

THERMODYNAMIC AND KINETIC ANALYSIS OF APTAMER-
LIGAND INTERACTIONS USING ISOTHERMAL TITRATION
CALORIMETRY

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Abstract

Aptamers are short single-stranded DNA or RNA oligonucleotide sequences capable of binding to a broad range of target molecules with high affinity and specificity. They can interact with a large variety of targets such as small molecules, ions, enzymes, and proteins employing all kinds of non-covalent interactions. Isothermal Titration Calorimetry was employed to explore the binding behaviour of aptamer-ligand interactions using the cocaine-binding aptamer as a model system. This dissertation is an assembly of two distinct research projects; In the first part, a bifunctional cocaine and deoxycholic acid-binding aptamer was constructed from individual cocaine-binding aptamer variants and the binding affinity and thermodynamics were measured using isothermal titration calorimetry. We show that the bifunctional aptamer binds its ligands with positive cooperativity, having a Hill coefficient of 1.2 -1.5, whether the ligands are added individually or as an equimolar mixture. A mechanism where dynamics at one ligand-binding site is affected by the presence of the ligand at the other is proposed to account for the cooperative binding.

The next chapter highlights the use of *kin*ITC to extract binding rates from ITC experiments. Determining the binding thermodynamics and kinetics between aptamers and ligands is important for understanding their recognition mechanisms as well as providing a framework for developing biosensors. One aptamer that has been particularly well studied is the cocaine-binding aptamer. There has been a lot of research conducted on the thermodynamics for the cocaine-binding aptamer however, one feature not reported yet is the binding kinetics. Here, we measure the kinetics of quinine binding to two sequence variants of the cocaine-binding aptamer, one with a short stem-one (MN19) and the other with a long stem-one (MN4). When stem-one of the aptamer is six base

pairs long, the aptamer retains its secondary structure in its free and bound forms. When the length is shortened to three base pairs, such as for MN19, the aptamer is loosely folded in its unbound state and undergoes structural changes upon binding quinine. Both binding kinetics and thermodynamic data were acquired as a function of different temperatures. A 1:1 binding model was applied to extract binding rates for both variants of the cocaine-binding aptamer. Arrhenius plots were derived to compare the activation energies for MN4 to that of MN19 since MN19 exhibits ligand-dependent conformational changes. The values obtained for the transition state enthalpy barrier were averaged to be 5.1 kcal mol⁻¹ for MN4 and -0.8 kcal mol⁻¹ for MN19. These results suggest that MN19 has an energy barrier that is almost negligible under standard conditions.

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List of Abbreviations

ΔH - Change in enthalpy

ΔG - Change in free energy

ΔS – Change in Entropy

ΔC_p – Change in heat capacity

DCA – Deoxycholic Acid

DNA – Deoxyribonucleic Acid

RNA – Ribonucleic Acid

NMR – Nuclear Magnetic Resonance

TRIS - Tris(hydroxymethyl) aminomethane

Standard ITC buffer - 20 mM Tris (pH7.4), 140 mM NaCl, 5 mM KCl

WC – Watson Crick

nt - Nucleotide

K_d – Dissociation constant

ddH₂O – Distilled deionized water

ITC – Isothermal Titration Calorimetry

ETC – Equilibration Titration Curve

k_{on} – Association rate constant

k_{off} – Dissociation rate constant

RSS – Residual sum of squares

Chapter One : Introduction to Aptamers and Isothermal Titration Calorimetry

1.1 Systematic Evolution of Ligands by Exponential Enrichment (SELEX)

Aptamers are short single-stranded DNA or RNA oligonucleotide sequences capable of binding to a broad range of target molecules with high affinity and specificity. They can interact with a large variety of targets such as small molecules, ions, enzymes, and proteins employing all kinds of non-covalent interactions. Aptamers can adopt a variety of topologies upon target binding, with the most common architecture being a hairpin structure, in which the target binding site is situated in the looped region. There are also three-way junctions like the cocaine-binding aptamer, where the ligand binding site is located at the branch point¹. Other conformations include G-quadruplexes, pseudoknots, and bulged structures^{2,3}.

Aptamers are selected *in vitro*, following the classical methodology of Systematic Evolution of Ligands by Exponential Enrichment, termed SELEX. This method was first introduced in the early 1990s by Gold and Szostak groups separately^{4,5}. SELEX is an iterative process that involves repetitive cycles of binding, partitioning and amplification. Figure 1.1 represents the fundamental steps within the SELEX procedure used for the selection of high affinity aptamers to their desired target molecule⁶. The process typically begins with a randomized pool of oligonucleotides consisting of about 10^{16} sequences, each flanked by two known sequences to facilitate primer binding. The combinatorial library is then incubated with the target molecule followed by a partitioning step which separates aptamer sequences that are bound to the target from non-specific sequences. Different techniques can be employed to enhance the efficiency of partition by

separating the target bound aptamer. These include chromatography, membrane filtration and centrifugation steps⁷. Lastly, the target bound oligonucleotides are amplified by PCR and the resulting library is subjected to repeated rounds of selection to generate aptamer sequences with high binding affinity and specificity. The general steps remain consistent, however, over the years the method has undergone numerous modifications and improvements that has made this technology faster, robust, and cost-effective.

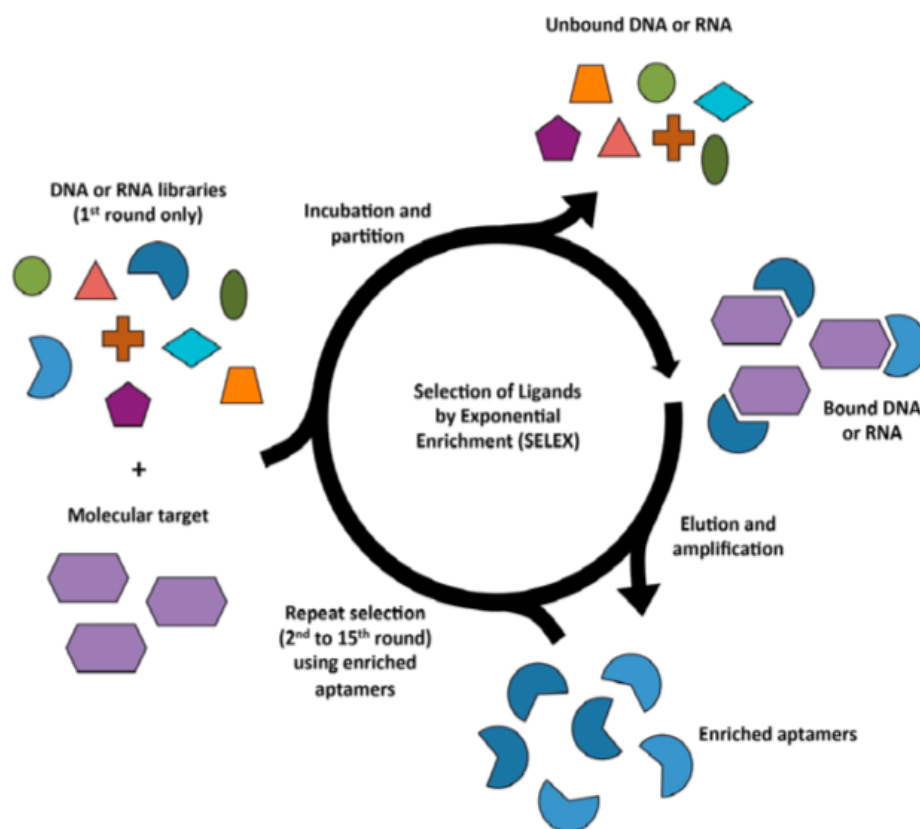


Figure 1.1: Schematic representation of the SELEX procedure. (Adapted from reference 6)

1.2 Applications of Aptamers

Aptamers have been applied in diverse fields of life sciences, for example diagnostic, drug delivery, food inspection and biosensor development. One of the most useful application of aptamers is for biosensor development, aptamers can form stable tertiary structures which has led to their widespread application in biosensors. A good example of this is the graphene-based biosensor targeting B-globulin for the detection of milk allergen⁸. Aptamers also play a role in inhibiting biofilm formation. Biofilms are a community of microbes that adhere to surfaces by embedding in the exopolysaccharide matrix. They have a complex antibiotic resistance profile, due to failure of antibiotics being able to penetrate the polysaccharide layer. Their treatment against human infections has become a serious problem since they are resistant to the human immune system. It was discovered that binding of a specific aptamer to the flagella of *Salmonella choleraesuis* can restrict the bacterial rotational frequency, therefore preventing biofilm formation⁹. Another important application of aptamers is using them to monitor environmental contamination. Aptamers have been developed against antibiotics like tetracycline¹⁰ and chloramphenicol¹¹ to target antibiotics used in farm animals that can cause human ingestion by accumulating in animal tissues.

Several important attributes of aptamers make them promising for use in clinical development. Aptamers can easily be synthesized, bear low manufacturing costs, have better thermal stability and little to no batch-to-batch variability. In addition, aptamers can easily be modified to increase their feasibility without reducing their affinity¹². Correspondingly, there are some challenges associated with the use of aptamers. Their low molecular weight presents significant challenges with renal filtration control, small aptamers can be excreted through renal filtration when

administered into the blood stream. This can be modified by adding specific functional groups to reduce their renal filtration and prolong their lifetime and stability. Aptamers can also be sensitive to degradation by nucleases, for example an anti-thrombin DNA aptamer was shown to have a half-life of 108 seconds in vivo¹³.

1.3 The Cocaine-Binding Aptamer: Sequence and Structural Characteristics

The cocaine-binding aptamer is a DNA aptamer composed of three stems built around a three-way junction. The original version of this aptamer, MNS4.1 was selected by Stojanovic et al in 2000¹ by classical SELEX using cocaine as the target molecule. It contains 38 nucleotides with the majority connected by Watson-Crick base pairing. The aptamer also consists of 5 non-canonical base pairs, 3 GA base pairs near the end of stem 1 and GA and GT base pairs in stem 3. The secondary structure of MNS4.1 was predicted based on mutation analysis, including random and site-specific mutagenesis¹. (Figure 1.2) Cocaine binds via a lipophilic cavity formed at the three-way junction. In the absence of cocaine, stem three is not formed, but in its presence the stems close and form the three-way junction. (Figure 1.3) This was reported through double end labelling with a fluorophore and a quencher.

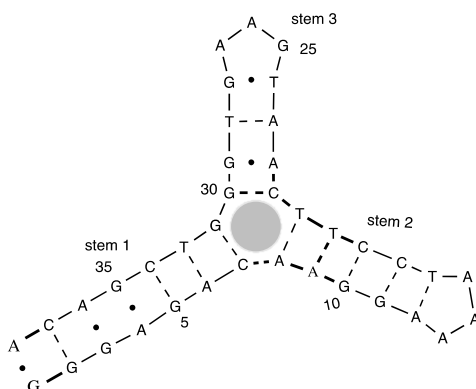


Figure 1.2: Secondary structure and sequence of the originally selected cocaine-binding aptamer, MNS4.1. Circled is the high-affinity binding site for cocaine. Dashed lines represent Watson-Crick base pairs and dots represent non-canonical base pairs. (Adapted from reference 1)

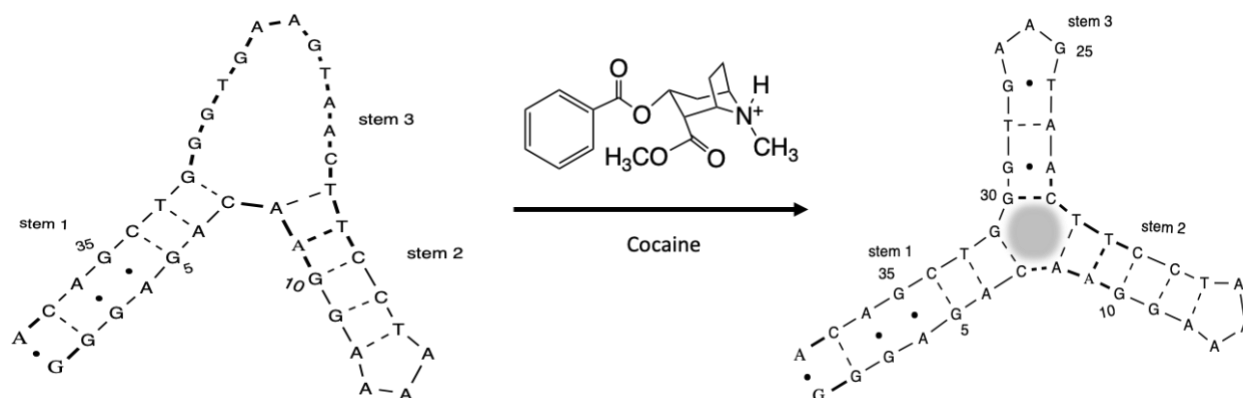


Figure 1.3: Binding mechanism proposed for the cocaine-binding aptamer. Stems 1 and 2 are completely folded while stem 3 folds upon ligand binding. The grey circle indicates the binding site for cocaine. (Adapted from reference 10)

Different derivatives of the MNS-4.1 cocaine-binding aptamer have been labelled with sensing elements such as fluorophore/quencher pairs and magnetic nanoparticles to achieve detection of cocaine. In 2001, Stojanovic et al designed a fluorescent sensor for cocaine from the originally derived MNS-4.1 aptamer. The new variant named F7.9D was created by engineering an instability in stem 1 of MNS-4.1 where both ends were labeled with a fluorophore and quencher. In the absence of cocaine, relatively high fluorescence was observed. On addition of cocaine, quenching of the fluorescence signal was observed, indicating that folding of the aptamer took place¹. The rapid detection of cocaine gains a considerable amount of attention due to its adverse effects on human health. In 2011, Kawano et al used split aptamers to detect cocaine. Split aptamers are made up of two or more independent fragments, that can assemble in the presence of a specific target. They were able to detect 5 μM concentrations of cocaine in approximately 60 seconds using split cocaine binding aptamers by employing nanopore amperometry¹⁴.

