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THERMODYNAMIC INVESTIGATION OF THE BINDING OF PLATINUM BASED COMPLEXES WITH 10 BASEPAIR DNA/RNA STRUCTURES

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THERMODYNAMIC INVESTIGATION OF THE BINDING OF PLATINUM BASED COMPLEXES WITH 10 BASEPAIR DNA/RNA STRUCTURES

Senior Project Submitted to

The Division of Science, Mathematics, and Computing

By

Humam Al Rubaye

Bard College
Annandale-On-Hudson, NY
May 2019

Dedication

This work is dedicated to my parents, Ebtesam and Jaafar, my brother, Ammar, and my close to the heart sister, Haddeer and her husband, Ali. It is dedicated to my extended, new family, in Philadelphia who always made me feel home and welcomed.

Without Dr. Swapan Jain, I would have regretfully given up on the dreams I'm pursuing now.

Thank you, everyone, who took a chance at me even at my lowest times.

Introduction

1.1 Chemotherapy and Genetics

Referring to blood-based medications functioning through unique mechanisms on inhibiting cell growth, chemotherapeutic agents have been in the front line of the fight against cancer [1]. Cisplatin (**Fig. 1a**) [cis-diamminedichloridoplatinum(II)] is one of the most commonly used chemotherapeutic agents mostly targeting (**G-G**) or (**rG-rG**) base pairs of DNA/RNA structures to form two covalent bonds with one N7 of each guanine [2]. Since DNA and DNA modifiers (epigenetics) are altered by chemotherapy, investigating long and short term gene regulation of Cisplatin and other chemotherapeutic agents is of critical importance to researchers [3].

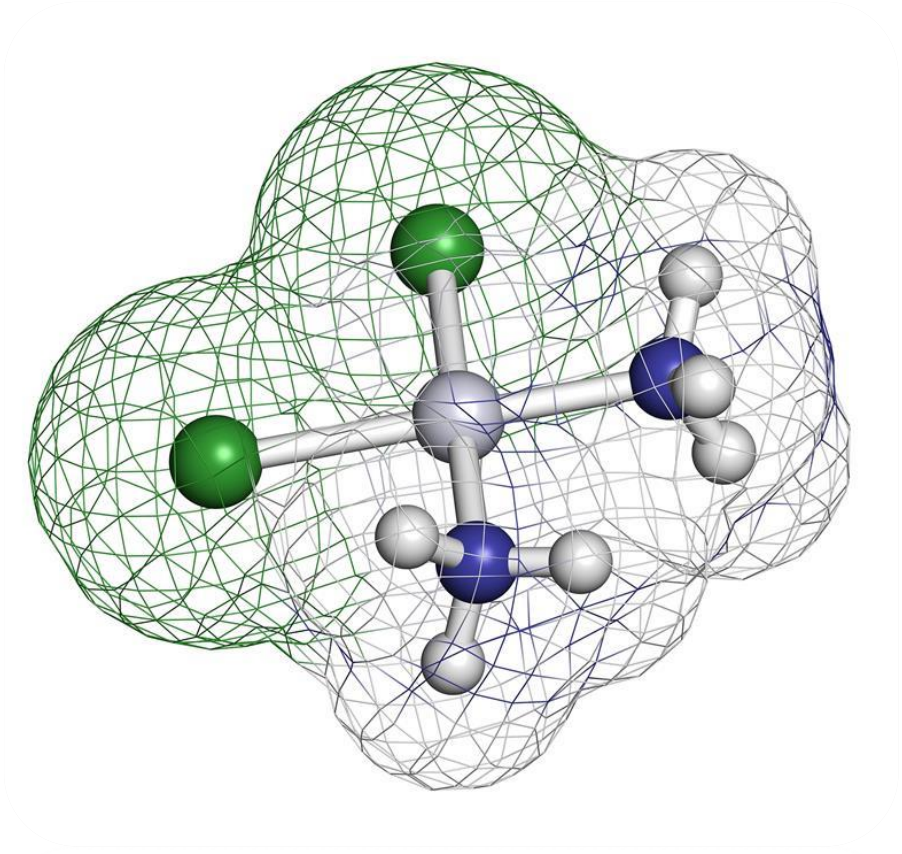


Figure 1a: 3-D Representation of Cisplatin. [6]

Testicular cancer, treated mainly by Cisplatin and Carboplatin [4, 5], is of interest due to its low fidelity rate and its targeting of young people [3] allowing scientist to observe gene regulation over a long period of time.

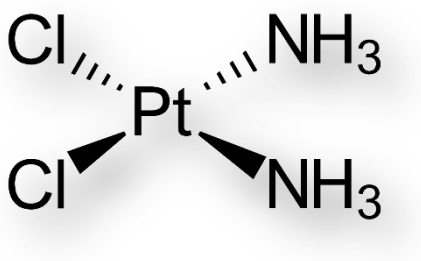
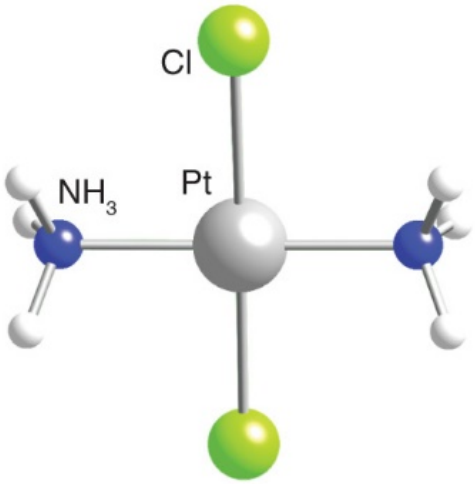


Figure 1b: 2-D Representation of Cisplatin

It is worth noting that the *trans* version (**Fig. 1c**) of this molecular is biologically inactive due to a lowered binding affinity to nucleic acid structures [15].

Figure 1(c):

3-D Representation of Transplatin



Intervention of Cisplatin in genetics comes from its ability to inhibit transcription and translation [1, 16, 23, 24]. As scientists describe different possible mechanisms of Cisplatin interaction with nucleic acids, old facts get either replaced or added to.

One debate of Cisplatin is its thermodynamic effects on DNA duplexes. Though studied extensively, thermodynamics stays a mystery; one to be explored here.

1.2 DNA/RNA Structures Binding to Cisplatin

The primary binding mood of Cisplatin to nucleic acids is the formation of an intrastrand link between two adjacent guanines [20] (**Fig. 2**). Cisplatin has shown that it can bind to either single-stranded DNA/RNA or duplexes and hybrids [10-12].

In the past, our research found that upon the binding of Cisplatin to Calf Thymus DNA, a thermal destabilization of the duplex take place (**Appendix A**) mainly due to thermodynamically unfavorable structural consequences causing the double helix to unwind [11, 2] and the DNA to stagger in one area forming shorter duplexes [2, 21].

Todd and Lippard [13] produced a computational model to visualize the structural effects of Cisplatin on DNA double helix [**Appendix B**].

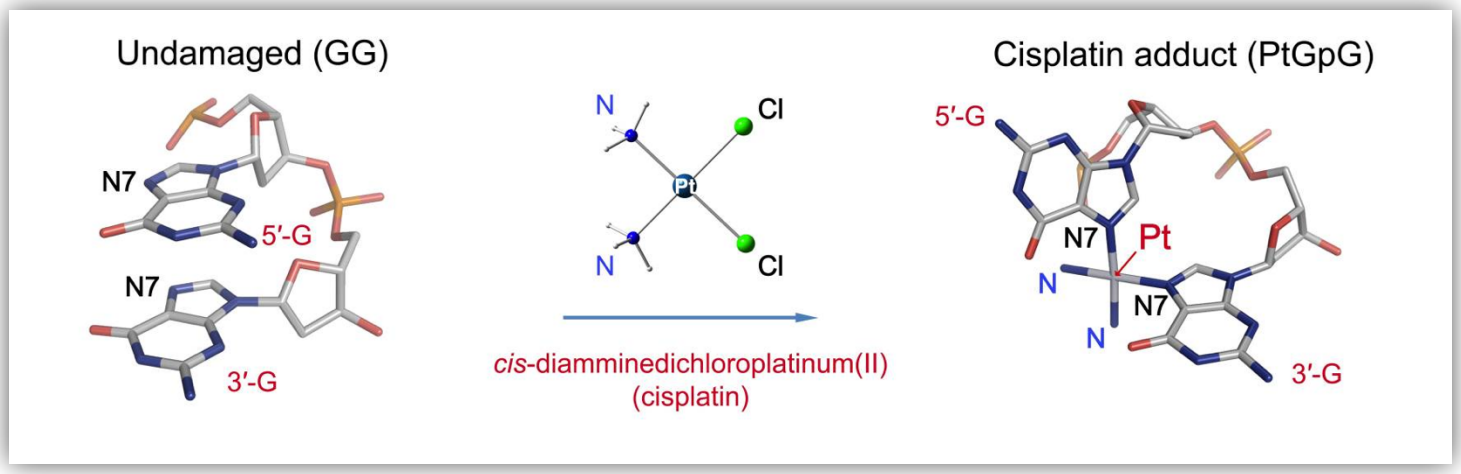


Figure 2: Formation of two covalent bonds (intrastrand linkage) between Cisplatin and **GG** base pairs of DNA/RNA causing the latter to lose thermal stability [9].

1.3 Thermodynamics

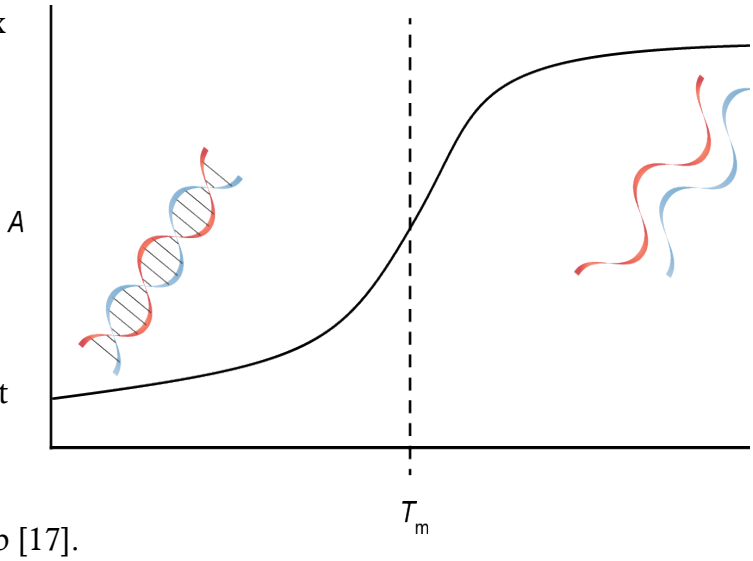
To measure thermodynamic properties Enthalpy, Entropy and Free Energy, our research used two analytical methods to measure heat of duplex/hybrid formation and heat of duplex/hybrid denaturation.

