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Determination of minimal sequence for binding of an aptamer. A comparison of truncation and hybridization inhibition methods

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Abstract: Nucleic acid aptamers are attracting increasing interest as sensing and therapeutic molecules as a consequence of their high affinity and specificity for their target molecules. Aptamers are selected from large random libraries and where structural data are available it appears that only a small fraction of the sequence is actually involved in direct contact with the target. As there are many advantages to minimizing the size of the aptamer a rapid method that can determine those parts of the sequence critical for the target binding would be very useful. In this paper we describe mapping the effective binding region of an aptamer selected against *electric eel* Acetylcholinesterase. As originally selected this aptamer is 77 nucleotides in length and was shown to bind the target with a high affinity (K_d = 174 ± 27 pM). Truncation to 39 nucleotides enhanced affinity for its target by an order of magnitude (K_d = 14 ± 1 pM). To further probe the relationship between sequence and affinity, we used two approaches: truncation and hybridization inhibition. Binding assays were performed with a number of truncated variants to determine a minimal binding sequence. A similar set of measurements for hybridization inhibition was also performed to allow a comparison of the two approaches. In general hybridization inhibition resulted in comparable conclusions to those found by truncation. The exception to this was where the former resulted in steric

clashes between double stranded DNA regions and the target. In this case the effect on affinity was less pronounced with truncation.

Key words: aptamers, truncation, affinity, hybridization inhibition, competitive probe

Introduction

In vitro selection of nucleic acid aptamers starts with a random sequence library typically with a length that varies from a couple of dozen to over a hundred nucleotides (the core) flanked by forward and reverse priming regions of further tens of nucleotides in length [1]. It is not necessary for every nucleotide of the sequences that emerge from selection to be involved in the target binding. Nucleotides in the priming regions have been shown to occasionally be involved in binding [2] but more often they have little or no effect [3]. In addition, not all the nucleotides in the random regions make a contribution to the aptamer-target interactions. For example, the most investigated DNA aptamer (to human α-thrombin) was selected from a library of 60 nucleotides in the random region flanked by 50 nucleotides in the two priming regions but subsequently was shown to require only 15 nucleotides [4]. Another example is the first approved aptamer-based drug, an RNA aptamer against VEGF, was selected from two RNA libraries with 61 and 71 nucleotides in total [5] and was trimmed to 27 nucleotides for its use as a drug [6]. More recently the single round selection followed by deep sequencing of the selected pool approach described by Hoon *et al.* found many naturally truncated sequences in the enriched pool [7]. It is often desirable to generate minimal length, binding sequences as reagents in binding assays and structural studies and subsequently for sensing or therapeutic uses [810]. There are a number of advantages in eliminating unnecessary nucleotides and decrease the aptamer size. Firstly it obviously reduces production cost of the aptamers. Secondly it was reported that removing those nucleotides not involved in binding could increase the aptamer affinities and subsequently

their performance as therapeutic or analytical reagents [11; 12]. In addition, structural studies would benefit from reducing the molecules' size.

Initially clues to identify potential binding sequences of selected aptamers could be obtained by sequence analysis to find common motifs, or alternatively identifying G-quadruplet structures and preference-binding motifs that had previously been discovered either in naturally occurring nucleic acids or arise frequently during *in vitro* selection [1317]. In most cases, where no such clues can be found, the experimental approach of sequence truncation is used. Truncations at either the 5'- or 3'- end of sequences are easily generated and the minimal binding sequences can be identified through the binding constants of differently truncated variants [11; 18]. The truncated variants are mainly chosen empirically [5; 19] but it has also been reported that predicted secondary structures can be used as guidance [20; 21]. However, as aptamers often undergo conformational changes upon binding to their targets, secondary structures in the unbound states can be very different from those in the bound state and therefore truncation using predicted secondary structures of the unbound forms could be misleading.