One of the most common variants of the cocaine binding aptamer studied in our lab is the MN4 aptamer. (Figure 1.4) This 36-nucleotide aptamer was selected as a strong binder to cocaine ($K_d = 5.5 \mu\text{M}$) over its metabolites benzoylecgonine and ecgonine methyl ester. Many subsequent studies including the ones published in our research group show that the binding affinity is dependent on different regions and nucleotides in the aptamer^{15,16}. Structural studies show that MN4 folds into a three-stem structure in the absence of cocaine, a more stable folded structure is thermodynamically favoured in the presence of cocaine.

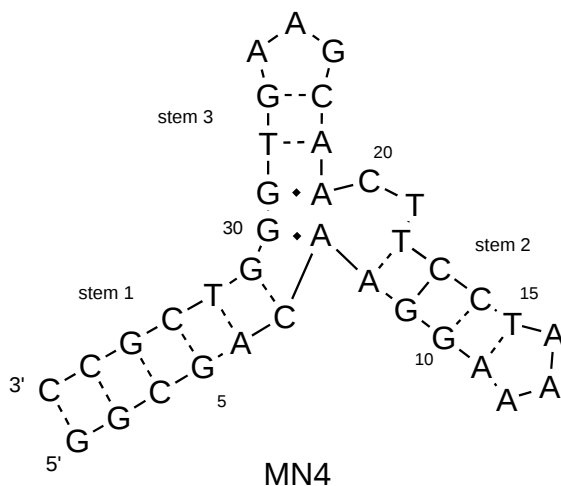


Figure 1.4: Secondary structure and sequence of the MN4 cocaine-binding aptamer. Dashed lines represent Watson-Crick base pairs while dots represent non-canonical base pairs.

1.4 Biophysical characterization of aptamer-target interactions

Aptamers can bind various kinds of ligands: ions, small molecules, peptides, proteins, viruses, bacteria, and even whole cells. They feature high affinity and specificity to their target molecule for which they require biophysical tools along with structural studies to understand their recognition mechanism. Over the last decade, many techniques have been developed to quantify biomolecular interactions, providing binding affinities, kinetics, and thermodynamics between an

aptamer and its target. Traditionally, the dissociation constant has been used to measure the strength of a biomolecular interaction, however, this does not reveal the underlying mechanism of the nature of the interaction.

X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy have been used to study the three-dimensional structures of macromolecules. Each approach owns its superiority and challenges. Structures of several small aptamers that lie within the 30 to 50-mer range have been resolved using NMR spectroscopy. On the other hand, structures of larger molecules can be determined by X-ray crystallography provided the crystals provide suitable diffraction data. Intensive work is needed to generate crystals that produce well diffracted data¹⁷. For example, Lin and Patel used NMR to study structure of ATP-binding DNA aptamer bound to AMP⁸¹⁸. Long et al. solved the crystal structure of the thrombin binding aptamer bound to thrombin at 1.9Å resolution¹⁹ The focus on my thesis relies on using ITC to understand the principles of aptamer-ligand interactions by dissecting thermodynamic and kinetic components that govern binding.

1.5 ITC for studying binding interactions

Isothermal titration calorimetry is a widely used biophysical technique for studying the formation or dissociation of molecular complexes. It is the only method where the binding constant (K_a), enthalpy (ΔH) and stoichiometry (n) can be directly determined from a single experiment.²⁰ ITC determines the equilibrium constant of the binding interaction from which the change in free energy of binding (ΔG) and entropy (ΔS) can be calculated. Quantification of these thermodynamic parameters is an important step to characterize aptamer-ligand interactions. The convenience of obtaining all this information from a single run has made ITC a valuable tool in

studying biomolecular interactions including protein/protein, protein/ligand, nucleic acid/small molecule, and protein/nucleic acid interactions²¹.

In the last few years, ITC has been used to characterize aptamer-target interactions by measuring binding rates. For example, in a paper from 2012, Burnouf et al proposed a method for collecting both thermodynamic and kinetic data from conducting ITC experiments that could be applied for simple 1:1 binding interactions to more complex processes²². They studied the binding of Nevirapine to HIV-1 reverse transcriptase, and the binding of thiamine pyrophosphate (TPP) to the *Escherichia coli* riboswitch present in the 5'-UTR of the thiC messenger RNA, which undergoes folding on binding of TPP

The advantage of ITC over other methods is that it is information-rich, a single run can quantify all the basic thermodynamic parameters of an aptamer-target interaction including binding rates. It is also a non-destructive label-free technique as opposed to Microscale Thermophoresis (MST) which requires your ligand to be fluorescently labelled, and Biolayer Interferometry (BLI) which requires surface-immobilization of the target molecule. There are some limitations associated with ITC such that it requires larger quantities of samples and has low throughput capabilities of about 2-4 runs in a day.

1.6 Thermodynamic characterization of Ligand-Aptamer binding

A typical ITC instrument (Figure 1.5 (a)) consists of two cells, a sample and reference cell housed in an adiabatic jacket. During a titration experiment, the ligand in the injection syringe is added in aliquots to the aptamer which is loaded in the sample cell. The reference cell is filled with buffer

solution that is used to prepare the ligand and aptamer solutions. When a binding event occurs, a feedback heater that is in contact with the sample cell detects any heat absorbed or released as a result of the aptamer-ligand interaction. The instrument maintains the temperature of both cells by means of a feedback heater, which is responsible for maintaining $\Delta T = 0$ throughout the course of the experiment. At the start of the experiment, the temperature of the reference and sample cell are the same. When an exothermic binding event occurs, the temperature in the sample cell rises upon addition of a ligand causing a downward peak in the signal. This newly established temperature difference between the two cells causes a decrease in power level to the feedback heater around the sample cell. For an endothermic reaction, the opposite occurs and a positive peak appears on the raw thermogram. As the injections continue, the ligand binding sites in the aptamer start to get occupied. This is reflected by a decrease in heat signal and saturation of the curve. The last few injections correspond to the heat of dilution of the ligand, which appear once the target is fully saturated.

The bottom panel of the ITC thermogram shows integrated heat pulses where the area of each peak is integrated to generate a binding isotherm that yields the heat per mole of injectant as a function of the molar ratio of ligand to aptamer (Figure 1.5 (c), bottom). The first few injections consist of large amounts of heats absorbed or released as all of the macromolecule is available for binding. The last few injections usually consist of unreacted titrant since most of the macromolecule has already reacted with the ligand to form a complex.

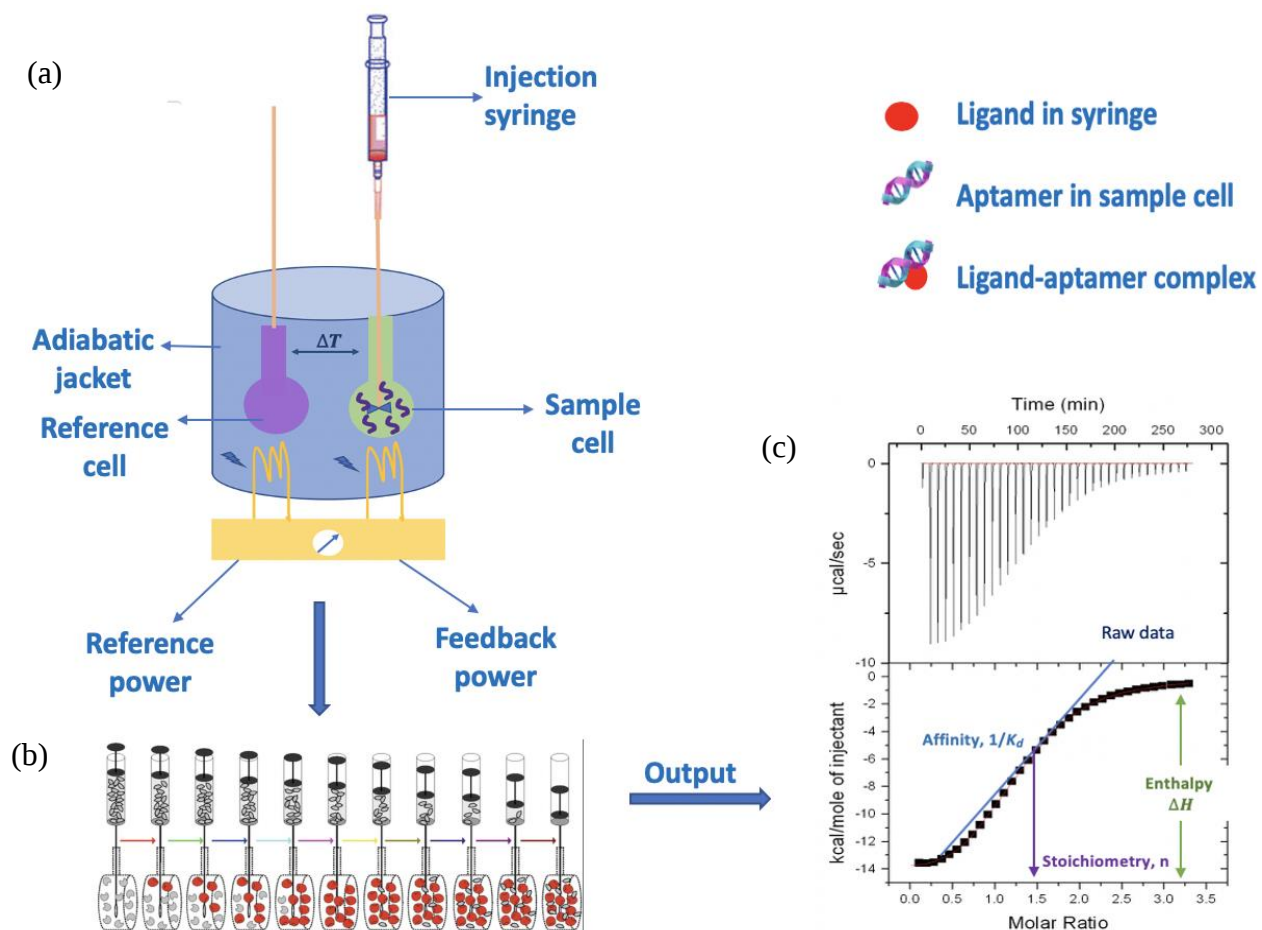


Figure 1.5: (a) Diagram of a typical ITC instrument showing two cells (sample and reference) surrounded an adiabatic jacket. (b) An automated syringe is used to deliver the titrant in known aliquots into the sample cell containing aptamer solution. (c) Sample ITC thermogram of an exothermic binding event measured by ITC. The top panel shows a sequence of peaks, each one corresponding to one injection of ligand solution into the sample cell. The peaks for each injection are integrated with respect to time resulting in the total heat per injection (bottom panel). By fitting the integrated curve to a suitable binding model, ΔH (y-intercept), K_a (association constant) and n (binding stoichiometry) can be determined.

1.7 Equilibrium Thermodynamics and Data Analysis using ITC

The affinity between an aptamer and its target can be defined by the equilibrium association constant, K_A

$$K_A = \frac{[AL]}{[A][L]} = \frac{1}{K_d}$$

Where K_d is the equilibrium dissociation constant of the complex, and $[A]$ and $[L]$ are the concentrations of free aptamer and ligand. The relationship between these constants can be mathematically described by the Gibbs equation where the standard Gibbs energy of binding can be further decomposed into its enthalpic (ΔH) and entropic (ΔS) components

$$\Delta G = RT \ln K_d = -RT \ln K_A$$

Following an ITC run, data is fit to either a one-site or multi-site binding model using Origin 7.0 which outputs all the thermodynamic parameters; K_A , n and ΔH for each binding event. The shape of the sigmoidal curve determines the binding affinity, K_A . The stoichiometry n is determined by the molar ratio of ligand to aptamer binding at equivalence point and ΔH is amount of heat released which is determined from the y-intercept value. Using this information, the Gibbs free energy change, ΔG and the entropy change, $-T\Delta S^\circ$ can be calculated using Equations 1 and 2.

$$\Delta G = RT \ln K_d \quad (1)$$

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (2)$$

The dissociation constant, K_d measures the strength of the interaction at equilibrium and can be calculated from K_A . ΔG° , ΔH° and ΔS° are Gibbs free energy, enthalpy and entropy of binding respectively. T is the absolute temperature and R is the ideal gas constant with a value of $1.98 \text{ cal mol}^{-1} \text{ K}^{-1}$.

1.8 Essential goals in this research

The focus on my thesis relies on using ITC to understand the principles of aptamer-ligand interactions by dissecting thermodynamic and kinetic components that govern binding. This research is an assembly of two independent projects. In the first part, a bifunctional cocaine and deoxycholic acid-binding aptamer was designed from individual cocaine-binding aptamer variants and the binding affinity and thermodynamics were measured using isothermal titration calorimetry. The second part highlights the use of kinetic ITC to extract binding rates from ITC experiments. Determining the binding thermodynamics and kinetics between aptamers and ligands is important for understanding their recognition mechanisms as well as providing a framework for biosensor development

Chapter Two : Cooperative binding by a bifunctional deoxycholic acid and cocaine-binding aptamer

2.1 Preface

All work presented in this chapter is currently under review for publication in the journal *Aptamers*. All the data presented in this chapter were acquired and analyzed by me however previous members in the lab have contributed to this research by conducting preliminary studies.