1.3.1 Observing DNA/RNA Structures Denaturation (Fig. 3)

Figure 3:

As temperature increases (x axis), the duplex undergoes a hyperchromic shift causing it to starts to disassociate forming two single strands.

A (y-axis) is absorbance. Purine and Pyridines have a tendency to absorb light but when they exist in a duplex formation, base stacking interferes with their ability to absorb [17].



To absorb denaturation of DNA/RNA structures, UV-Spectroscopy (**Fig. 4**) was performed in order to calculate the absorbance at 260nm at a temperature range of 10-75 C.

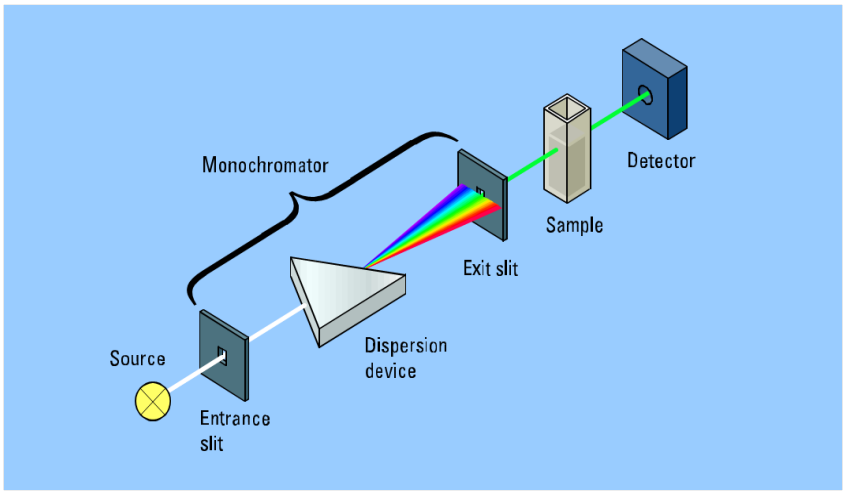


Figure 4

1.3.2 Observing DNA/RNA Structures Formation

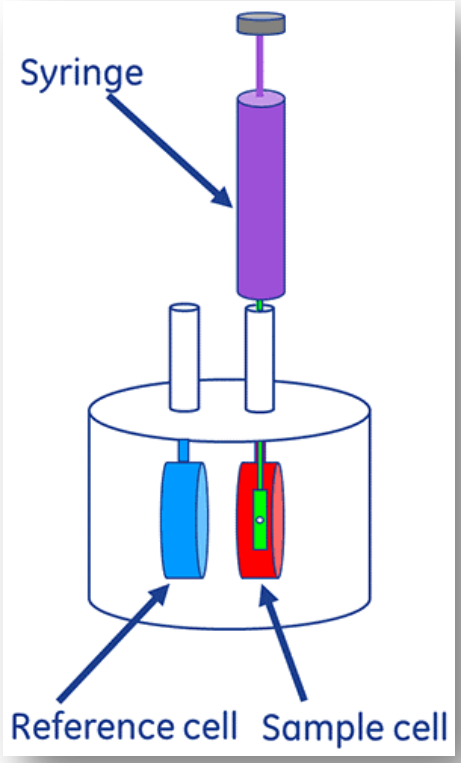
Though there is no debate against Cisplatin's ability to destabilize a DNA duplex enthalapically [11, 18, 19] after being bond to the duplex, there is a question of stability/destibility of formation of duplexes that need to be addressed when cisplatin is in the solution or bond to a single strand of DNA/RNA. One reason why this is relevant is due to Cisplatin ability to inhibit translation and transcription inside the cell [16, 23]. The mechanism with which that happen is still unclear. Our research in this article is investigating the hybrid formation thermodynamically and comparing the results to the thermodynamics of denaturation [15, 17, 18] to insure consistency and avoids the thermodynamic subjectivity of nucleic acids.

Hence, a calorimetry study using Isothermal Titratation Calomitery (ITC) (**Fig. 5a**) [22] investigated the thermal stability and binding association of RNA/DNA structures formation with and without cisplatin.

The reason why ITC was used is due to its ability to calculate direct binding affinity (**Fig. 5b**) of two molecules and the thermodynamics of that reaction by gradually introducing one single strand of DNA/RNA from the syringe into the sample cell which contains the complimentary single strand of DNA/RNA.

Figure 5a:

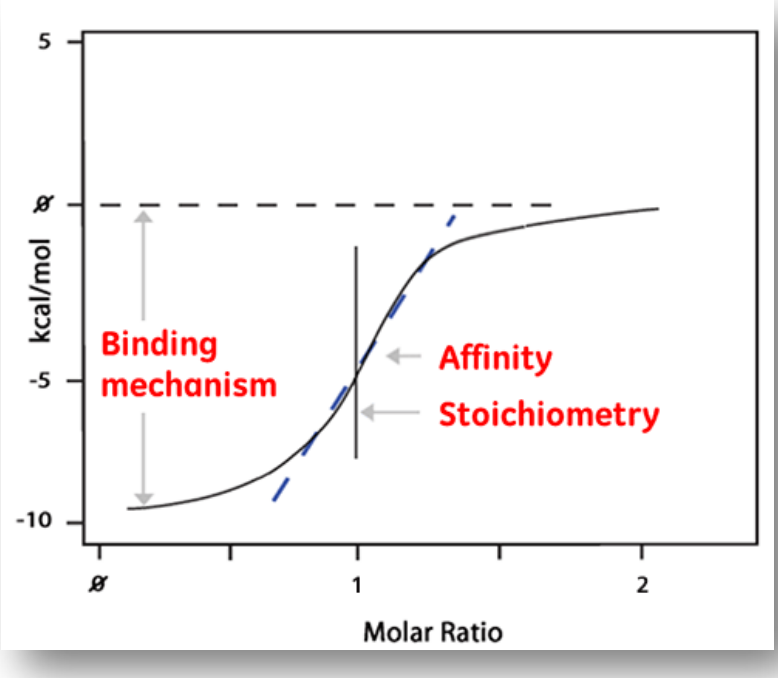
Visual representation of Isothermal Titration Calorimetry (ITC).



- The reference cell is usually contains filtered di-Water.
- The green metal in the sample cell is a spinner that allows for a good mixture of molecular.

Figure 5b:

The premise of ITC is to calculate the heat required to keep the sample cell at the same temperature as the reference cell. Hence, when a reaction is happening at its fullest scale, more heat is required to keep the isotherm.



- Y-axis = heat required to keep the isotherm.
- X-axis = molar ration OR injection number.

1.4 Effects of Cisplatin on Transcription and Translation

1.4.1 Transcription (Fig. 6) is the process in which RNA polymerase, an enzyme, copies out a DNA sequence of a specific gene in the similar alphabet of RNA to produce mRNA [16] to be used for protein production later.

Jean Ann [23] has shown that the pre-structures of single stranded DNA/RNA are of critical importance to their binding affinities to their complimentary strands. Herein, an investigation of Cisplatin modified single stranded structures in comparison to unmodified ones is relevant to processes dependent on the formation of duplexes and hybrids.

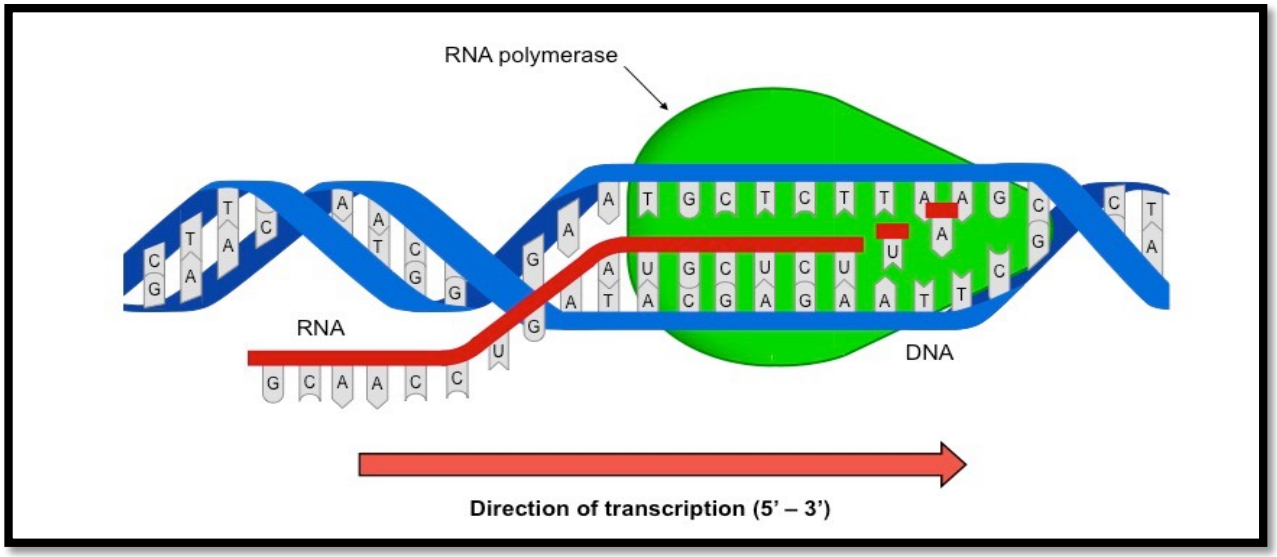


Figure 6: Starting from 5' position, a single-stranded RNA approaches a DNA duplex of a specific gene to form a hybrid structure with the non-coding DNA strand while pushing the coding strand away. Transcription continues till a breakage point happens. Termination of transcription is subjective from one case to another; in Bactria, termination depends mainly on enzymatic activity [16].

1.4.2 Translation (Fig. 6) is the process in which the sequence on the messengerRNA (mRNA) molecule, created after going through transcription, is used to create a protein by binding to transferRNA (tRNA) [24].

Similarly to transcription, translation requires the formation of hybrids although in translation, the hybrid formation does not need to thermodynamically compete with a duplex formation reaction or to break a duplex as it is the case with transcription [16, 24].