Typically truncation studies were done by synthesizing new sequences with nucleotides eliminated from the full length sequences, which requires synthesis of a whole new sequence for each truncated variant [5; 19; 22]. Alternatively mapping sequences can also be done through hybridization inhibition using competitive probes, which only requires preparing a short complementary oligo to the sequence being investigated [23; 24]. As no direct comparison of truncation with hybridization inhibition has been made previously, we assessed both approaches with a high affinity aptamer selected against *electric eel* Acetylcholinesterase (AChE) and which has a K_d value of 174 ± 27 pM. Binding assays with

both truncated and inhibited variants were performed. When combined with calculation of the binding of competitive probes to the aptamer this offers a free energy-guided design for effective competitive probes for hybridization inhibition in determination of minimal sequence for binding.

Materials and Methods

Selection of the aptamer

A DNA library with the central region containing 41 randomized nucleotides and 18 nucleotide priming regions (AAGCA TCCGC TGGTT GAC–N41-GATCT TGGAC CCTGC GAA) was used as the starting pool for the selection. Details of the selection condition are described in [25]. After 15 rounds of selection, the enriched pool was cloned and individual colonies were picked for sequencing. One particular sequence (R15C19) was found with high frequency (40%) and used for the subsequent truncation studies.

Hybridization of the probes and the aptamer

Reverse and complementary oligos were used for completive hybridization experiments. Initial solutions containing 1 μM of the biotinylated R15C19 full length and 1.25 μM of a probe in the selection buffer were heated at 95^{o}C for 5 min and then lowered to 50^{o}C for 10 min and then cooled at room temperature for 20 min. The solutions of desired concentrations of the aptamer for binding assays were diluted from these initial solutions.

ELONA assays

K_d values were determined using an Enzyme Linked Oligonucleotide Assay (ELONA). In this assay the target (AChE) is adsorbed in the wells of a microtiter plate and titrated with biotinylated aptamer, incubated and then the wells washed with buffer. The amount of captured aptamer is then determined by adding streptavidin-HRP and suitable substrates and measuring the absorbance.

For immobilization of the target, 100 µL of 10 nM AChE solution in PBS was added to each well of 96-well polystyrene plates and incubated at 4°C overnight. The AChE solutions in the wells were discarded and the wells were then washed 4 times with 150 µL of PBS supplemented with 0.05% (v/v) Tween 20 (PBST) to remove weakly adsorbed molecules before being blocked with 1% (w/v) BSA. 100 µL of selection buffer (for background) or a solution of the biotinylated aptamer in the selection buffer was then added and incubated at room temperature for 2 h. The unbound DNA was removed by washing 4 times with 150 µL of the selection buffer supplemented with 0.05% (v/v) Tween 20. The bound DNA was determined by adding 100 µL of a streptavidin-HRP solution and incubated at room temperature for 30 min. Streptavidin-HRP is bound to the DNA through biotin incorporated on the aptamer. Excess streptavidin-HRP was removed by washing 4 times with 150 µL of the selection buffer supplemented with 0.05% (v/v) Tween 20 and 0.1% (w/v) BSA. The amount of HRP retained corresponds to the amount of bound aptamer. Quantification of the HRP, hence the bound aptamer, was done by measuring HRP activity using 100 µL of TMB substrate solution. The enzyme reaction was quenched by addition of 100 µL of 2 M H_2SO_4. Finally the absorbance at 450 nm is measured.

Calculation of the ΔG values

The free energies of formation of the aptamer-AChE complexes were established from the experimentally-obtained equilibrium constants. The free energies of hybridization were calculated based on the nearest-neighbor method using the UNAfold [26].

Melting temperature measurements

Aptamer solutions of around 15ng/µL were prepared for the melting experiments. The melting temperature measurements were performed using a Perkin Elmer Lamda 25 with a Peltier for controlling temperature. The temperature was ramped 0.5°C per minute and the absorbance was recorded minutely.

Results and discussion

The aptamer selection against AChE resulted in the following sequence (R15C19); AAGCATCCGC TGGTTGACTG TAGCTCTGGC AGACGTAGTG TGAAGGTACC AGCTATTGGG ATCTTGGACC CTGCGAA (primer regions underlined). ELONA binding assays showed R15C19 has a high affinity towards the target, with a K_d value of 174 ± 27 pM (Figure 1).