Nusaibah Dawood and Philip E. Johnson*

2.2 Introduction

Aptamers are typically selected to bind to one specific target molecule^{23,24} Aptamers that can bind two separate molecules, bifunctional aptamers, can be generated by fusing together two different aptamers that each bind a single target. Bifunctional aptamers can bind two copies of the same molecule, such as seen for the cooperative binding split aptamer developed by Xiao and co-workers for cocaine^{25,26}. Alternately, and more common, bifunctional aptamers can bind two different molecules as shown in several examples in recent years. Bifunctional aptamers are useful when using one aptamer to target a particular binding location, for example a cell or protein, and use the second aptamer to signal binding or deliver a “payload”, such as a therapeutic agent, to that target^{27,28,29}. Bifunctional aptamers, where two separate molecules are bound by the aptamer are distinct from bivalent aptamers, or multivalent aptamers, where multiple aptamers bind the same target, usually as a way to increase affinity for a ligand for an aptamer^{30,31,32} While the

number of different aptamers that can be fused together is only limited by the length of DNA that can be made longer chains of aptamers can be assembled by non-covalent interactions^{33,34,35}

The cocaine-binding aptamer is one of the most well-studied DNA aptamers. This aptamer has a three-way junction structure with a tandem AG mismatch and a dinucleotide bulge adjacent to the junction³⁶ One of the most commonly studied versions of the cocaine-binding aptamer in our lab is a construct referred to as MN4 (Figure 2.1). One interesting feature of the cocaine-binding aptamer is that changing one of the adenine nucleotides in the AG mismatch to a cytosine (A21 in MN4) results in the reduction in affinity for cocaine and the introduction of the ability to bind the steroid deoxycholic acid (DCA) in place of cocaine^{37,38,39}. Prior studies in our laboratory have quantified the affinity and binding thermodynamics, by ITC methods, of a version of the DCA-binding aptamer we referred to as the WC aptamer (Figure 2.1)

In this manuscript we build on our work with the individual MN4 and WC aptamers to investigate how they work fused together as a bifunctional aptamer (WC-MN4; Figure 1). Would the two sites be functional in a bifunctional aptamer? Would the aptamer bind its ligands individually, or in a cooperative manner? Some hint of how they would function was provided by the report of the cooperative binding split aptamer for cocaine by Xiao and co-workers^{25,40}. Here, two cocaine-binding aptamers were joined in a manner similar to our WC-MN4 aptamer by linking a stem 1 to a stem 2. The authors found the bifunctional aptamer bound two molecules of cocaine in cooperative fashion and when engineered to function as a sensor for cocaine, to have a lower concentration limit of detection (C_{LOD}) than seen for an individual aptamer. In this study, we also

find cooperativity between the two binding sites and compare the binding parameters of the free WC and MN4 aptamers with the similar sites as part of the WC-MN4 aptamer.

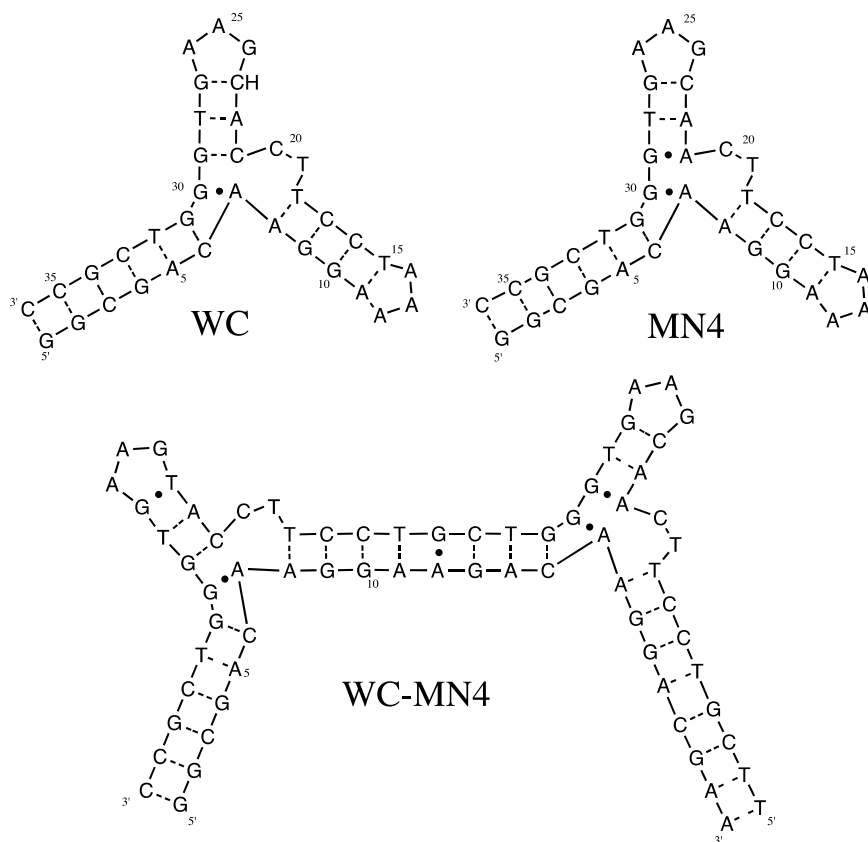


Figure 2.1: Secondary structure of the individual MN4 and WC aptamers as well as the bifunctional WC-MN4 aptamer. Circled are the ligand-binding sites. Watson-Crick base pairs are indicated by dotted lines and non-Watson-Crick base pairs are indicated by dots between the nucleotides.

2.3 Materials and Methods

Sample Preparation

All aptamer samples were obtained from Integrated DNA Technologies (IDT). DNA samples were dissolved in distilled deionized H₂O (ddH₂O) and then exchanged three times using a 3 kDa molecular weight cut-off concentrator with sterilized 1 M NaCl, followed by three exchanges into distilled and deionized H₂O. All DNA samples were exchanged with standard ITC binding buffer (20 mM Tris (pH7.4), 140 mM NaCl, 5 mM KCl) three times before use. Aptamers were heated in a boiling water bath for 3 min, followed by cooling in ice water to allow aptamer to anneal. Aptamer concentrations were determined by UV absorbance spectroscopy using extinction coefficients supplied by the manufacturer. Sodium deoxycholate (DCA) was obtained from Sigma-Aldrich and cocaine hydrochloride from Toronto Research Chemicals. Stock solutions of DCA were prepared by weight and dissolved in binding buffer. The pH was confirmed to be 7.4.

Isothermal Titration Calorimetry Experiments

Isothermal titration calorimetry (ITC) experiments were performed using a MicroCal VP-ITC instrument following a procedure described previously²⁰. Samples were degassed prior to use with a MicroCal ThermoVac unit, and all experiments were corrected for the heat of dilution of the titrant. All titrations were performed with the aptamer in the sample cell and ligand in the injection syringe. All ITC experiments were conducted at 15°C with aptamer solutions ranging from 0.12 mM to 0.16 mM. The ligand solutions were 2.5 mM for DCA titrations and equimolar DCA:cocaine titrations, and 1.87 mM for cocaine titrations. Each ITC experiment consisted of 35 injects of 8 µL spaced every 600 s with the first injection being 2 µL in order to account for diffusion from the syringe into the cell during equilibration.

Data Fitting

One-site ITC data were fit using the Origin 7.0 software package provided by the manufacturer. The data for two-site binding was fit to both independent and cooperative models developed by Freiburger *et. al*⁴¹. using Matlab 2019 software package and employed by us in previous studies^{42,43}. The binding cooperativity was determined by calculating Hill coefficients using a method developed by Cattoni *et al*⁴⁴ and Freire *et.al*⁴⁵.

2.4 Results and Discussion

The binding affinity and thermodynamics of cocaine, DCA and an equimolar mixture of cocaine and DCA to the WC-MN4 aptamer were determined using ITC methods (Table 2.1). In our initial studies on this project, we attempted to fit the data of the individual cocaine and DCA titrations to a 1:1 binding model but the data never fit this model well. We also tried to fit the cocaine/DCA mixture to the two independent sites binding model, but again the fits were not satisfactory. By fitting each of the titration experiments to a two-site cooperative model we obtained satisfactory fits for all the data sets (Figure 2.2).

For both the individual cocaine and individual DCA titrations we were not able to obtain satisfactory fits to a 1:1 binding model as the WC-MN4 aptamer has two binding sites for each ligand, a high affinity site and a low affinity site. In the WC-MN4 aptamer there is a primary binding site for DCA in the WC portion of the bifunctional aptamer (site 1) and a weaker secondary binding site with an appreciable binding affinity for DCA in the MN4 portion of the bifunctional aptamer (site 2). Similarly, for cocaine binding by WC-MN4 aptamer there is a primary, higher

affinity, binding site for cocaine in the MN4 portion (site 1) and a weaker secondary binding site for cocaine in the WC portion of WC-MN4 (site 2).

For both ligands titrated into the WC-MN4 aptamer individually, the data is best fit using the two-site cooperative model. This is shown by this model having the lowest residual sum of squared differences (RSS) between the experimental and calculated data points (Table 1). This is also indicated by the errors in the fit to the independent model being much larger than for the cooperative model. For DCA binding the WC-MN4 aptamer the affinity at its primary site is $(25 \pm 2) \mu\text{M}$. This is close to the previously reported value of DCA being bound by the individual WC aptamer with an affinity of $(16 \pm 3) \mu\text{M}$ ⁴⁶. Both the enthalpy (ΔH_1) and entropy ($-T\Delta S_1$) of DCA binding at the primary site are within the error range of what was previously reported.

For cocaine binding by WC-MN4 aptamer, the data are also best fit by the cooperative binding model. The affinity for cocaine at the primary site (K_{d1}) is $(6 \pm 2) \mu\text{M}$ which is within the error range of our previously reported value for cocaine binding the individual MN4 aptamer of $(7 \pm 1) \mu\text{M}$ as measured by ITC methods⁴⁷ and $(3.92 \pm 0.07) \mu\text{M}$ as measured by fluorescence quenching methods⁴⁸. While having enthalpy as the driving force for binding is the same between the individual MN4 aptamer and primary binding site in WC-MN4 the values of ΔH and $-T\Delta S$ values are significantly different between these two aptamers (Table 2.1)

For cocaine binding at the secondary site in the WC-MN4 aptamer the affinity of $(114 \pm 53) \mu\text{M}$ is significantly tighter than our previous measurement of cocaine to the individual WC aptamer of

$(204 \pm 6) \mu\text{M}$ ⁴⁹. This increase in affinity at the WC site in WC-MN4 likely reflects the positive cooperativity detected in the data fitting.

For the equimolar mixture of cocaine and DCA these data are also best fit by the cooperative binding model (Table 2.1). The affinity of WC-MN4 for cocaine is increased when the mixed ligands are added $((2.4 \pm 0.1) \mu\text{M})$ compared to when cocaine was added individually, but the affinity for DCA is reduced when added as part of the ligand mixture compared to when added on its own. This reduction in affinity at the higher affinity site is a bit counterintuitive as it may be expected that the high affinity ligand binds first and the second site would show an increase in affinity. However, it may be that the on rate for DCA binding is faster than for cocaine binding and that the difference in affinity is dictated by the off rate. Very little is currently known about binding rates for cocaine or DCA to their aptamers.

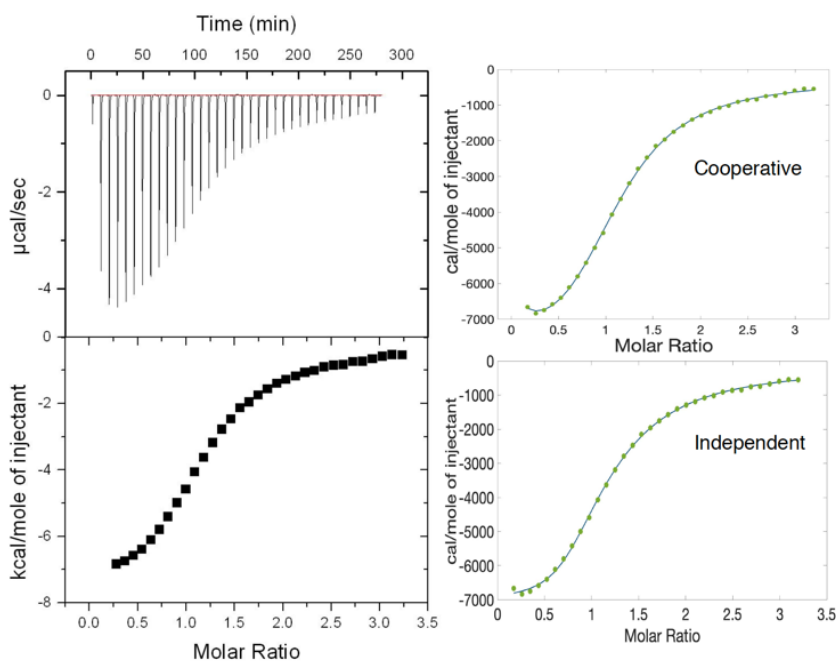
In both this study and the previous study of the cooperative binding split aptamer both of these bifunctional aptamers show positive cooperativity in ligand binding. The origin of this cooperativity is not yet defined but may result from a structural linking of the two binding sites through the intervening helix. In the free state the ligand-binding site in MN4 is more dynamic than in either the cocaine- or quinine- bound states as demonstrated by imino proton exchange rates⁵⁰. When the first ligand binds to the WC-MN4 aptamer the dynamics at that site should be reduced. If this reduction in dynamics gets transmitted to the second site, the binding affinity at the second site could increase as less of the free energy of binding would need to reduce the dynamics at this site, and more binding free energy could go into the observed binding affinity. A similar mechanism linking dynamics and cooperativity could also be present in the ATP-binding