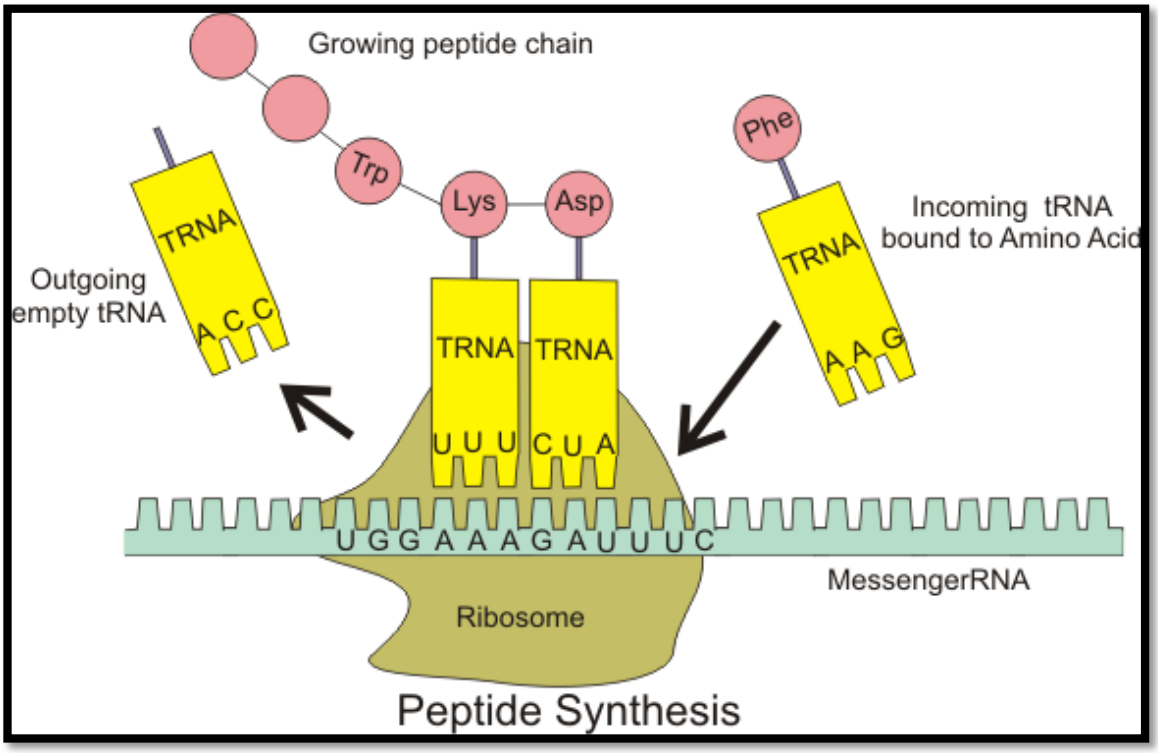
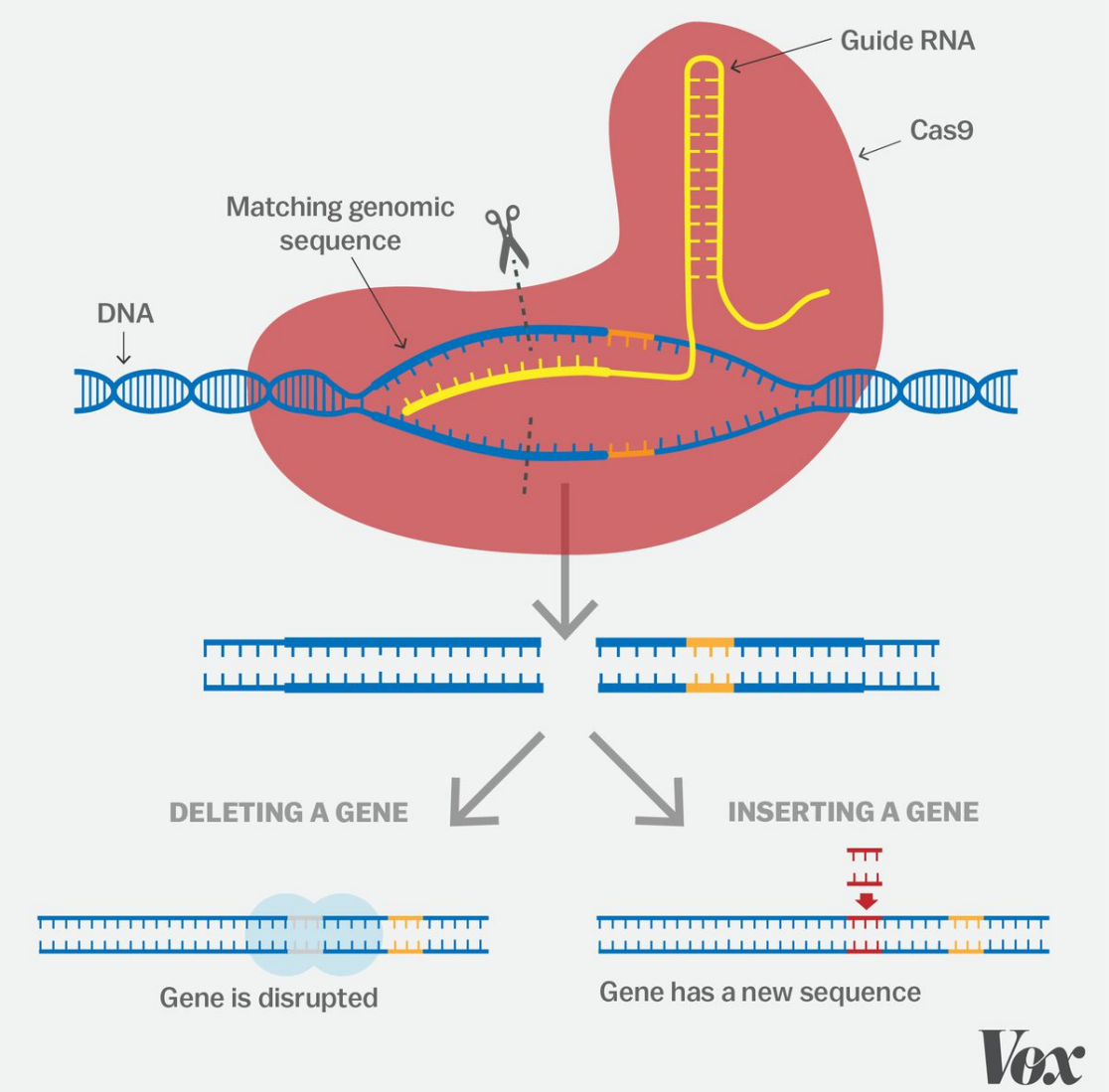


Figure 6: Translation or protein synthesis by the interaction of mRNA and tRNA inside the ribosome

1.5 Future Work: CRISPER

Our project works closely to Hybrid formation/denaturation through experimental utilization of Isothermal Titration Calorimetry and UV-Vis Spectroscopy and computational utilization of Prism, Excel and Schrodinger software. Therefore, a CRISPER oriented project is not out of reach from Bard College Chemistry and Biochemistry Program.



END OF CHAPTER 1

Materials and Methods

2.1 Materials

- The purified single stranded 10 base pair DNA oligonucleotide sequence (DNA1), 5'- CGT TGG CAT G - 3', and its complementary sequence (DNA2), 5'- CAT GCC AAC G - 3', were purchased from Integrated DNA Technologies (Coralville, IA).
- The purified single stranded 10 base pair modified (highlighted) DNA oligonucleotide sequence (RNA1), 5'- CGT T**rGrG** CAT G - 3', and its complementary sequence (RNA2), 5'- CAT G**rCrC** AAC G - 3', were purchased from Integrated DNA Technologies (Coralville, IA).
- The 10xPBS buffer was purchased from National Diagnostics (Atlanta, Georgia).
- Cisplatin and Calf Thymus DNA were purchased from Sigma-Aldrich Corporation (St Louis, Missouri).
- All stocks were stored in filtered di-water. DNA/RNA were stored at -20C while others at 4C.
- Table 1 illustrates nucleic acids sequences 5' to 3'

Table 1

DNA 1 (D1)	CGT TGG CAT G
DNA 2 (D2)	CAT GCC AAC G
RNA 1 (R1)	CGT TrGrG CAT G
RNA 2 (R2)	CAT GrCrC AAC G

2.2 UV-Vis Spectroscopy - Thermal Denaturation

- All the measurements were done using Cary 100 UV-Vis spectrophotometer (Varian). Samples (3 mL) were placed in a stoppered quartz cuvette (1 cm path length), and thermal denaturation was carried out.
- A temperature range of 10-75℃ for 10 base pair DNA/RNA structures and a range of 10-95℃ for Calf Thymus DNA.
- Heating/cooling rate of 0.25 ℃/min were used to monitor the absorbance profile of the sample at 260 nm.
- The melting temperature (Tm) and thermodynamic parameters were determined from the changes in the absorbance as a function of temperature. In this project, the data was analyzed using the implemented software Thermal Application of Agilent Technologies. Other wise one can get fairly similar results using the nonlinear least-square curve fitting method [25].

2.3 NanoDrop- Concentration Calculations

- Concentration of DNA/RNA structures were obtained through measuring absorbance of stocks at 260nm and translating that to concentration through The Beer-Lambert law[26].
- Concentration of Cisplatin was obtained through measuring absorbance of stocks at 220nm and again using The Beer-Lambert law [26] to translate that to concentration.

Table 2 and Table 3 demonstrate the representative experimental conditions for the calf thymus and the 10-bp DNA/RNA structures respectively.

Table 2	**Concentration**		**Stock Volume (µL)**
	Stock	**Sample**	
Calf Thymus DNA	2mg/ml	500µg/ml	750
NaCl	500mM	50mM	300
KH_2PO_4/K_2HPO_4	100mM	10mM	300
Cisplatin	2000µM	500µM	750
Filtered di-H_2O			900
Total Volume (µL)			3000

Table 3	**Concentration**		**Stock Volume (µL)**
	Stock	**Sample**	
dsDNA/RNA/Hybrids	100µM	5µM	150
NaCl	500mM	50mM	300
$MgCl_2$	50mM	5mM	300
KH_2PO_4/K_2HPO_4	100mM	10mM	300
Cisplatin	2000µM	500µM	750
Filtered di-H_2O			120
Total Volume (µL)			3000

2.4 Isothermal Titration Calorimetry (ITC) - Thermal Formation

- Thermodynamics Analysis All experiments were performed on the Low Volume Affinity ITC (TA Instruments). The concentration of the samples, the buffer conditions, and the trial settings in both the cell and syringe are optimized using centrifuging, degassing, similar concentration of molecules used along with consistent settings of trials.
- In order to attain thermodynamic parameters with minimal error, the generated heat values from the blank titration. The blank titration between ssDNA1/RNA1 ± Cisplatin in the syringe and the buffer used in sample cell was conducted before running the titration again with ssDNA2/RNA2 in sample.
- All samples were degassed under high vacuum for 15 minutes before the experiment.
- All samples were centrifuged for 1 hour @ 37C before the experiment.
- Temperature = 25C; Spin Rate = 75rpm
- The ITC data were analyzed on the NanoAnalyzer software from TA instruments.
- All data are fitted with the independent model installed in the software.