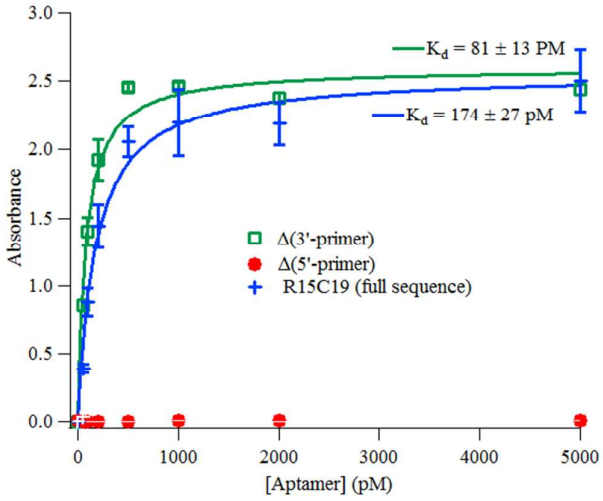

Fig. 1: Binding data for the full length aptamer is shown by the crosses (K_d = 174 ± 27 pM), the aptamer with the 3'-primer truncation (Δ(3'-primer)) is shown by the squares (K_d = 81 ± 13 pM) and the aptamer with the 5'-primer truncation (Δ(5'-primer)) is shown by the circles. Removing the whole 5'-priming sequence results in a complete loss of affinity The K_d values were calculated by fitting the binding curves to a hyperbolic equation (Langmuir model) using Igor Pro (WaveMetrics).

Truncation of the AChE-binding aptamer

The full length sequence binds AChE with high affinity (K_d = 174 ± 27 pM) as shown in Figure 1. Truncation of the 3'-priming sequence resulted in no reduction in affinity (K_d = 81 ± 13 pM) whilst removing the 5'-priming sequence caused a complete loss of affinity. This suggests that a part or all of the 5'-priming region is involved in the binding to AChE whilst the 3'-priming region is not. These observations confirmed that a number of nucleotides of

the selected aptamer are not required for binding to the target. In order to further refine the sequence, we measured the binding constants of different truncated variants where nucleotides were removed from the 5'- and 3'-ends. The sequences of the truncated variants are listed in the Supplement data.

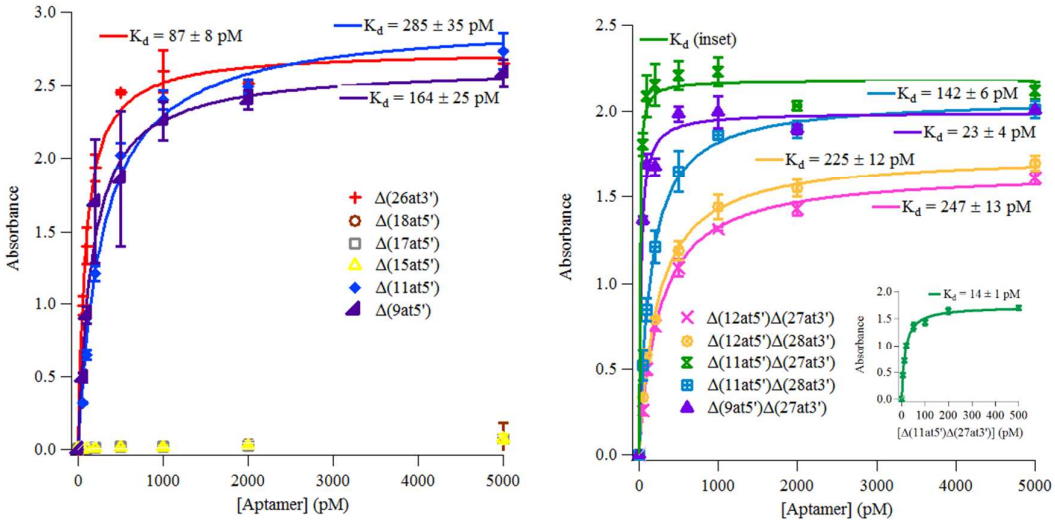

Fig. 2: Binding curves of the truncated variants to AChE. Truncation (Δ) of a 3'- or a 5'-end and of dual 3'- and 5'-ends. The K_d values were obtained using ELONA. The binding curves were fitted to a hyperbolic equation (Langmuir model) using the Igor Pro.