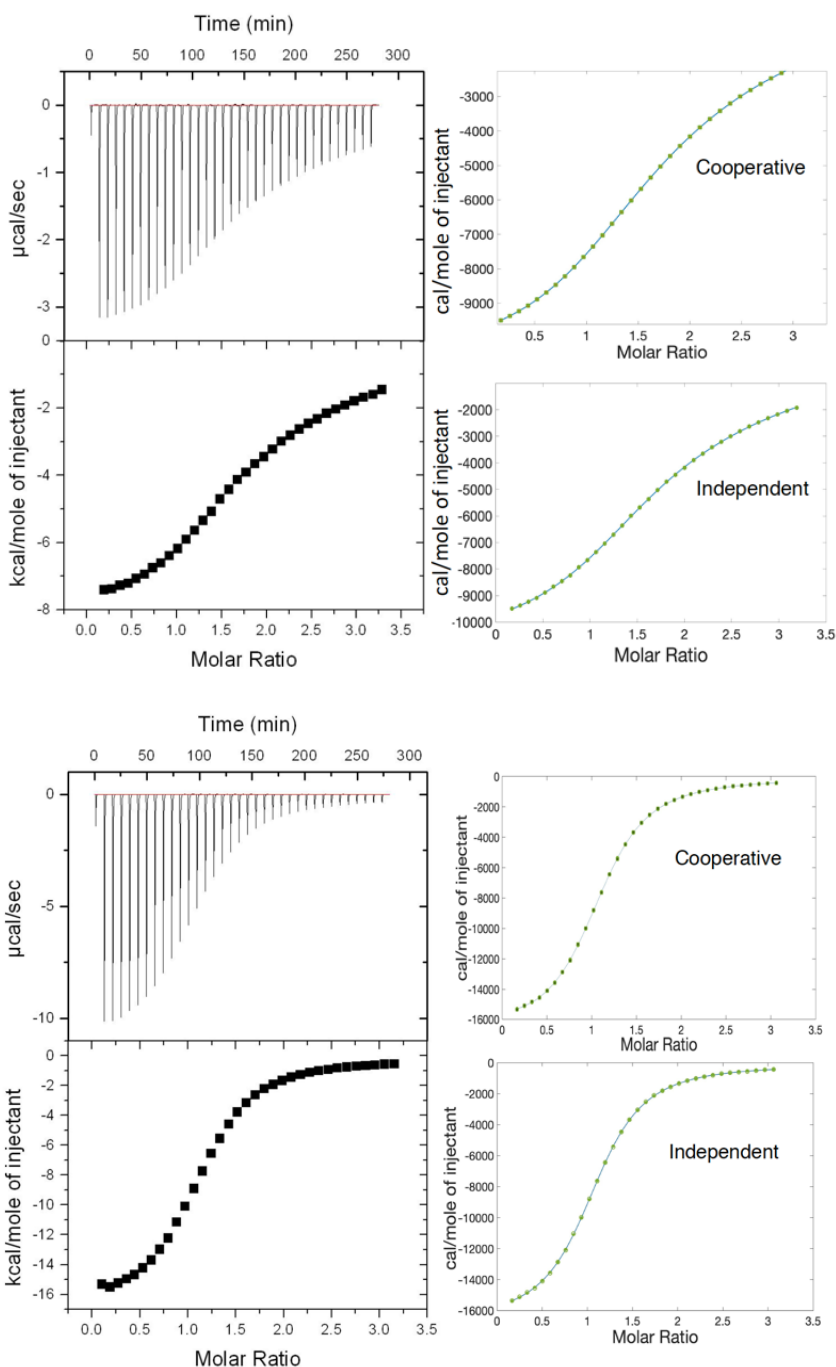
DNA aptamer⁵¹ that also exhibits positive cooperativity in binding its two ligands and a reduction in imino exchange rates with ligand binding^{52,53} It is possible that positive cooperativity is common in bifunctional aptamers, though more study is needed to demonstrate this.

Table 2.1: Dissociation constants and thermodynamic parameters of different ligands titrated with the AND bifunctional aptamer construct used in this study.

Ligand	Model type	K_{d1} (μM)	ΔH_1 (kcal mol^{-1})	$-T\Delta S_1$ (kcal mol^{-1})	K_{d2} (μM)	ΔH_2 (kcal mol^{-1})	$-T\Delta S_2$ (kcal mol^{-1})	RSS value	n_H
DCA	Independent	4 ± 4	-6.8 ± 0.1	-0.3 ± 0.2	72 ± 25	-9 ± 9	3 ± 1	3.7×10^{10}	1.3
	Cooperative	25 ± 2	-8.0 ± 0.4	1.9 ± 0.7	46 ± 5	-11.7 ± 0.5	6.1 ± 0.7	2.1×10^8	
Cocaine	Independent	8 ± 7	-10.3 ± 0.5	17 ± 14	79 ± 68	-6 ± 6	12 ± 10	9.6×10^9	1.2
	Cooperative	6 ± 2	-7.9 ± 0.5	1.0 ± 0.3	114 ± 53	-8 ± 2	3 ± 1	3.0×10^8	
1:1 Cocaine :DCA	Independent	3.7 ± 0.7	-16.5 ± 0.1	9 ± 2	36 ± 5	-4 ± 3	-2.1 ± 0.3	2.7×10^9	1.5
	Cooperative	2.4 ± 0.1	-14.11 ± 0.06	6.7 ± 0.4	34 ± 1	-10.7 ± 0.3	4.8 ± 0.2	6.0×10^8	

Data was acquired at 15°C in 20 mM TRIS (pH 7.4), 140 mM NaCl, 5 mM KCl. The values reported are from an average of 2-4 individual experiments. The data was fit to a two-site independent and cooperative binding model and the Hill coefficient was calculated to assess cooperativity of the bifunctional aptamer.





Cocaine

1:1 Cocaine and DCA

Figure 2.2: ITC thermogram showing the interaction of DCA, cocaine and an equimolar mixture of DCA and cocaine titrated with the AND aptamer. The top panel indicates raw titration data showing the heat resulting from each injection of cocaine into the aptamer solution. On the bottom are the integrated heats after correcting for the heat of dilution (b) Cooperative fit (c) Independent fit. Binding experiments were performed at 15 °C with aptamer solutions of 0.16 mM using cocaine concentrations of 2.5 mM. The aptamer and ligand solutions were prepared in a buffer of 20 mM Tris (pH 7.4), 140 mM NaCl, 5 mM KCl

2.5 Conclusion

In this study we show that the bifunctional WC-MN4 aptamer has generally similar binding affinity and thermodynamics with cocaine and DCA as seen in the individual aptamers. However, ligand binding by the bifunctional aptamer displays positive cooperativity indicating that binding at one site is affected by binding at the other. We suggest that this cooperativity may be linked to dynamics at the two binding sites where binding at one site reduces the dynamics at the other allowing the second ligand to more readily bind.

Chapter Three : Kinetic analysis of the Cocaine-Binding Aptamer by Isothermal Titration Calorimetry

3.1 Preface

All work presented in this chapter is currently being prepared for publication. The experimental methods and data analysis shown here are also included on the paper.

Nusaibah Dawood and Philip E. Johnson

3.2 Introduction

3.2.1 Quinine binding by the Cocaine-Binding Aptamer

We used ITC to measure the binding kinetics of quinine to two sequence variants of the cocaine-binding aptamer: MN4 and MN19 (Figure 3.2). In the cocaine-binding aptamer, the length in stem-one controls whether the structure-switching binding mechanism in the aptamer occurs or not. In 2010, A study by Neves *et al.* looked at a series of cocaine-binding aptamers with varying lengths in stem one, those with a longer stem 1 (6 bp, MN1, MN4) and aptamers with a short stem 1 (3 bp, MN6, MN19). It was found that the structural transition from the unfolded to folded in the unbound aptamer occurred when the length of the base pair in stem one was increased from 3 to 4 base pairs⁵⁴. Originally the aptamer was selected to bind cocaine, but it also binds quinine and its analogues up to 50 folds tighter than cocaine⁵⁵. Quinine can provide enough free energy to both fold and bind the two-base pair OR8 aptamer while cocaine does not⁵⁵.

The cocaine-binding aptamer switches from a one-site to two-site ligand binding depending on the concentration of NaCl. At low NaCl concentrations, the cocaine-binding aptamer binds two molecules of its ligand, which is evident from the non-sigmoidal nature of the binding curve in 0

mM NaCl. As the concentration of NaCl is increased, the shape of the thermogram shifts from a two-site to one-site binding event (Figure 3.1) It is possible that at higher Na⁺ concentrations, the cation shields the electrostatic interactions between the polyanionic DNA and positively charged quinine⁵⁶. The binding at the second low-affinity site can be disrupted by adjusting the concentration of NaCl in the buffer, which is suitable for conducting kinITC experiments since it only applies to simple 1:1 binding models.

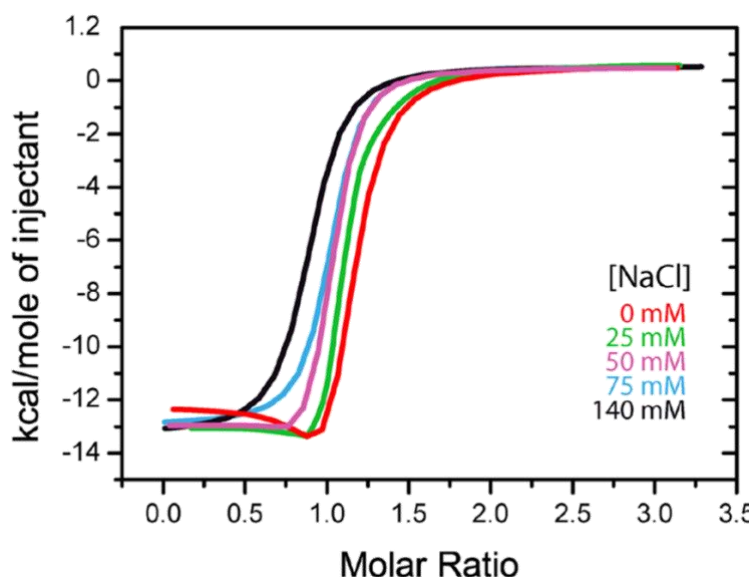


Figure 3.1: Effect of NaCl concentration on the shape of the binding curve. Shown are MN4 titration curves binding to quinine at concentrations ranging from 0 mM NaCl to 140mM NaCl. (Adapted from reference 57)

We looked at two variants of the cocaine-binding aptamer, MN4 and MN19 (Figure 3.2). The secondary structure of MN4 is pre-formed in its bound and unbound forms. If the length of stem one is shortened from 6 base pairs to 3 base pairs, such as for MN19, the aptamer is loosely structured in its unbound state and undergoes conformational changes upon ligand binding⁴⁷. In the absence of cocaine, the MN19 aptamer exists in an equilibrium state consisting of both folded

and unfolded conformations with stem 1 being mostly unfolded⁵⁷. When bound to cocaine, the unfolded parts of the aptamer undergo target-induced conformational changes. This ligand-induced structural switching binding mechanism of MN19 is also retained with quinine as a ligand. These structural comparisons between the free and bound forms reveal that aptamers can undergo an induced fit upon binding their target. Here, we compare the binding kinetics of MN4 and MN19 with quinine by ITC. To our knowledge, this study is the first kinetic binding characterization reported for the cocaine-binding aptamer. It is known that kinetic ITC (kinITC) can dissect thermodynamic and kinetic data for two separate events; primary ligand binding and aptamer folding⁵⁸.

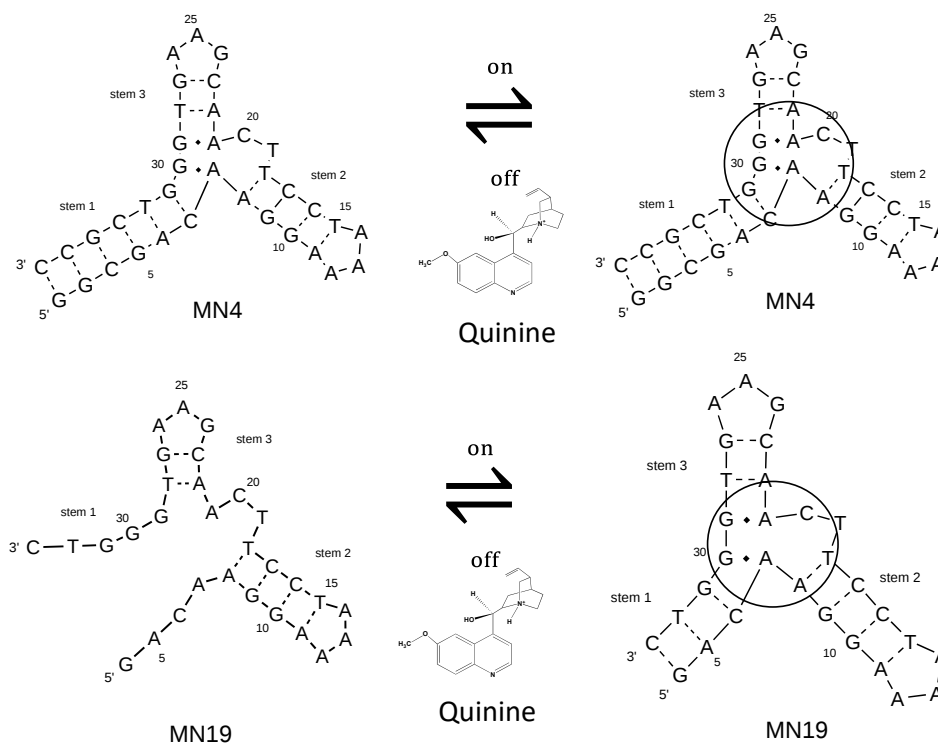


Figure 3.2: Secondary structures and sequences of the MN4 and MN19 cocaine-binding aptamer and chemical structure of the quinine ligand used in this study. MN19 has three base pairs removed from the end of stem-one. Watson-Crick base pairs are indicated by dotted lines and non-Watson-Crick base pairs are indicated by dots between nucleotides. Circled are the high affinity ligand binding sites for quinine

3.2.2 kinITC assay to capture both thermodynamic and kinetic information

ITC is predominantly used to measure the thermodynamics of binding, however, there is growing interest in using it to measure the kinetics of binding as well. kinITC is a recently developed tool that can jointly determine thermodynamic and kinetic parameters. The ability to extract kinetic data relies on real-time power response curves from ITC experiments⁷. The raw output generated from conducting an ITC experiment allows real-time monitoring of the heat generated or absorbed in the sample cell.

