is inserted between the G-quartets and drastically increases the inter-strand distance from ~12 Å in 1HAO and 1HAP to ~21 Å in 4DIH and 4DII. More details about interaction modes have already been described

<sup>15,16</sup>. Concerning HD22, the X-ray structure recently solved by Krauss et al. with a 2.80 Å resolution was used in the calculations (PDB code: 4I7Y) <sup>14</sup>.

All calculations were performed with Amber 10 suite of programs <sup>17</sup> and the parm99/bsc0 force field <sup>18</sup>. As a first step, TBAs were submitted to molecular mechanics energy minimization. Simulations were performed in implicit solvent (Generalized Born approach <sup>19</sup>), and using the sander module of Amber, with 200000 steps of conjugate-gradient minimization without restraints and a cutoff of 12 Å. Resulting energies are presented in Table I. Despite changes in folding associated to the lower compaction of 4DII and 4DIH vs. 1HAO and 1HAP, the four HD1 energies are similar, spanning -3.15 to -3.02 kcal.mol<sup>-1</sup>. It is therefore impossible to ensure that one HD1 conformation prevails over the other ones. Moreover, the energy of HD22 is -5.98 kcal.mol<sup>-1</sup>, *i.e.* twice as large as that of HD1. This result is expected due to the existence of a longer G-quadruplex motif in comparison to HD1, plus four base-pair duplex. For this reason, HD22 is less likely to undergo conformational changes; however, there is no other PDB data to support this hypothesis.

As a second step, the binding energy of TBA with thrombin was estimated with molecular mechanics Poisson-Boltzmann surface area (MM-PBSA) method. This method combines molecular mechanics with continuum solvation models to exploit multiple snapshots extracted from molecular dynamics trajectories. It presents the advantage to be faster than other usual techniques <sup>20,21</sup>. Each aptamer-thrombin complex was solvated in a cubic box of water molecules extending at least 10 Å outside the structures (TIP3P model <sup>22</sup>). We first equilibrated the solvated complexes with a short 2000 steps of conjugate-gradient minimization run, followed by 50 ps of heating up to 300K with MD, 50 ps of density equilibration with weak restraints (2 kcal.mol<sup>-1</sup>.Å<sup>-2</sup>) and 500 ps of constant pressure equilibration with no restraint. All simulations were run using a cutoff of 8 Å. The equilibration and subsequent production were run with Langevin dynamics for temperature control, and the shake algorithm to impose constraints on bond lengths involving hydrogen atoms, allowing for a 2 fs time step. The production runs were performed during 10 ns of fully unrestrained MD and the collected results for each run, recorded every 5 ps, were used to estimate the aptamer binding energy. The resulting estimated binding energies are reported in Table I. The binding energies of 1HAO and 1HAP complexes is very similar (-47.03 and -48.81 kcal.mol<sup>-1</sup>, respectively), but approximately twice lower than that of HD22-thrombin complex (-88.37 kcal.mol<sup>-1</sup>). However, in presence of cations and more specifically of K<sup>+</sup>, HD1-thrombin complexes gains in stability

with binding energies of  $-60.29$  and  $-66.73$  kcal.mol<sup>-1</sup>. Because monovalent cations are ubiquitously used in molecular biology assays, HD1 is unlikely to be found unbound to cations. Therefore 4DIH and 4DII complexes are expected to prevail over 1HAO and 1HAP, and at least two states likely co-exist in experiments. At this step we wished to test this idea further with simple experiments.

