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MINIREVIEW

Conformational flexibility and structural dynamics of aptamers

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ABSTRACT

This minireview summarizes two important features of aptamer structures: conformational flexibility in their free and bound states, and their structural dynamics that can be influenced by sample preparation and experimental conditions. Examination of aptamer structures in the literature supports the notion that aptamers are pre-disposed to adopting secondary and tertiary structures that can undergo conformational changes of different degrees upon binding to their ligand. While it is difficult to generalize the structural similarity between the free and bound state, as they vary from aptamer to aptamer, such knowledge can be useful for aptamer sequence design and optimization. On the other hand, aptamer sequences can adopt different secondary structures in their ligand-free state, which can be re-populated when thermally treated. This aspect of structural dynamics of aptamers should be considered in sample preparation to ensure experimental reproducibility involving aptamers.

KEYWORDS: Aptamer, conformational flexibility, adaptive recognition, secondary and tertiary structure, ligand

INTRODUCTION

Interest in the use of aptamers has increased rapidly in recent years. A survey in Web of Science showed that the number of publications containing “aptamer” in the title or topic (abstract) rose from 8 and 10 in 1993 to 787 and 2312 in 2022 (Figure 1), respectively. This trend is not surprising, given the advantages and versatility of aptamers, especially in comparison with antibodies (Keefe et al, 2010). It is well known that single-stranded nucleic acids can form intramolecular secondary and tertiary structures (Gupta et al, 1980; Kaushik et al, 2016; Hu et al, 2019). This nucleic acid conformational flexibility remains true for nucleic acid-based aptamers. Thus, a clear understanding of aptamer structures in their ligand-free and bound states is of crucial importance. This knowledge is limited at this point, as the number of aptamers with their structures resolved for both ligand-free and bound states is small. Furthermore, the difficulty to reproduce binding properties of some aptamers has prompted the recommendation of “publication standards” on aptamer research from the International Consortium of Aptamers (Mckeague et al, 2022). In this respect, interest in our laboratories in studying nucleic acid conformational plasticity (Sun et al, 2020; McAdorey et al, 2021; Bennett et al, 2023) drew our attention to evaluate the possible contribution of structural dynamics on aptamer binding properties. Here, we summarize examples of aptamer conformational flexibility and structural dynamics, highlighting the importance of these structural aspects in aptamer research.

Conformational flexibility of aptamers - structural comparison between free and bound aptamers

Secondary and tertiary structures are necessary for the binding of an aptamer to its ligand. Thus, an important aspect of aptamer research focuses on understanding whether aptamers are innately folded in their ligand binding conformation even in the absence of any ligand, or they only fold upon interaction with their target molecule. Both scenarios are observed in the literature and the aptamer