Dumas et al partnered with S4SD to include kinetic analysis as an extended feature in the AFFINImeter-ITC software package. The kinITC-ETC software implemented by AFFINImeter is based on determining the effective end time for each injection to yield an ETC that measures binding rates, k_{on} and k_{off} ⁶⁰. This software has been used to study the binding kinetics of aptamers and various inhibitors. For example, in a paper from 2012, Burnouf et al proposed a method for collecting both thermodynamic and kinetic data from conducting ITC experiments that could be applied for simple 1:1 binding interactions to more complex processes²⁷. They studied the binding of Nevirapine to HIV-1 reverse transcriptase, and the binding of thiamine pyrophosphate (TPP) to the *Escherichia coli* riboswitch present in the 5' -UTR of the thiC messenger RNA, which undergoes folding on binding of TPP⁶¹. They used surface plasmon resonance (SPR) to compare the binding rates with those obtained by kinITC. It is important to note that collecting kinetic results from ITC experiments is attractive since it does not require target immobilization to a solid support compared to SPR, this helps mitigate some of the challenges associated with conventional label-based methods. The research presented in this chapter focuses on determining binding rates for a one-site interaction of MN4 and MN19 binding quinine.

The ability to extract kinetic data relies on real-time power response curves from ITC experiments. The primary signal recorded by ITC is the heat-power in microcalories per second. Each negative peak in the raw thermogram corresponds to the heat released in the sample cell resulting from an interaction between the ligand and aptamer. These power peaks are further integrated to obtain the heat evolved during each titration step Figure 3.3 shows an illustrative representation of a sample ETC obtained from the thermogram of a 1:1 interaction of MN4 binding quinine. The ETC on the right shows the equilibration time for each peak as a function of the molar ratio of ligand and aptamer. Many ETCs for 1:1 binding interaction contain a distinctive feature where peaks become broader near the equivalence point of titration⁶². This can be explained in terms of the binding constants where the concentration of free component drops throughout the titration as the ligand-aptamer complex is being formed. This peak broadening observed at mid-titration (injection 13) is an indication that the thermogram contains kinetic information that can be determined by ITC. The equilibration time starts from a minimum value at the first injection and reaches its maximum at mid-titration²⁷. AFFINImeter fits the ETC to a 1:1 binding model and uses the kinITC method developed by Dumas *et al.* which uses k_{off} and the instrument response time as fitting parameters. k_{on} can then be calculated as a product of $K_A * k_{\text{off}}$.

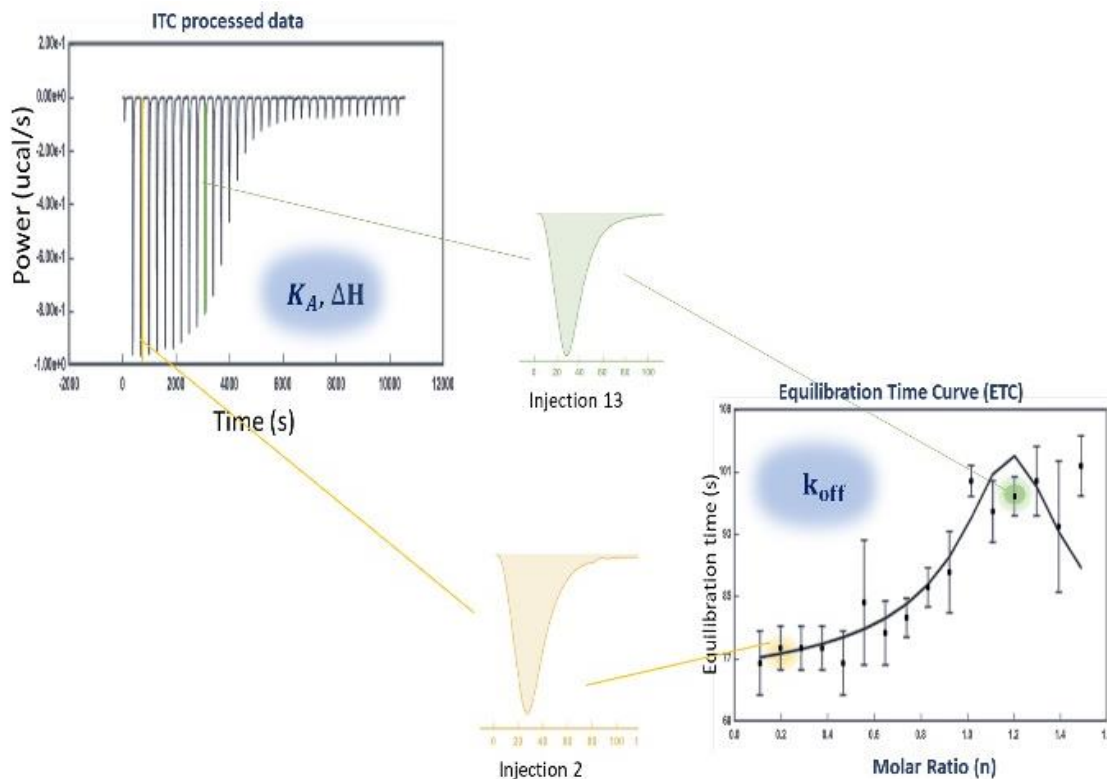


Figure 3.3: Schematic illustration of thermodynamic and kinetic parameters obtained from a single ITC experiment (A) ITC power trace following baseline subtraction. Integration of each peak yields the heat evolved by binding, which is plotted against the molar ratio of ligand to aptamer. The binding enthalpy (ΔH) and equilibrium constant (K_A) are determined by fitting the isotherm to a one-site binding model B) ETC generated from a 1:1 interaction of MN4 binding quinine at 15 °C. The equilibration time for each peak is plotted against the molar ratio of the ligand to aptamer. k_{off} is determined by fitting the ETC curve from which k_{on} is calculated.

3.3 Experimental Methods

Sample Preparation

Aptamer samples were obtained from Integrated DNA Technologies (IDT, Coralville, Iowa) with their sequences confirmed by mass spectrometry. The DNA aptamer samples were dissolved in distilled deionized H₂O (ddH₂O) and then exchanged three times using a 3-kDa molecular weight cut-off concentrator with sterilized 1 M NaCl followed by three exchanges into ddH₂O. The samples were then exchanged with Tris buffer (20 mM Tris (pH7.4), 140 mM NaCl, 5 mM KCl) three times before use. Aptamers were heated in a boiling water bath for 3 min, followed by cooling in an ice-water bath for 5 min to favor intramolecular folding. Quinine hemisulfate monohydrate was obtained from Sigma Aldrich (catalog number 145912) Ligand solutions were prepared by dissolving an appropriate weight of ligand in buffer. The concentrations of the aptamer and ligand were calculated using Beer Lamberts Law by measuring the absorbance at A₂₆₀ with a Cary 100 Bio ultraviolet (UV) spectrophotometer.

Isothermal Titration Calorimetry Experiments

ITC experiments were performed using a MicroCal VP-ITC instrument at temperatures ranging from 5 °C – 20 °C. Samples were degassed prior to use with a MicroCal ThermoVac unit, and all experiments were corrected for the heat of dilution of the titrant. All titrations were performed with the aptamer in the sample cell and ligand in the injection syringe. Binding experiments were performed with aptamer concentrations of 20 μM and quinine at a concentration of 310 μM. In all experiments, the aptamer and ligand were dialyzed in the same buffer of 20 mM Tris (pH7.4) and ddH₂O was used to prepare all the buffers, ligand, and aptamer solutions. Each binding

experiment consisted of 35, 8 μL injections spaced every 300 s with the first injection being 2 μL , to account for diffusion from the syringe into the cell during equilibration.

Data Fitting

ITC data was fit to a one-site binding model using the Origin 7.0 software package provided by the manufacturer. Kinetic parameters were determined by processing raw ITC thermograms using AFFINImeter to generate ETCs for standard 1:1 binding models.

3.4 Results and Discussion

3.4.1 ITC-derived thermodynamics of quinine binding to the cocaine binding aptamer

ITC experiments were performed to quantify the binding affinity of quinine binding to MN4 and MN19 at temperatures ranging from 5 $^{\circ}\text{C}$ – 25 $^{\circ}\text{C}$. Both sets of aptamers contain two binding sites for quinine: a high-affinity site and a low-affinity site. Binding at the second site can be controlled by adjusting the concentration of NaCl in the buffer. At high salt concentrations the thermogram shifts from a two-site to a one-site binding event. For all ITC experiments conducted, thermodynamic and kinetic data were collected under high salt concentrations of 140 mM NaCl to promote one-site binding to the high-affinity site. Figures 3.4 and 3.5 depict binding curves obtained from titrating MN4 and MN19 with quinine at various temperatures. The thermograms were processed and fit to a one-site binding model to determine the affinity and binding enthalpies. The sigmoidal curve observed when both MN4 and MN19 bind quinine is indicative of one-site binding.

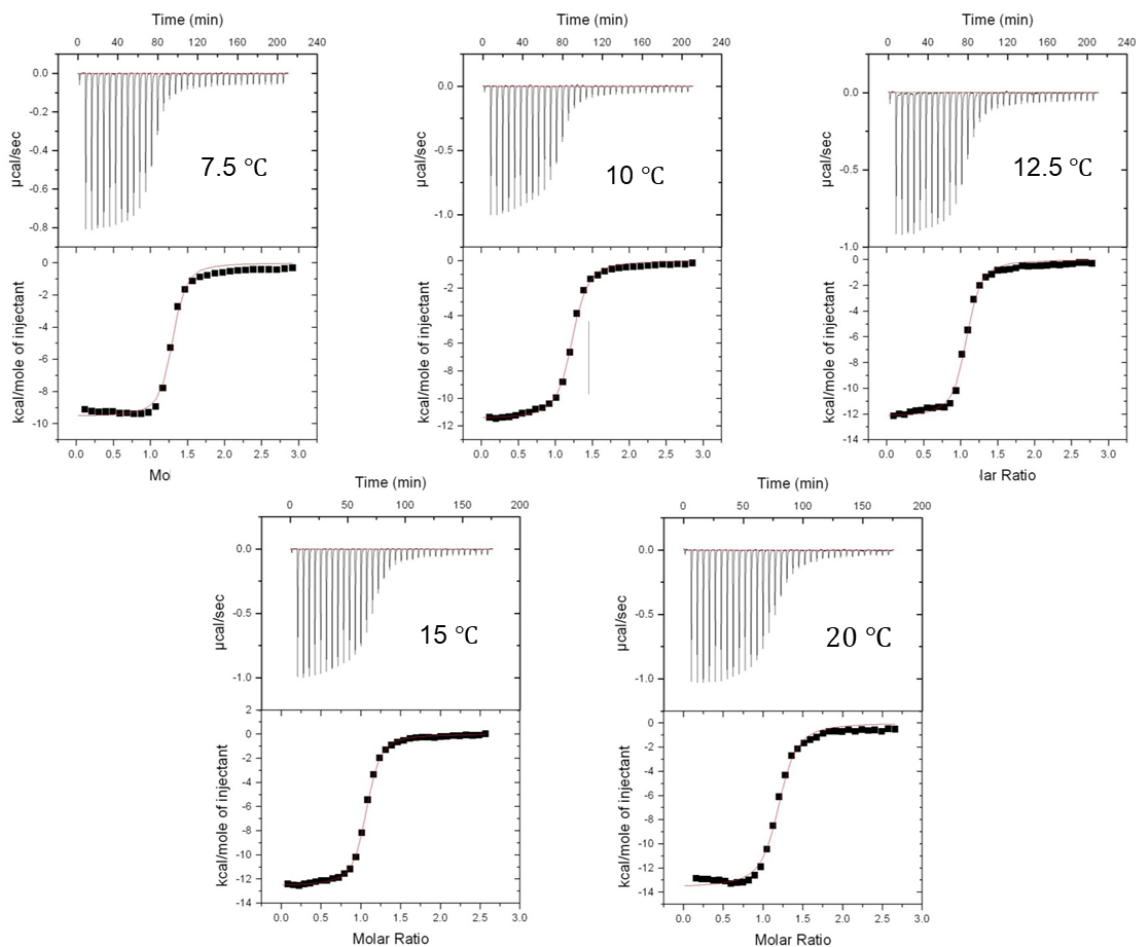


Figure 3.4: ITC thermograms showing the interaction of MN4 with quinine at different temperatures. Top: ITC power trace showing the heat resulting from each injection of quinine into the MN4 aptamer solution. Bottom: Integrated heat plots after correcting for the heat of dilution. Integration of each peak yields the heat evolved by binding, which is plotted against the molar ratio of ligand to aptamer. Binding experiments were performed at temperatures ranging from 7.5 °C to 20 °C in a buffer of 20 mM Tris (pH7.4), 140 mM NaCl, and 5 mM KCl. Data was processed and fit to a one-site binding model.