Table 4 and Table 5 demonstrate the representative experimental conditions for the syringe and the sample cell respectively.

Table 4 (Syring)	**Concentration**		**Stock Volume (µL)**
	Stock	**Sample**	
PBS Buffer	10X	1X	50
ssDNA1/ssRNA1	500µM	60µM	60
Cisplatin	2000µM	120µM	30
Filtered di-H_2O			360
Total Volume (µL)			500

Table 5 (Sample Cell)	**Concentration**		**Stock Volume (µL)**
	Stock	**Sample**	
PBS Buffer	10X	1X	50
ssDNA2/ssRNA2	500µM	20µM	20
Filtered di-H_2O			430
Total Volume (µL)			500

NOTE:

ITC experiments are very sensitive to contaminations as they the leading cause for error. Care should be taken by cleaning the syringe with 20-25ml of water and the sample cell with 100ml [27]. Learning how temperature, spin rate, concentrations, buffer used, and volume used was a journey. Scientific research [28], Hoang [29] and Dr. Swapan Jain have made the latter task more approachable and appealing to me.

END OF CHAPTER 2

Results and Discussion

3.1 Thermodynamics in Context

3.1.1 Enthalpy and Stiffness

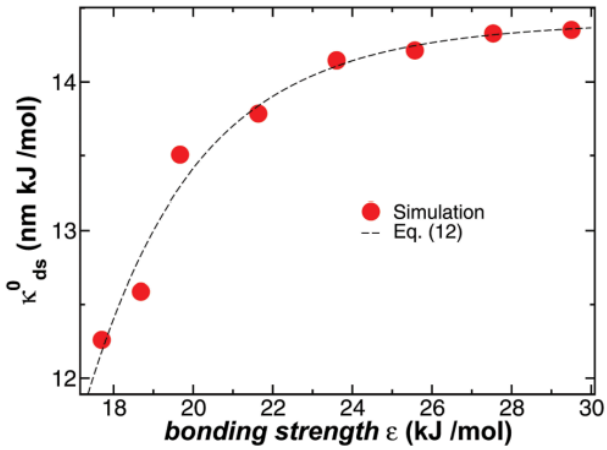
Linguistically translated to "heat inside" and mathematically prescribed as (**Eq1**):

$$Change\ in\ Enthalpy = Internation\ Energy + (Pressure \times Volume)$$

Enthalpy is the heat available for molecules to do work in addition to the cost of making the space for these molecules to expand or contract. Closely related, stiffness is simply defined as the resistance of an object or a system to a change in length. Their relation was depicted by *F. Vargas-Lara* computationally [30] (**Fig. 8**). Higher the stiffness (k^0), the higher change in enthalpy (bonding strength) is.

Figure 8:

Computational representation of Enthalpy's (Bonding Strength/x-axis) relation to Stiffness (y-axis).



Enthalpy cannot be directly calculated due to its mathematical dependence on the change of a system's temperature and humans' inability to calculate an absolute zero of temperature. Hence, a system needs a change of temperature for enthalpy to be calculated. For DNA, **+ΔH** (Final Enthalpy – Initial Enthalpy) indicates higher internal energy [31], thermal destabilization [19], greater intrinsic stiffness [32, 30], and an endothermic reaction.

With force applied on the DNA/RNA duplexes held constant, the bending of the double helix of the DNA/RNA directly affects their stiffness (**Eq2**). Theoretically, an increase in stiffness hints an enthalpic thermal destabilization of the DNA/RNA structures (**R1**).

$$\boldsymbol{Stiffness\ (k) = \frac{Force}{Deflection}} \qquad \textit{Equation 1}$$

$$\boldsymbol{+\Delta k \sim\sim +\Delta G} \quad \textit{Relation 1}$$

Researchers showed that upon binding Cisplatin to DNA, the DNA structure got destabilized (+ΔG) [19, 18, 11] without investigating the stiffness of DNA. However, a recent study by *X.-M. Hou* [32] has showed that Cisplatin binding to a single molecular of DNA decreased the structural stiffness of DNA (-Δk) and consequently increased the thermal stability of that DNA (-ΔG). With temperature held constant, the only other state function that plays a role in thermal stability is Entropy (ΔS).

3.1.2 Effects of Cisplatin on The Entropy of a DNA Duplex

More commonly defined as the disorder of a system, Entropy is a measure of the unavailable energy in a closed thermodynamic system [14]. Similarly to enthalpy, entropy shares different correlation relationships with stiffness [30]. One is involving extrinsic constraints (ø) meaning volume fraction of crowding agents (**R2**).

$$\boldsymbol{+\Delta ø \sim\sim -\Delta S \sim\sim +\Delta k \sim\sim +\Delta G} \quad \textit{Relation 2}$$

To help understand how Cisplatin affects the DNA structure, multiple studies provided experimental evidences indicating that Cisplatin bend the DNA duplex to the major grove [11, 19]. Other studies looked at the DNA at a bigger scale visualizing how Cisplatin control the DNA movement in space [2, 21] (**Fig.8**).

Our lab ran denaturation studies of a 10 base pairs and Calf Thymus DNA. Both DNAs became less stable upon introduction of Cisplatin producing the following parameters:

Table 6: Thermodynamic parameters produced after introduction of Cisplatin to the 10 base pairs D1/D2 (see materials for sequences).

$+\Delta S$	$+\Delta H$
$+\Delta G$	

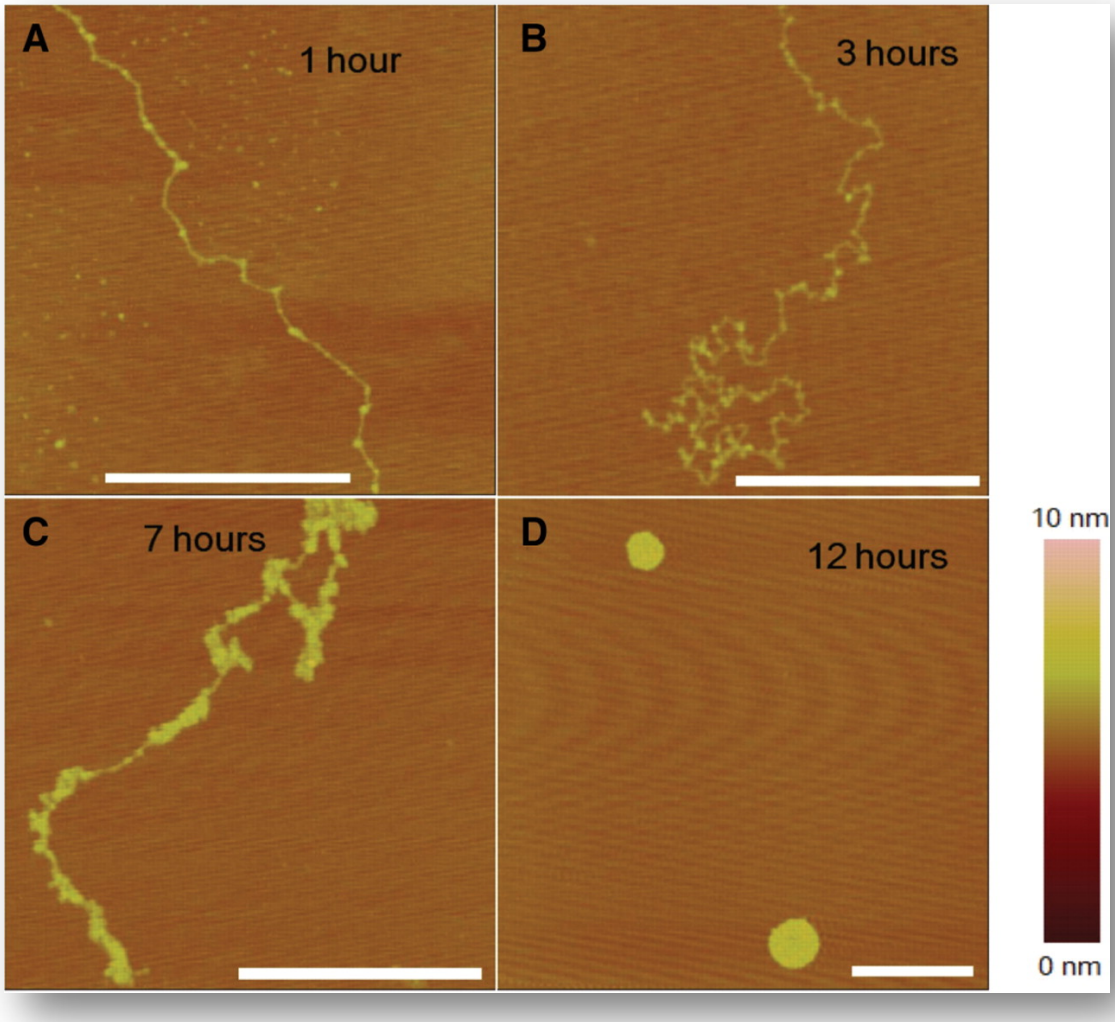


Figure 8:

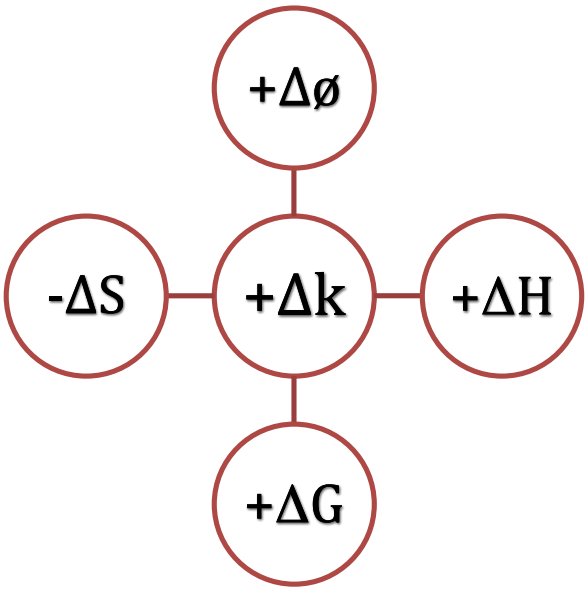
Atomic force microscopy of a DNA duplex incubated with 770μM Cisplatin hints at DNA globe clusters formation [21].