As shown in Figure 2, the affinities of variants with deletion of 18, 26, 27, 29, 35 nucleotides from the 3'-end and 18, 17, 15, 13, 11, 9 nucleotides from 5'-end were determined. The K_d values in Table 1 showed that not only was the entire 3'-priming region not involved in the binding to AChE but also several nucleotides at the 3'-end of the core could also be removed without loss of affinity. Removing 27 nucleotides at the 3'-end, including 9 nucleotides in the core, resulted in no loss of binding affinity suggesting all of these 27 nucleotides do not make any 'binding contribution' to the target. However, eliminating a further 3 nucleotides gave a 30-fold loss in affinity whilst removal of an additional 6 nucleotides showed a loss of around 80-fold in affinity indicating that the nucleotides in this region are involved in binding.

Truncation studies at the 5'-end showed that removal of up to 15 nucleotides resulted in a complete loss of the binding whilst removal of 13 nucleotides at the 5'-end caused a 3 order of magnitude reduction in binding. Removal of 9 and 11 nucleotides, respectively, resulted in no loss in binding suggesting that 7 nucleotides of the 5'-priming sequence were involved in the binding of the aptamer to the AChE.

As there were clear differences between the K_d values of truncation of 27 to 29 nucleotides at the 3'-end and 11 to 13 nucleotides at the 5'-end, truncated variants 27, 28 nucleotides at the 3'-end and 11, 12 nucleotides at the 5'-end were then tested to establish the minimal binding sequence.

From the binding data shown in Table 1, it can be seen that the minimal binding sequence comprises 11 nucleotides fewer at the 5'- plus 27 nucleotides fewer at the 3'- ends. This sequence is only just over a half of the length of the sequence that was originally selected (39 nucleotides compared to 77 nucleotides of R15C19) but also has an order of magnitude higher affinity. This will be discussed in more detailed latter.

Identifying the minimal binding sequence by hybridization inhibition

As previously mentioned identification of the minimal binding sequence has often been achieved through truncation [5; 19; 22] but hybridization inhibition using competitive probes has also been used [23; 24]. In addition, hybridization inhibition was recently employed for eliminating binding interference of priming regions during SELEX [27]. We directly compare, for the first time, the two approaches. Based on the truncation results, a corresponding set of competitive probes for hybridization inhibition were performed to the respective regions of the original aptamer sequence as shown in Figure 3.

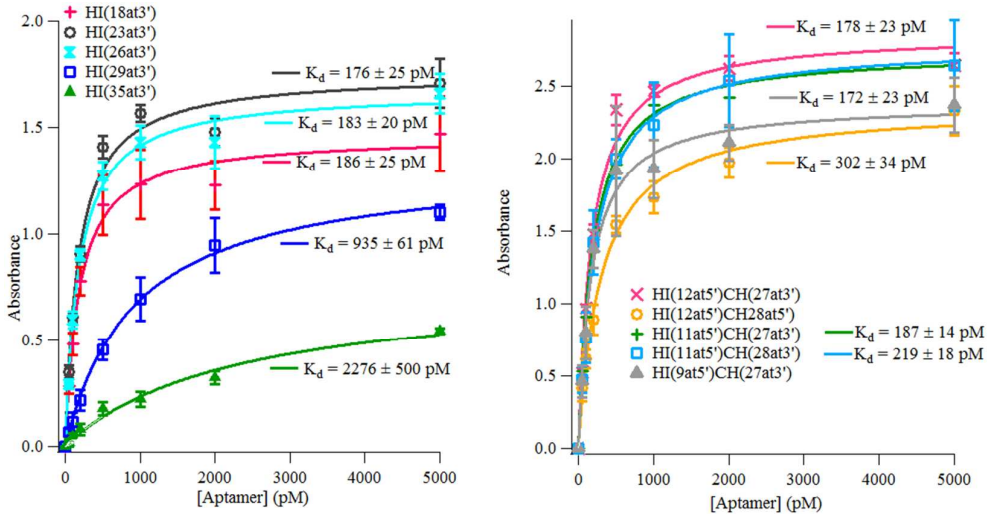

Fig. 3: Binding curves of the variants by hybridization inhibition (HI) toward AChE. The complementary probes were used to hybridize with parts of the original aptamer. The K_d values were calculated from binding data obtained using ELONA. The binding curved were fitted to a hyperbolic equation using the Igor Pro.