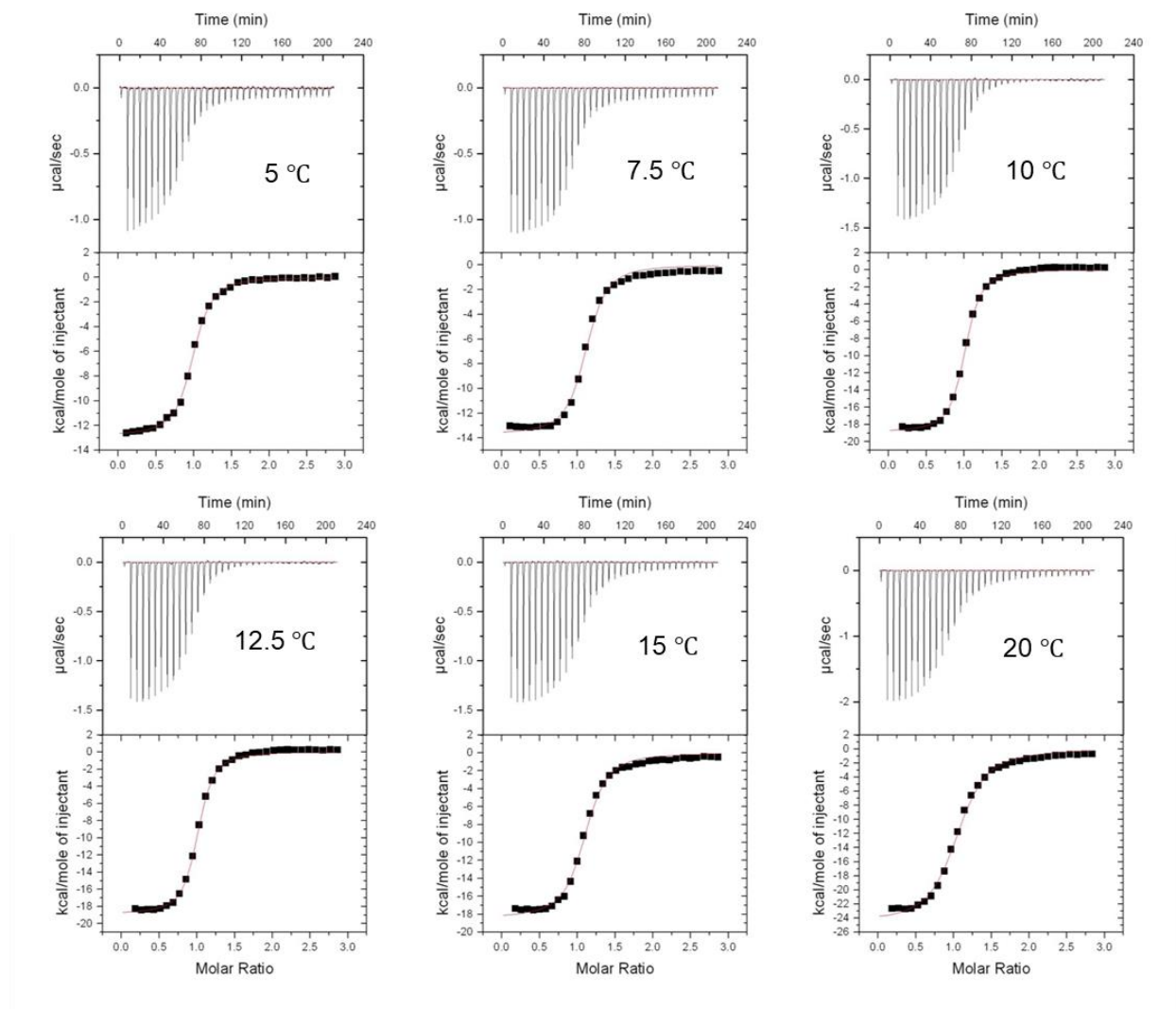


Figure 3.5: ITC thermograms showing the interaction of MN19 with quinine at different temperatures. Top: ITC power trace showing the heat resulting from each injection of quinine into the MN19 aptamer solution. Bottom: Integrated heat plots after correcting for the heat of dilution. Integration of each peak yields the heat evolved by binding, which is plotted against the molar ratio of ligand to aptamer. Binding experiments were performed at temperatures ranging from 7.5 °C to 20 °C in a buffer of 20 mM Tris (pH7.4), 140 mM NaCl, and 5 mM KCl. Data was processed and fit to a one-site binding model.

The thermodynamic analysis and kinetic parameters for quinine binding MN4 and MN19 are summarized in Tables 3.1 and 3.2. Binding data for MN4 was acquired from 7.5 °C to 20 °C and binding data for MN19 was acquired from 5 °C to 20 °C. Both sets of data were obtained under the same pH and buffer conditions of 20 mM Tris (pH7.4), 140 mM NaCl, and 5 mM KCl. The affinity for MN4 binding quinine gets tighter at lower temperatures with the K_d value being (0.07 ± 0.01) μM at 5 °C compared to 20 °C which yields a K_d value of (0.27 ± 0.01) μM . A similar trend is also observed with MN19 where the binding affinity increases at lower temperatures. In both variants analyzed, MN4 exhibits the tightest affinity for quinine compared to MN19 which has three base pairs removed from the end of stem-one. This observation corroborates the results that were published previously for quinine binding under the same buffer conditions⁵⁵.

Our binding experiments also show that the enthalpies become more negative at higher temperatures until you approach the T_m of the aptamer where parts of the aptamer begin to unfold. It is known that at temperatures greater than 17.5 °C, some parts of MN19 start to unfold resulting in the enthalpy becoming less negative at higher temperatures⁴⁸. It is evident from the table that the exothermicity increases as a function of temperature for MN4 and MN19 for the temperature conditions studied.

Table 3.1. Binding affinity and kinetic parameters of MN4 titrated with quinine at different temperatures as determined by ITC

Temperature (°C)	K_d [μM]	k_{on} [$\text{M}^{-1}\text{s}^{-1}$]	k_{off} [s^{-1}]	ΔH [kcal mol^{-1}]
7.5	0.07 ± 0.01	$(4.8 \pm 0.3) \times 10^4$	$(3.2 \pm 0.2) \times 10^{-3}$	-9 ± 0.02
10	0.17 ± 0.001	$(2.7 \pm 0.1) \times 10^4$	$(4.8 \pm 0.2) \times 10^{-3}$	-12 ± 0.1
12.5	0.19 ± 0.01	$(2.7 \pm 0.1) \times 10^4$	$(3.3 \pm 0.2) \times 10^{-3}$	-12 ± 0.1
15	0.21 ± 0.01	$(3.1 \pm 0.2) \times 10^4$	$(4.9 \pm 0.3) \times 10^{-3}$	-12 ± 0.1
20	0.27 ± 0.01	$(2.2 \pm 0.2) \times 10^4$	$(4.1 \pm 0.3) \times 10^{-3}$	-14 ± 0.2

Binding data was acquired at temperatures ranging from 7.5 °C – 20 °C in a buffer solution of 20 mM TRIS (pH7.4), 140 mM NaCl, and 5 mM KCl. The errors for 7.5 °C and 15 °C are standard deviation from replicates and the rest are individual fits. The initial concentration of MN4 for all binding experiments was 20 μM and the concentration of quinine was 310 μM

Table 3.2. Binding affinity and kinetic parameters of MN19 titrated with quinine at different temperatures as determined by ITC

Temperature [°C]	K_d [μM]	k_{on} [$\text{M}^{-1}\text{s}^{-1}$]	k_{off} [s^{-1}]	ΔH [kcal mol^{-1}]
5	0.5 ± 0.004	$(2.6 \pm 0.1) \times 10^4$	$(4.7 \pm 0.2) \times 10^{-3}$	-14 ± 0.2
7.5	0.3 ± 0.001	$(2.6 \pm 0.2) \times 10^4$	$(5.9 \pm 0.6) \times 10^{-3}$	-14 ± 0.2
10	0.2 ± 0.01	$(2.8 \pm 0.2) \times 10^4$	$(4.6 \pm 0.3) \times 10^{-3}$	-15 ± 0.2
12.5	0.3 ± 0.01	$(2.3 \pm 0.2) \times 10^4$	$(6.8 \pm 0.6) \times 10^{-3}$	-19 ± 0.1
15	0.4 ± 0.01	$(2.2 \pm 0.2) \times 10^4$	$(7.5 \pm 0.7) \times 10^{-3}$	-19 ± 0.2
20	0.7 ± 0.01	$(2.9 \pm 0.4) \times 10^4$	$(1.5 \pm 0.2) \times 10^{-2}$	-26 ± 0.3

Data was acquired at temperatures ranging from 5°C – 20°C in a buffer solution of 20 mM TRIS (pH7.4), 140 mM NaCl, and 5 mM KCl. The errors reported for 7.5 °C are standard deviation from replicates and the rest are individual fits. The initial concentration of MN19 for all binding experiments was 20 μM and the concentration of quinine was 310 μM

Furthermore, the binding heat capacity change ($\Delta C(p)^\circ$) of MN4 and MN19 was computed from the ITC data in the form of a Van't Hoff plot. The tangent to the curve at any point of the Van't Hoff plot is the ΔH at that precise temperature. The $\Delta C(p)^\circ$ values for both aptamers are in good agreement with the calorimetry data that was previously reported in our lab. According to the study performed by Oren Reinstein, the aptamer with the long stem-one (MN4) has a heat capacity $\Delta C(p)^\circ$ value of $-557 \pm 29 \text{ cal mol}^{-1} \text{ K}^{-1}$ and the aptamer with a short stem-one (MN19) has a $\Delta C(p)^\circ$ value of $-922 \pm 51 \text{ cal mol}^{-1} \text{ K}^{-1}$ ¹¹. The trend in enthalpy values is consistent with the results obtained from our binding experiments, which indicate MN19 having a more negative $\Delta C(p)^\circ$ value of $-300 \text{ cal mol}^{-1} \text{ K}^{-1}$ compared to MN4 with a $\Delta C(p)^\circ$ value of $-200 \text{ cal mol}^{-1} \text{ K}^{-1}$. This difference in $\Delta C(p)^\circ$ values for MN4 and MN19 is attributed to the structural changes that occur in the aptamer upon ligand binding. For MN4 it was known that there were no secondary structural changes that occurred with binding hence reflecting the less negative $\Delta C(p)^\circ$ value compared to MN19.

3.4.2 Determining binding rates of the cocaine binding aptamer by kinITC

Kinetic constants were determined using AFFINImeter to yield equilibration time curves for standard one-site binding data. Figures 3.6 & 3.7 show the ETCs generated from a 1:1 interaction of MN4 and MN19 binding quinine. From the fit of the experimental ETC's we obtained k_{on} and k_{off} rates at varying temperatures and compared values for MN4 with those from MN19. From visually looking at the fits, the ETC adopts a bell-shaped curve signaling the presence of valuable kinetic data. The kinetic constants were experimentally determined to be within the range of $(2.2 \pm 0.2) \times 10^4 - 4.8 \pm 0.3) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for k_{on} and $(3.2 \pm 0.2) \times 10^{-3} - 7.5 \pm 0.7) \times 10^{-3} \text{ s}^{-1}$ for k_{off} .

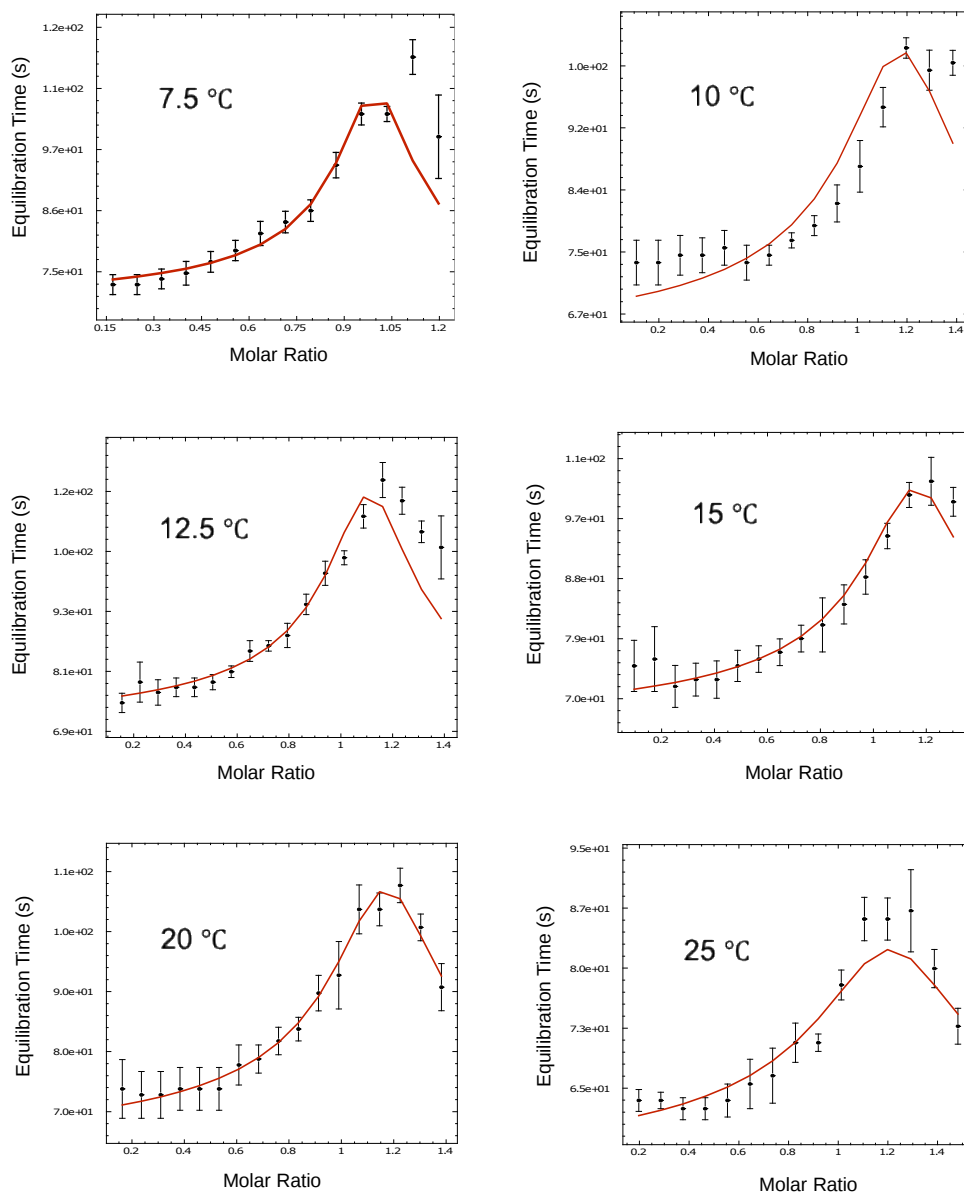


Figure 3.6: Equilibration-time curves obtained from the interaction of MN4 with quinine at different temperatures. The solid red line corresponds to the fit of the experimental data to yield k_{off} value. The experimental equilibration times and their errors were automatically determined by AFFINImeter and the error bars are increased for noisier injections following mid-titration

