Development of a Nucleic Acid Based Fluorescence ATP Biosensor

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Abstract

Secondary structures formed by single-stranded DNA aptamers can allow for the binding of small molecule ligands. Some of these secondary structures are highly stable in solution and are great candidates for use in the development of biosensors for disease markers, environmental impact, and many other applications. In this research, we explored these unique properties of aptamers in developing a fluorescence-based biosensor for ATP (adenosine triphosphate) and related small molecules. The effectiveness of the biosensor was determined by measuring the binding affinity and specificity of the ATP biosensor on a molecular level, towards different, but structurally similar, ligands. We observed strong and similar binding affinity towards ATP and ATP analogs with K_d range (73-347 µM). However, when probed against other deoxyribonucleotide triphosphates (dNTPs), little to no binding was observed indicating the biosensor specifically targets only ATP analogs. The ATP aptamer sequence can also form noncanonical G4 secondary structure depending on the solution conditions. We investigated the involvement of the G-quartets in the aptamer sequence in ligand binding and found that both G-quartets contribute to ligand binding.

Background

DNA is a complex and versatile molecule that can adopt many alternative structural conformations. Some of these nucleic acid secondary structures are less familiar and include single-stranded, hair pinned, triple-stranded, and even four-stranded[1] structures. The four-stranded nucleic acid structures include the G-quadruplex, i-motif[2], three-way junctions, and cruciforms[3,4]. One important and practical application of nucleic acid secondary structures is in sensing molecules whereby synthetic RNA and DNA sequences, also called aptamers, are systematically generated to specifically bind molecules including non-nucleic acid targets such as drug molecules, proteins, and cells[5, 6, 7].

Nucleic acid aptamers are short, single-stranded DNA or RNA[8] sequences, which bind target molecules with comparable affinity and selectivity to antibodies. Aptamers are easier to make and more resistant to biodegradation than antibodies and therefore, are ideal for use in designing biosensors for biomarkers, environmental monitoring, and various applications[9]. One such aptamer has been identified to specifically target ATP molecules[10]. Sequence analysis of the binding domain of this ATP aptamer revealed highly conserved guanine-rich regions suggesting the formation of a stack of G-quartets (G-quadruplex also called G4) in the ATP-binding motif of the aptamer.

Guanines in nucleic acid sequences tend to self-associate into highly stable four-stranded structures. This structure is a group of four (4) guanosine bases coming together to form a three-dimensional box-shaped stack, due to hydrogen bonding and monovalent cation stabilization (Figure 1). Guanine and its typical base pair, cytosine, form three (3) hydrogen bonds with each other but guanine-guanine pair has four(4) hydrogen bonds, making this bonding more stable than typical base pairing.

Figure 1: Structure of G-quadruplex- The figure above illustrates the structure of the G-quadruplex. It is shown to be stabilized by a monovalent cation, M^+ which in our case is K^+ (potassium monocation).

We designed a fluorescent biosensor for sensitive detection of binding activity by labeling the DNA aptamer sequence with a fluorophore (Figure 2). A biosensor is a biochemical detection device for biomolecular interactions and can be used for medical and environmental applications such as detecting biomarkers for diseases in cells or testing water for heavy metals[11]. With this design, the binding between the aptamer and the ligand results in increase in the fluorescence signal through a ligand turn-on mechanism by which a conformational change induces a signal[12].

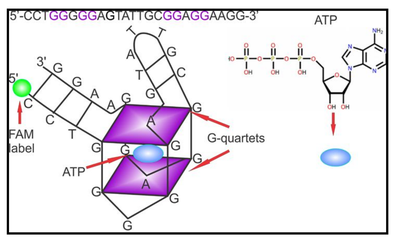

Figure 2: ATP aptamer sequence (top left) and a schematic of the folding of the aptamer sequence into a secondary structure consisting a stem, hairpin, G-quartets and a binding pocket for small molecule ligands (blue oval representing ATP ligand). The structure of ATP is shown on the top right. The aptamer sequence was labeled with 6-carboxyfluorescein (FAM) (green solid circle) for fluorescence detection.

Methods

G4 Formation: The DNA ATP aptamer-forming sequences, labeled with the fluorescent dye FAM (Integrated DNA Technologies) was be dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and used without further purification. Aliquots of the dissolved oligo was added to 50 mM Tris buffer containing 100 mM potassium chloride (KCl) and heated to 95 °C for 10 min in a thermal cycler in order to denature random structures within the DNA and slowly cooled to 4 °C in the presence of KCl to form the G4 structure.

Ligand Binding Assay: To test the activity and specificity of the biosensor, we performed equilibrium ligand binding assays using ATP, ATP analogs, and other NTPs (nucleotide triphosphates). Due to binding kinetics, the solutions containing varying concentrations of the ligand were incubated with about 0.5 µM of the aptamer and thermal cycled in order for the G4 to form in the presence or absence of the ligand. This ensured complete binding even at low ligand concentrations. After incubation, sample solutions were transferred from the tubes into a 96 well microplate and placed into a POLARstar Omega® fluorescent plate reader to measure the fluorescence intensity.