These probes were designed to hybridize to the 5'-end and the 3'-end. As shown in Table 1, the K_d values for inhibited variants using competitive probes showed a similar pattern in affinities as found with the corresponding truncated variants. Hybridization inhibition using competitive probes for each end as well as both ends of up to 11 nucleotides at the 5'-end and up to 27 nucleotides at the 3'-end yielded K_d values ~ 130-190 pM, closed to the K_d value (174 pM) of the original sequence, showing these nucleotides do not contribute to the affinity for the target and so allows identification of the minimal binding sequence of the aptamer. This is consistent with the truncation results in indicating those nucleotides are not involved in aptamer-target binding. However, truncation generally has higher affinity than hybridization inhibition in this case. Truncation at both 5'- and 3'- ends shows approximately an order of magnitude higher affinity than the full length and the hybridized forms. We attribute the higher affinity to the loss of intramolecular hydrogen bonds. That effect is not

seen on the hybridization may be owing to the fact that the 5'- and 3'- ends become much more rigid due to their double stranded nature and that this loss of flexibility leads to an increased effect of steric clashes between the ends of the aptamer and the target when the intervening sequence binds to the target. This argument implies that hybridization of inhibitory probes could either increase or decrease affinity depending upon the balance between eliminating unproductive intramolecular hydrogen bonds and increasing steric clashes between the double stranded regions and the target.

Table 1: K_d values for different variants of truncation (Δ) and hybridization inhibition (HI). The highlights show the primer regions. The K_d values were obtained using ELONA. The sequences with no detectable binding were indicated as ND whilst the sequences with K_d values that could not be determined by the Langmuir binding model are shown as ND*.

Variant	K_d (pM)	
	Truncation (Δ)	Hybridization Inhibition (HI)
R15C19 (full sequence)	174 ± 27	
18at3'	81 ± 13	186 ± 25
26at3'	86 ± 8	183 ± 20
27at3'	102 ± 14	176 ± 20
29at3'	(4.7 ± 0.3) x10^3	935 ± 61
35at3'	(12.2 ± 2.4)x10^3	(2.3 ± 0.5)×10^3
18at5'	ND	ND
17at5'	ND	ND
15at5'	ND	ND*
13at5'	(68.8 ±4.4) x10^3	ND*
11at5'	285 ± 35	134 ± 11
9at5'	164+25	164 ± 23
(12at5')(27at 3')	247 ± 13	178 ± 23
(12at5')(28 at 3')	225 ± 12	302 ± 34
(11at5')(27at3')	14 ± 1	187 ± 14
(11at5')(28at3')	142 ± 6	219 ± 18
(9at5')Δ(27at3')	23 ± 4	172 ± 23

Furthermore, with variants where truncation caused loss of binding affinities as in removing of 29 and 35 nucleotides at the 3'-end, hybridization inhibition using the matching competitive probes had K_d values around 5-fold higher. Hybridization inhibition using competitive probes of 18 and 17 nucleotides at the 5'-end showed no binding of the variants to AChE consistent with the truncation results in indicating removal of these nucleotides causing a complete loss in binding affinities. The loss of affinity showed in both truncation and hybridization inhibition indicating the involvement of these nucleotides. The consistency in showing whether or not the nucleotides were involved to the binding of the aptamer and its target by truncation and hybridization inhibition suggests that the latter can also be efficiently used in determining nucleotides that do not contribute to the binding. With competitive probes of 15 and 13 nucleotides at the 5'-end, the data could not be fitted to a simple Langmuir isotherm and therefore no K_d values were obtained.

Effectiveness of the competitive probes for hybridization inhibition

A common design for aptamers used in sensing applications is the so-called aptamer beacon [2830]. In this design the aptamer is incorporated into a stem-loop structure, such that target binding results in disruption of the double stranded stem. Unlike aptamer beacons, in hybridization inhibition, a competitive probe has to have a more stable structure with aptamer than the aptamer-target complex. Thermodynamically, those DNA hybridized complexes should have lower free energy (ΔG) values. Table 2 shows the calculated ΔG values and the derived calculated K_d values of the aptamer-probe complexes. The values were calculated as the hybridization free energy of the probes upon forming double stranded DNA.