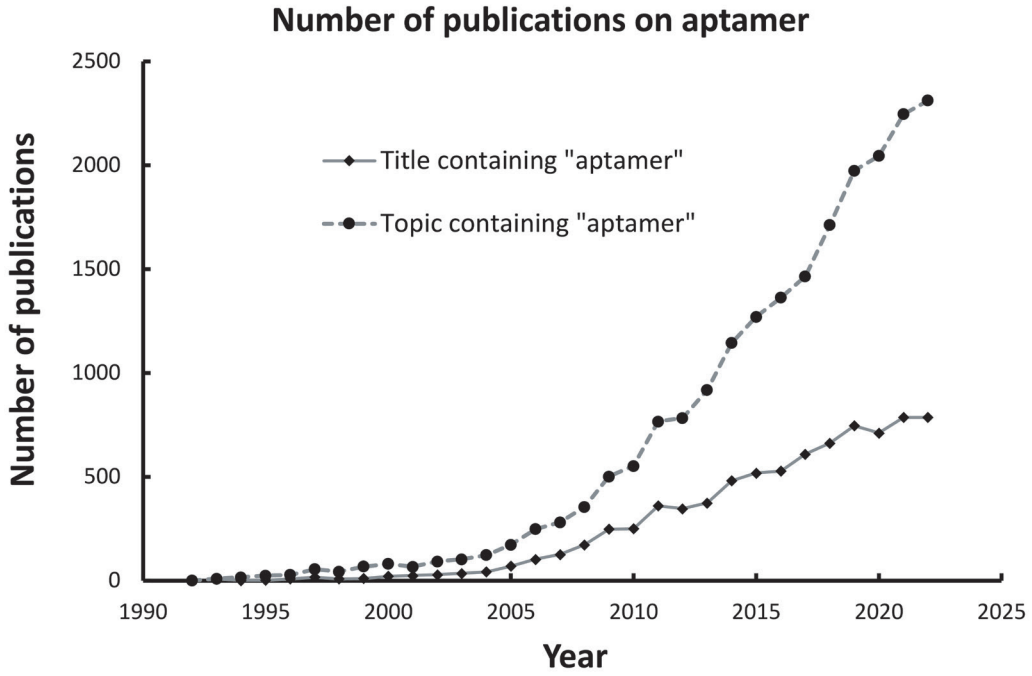

Figure 1. Number of publications containing "aptamer" in the title or topic from 1993 to 2022. Numbers were retrieved from the Web of Science.

conformational changes likely depend upon the type of aptamer-ligand pair and the nature of their interaction (Hermann and Patel, 2000). To date, however, only a few aptamer structures have been resolved in the absence and presence of ligands, allowing clear understanding of the structural conformations present in free and bound states.

During the SELEX (systematic evolution of ligands by exponential enrichment) process for the evolution of aptamers, nucleic acids are subjected to evolutionary pressures to select for those that can bind to the desired target, a process that has been used in the past to support the notion that aptamers fold in response to ligand binding (Hermann and Patel, 2000). When free in solution, aptamers may possess unpaired and/or disordered regions which become defined when ligands are present, a process termed adaptive recognition (Hermann and Patel, 2000). The thermodynamic cost required to undergo conformational changes upon binding to ligands is compensated by the binding affinity and shape complementarity of the aptamer to its ligand (Amano et al, 2016; Gelinas et al, 2016; Sakamoto et al, 2018). This conformational change upon ligand binding has been shown to occur with some aptamers targeting protein ligands.

Examples of aptamer conformational flexibility discussed in this section are limited to those where the 3D structures of ligand-free and bound aptamers are available. Superposition of ligand-free and ligand-bound structures will provide insight into the structural relationship of aptamers between these two states.

RNA aptamer against ribosomal protein S8 from Bacillus anthracis

The structures of ribosomal protein S8 from *Bacillus anthracis* have been resolved for both free and bound aptamers, where the RNA aptamer undergoes substantial conformational changes upon binding the target (Davlieva et al, 2014). A SELEX experiment identified an aptamer which binds to S8 protein with high specificity and high affinity (Davlieva et al, 2014). As shown in the free state structure resolved by NMR, the aptamer forms a well-ordered helical duplex including some non-canonical base pairs. When the RNA aptamer binds to S8 protein, the crystal structure shows that multiple base pairs are disrupted, and some are re-paired with different nucleotides. Interestingly, some duplex bases rearrange into a triplex and quartet (see Figure 8 in Davlieva et al, 2014).

It should be noted, however, that the sequences of free and bound S8-aptamer are not identical (Figure 2a). Despite the consensus sequence in the core stem-loop region, the stem sequences are different. As such, alignment using the RNAalign program (Gong et al, 2019) failed to recognize the common fold derived from consensus sequence, and thus gave a rather low RMSD (3.48 Å, Figure 2b), which does not reflect the rather large overall differences between the free and bound structures. Superposition of the free and bound structures in Maestro against RNA backbone, on the other hand, aligned the consensus sequence (Figure 2c), but with a large RMSD (16.18 Å), which resembles the large conformational changes of this aptamer upon binding to its ligand. The authors (Davlieva et al, 2014) suggested that this aptamer is the first instance where an RNA aptamer structure in the free and bound state was found to be largely different, highlighting the prevalence of RNA sequence polymorphism and the influence that ligand has on structure.