Data Analysis: Experiments were performed by triplicate measurements unless stated otherwise. The fluorescence intensities with varying ligand concentrations were blank-corrected, averaged and the normalized fraction bound calculated as ($F-F_0$ / $Fmax-F_0$) where, F_0 and F are the fluorescence intensity without ligand and with varying concentrations of ligand respectively and Fmax is the fluorescence intensity with saturated ligand. The data analysis and normalization were done using Excel and the graphs plotted using Origin Lab and fitted to one binding site equation $Y = Bmax*X / (K_d+X)$ where K_d is the equilibrium dissociation constant or the binding affinity of the ligand for the aptamer and Bmax is the maximum fraction of ligand bound. Maximum binding describes the point at which the binding plateaus and binding sites are saturated. Smaller dissociation constants are associated with ligands that are tightly bound to their target receptors.

References

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Results

ATP has high affinity for the aptamer

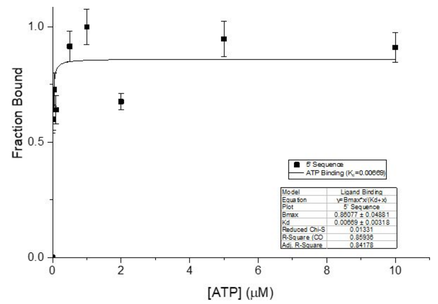

Figure 3: Equilibrium binding measurements for ATP and fluorescently labeled ATP aptamer. The dissociation constant value K_d indicated in the figure is a lower limit estimate due to the low range of ATP concentrations tested.

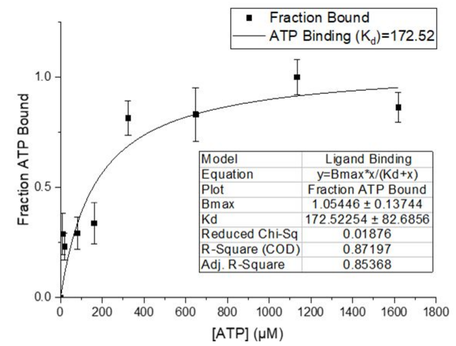

Figure 4: The graph above illustrates the binding of ATP to the 5'-ATP binding sequence. There is a very drastic increase in binding between ATP concentrations of 0 µM and 1620 µM. Overall, the binding shows a general increase as ATP concentration is increased. The dissociation constant, K_d, is shown to be 172.52 µM.

ATP and ATP analogs show strong affinity to the aptamer

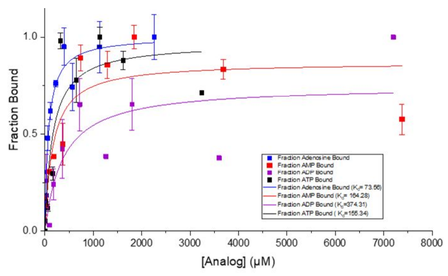

Figure 5: The plot above shows the normalized binding of ATP along with the analogs Adenosine, AMP, and ADP with concentrations between 0 µM and 7380 µM. The K_d of ATP was found to be 172.5. Adenosine was found to have the highest binding affinity with a K_d of 73.56 µM followed by AMP with a Kd value of 164.2 µM while the lowest binding affinity is seen from ADP with a K_d of 374.3 µM.

Specifically of the aptamer towards ATP compared to other dNTPs

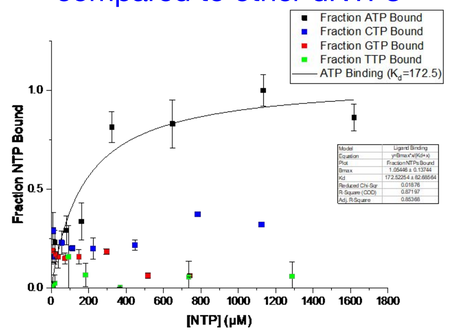

Figure 6: The figure above demonstrates the binding of ATP, TTP, CTP, and GTP (dNTPs) to the ATP aptamer. Following the normalized data, we see that ATP has a far stronger binding affinity than the other dNTPs. The ATP binds to the aptamer in a consistently increasing manner, whereas TTP, CTP, and GTP binding is negligible.

Both top and bottom G-quartets are involved in ligand binding

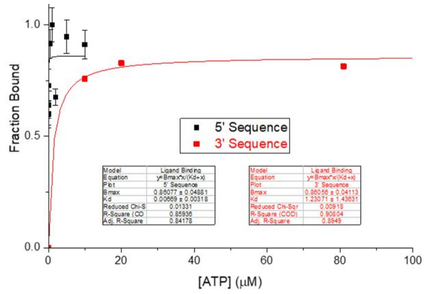

Figure 7: The figure above shows the normalized binding of the 3' and 5' ATP aptamer sequences to varying concentrations of ATP. Note that the 3' aptamer data is only one experiment at low ATP concentrations and the 5' aptamer plot is the low ATP data plot from Figure 3 for comparison. Both aptamers showed increased binding with increased concentration with significantly lower Kd values under these conditions, indicating strong ATP binding.

Conclusions

We have demonstrated the binding between ATP aptamer and ATP analogs and other dNTPs and determined their dissociation constants. We observed that the binding affinity of the aptamer is specific toward ATP and ATP analogs with no affinity towards the other dNTPs. The binding affinity was highest for adenosine, which lacks a phosphate group. It is possible that the adenosine molecules are sterically more favored at the binding site due to lack of phosphate groups. Also, the negatively charged phosphate groups on the other analogs may encounter electrostatic repulsion from the phosphate groups on the binding site. Our studies on the role of the G4 motif in the ATP aptamer binding site suggest that both G-quartets are equally involved in ligand binding even at a fairly low ligand concentration.

Acknowledgments

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