We thus carried out experiments involving TBA-capped gold nanoparticles of 80 nm (AuNPs). We monitored the dynamics of aggregation, which occurs whenever thrombin mediates the formation of bonds between two functionalized AuNPs<sup>23,24</sup>. Aggregation kinetics was monitored by dynamic light scattering (DLS), which provides measurements of average hydrodynamic radius over time. In a typical experiment, TBA-AuNPs diluted in 100  $\mu$ L PBS buffer at the final concentration 0.1 OD at 540 nm were covered with 0.85 nmol of thiol modified TBA (Eurogentec). The initial size of the NP was measured as the control assuring that there was no aggregation without thrombin (Sigma-Aldrich), which was then introduced at a concentration of 50 nM. Thrombin injection into HD1-AuNP and HD22-AuNP mixture led to rapid aggregation, as expected and shown in many other experiments (Fig. 2). Next the same experiment carried out with only one TBA on AuNPs showed rapid aggregation for HD1 and a slight onset in average diameter of  $3 \pm 0.5$  nm for HD22, which is consistent with the binding of thrombin to NPs. This result therefore shows the existence of multiple binding sites for HD1, in agreement with earlier reports on the weak association of HD1 to thrombin exosite II<sup>12,25</sup>. Further complementing experiments were conducted to clarify whether the second binding site for HD1 was indeed exosite II. We reasoned that the specificity of HD22 binding to thrombin exosite II could be used to block the access to this site by pre-incubation of thrombin with an excess of HD22 for 20 min. This HD22-thrombin complex was then injected into the HD1-AuNP solution, and aggregation did not take place. Rather the average diameter increased by  $15 \pm 2$  nm, in agreement with the formation of ternary complex between HD1-AuNP, thrombin and HD22. Altogether our results indicate that HD1 has binding sites with thrombin towards exosite I and also at the vicinity of exosite II. We made a final round of experiments to estimate the binding energy of HD1 to these two sites. HD1-AuNP was incubated with excess of single-stranded DNA complementary to HD1 (ssHD1'), and this mixture was then injected to a solution of HD22-AuNP and thrombin or only thrombin. Aggregation occurred normally in the latter case, but not in the former one (not shown). The successful aggregation suggests that HD1 binding to exosite I is favored energetically over HD1-HD1' pairing, whereas thrombin could not displace HD1' for binding HD1 to exosite II leading to a

constant hydrodynamic radius. Given the energy of HD1-HD1' pairing of  $-29.35 \text{ kcal.mol}^{-1}$  (OligoAnalyzer IDT), our simulations are consistent with these estimates of HD1 binding to exosite I, since all calculated binding energies range from  $-66.73$  to  $-47.03 \text{ kcal.mol}^{-1}$ . Moreover the low energy of the secondary binding site of HD1 to exosite II of less than  $30 \text{ kcal.mol}^{-1}$  likely accounts the absence of crystals of this complex.

In this paper, a theoretical study of all different thrombin-aptamer complexes available in the PDB is conducted to investigate the possible binding modes of HD1 and HD22 to thrombin. Simulations show that HD1 has multiple modes of interaction to thrombin exosite I, which possibly co-exist, and HD22 has higher affinity for exosite II. Experiments confirm that the binding landscape of HD1 to thrombin is not limited to exosite I, and suggest a secondary low-affinity interaction to the vicinity of exosite II. These observations call into question the relevance of HD1 for sensing thrombin in real conditions involving abundant proteins with residual non specific interactions. Consequently the selection of aptamers through the SELEX protocol is not sufficient to guarantee the successful design of biosensing systems. This result is consonant with our previous observation that the attachment of an aptamer to a surface was interfering with its folding and potentially with its interaction properties<sup>26</sup>. Altogether, we argue that structural data, molecular modeling, and kinetic experiments are required to collect the full benefits of aptamers in technological developments.

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## **SUPPLEMENTARY MATERIAL**

See supplemental material at [URL will be inserted by AIP] for more information about the aggregation assays described in this letter.