Thrombin-binding DNA aptamer

The thrombin-binding DNA aptamer is an example where a defined structure is formed prior to ligand binding,

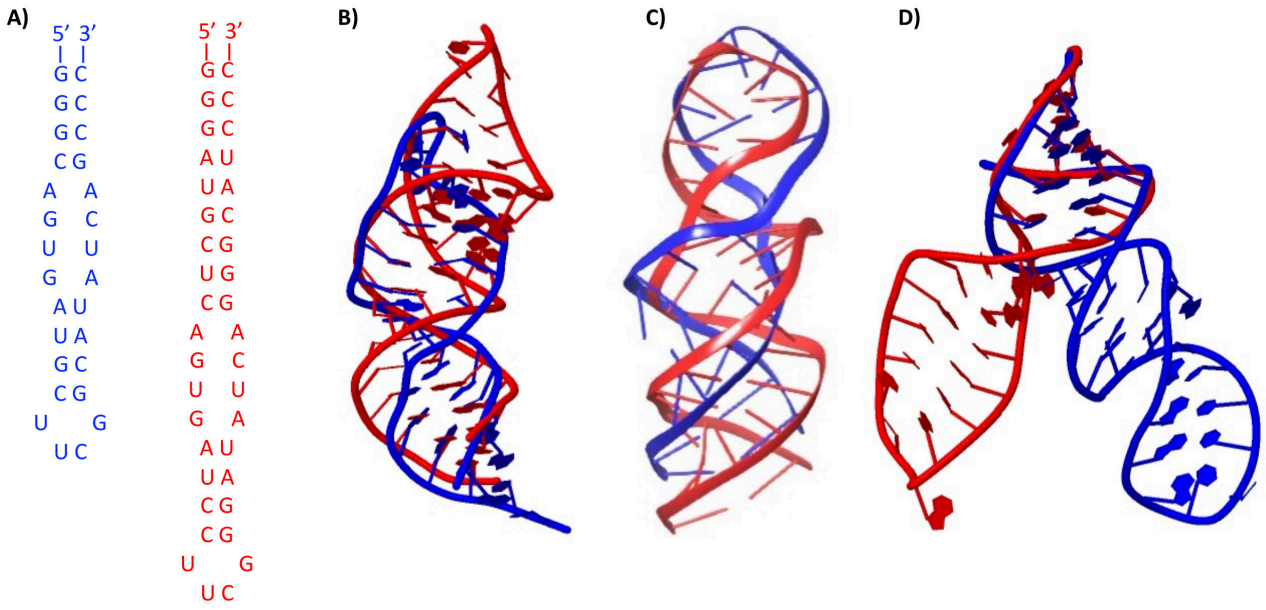

Figure 2. Secondary and tertiary structures of ligand-free and bound S8 aptamer. **A**. Secondary structures of S8-binding aptamer (Blue: structure 6 from 2LUN (NMR structure of ligand-free aptamer, structure 6 was determined to be closest to average structure by the WHAT IF program); red: 4PDB (ligand-bound aptamer)). **B.** Ligand-free and bound structures superimposed using RNA-align, which gives an RMSD of 3.48 Å. **C.** Ligand-free and bound structures superimposed using Maestro (Version 13.5.128, Schrödinger Release 2023-1) against RNA backbone, with an RMSD of 16.18 Å. **D.** A control superposition of 4PDB (Blue) with 1OOA (red), an RNA aptamer against the $p50_2$ mammalian transcription factor NF-kB, showing poor alignment of the two aptamers.

although minor changes are observed upon binding (Figure 3). The structure of the 15-mer thrombin aptamer was solved in solution by NMR as unbound (Macaya et al, 1993) and bound to human thrombin by X-ray crystallography (Padmanabhan et al, 1993). NMR structure of the ligand-free aptamer in solution showed that the aptamer forms a defined G-quadruplex structure (Macaya et al, 1993). The crystal structure of the aptamer in complex with thrombin was also found to be a G-quadruplex (Padmanabhan et al, 1993). The 15-mer aptamer forms a stacked G-quartet with a TGT loop on one face of the quartet, and two TT loops on the opposite face. Only minor structural changes were observed upon complexation with thrombin, that is, changes in the positioning of the T bases in the loop regions. This change is likely due to the interaction of various T bases with amino acid residues in thrombin to stabilize the complex (Padmanabhan et al, 1993).