Table 2: ΔG values of the probes hybridized with the aptamer regions. The values at 25°C (298 K) were computed using the UNAFold [26].

Competitive probe	Sequence	Calculated $\Delta G_{298,\ hyb}$ (kcal×mol^{-1})	Calculated $K_{d,\ hyb}$ (M)
CH(9at5')	AAGCATCCG	-11.5	4.2×10^{-9}
CH(11at5')	AAGCATCCGCT	-15.2	8.4×10^{-12}
CH(13at5')	AAGCATCCGCTGG	-18.9	1.7×10^{-14}
CH(15at5')	AAGCATCCGCTGGTT	-21.5	2.2×10^{-16}
CH(17at5')	AAGCATCCGCTGGTTGA	-24.9	7.2×10^{-19}
CH(18at5')	AAGCATCCGCTGGTTGAC	-26.3	6.8×10^{-20}
CH(18at3')	GATCTTGGACCCTGCGAA	-25.6	2.2×10^{-19}
CH(23at3')	ATTGGGATCTTGGACCCTGCGAA	-33.2	6.4×10^{-25}
CH(26at3')	GCTATTGGGATCTTGGACCCTGCGAA	-37.9	2.4×10^{-28}
CH(29at3')	CCAGCTATTGGGATCTTGGACCCTGCGAA	-42.9	5.5×10^{-32}
CH(35at3')	AAGGTACCAGCTATTGGGATCTTGGACCCTGCGAA	-51.3	4.2×10^{-38}

The longer the competitive probe, the more stable the duplex is and therefore the more effective in hybridization inhibition. Hybridization of the original aptamer sequence with the probe of 9 nucleotides at the 5'-end has a calculated K_d value of -4.2×10^{-9} M, which is 25-fold larger than the K_d value of the original aptamer-target binding (174 pM) and therefore should not be effective at inhibiting target binding. Those probes have calculated K_d values for aptamer hybridization significantly lower than the aptamer-target binding's values would be expected to be effective in hybridization inhibition for identifying the minimal binding sequence. As shown in Table 2, hybridization of the 17-nucleotide competitive probe and the full length aptamer has a calculated K_d value of 7.2×10^{-19} M, which is 8 orders of magnitude lower than K_d values of the aptamer-target binding. Our hybridization inhibition results showed binding affinity had a significant reduction by the probes of 29 and 35 nucleotides at the 3'-end and a complete loss by the probes of 17 and 18 nucleotides at the 5'-end and was consistent with the truncation indicating that they are efficient as the competitive probes.

Finally, with the probes of 13 and 15 nucleotides at the 5'-end where the ELONA data showed evidence of binding whilst the predicted Kd values for hybridization where 2-4 orders of magnitude smaller, we attributed this to uncertainties in the calculation of $K_{d,hyb}$. Calculations of the ΔG values of DNA hybridization are known to be different from the experimentally obtained values. For example Breslauer *et al* reported variation between neighbour-nearest calculations and calorimetric measurements [31].

Improvement in binding affinity of truncated sequences over the full sequence

The truncated aptamer Δ(11at5')Δ(27at3') has a K_d = 14 ± 1 pM, which is an order of magnitude lower in K_d value of the original aptamer (K_d = 174 ± 27 pM). The decrease in K_d values of the truncated aptamers compared to the longer full length aptamer has been reported before. Examples include a truncated DNA aptamer for VEGF that had over 200-fold lower K_d value compared to its parental sequence [11] and a 4-fold decrease in K_d for a truncated RNA aptamer against human α-thrombin [32].

Some studies have attributed reduced steric hindrance as the reason for the improvement of the truncated aptamers compared to their parental sequences [21; 33] and this is not unexpected as reducing the size of the aptamers wold give less opportunity for steric clashes. However, apart from the effect of steric hindrance, it could be that the difference in free energy of binding of truncated versus the full length aptamers is due to unproductive intra-molecular interacting in the latter which would have to be broken to form productive inter-molecular interactions in the aptamer-target complexes as aptamers have been known to undergo conformational changes upon binding to their targets. To estimate the extent of intramolecular based pairing we determined the melting temperatures of the R15C19 and the truncated derivative Δ(11at5')Δ(27at3') that has over an order of magnitude higher affinity

for AChE. The predicted structures of these aptamers and the melting curves are shown in Figure 4.