**Figure 1**

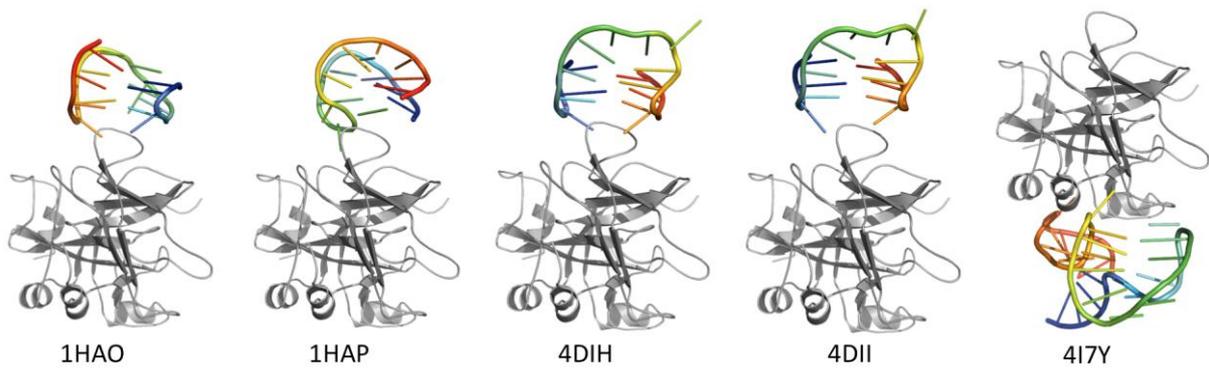


FIG. 1. Molecular structures of four HD1-thrombin complexes (1HAO, 1HAP, 4DIH, 4DII) and one HD22-thrombin complex (4I7Y) used in the calculations. Thrombin is colored in gray and aptamers in spectrum colors from blue to red to indicate the sequence orientation (5' to 3').

**Figure 2**

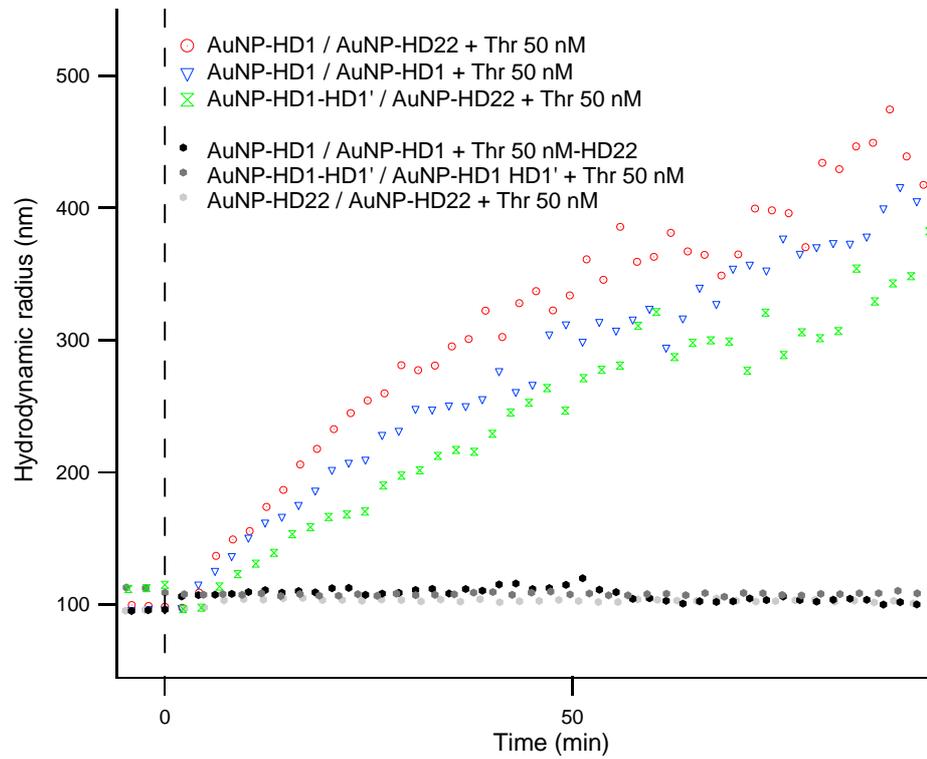


FIG. 2. Aggregation assay of 80 nm AuNPs coated with HD1 or HD22 aptamers. Initial time corresponds to the addition of 50 nM thrombin in each vial. Aggregation is monitored by dynamic light scattering, which yields the average hydrodynamic radius over time.

**Table 1**

	HD1 (1HAO)	HD1 (1HAP)	HD1 (4DIH)	HD1 (4DII)	HD22 (4I7Y)
Energy (kcal.mol <sup>-1</sup> )	-3.0241	-3.0715	-3.1594	-3.1483	-5.9810
$\Delta G$ Binding (kcal.mol <sup>-1</sup> )	-47.0313	-48.8083	-60.2850	-66.7253	-88.3688

Table I. Minimized energy of five aptamer structures extracted from the PDB, calculated with Amber 10 package, with the parm99/bsc0 force field and in implicit solvent. Binding energies of TBAs to thrombin are estimated with the MM-PBSA method.