RNA aptamer against the $p50_2$ mammalian transcription factor NF-kB

Another example of an aptamer that is present in similar general conformations in the free and bound states is an RNA aptamer against the $p50_2$ mammalian transcription factor NF-kB (Reiter et al, 2008). The aptamer folds into a stem-loop containing an asymmetric internal loop (Reiter et al, 2008). The structure of the free aptamer determined by NMR spectroscopy possesses some structural elements that are quite similar to that of the structure in the ligand-bound complex resolved by X-ray crystallography, but still with considerable differences, as a result of induced fit upon binding (Reiter et al, 2008). The overall structural comparison is best illustrated in the two superimposed views shown in Figure 4. By superimposing the free (red) and bound (blue) structures against RNA backbone in Maestro (Figure 4a), the overall folds of the two states showed a large degree of overlap, with an RMSD of 6.43 Å. Superposition of the two structures using RNA-align (Gong et al, 2019), however, clearly demonstrated a bending within the internal loop to alter stem orientation, and a perturbation of the loop to accommodate the protein ligand (Figure 4b) (Reiter et al, 2008). It should be noted, however, that the free and bound structures are different by swapping the 3'- and 5'-end nucleotides, which should not affect the overall structure of the aptamer.

Binding of different nucleic acid sequences to the same target ligand

Sequence similarity is an important aspect in evolutionary and functional analyses of proteins (Pearson, 2013). Homologous proteins that show similar binding properties to their ligands are well studied. Similar comparative analyses for aptamers would be helpful in aptamer sequence design and optimization. With the knowledge of key interactions between aptamers and their ligand, sequence alignments of these "homologous" aptamers may allow for the identification of redundant regions or regions to introduce mutations in order to optimize binding properties. Indeed, the possibility of different aptamers to bind to the same target has been examined in the literature. The fact that these homologous aptamers are capable of binding to the same ligand reflects the plasticity of aptamers in the 3D space. Some examples of aptamers with different sequences that bind to the same target ligands are shown in Supplementary Table 1. Similarity analyses of the tertiary structures of the aptamers were carried out using RNA-align (Gong et al, 2019) based on the 3D-structures obtained from the PDB

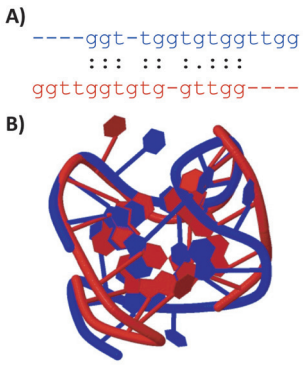

Figure 3. Comparison of free and bound thrombin-binding DNA aptamer structures. **A.** Alignment and, **B** superposition of 1HUT (thrombin-bound, blue) with 148D (free aptamer, Model 10 as most similar to average structure determined by NMR, red). An RMSD of 2.24 Å was determined between the free and bound structures by RNA-align.