Looking at **Fig 8** and thinking of entropy simply as the disorder of a system, one would assume -ΔS upon addition of Cisplatin due to condensation of DNA. To explain why experimental ΔS is positive (**Table 6**), a connection between ΔG, Δk and Δø in relation to ΔS is provided in the next section.

3.1.3 Free Energy ΔG

$$\Delta G = \Delta H - T\Delta S \quad \textit{Equation 2}$$

ΔG is a measure of the thermal stability of a system with -ΔG meaning greater stability and +ΔG meaning smaller stability. So far, we have established the following direct and in-direct relations [21, 30]:



Process 1: Single molecular of DNA thermal and structural response to an increase in stiffness due to formation of two covalent bonds with Cisplatin.

As time progresses, the DNA shortens in length [21, 33] due to loop formation that could lead to fragmentation of a DNA molecular (**Fig. 9**).

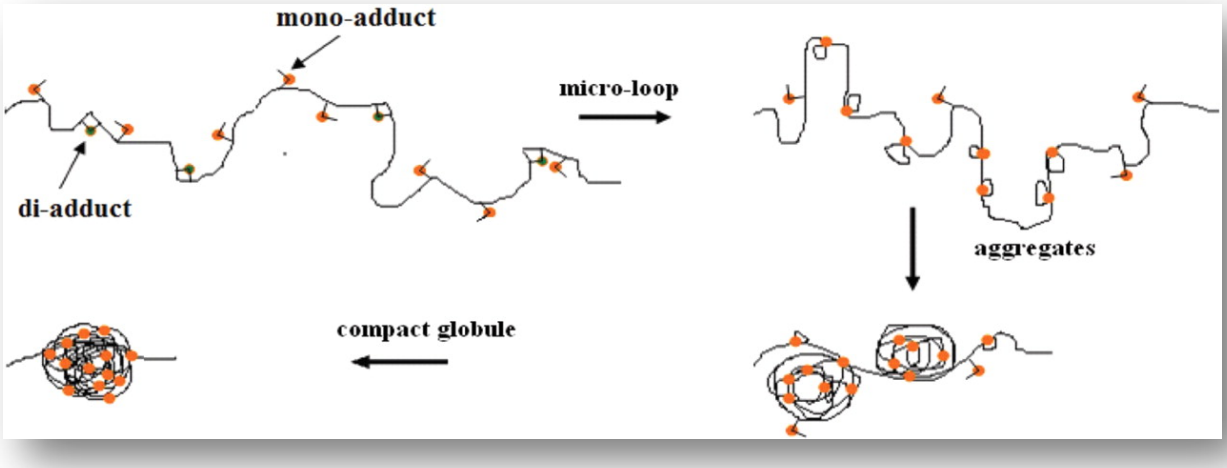


Figure 9: Formation of micro loops by di- and mono- Cisplatin-dsDNA adducts.

At this point, it is impossible to look at the DNA as a single molecular as DNA shortening produces new sequences; hence, new differing sequences of DNA. Computationally, DNA's length is directly related to its stiffness, Δk [21] (**Fig. 10**).

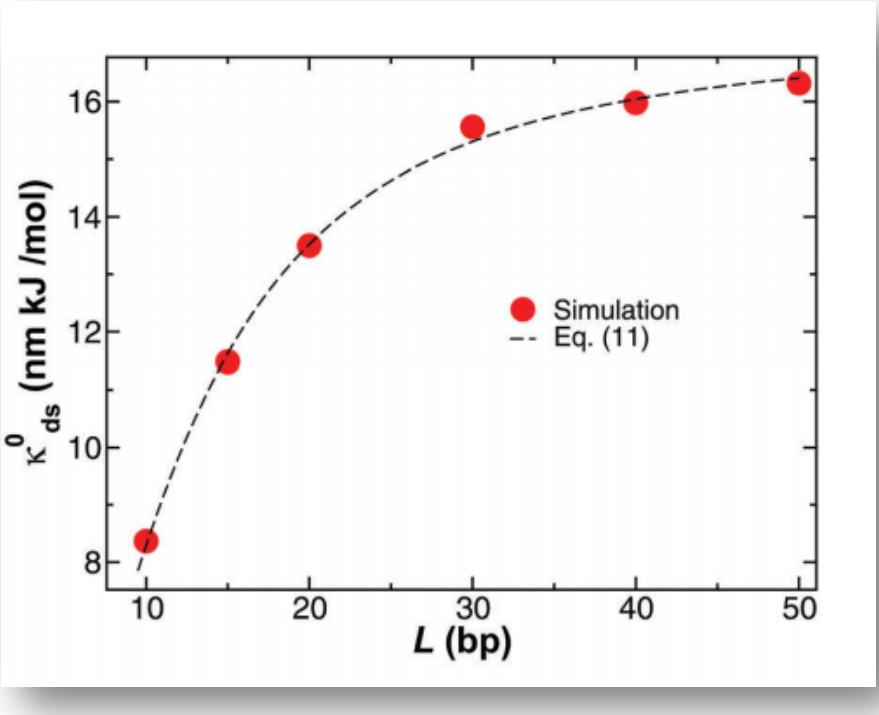
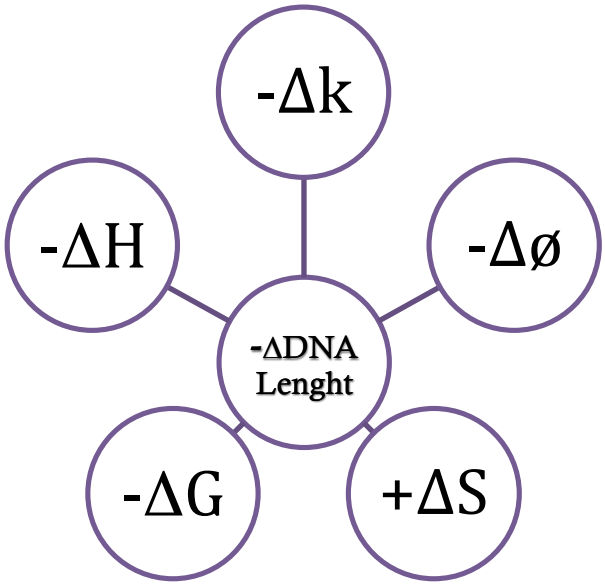


Figure 10:

Representation of the mathematical relationship of DNA sequence length (x-axis) and DNA structure stiffness (y-axis).

With the introduction of DNA shortening, DNA thermal and structural response changes accordingly. In **Process 2**, we demonstrated these new effects of Cisplatin on DNA duplexes collectively.



Process 2: DNA duplexes' response to DNA shortening by Cisplatin [35, 36].

It is important to realize that Processes 1 & 2 can take place at any-time when their conditions, Cisplatin decrease of DNA's stiffness through formation of micro loops and Cisplatin shortening of DNA for 1 & 2 respectively, are met. Both process will take place till they reach the thermodynamically favored state.

Cisplatin binding to DNA results in irreversible damage causing irreversible changes of state functions like ΔS and ΔH [34, 11, 21] make it impossible to balance one process by promoting the other.

3.2 DNA Duplexes Binding to Cisplatin

3.2.1 Calf Thymus DNA Denaturation

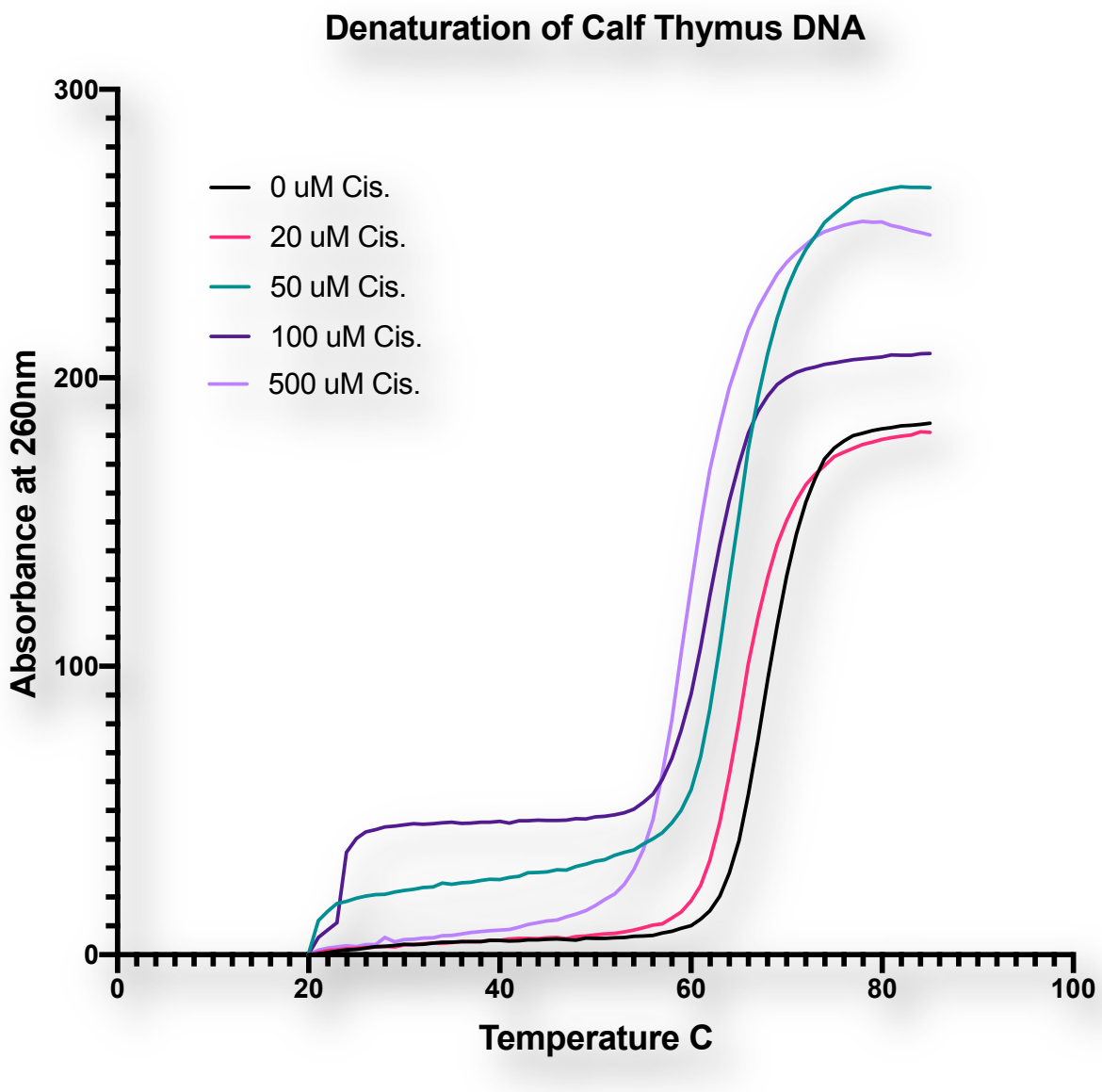


Figure 11: Melting profile of CT DNA in different concentration of Cisplatin.