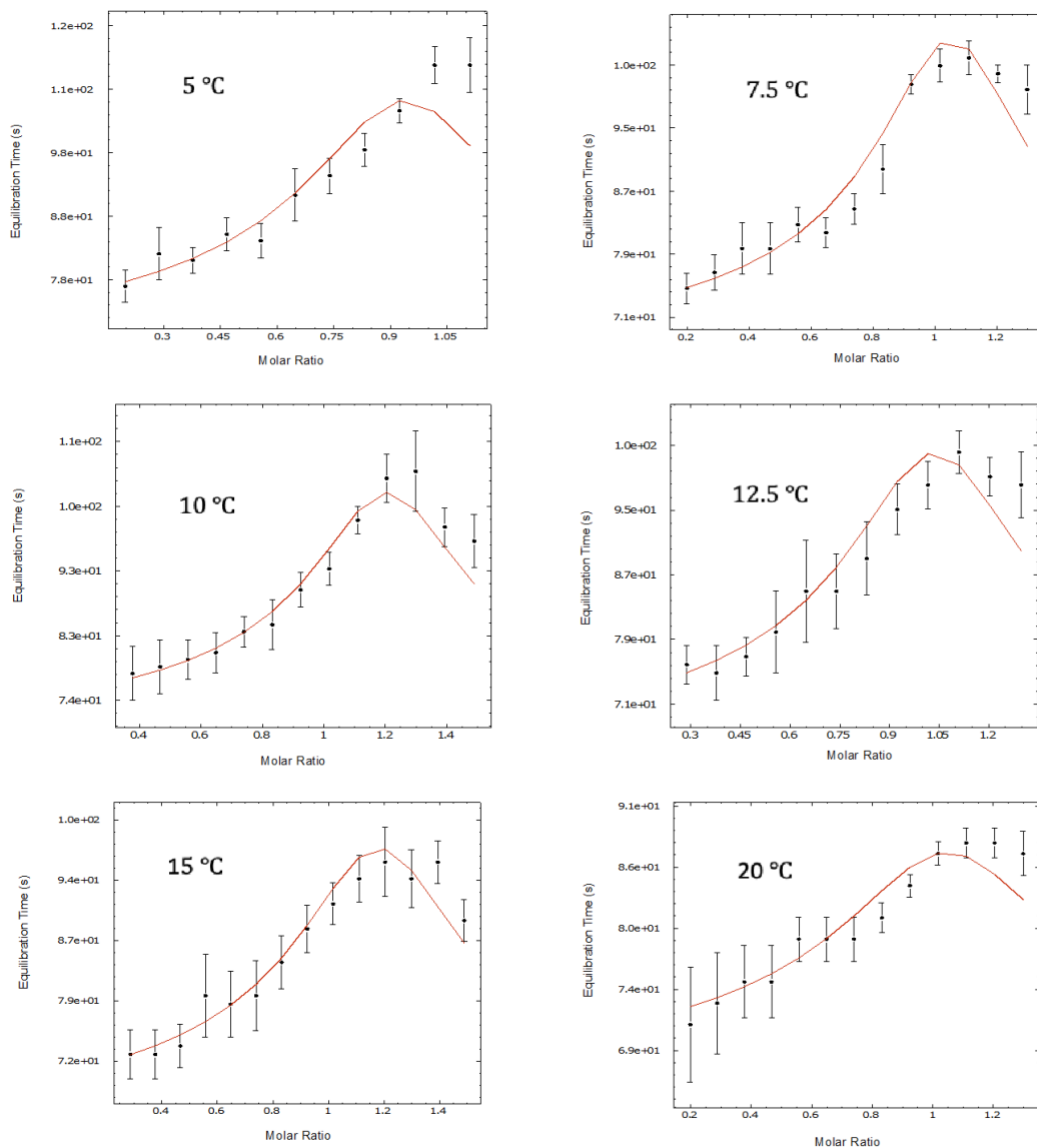


Figure 3.7: Equilibration-time curves obtained from the interaction of MN19 with quinine at different temperatures. The solid red line corresponds to the fit of the experimental data to yield k_{off} value. The experimental equilibration times and their errors were automatically determined by AFFINImeter and the error bars are increased for noisier injections following mid-titration.

The temperature dependence on the enthalpy of binding is modeled in the form of an Arrhenius equation, which is plotted as the natural logarithm of the binding constant vs the reciprocal of the absolute temperature. Figure 3.8 shows the temperature variation of k_{on} for MN4 as a function of different temperatures. The slope of the line represents $\frac{-E_a}{R}$ which is used to determine the activation energy of the reaction. This equation is based on the collision theory which states that the rate constant increases as a function of temperature which will give you a negative slope but this is not the trend that we observe here. The positive slope observed for MN4 could be attributed to the fact that the aptamer adopts multiple conformations at higher temperatures which are not capable of binding. This is consistent with the data that was produced seven years ago by Oren Reinstein where the rate is slowing down as a function of increasing temperature. (Figure 3.8)

For MN19, we wanted to get an insight on the structure of the unbound state, whether it is completely unfolded or loosely structured but dynamic. Figure 3.9 proposes two different models for MN19. The folding pathway for MN19 is similar to what we observe for proteins. At the top, we have a completely unfolded conformation for MN19 with no energy barrier to binding and folding. and somewhere lower than that, we have partially folded intermediate structure with an energy barrier that was calculated to be $2.5 \text{ kcal mol}^{-1}$. Because we have an energy barrier, we think that the unbound structure for MN19 is partially folded and not completely unfolded. This partially folded intermediate agrees with the data from our recent publication which confirms the presence of formed base pairs in free MN19 structure. The Arrhenius plot for MN19 results in a negative slope that aligns with the collision theory where increasing temperatures result in increased reaction rates (Figure 3.10) Overall our results demonstrate that the binding rates

increase at higher temperatures for MN19 compared to MN4 where the rates decrease with increasing temperatures.

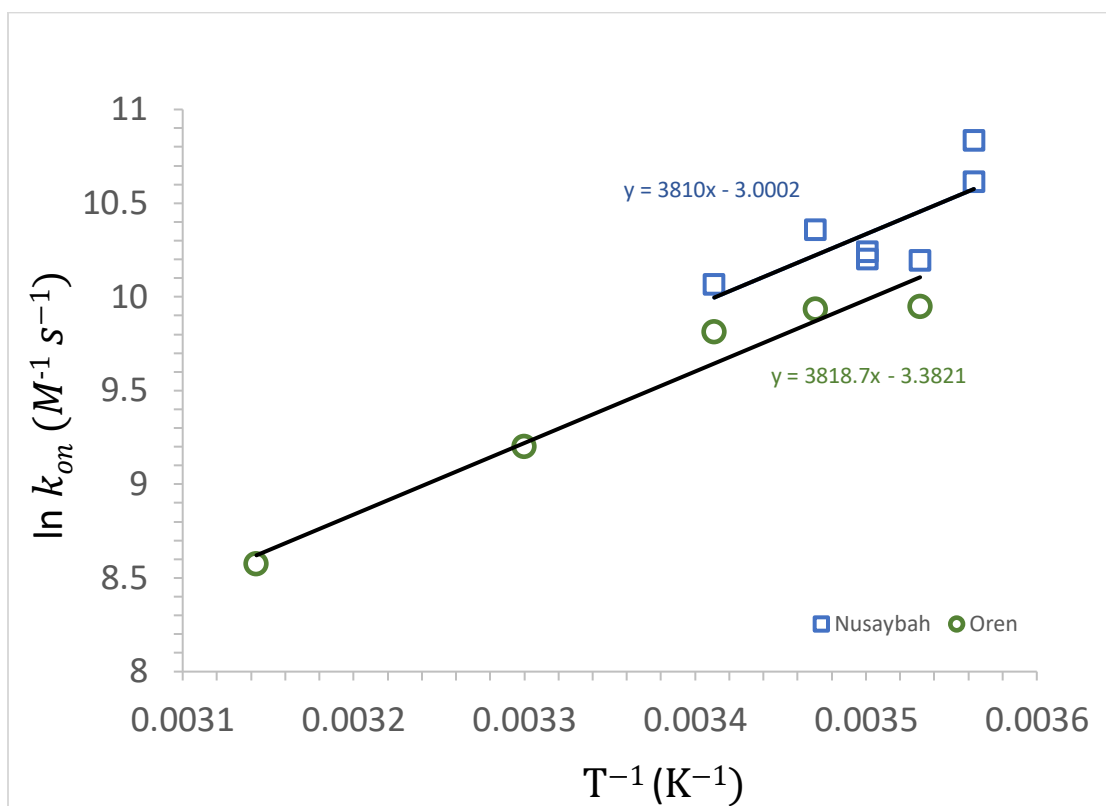


Figure 3.8: Arrhenius plots for MN4 binding quinine at temperatures ranging from 5°C – 25 °C. The slope of the Arrhenius plot represents $\frac{-E_a}{R}$ which can be used to calculate the activation energy.

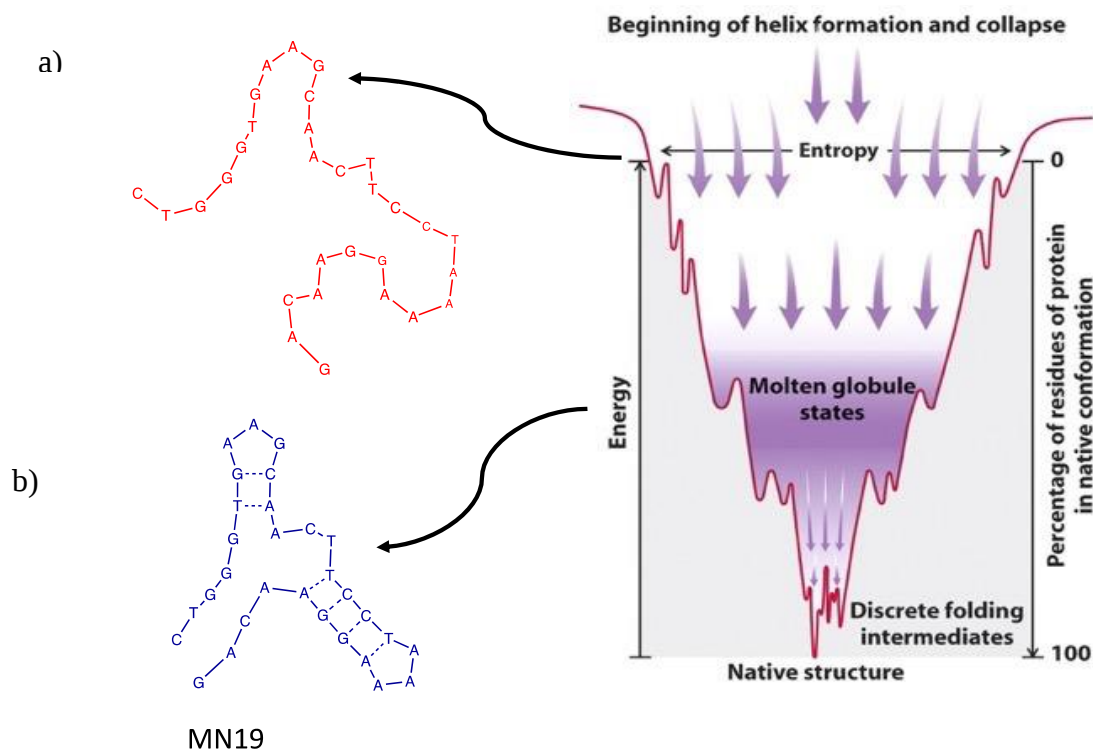


Figure 3.9: Transition state theory analysis for MN19. a) Unfolded MN19 b) Partially folded intermediate of MN19

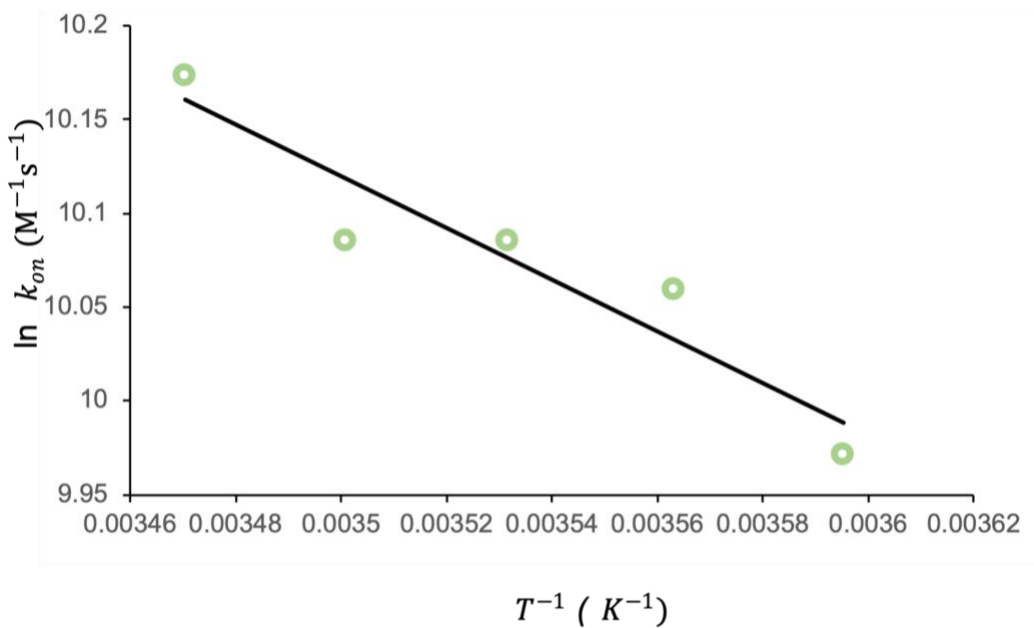


Figure 3.10: Arrhenius plots for MN19 binding quinine at temperatures ranging from 5°C – 25 °C. The slope of the Arrhenius plot represents $\frac{-E_a}{R}$ which can be used to calculate the activation energy.