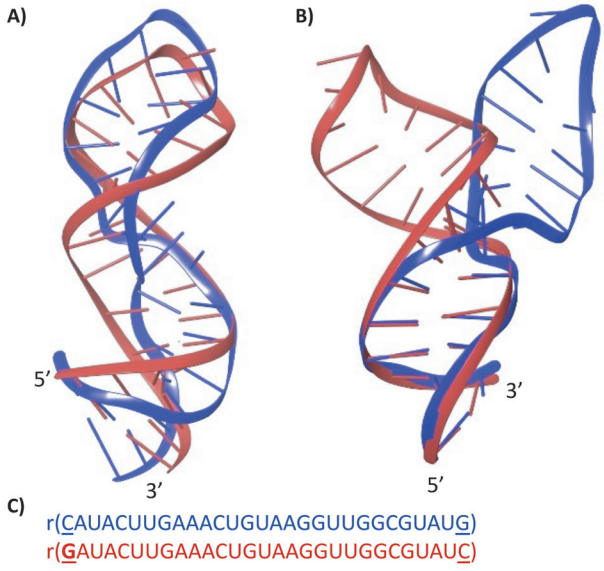

Figure 4. Superimposed structures of free (red, 2JWV) and bound (blue, 1OOA). **A.** Superimposed structure of free (red, structure 7 from 2JWV, as this structure is the closest to the average NMR structure, as determined by WHAT IF) and bound (blue, structure D from 1OOA) against RNA backbone in Maestro. **B.** Superimposed structure of free (red, structure 7 from 2JWV, as this structure is the closest to the average NMR structure, as determined by WHAT IF) and bound (blue, structure D from 1OOA) against RNA backbone in RNA-align. **C.** The sequences of the free and bound aptamer are different by swapping the 3′- and 5′-nucleotides, as shown underlined.

databank. RNA-align was chosen for this analysis as it aligns oligonucleotides by comparing 3D structures in an optimal nucleotide-to-nucleotide manner, enabled by distance-based secondary structure assignments (Gong et al, 2019). The aptamers listed in Table 1 that bind to the same target ligand were identified in a same selection process, and thus subjected to the same selection pressure. The ones that bind to the HIV-1 Rev peptide, however, were considered from two distinct families. As shown by the superimposed structures and root mean square deviation (RMSD) determined for the structures, these aptamers share overall quite similar tertiary structural features despite their differences in sequence, some of which are quite significant. Binding of these homologous aptamers with different sequences to the same target highlights another level of structural flexibility, which can be of significance for the sequence design and optimization of aptamers, and to study the evolutionary convergence of regulatory RNAs, especially by machine-learning approaches (Andress et al, 2023).

Structural dynamics of aptamers – potential importance in improving the reproducibility of experiments involving aptamers

Some recent literature highlighted a few examples of aptamers where the binding properties originally reported were found irreproducible. This irreproducibility was partially attributed to the method of choice in determining binding constants, along with deficiency in documenting detailed experimental procedures and others.

Table 1 shows selected aptamers that were found not to bind in the way originally reported. Among these, aptamers that were originally reported to bind arsenic (Mina et al, 2009), chloramphenicol (Yadav et al, 2014), ampicillin (Song et al, 2012), and pesticides such as Profenofos and Isocarbophos (Wang et al, 2012; Zhang et al, 2014), were later found not to show specific binding affinity toward their intended ligand. It was speculated that the inability to reproduce original binding properties might be results of multiple factors, such as non-specific binding during aptamer selection, non-specific binding during the measurement of binding affinity, and lack of proper control experiments. Taken together, the examples of irreproducibility in aptamer binding properties demonstrate the importance of the choice of method for analyzing aptamer binding and suggest that multiple complementary methods of analysis should be carried out to properly characterize aptamers with sufficient controls.

Another potential source of irreproducibility of aptamer properties can be attributed to their structural dynamics, which can be related to the way samples are prepared for binding assays. The literature has clearly shown that free and bound aptamers can acquire different folding, and that induced fit can take place when free aptamers are bound to their ligand. The latter aspect was examined with three aptamers, adenosine-, ampicillin-, and quinine-binding DNA aptamers, in a recent publication from our laboratories (Bennett et al, 2023). Using non-denaturing anion exchange high performance liquid chromatography, it was revealed that all three aptamers show more than one conformation when not thermally annealed in the buffer in which they were originally selected. In all three cases, the dominating species are single stranded in nature while the minor species are likely mis-matched double strands. Upon appropriate thermal annealing, however, the majority of double-stranded species were converted to single stranded. Furthermore, addition of ligands to unannealed aptamers did not lead to significant shift in the portions of the double- and single-stranded species. Taking the quinine-binding aptamer MN4 as an example, four different species were eluted off non-denaturing anion exchange HPLC, when the aptamer was not heat-annealed. When the aptamer was annealed at 95°C, however, greater than 97%

Table 1. Aptamers recently shown not to bind to their ligand in ways originally reported.