Destabilized.

3.2.2 D1/D2 Duplex Denaturation

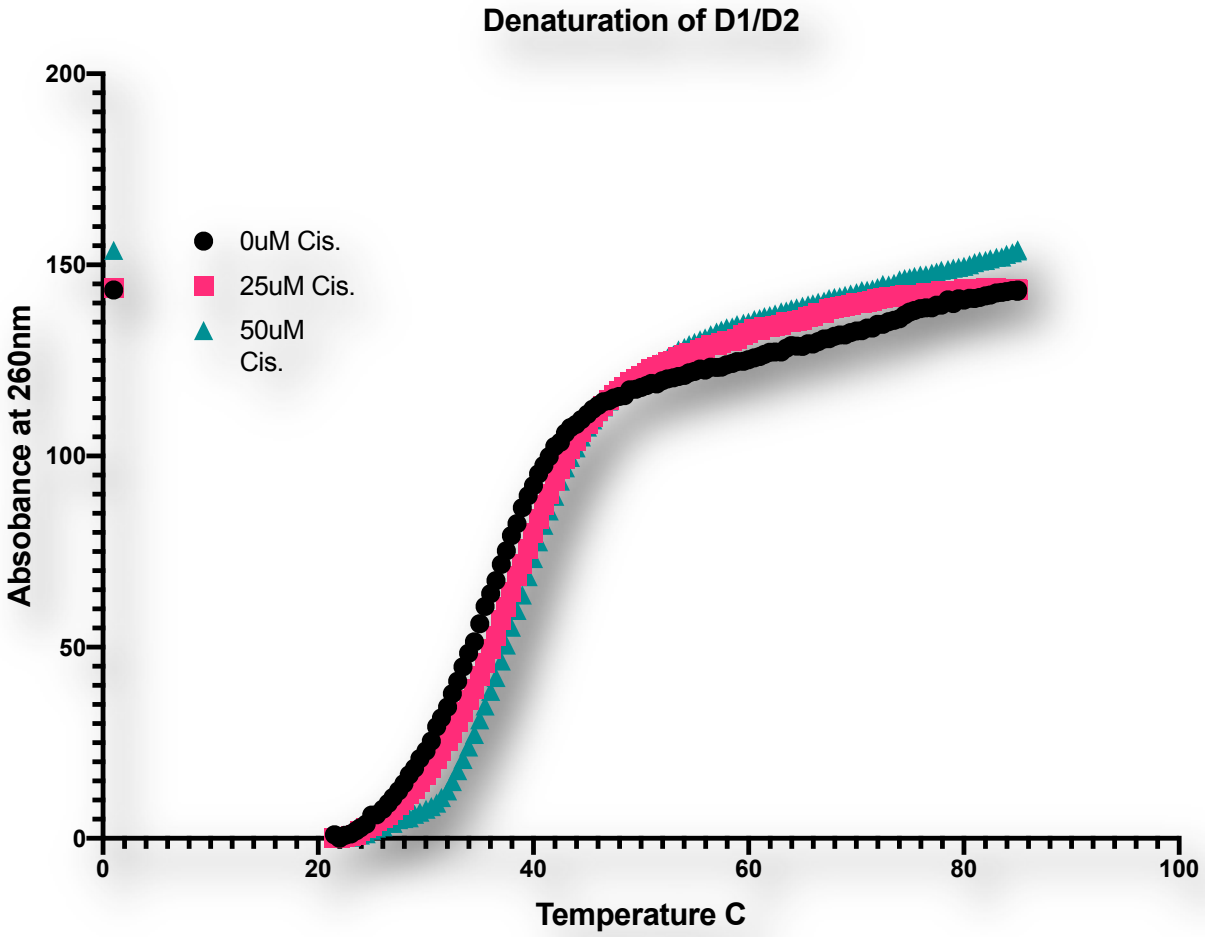
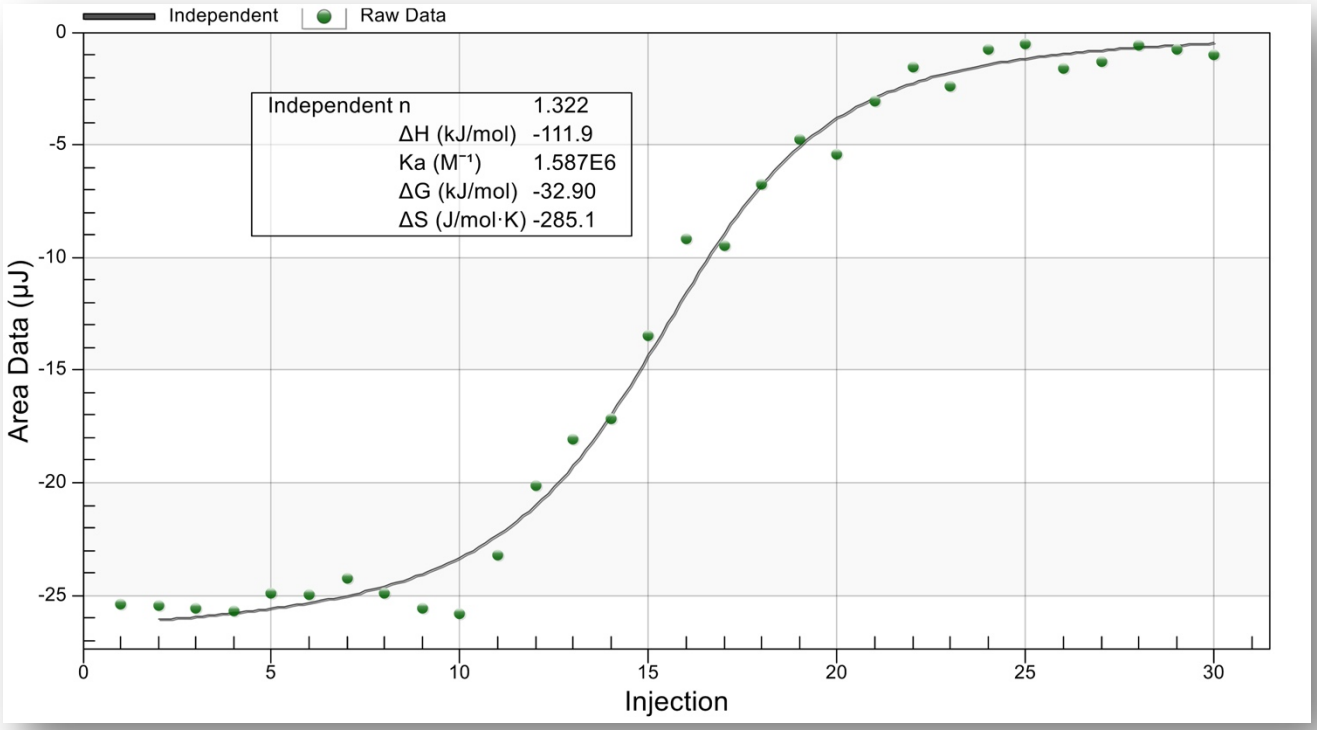
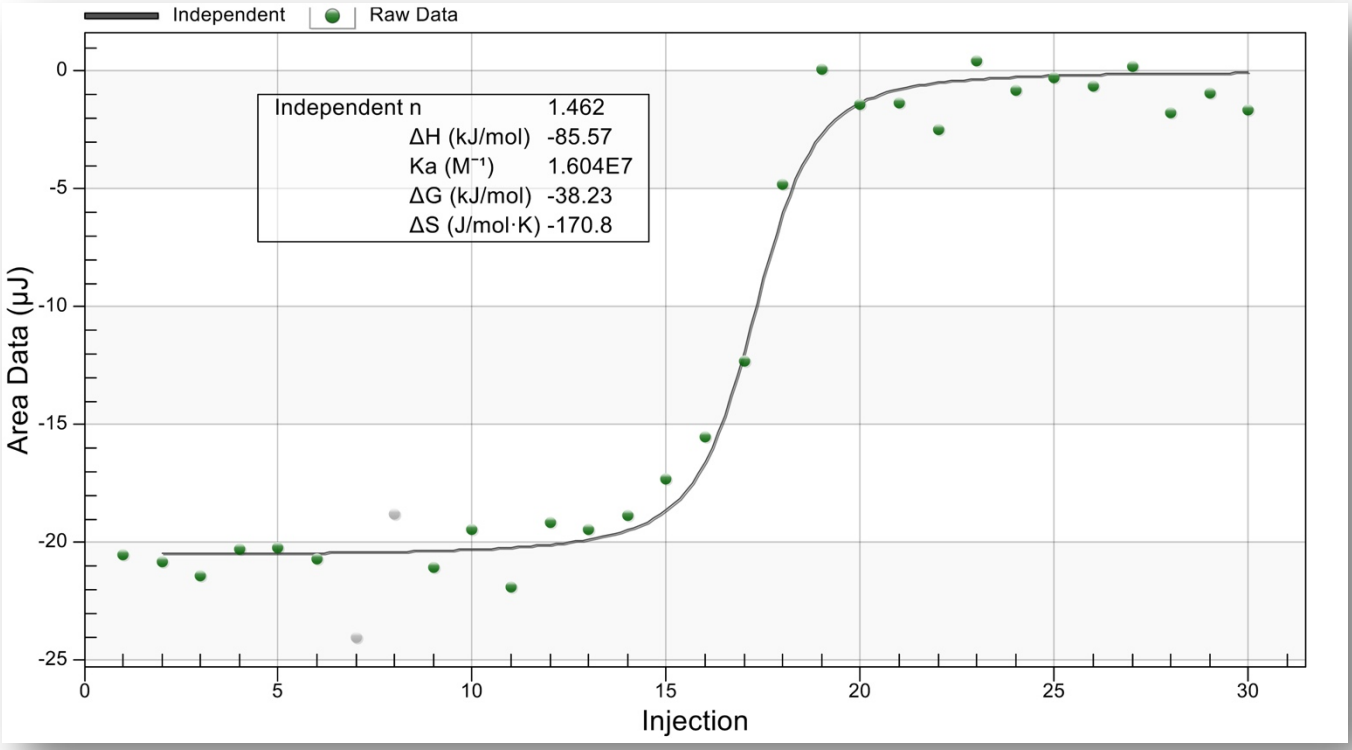


Figure 12: Melting Profiles of D1/D2 Duplex in Different concentration of Cisplatin. Stabilized.