The extensive hydrogen bonded stem predicted for the full length sequence is predicted to be absent in the truncated form potentially allowing the latter to more readily interact with the AChE. It is worth noting that most of one strand of the stem is the 3'-priming sequence. The full length aptamer had a melting temperature of 87.6°C compared to a melting temperature of 66.4°C for the truncated aptamer, consistent with the greater degree of intramolecular base stacking in the former by the predicted structures. In addition, calculations based on the changes in absorbance over DNA denaturation resulted in the R15C19 had 57% of its nucleotides in single stranded state at the lower plateau temperatures (before melting) compared to 76% for the truncated Δ(11at5')Δ(27at3').

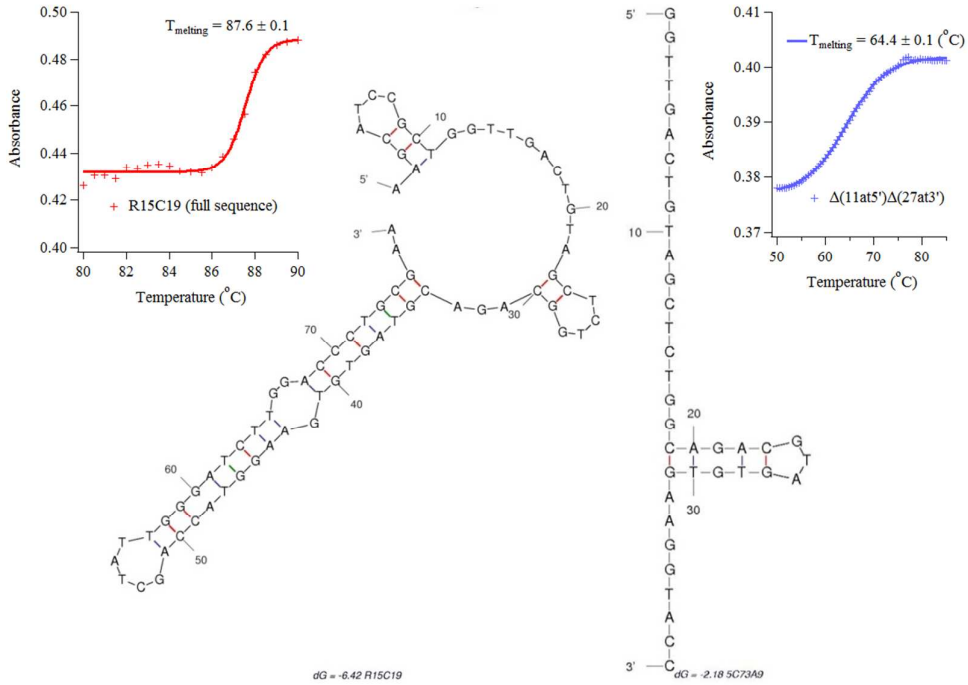

Fig. 4: Melting curves along with predicted structures of the full length R15C19 aptamer and its truncated version Δ(11at5')Δ(27at3'). The predicted structures and the computational ΔG values were obtained using MFold [34].

Conclusions

A 77-nucleotide DNA aptamer was selected against AChE with and a K_d value of 174 ± 27 pM and when truncated to 39 nucleotides it had over one order of magnitude lower K_d value (14 ± 1 pM). Truncation revealed that part of the 5'- primer sequence was involved in binding but the 3'-primer sequence along with 9 adjacent nucleotides in the core region could be removed without loss of affinity. Using this high affinity aptamer, we studied the effect of both truncation and hybridization inhibition using competitive probes on the target's affinity. The experimental data showed that when suitable complementary competitive probes were chosen, hybridization inhibition could be used to identify the nucleotides that do not contribute to the binding of the aptamer and the target and with the added advantage of needing only short probe sequences. We used free energy calculations in guiding the design of the probes. Our data also support the idea that apart from steric hindrance, a significant contribution to the improvement in K_d values of truncated aptamers is due to eliminating unproductive intra-molecular interactions.

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