Aptamer (reference)	Target ligand	Dissociation constant (method of measurement)	Updated binding properties
Ars-3 99-nt DNA aptamer (Mina et al, 2009)	arsenic	~7 nM for As(III) ~5 nM for As(V) (surface plasmon resonance)	no specific binding of As(III) was observed (Zong and Liu, 2019)
40-mer DNA aptamer (Yadav et al, 2014)	Chloramphenicol	Truncated from the original (Mehta et al, 2011) 80-mer DNA aptamer with a K_d of 0.766 µM (fluorescence-based affinity assay)	no specific binding of chloramphenicol was observed (Tao et al, 2020)
AMP4 (21-mer), AMP17 (19-mer), AMP18 (19-mer) DNA aptamers (Song et al, 2012)	Ampicillin	AMP7 (9.4 nM), AMP17 (13.4 nM), and AMP18 (9.8 nM) AuNP aggregation-based colorimetric method	None of the three aptamers showed any specific binding with ampicillin (Bottari et al, 2020)
SS2-55 (55-mer DNA aptamer) (Wang et al, 2012)		Profenofos (1 µM) Isocarbophos (0.83 µM) (fluorescence-based affinity assay)	No specific binding with profenofos and isocarbophos (Zara et al, 2021)
SS24-S-35 (35-mer DNA aptamer) (Zhang et al, 2014)		Shown to bind to profenofos and isocarbophos, but dissociation constants were not determined	No specific binding with profenofos and isocarbophos (Referred to as SS24-35 in Zara et al, 2021)

of the DNA was eluted at a retention time that corresponds to single stranded species, likely the three-way junction structure that are competent in binding quinine (Bennett et al. 2023). These observations are interesting, as very little "induced fit" was seen when ligands were added to free aptamers. It follows that, thermal annealing and resulting redistribution of species in aptamers are expected to affect the binding properties of aptamer. This aspect is currently under examination in our laboratory.

FINAL THOUGHTS

After more than three decades of research, aptamers have shown potential to complement antibodies in many areas of applications. Significant efforts have been made to establish the details of aptamer-ligand interactions, however, only limited number of structures have been resolved by NMR or crystallography (at the time of writing, 414 entries were recorded in the Protein Data Bank with "aptamer" as the search term). While it is commonly believed that aptamer-ligand binding induces conformational changes to aptamers, the extent of these induced conformational changes is not well understood, with the exception of a few aptamers. This is in sharp contrast to riboswitches, where ligand-induced conformational changes are well documented (Breaker 2011). It is perhaps reasonable to argue that aptamers adopt pre-formed secondary and tertiary structures in their unbound state, some of which can be very similar to those in the bound states; others, however, can experience rather significant conformational changes upon binding, especially when ligands are macromolecules such as proteins or whole cells. An understanding of the roles of secondary structures in the unbound form will then be useful toward rational sequence design and optimization of aptamers, as well as development of *in silico* aptamer simulation tools. Given the nature of aptamer selection, that is, aptamers are selected from specific starting pools, it then becomes difficult to generalize the secondary structural features of aptamers generated from different starting pools.

On the other hand, the adaptive nature reflects the conformational flexibility of aptamers. As single stranded nucleic acids, aptamers are pre-disposed to adopt various local minimum energy states. It would be necessary to characterize whether these local minimum energy states are competent in ligand-binding, or they can undergo induced conformational changes in the presence of ligand in order to bind. Alternatively, if the global minimum energy state is required for binding, then appropriate annealing processes will be necessary to ensure optimal aptamer binding.

Overall, aptamers provide excellent tools to complement antibodies in ligand binding, together with unique advantages that are difficult to get around with antibodies. Given the recent demonstration of difficulty in reproducing binding properties in a few aptamers, well defined and more consistent practices will benefit future aptamer research.

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STATEMENT OF COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

AMP4, AMP17 and AMP18: Ampicilline-binding aptamers of different lengths
MAPS: the Minimum Aptamer Publications Standards
NMR: Nuclear Magnetic Resonance
PCR: Polymerase Chain Reaction
RMSD: Root Mean Square Deviation

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