3.2.3 D1/D2 Duplex formation (ITC)



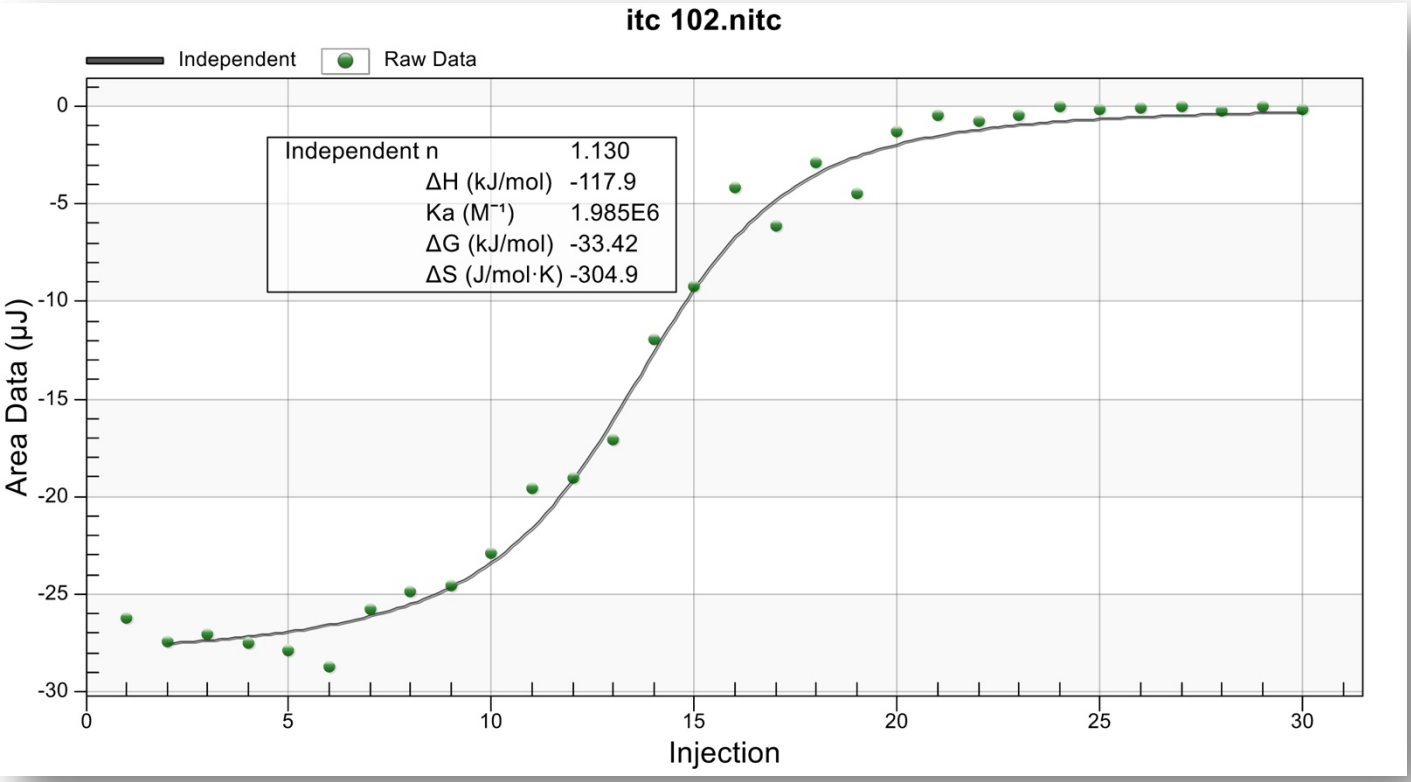
3.2.3 D1/D2 Duplex formation + Cisplatin (ITC)



Ratio of cisplatin to DNA 2:1

3.2.3 D1/D2 Duplex formation + Carboplatin (ITC)

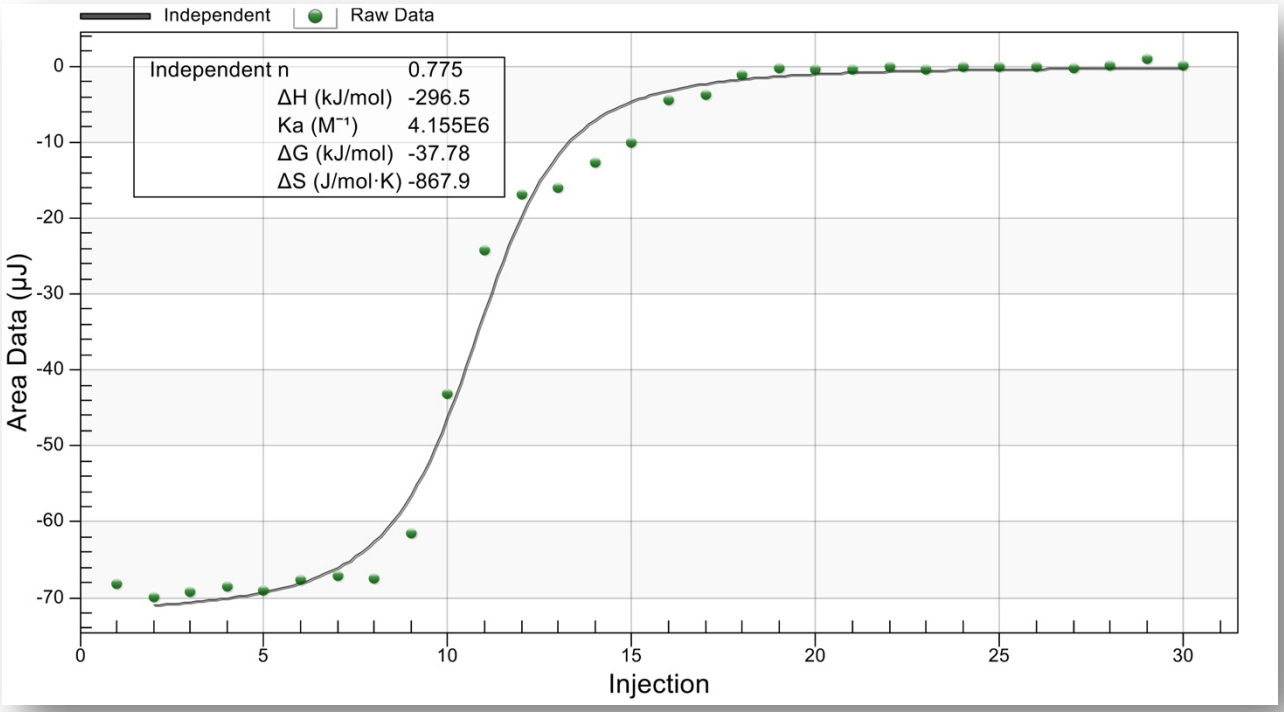
Ratio of carboplatin to DNA 2:1



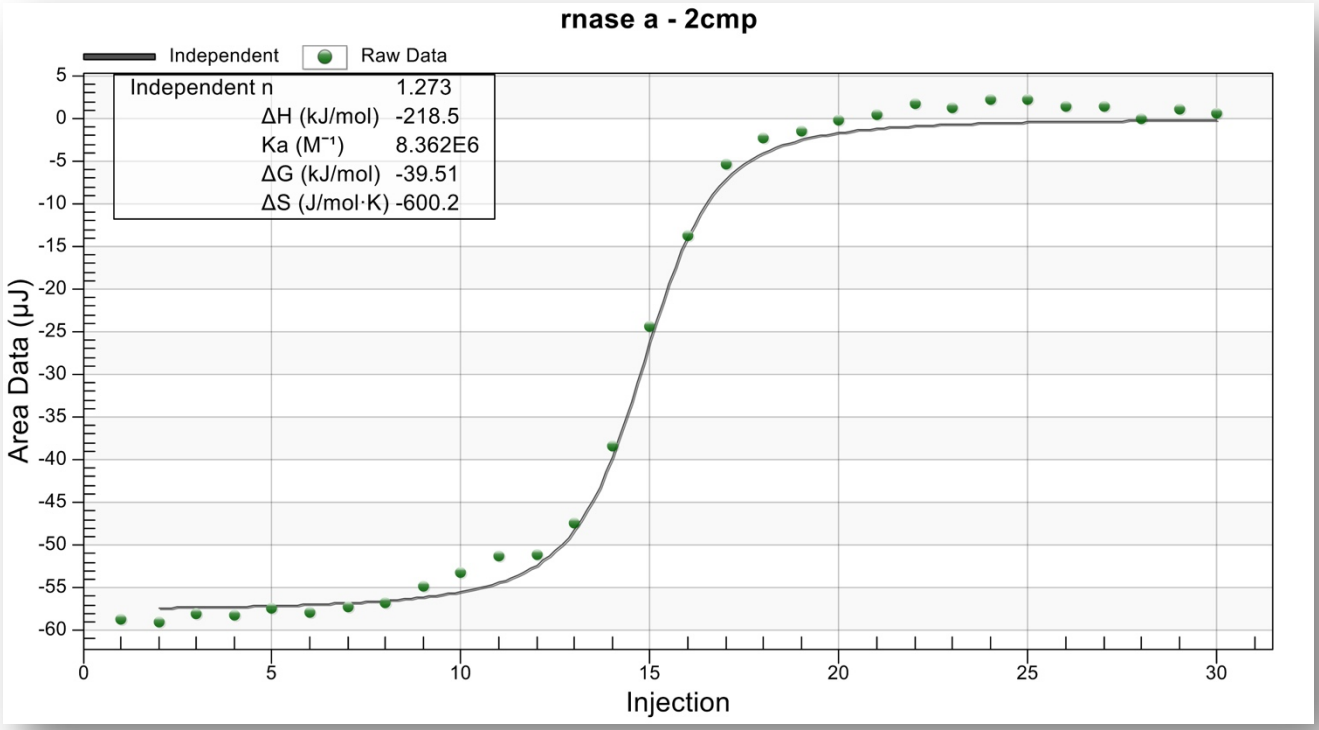
3.3 RNA Duplexes and Hybrids Binding to Cisplatin