3.5 Conclusion

This simplified ETC-based kinITC technique proved to be valuable in providing kinetic data along with accurate thermodynamic information. We have shown that kinITC is able to provide thermodynamic and kinetic data for both aptamers studied. The thermodynamic analysis supports prior studies that were published on the cocaine-binding aptamer and the transition-state enthalpies further highlight the differences in energy barrier between MN4 and MN19. Analyzing kinITC data from ITC experiments offers great benefits since it does not require any additional sample preparation steps, the binding rates can be directly retrieved from ITC power curves. One of the limitations for performing kinITC experiments is that only simple 1:1 binding interaction can be analyzed. This is still an area for ripe advancement as more studies are increasingly recognizing the value of obtaining kinetic data from ITC experiments.

Future Work

In this project, we designed the WC-MN4 bifunctional aptamer from two different cocaine binding aptamer variants, WC and MN4. The aptamer was shown to bind both ligands through positive cooperativity with hill coefficients ranging from 1.2-1.5. In terms of where this project is headed next, the focus would be to develop bifunctional aptamers with larger hill coefficient values. An example of this would be the MN24 and MN28 dimeric bifunctional aptamers where both binding sites are specific to the same ligand, cocaine.

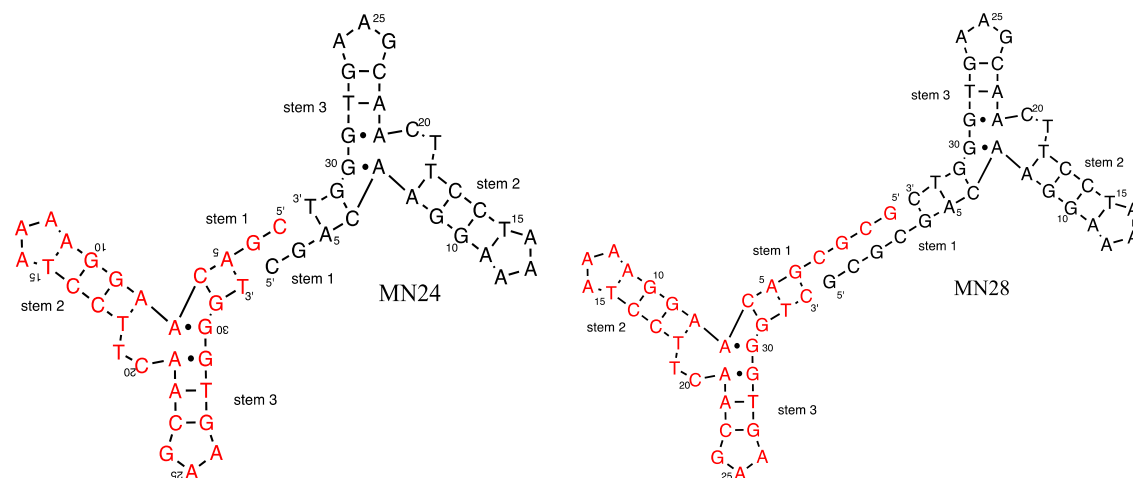


Figure 4.0: Secondary structure and sequence of two bifunctional aptamers that were designed by fusing two cocaine-binding aptamers.

The kinITC results presented on this thesis are one of the first kinetic studies that have been reported on the cocaine-binding aptamer. Future students who will follow up on the project can analyze kinetics for one-site interactions with cocaine and quinine binding aptamers from ITC experiments conducted in the lab from previously acquired data. In this report, the binding rates were analyzed as a function of different temperatures to compare the Arrhenius plots for MN4 to

MN19 since MN19 exhibits ligand-dependent conformational changes. The next step would be to look at the kinetics as a function of different aptamer concentrations to compare whether the ligand association is through conformational selection or induced fit. This is an important question yet to be determined and can only be addressed by conducting further kinetic analysis. Other one-site binding ligands and quinine antibiotics can also be analyzed to dissect kinetic parameters.

Supplementary information

In order to determine the cooperative behaviour of the aptamer, the Hill coefficient was calculated for data that was fit to a cooperative binding model. A cooperative site is described by the following data-fitting model developed by Frieburger et al and Cattoni *et al*^{11,12}

The first ligand can bind to either site of the bifunctional aptamer and is described with a dissociation constant, K_{d1} ($L \rightarrow LA$)

$$K_{d1} = \frac{[L][A]}{[LA]} \quad (1)$$

The second ligand binding site binds to the unoccupied site on the aptamer with a dissociation constant K_{d2}

$$K_{d2} = \frac{[LA][L]}{[LA_2]} \quad (2)$$

$[X]_T$ and $[P]_T$ are determined by the Origin 7.0 software package following an ITC experiment.

$$[L]_T = [L]_0 \left(1 - \left(1 - \frac{V_i}{V_c} \right)^n \right) \quad (3)$$

$$[A]_T = [A]_0 \left(1 - \frac{V_i}{V_c} \right)^n \quad (4)$$

V_i = volume of the i th injection

V_c = working volume of the sample cell ($1.42 \times 10^{-3}L$)

$[L]_T$ = concentration of ligand in working volume of the cell after n^{th} injections

$[A]_T$ = concentration of aptamer present in working volume of the cell after n^{th} injections

$[L]_0$ = initial concentration of ligand in the syringe

$[A]_0$ = initial concentration of aptamer

n = number of injections

Using the initial aptamer, $[A]_0$ and ligand $[L]_0$ concentrations together with the injection volumes, V_i and sample cell volume V_c , equations (3) and (4) can be used to calculate $[L]_T$ and $[A]_T$, which are the concentrations of the ligand and aptamer in the calorimeter cell after each injection.

The following cubic expression is generated from the fraction of aptamer in the singly and doubly bound states

$$a + b[X] + c[X]^2 + [X]^3 = 0 \quad (5)$$

$$\begin{aligned} a &= -K_{d1}K_{d2}[L]_T \\ b &= K_{d1}K_{d2} + 2K_{d2}([A]_T - [L]_T) \\ c &= 2K_{d2} + 2([A]_T - [L]_T) \end{aligned}$$

For a biomolecule with two binding sites, the average number of ligands bound per macromolecule is:

$$n_{LB} = \frac{K_1[L] + 2K_2[L]^2}{1 + K_1[L] + K_2[L]^2} \quad (6)$$

Where K_1 and K_2 are the binding constants directly obtained from Origin 7.0, $[L]$ is the concentration of free ligand at the n^{th} injection calculated by taking the positive real root of Equation 5

The Wyman's Hill plot for ligand binding two sites is generated by plotting $\ln\left(\frac{n_{LB}}{2-n_{LB}}\right)$ as a function of the free ligand concentration, $\ln [L]$. The Hill coefficient describes the fraction of the macromolecule saturated by ligand as a function of the ligand concentration. In order to calculate the Hill coefficient, the transition region in Wyman's Hill plot is fit to a third order polynomial function with fitting parameters a_1 , a_2 and a_3

$$y = a_3(x)^3 + a_2(x)^2 + a_1(x) + a_0 \quad (6)$$

The first derivative of equation above gives a maximum for positive cooperativity and minimum for negative cooperativity at the inflection point

$$\frac{dy}{dx} = 3a_3(x)^2 + 2a_2(x) + a_1 \quad (7)$$

The coordinates at the inflection point can be determined by equaling the second derivative of equation 7 to zero

$$\frac{d^2y}{dx^2} = 6a_3x + 2a_2 = 0 \quad (8)$$

$$so \ x = \frac{2 \cdot a_2}{6 \cdot a_3}$$

The Hill coefficient can be estimated by replacing the value of x in Equation 9

$$n_H = a_1 - \frac{a_2^2}{3a_3} \quad (9)$$

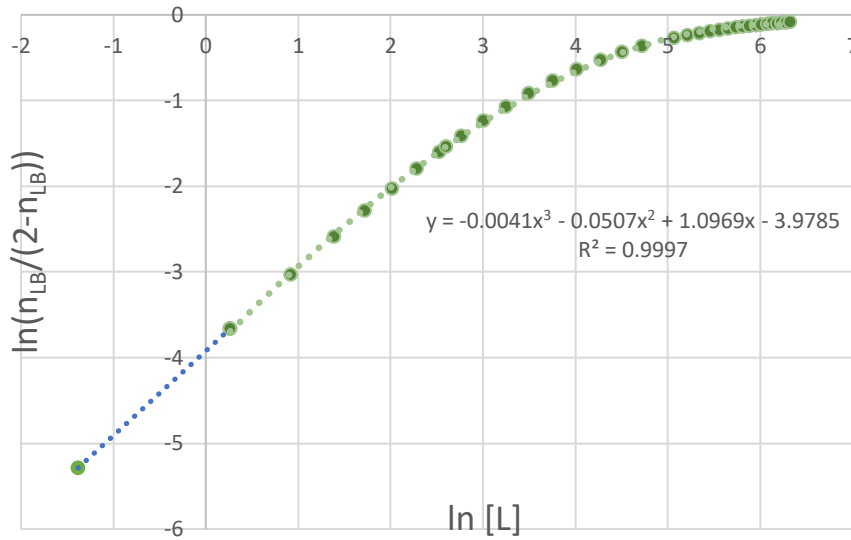


Figure 9 Wyman's Hill plot for DCA binding to two sites exhibiting positive cooperativity. The green trendline represents a third order polynomial fitted to data in the transition region where half saturation occurs and the blue trendline line represents data fit to a linear slope. The Hill coefficient was $n_H = 1.31$, which was obtained from the fitting parameters in the polynomial equation; $a_1 = 1.069$, $a_2 = -0.0507$ and $a_3 = -0.0041$

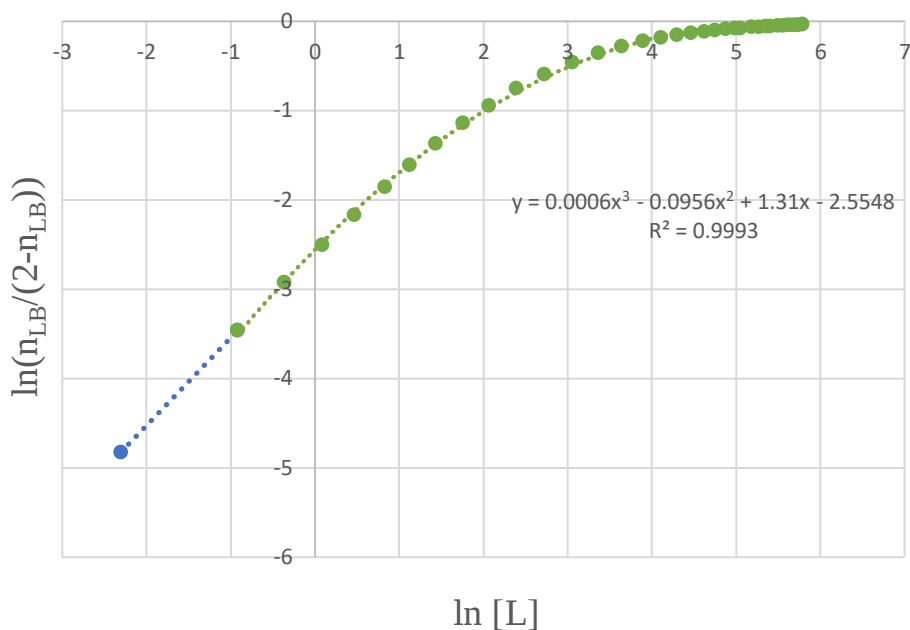


Figure 10 Wyman's Hill plot for cocaine binding to two sites exhibiting positive cooperativity. The green trendline represents a third order polynomial fitted to data in the transition region where half saturation occurs and the blue trendline line represents data fit to a linear slope. The Hill coefficient was $n_H = 1.25$ which was obtained from the fitting parameters in the polynomial equation; $a_1 = 1.31$, $a_2 = -0.00956$ and $a_3 = -0.0006$

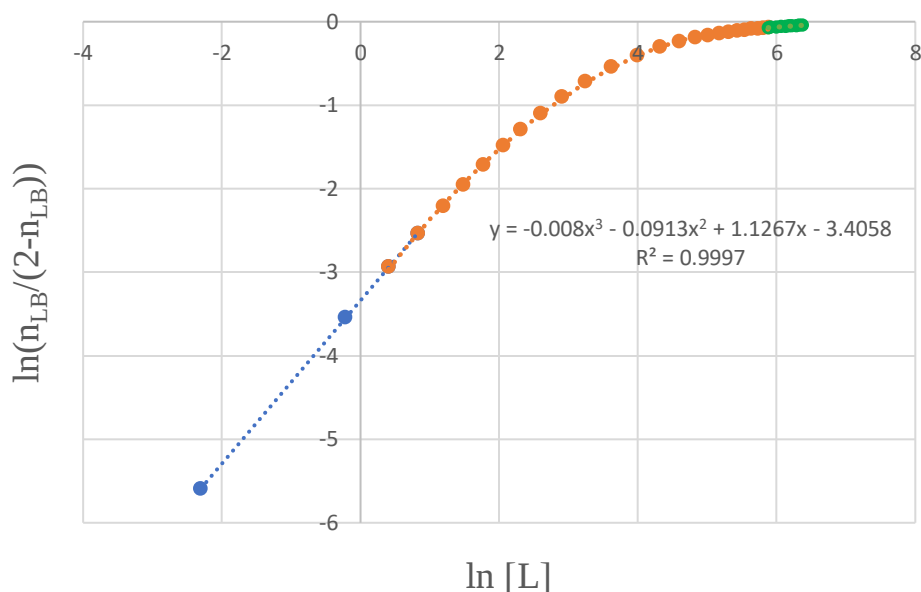


Figure 11 Wyman's Hill plot for Cocaine and DCA binding to two sites exhibiting positive cooperativity. The orange trendline represents a third order polynomial fitted to data in the transition region where half saturation occurs and the blue trendline line represents data fit to a linear slope. The Hill coefficient was $n_H = 1.52$, which was obtained from the fitting parameters in the polynomial equation; $a_1 = 1.176$, $a_2 = -0.09$ and $a_3 = -0.008$

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