3.3.1 RNA Duplex Formation

Without Cisplatin

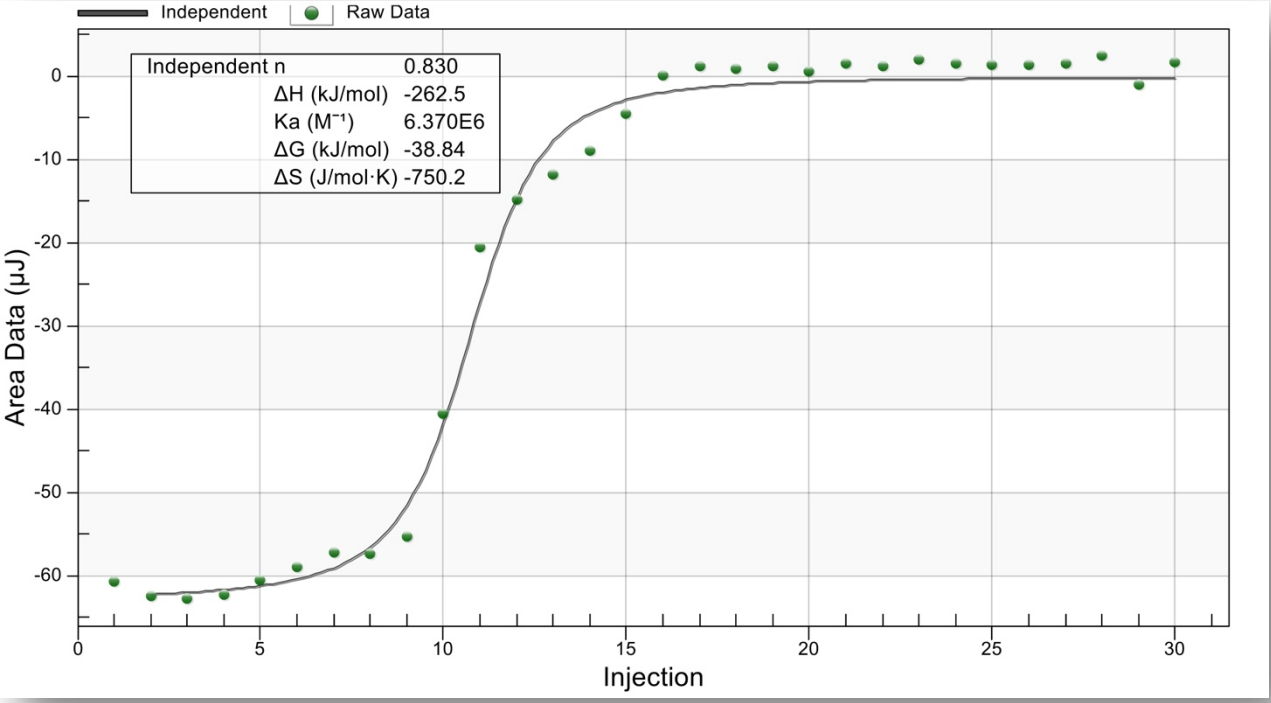


With Cisplatin

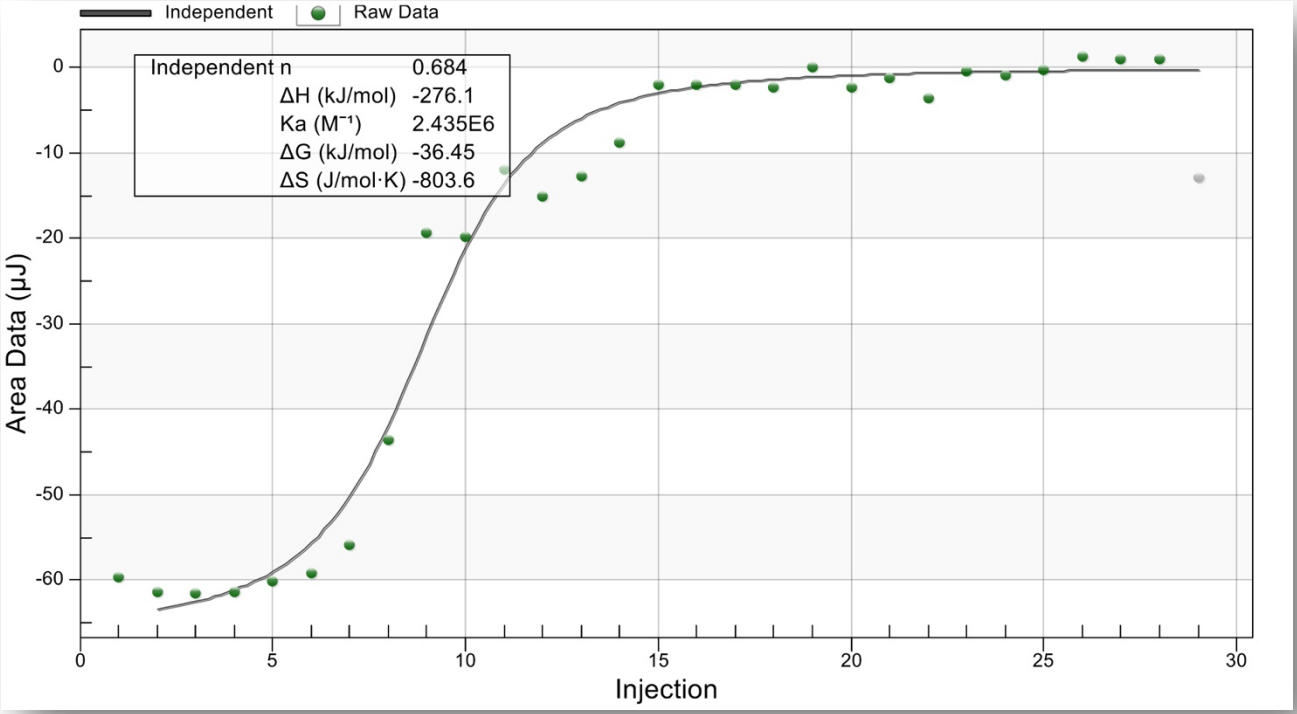


3.3.2 Hybrid Formation

Without Cisplatin



With Cisplatin



3.3.3 UV-Melting of Hybrids

Run 1 (1 hour)	Melting Temperature (C)	Delta G @ 25C (kJ/mol)	Delta S (J/mol*k)	Delta H (kJ/mol)
D1/R2	47.88	-56.174	-969.6	-345.3
D1/R2 + Cisplatin	47.58	-50.087	-722.8	-265.6
R1/D2	47.60	-56.528	-993.9	-352.9
R1/D2 + Cisplatin	47.59	-52.219	-796.6	-289.7

Run 2 (24 hours)	Melting Temperature (C)	Delta G @ 25C (kJ/mol)	Delta S (J/mol*k)	Delta H`` (kJ/mol)
D1/R2	46.77	-53.037	-995.7	-317.1
D1/R2 + Cisplatin	40.88	-48.088	-804.6	-288.0
R1/D2	46.92	-53.432	-910.9	-325.0
R1/D2 + Cisplatin	40.66	-42.606	-602.4	-222.2

END OF CHAPTER 3

Conclusion

The melting profiles of DNA duplexes has suggested two different mechanisms of action depending on the kind of DNA used. D1/D2 undergoes stabilization mainly due to its short length, hence greater flexibility or smaller stiffness making the conditions for Process 2 easier to meet as DNA shortening -ΔLength is directly proportional to Stiffness. Opposite destabilization effects were observed for the long Calf Thymus DNA when interacted with Cisplatin confirming literature [11] with ΔΔH/ΔΔS of [11] differing only 0.002 from results presented.

The melting profiles of Hybrids D1/R2 and R1/D2 indicate one mechanism of action in which thermal destabilization is taking place (+ΔG) though R1/D2 (binding site of cisplatin on RNA) destabilized much more greatly. It is suspected that because of greater binding affinity of Cisplatin to RNA comparing to that of DNA, R1/D2 will create more crowding +Δø leading to +ΔG. Also, it is worth noting that shortening of sequences was only observed for DNA-Cisplatin adducts; hence, DNA shortening will be more effective on D1/R2 leading to -ΔG.

ITC data shows that duplex formation is more favorable when cisplatin is introduced to one strand before the formation (-ΔG); however, this behavior is reversed in hybrids as their formation rate constant (association constant) decreases upon the introduction of Cisplatin to one strand before the formation (Fig. 14).

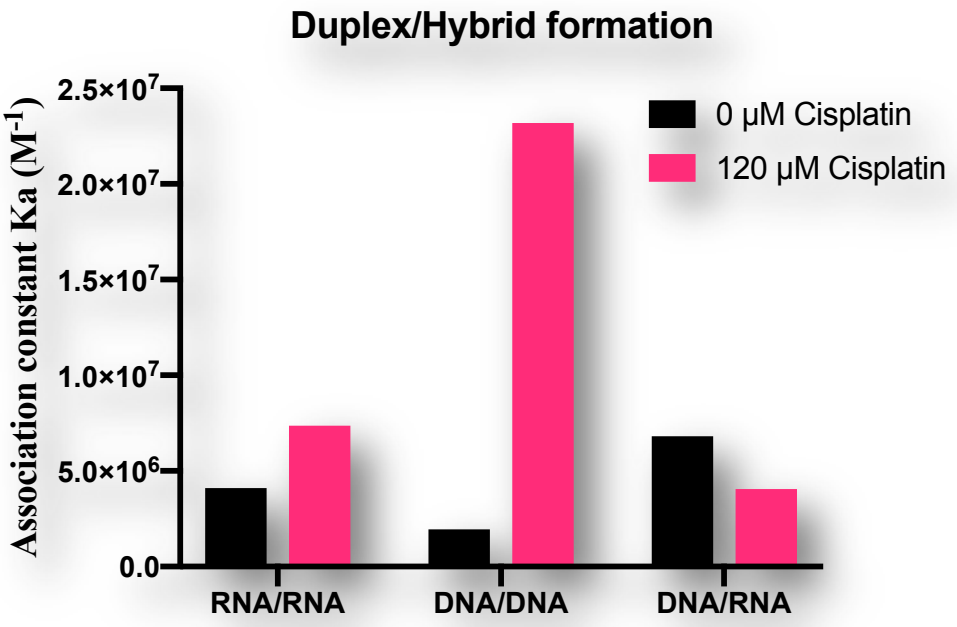


Figure 13: Association Constants of DNA/RNA Structures formation. Left to right:

- RNA duplex formation: 125mM NaCl
- DNA duplex formation: 50mM NaCl *caused greater binding of Cisplatin to DNA.
- Hybrid formation: 124mM NaCl

It is worth noting that in DNA duplex formation, the ionic strength of the buffer was reduced by half to facilities DNA-Cisplatin adducts formation.

This tendency of duplexes formation over hybrids when Cisplatin is introduced is suspected to greatly affect transcription initiation and elongation. Translation could also be inhibited by distributing the mRNA and leading it to bind.

END OF CHAPTER 4

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