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Preventing Gene Expression through the Interruption of DNA Binding

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Preventing Gene Expression through the Interruption of DNA Binding

An Honors Thesis submitted in partial fulfillment of the requirement for Honors in Chemistry

By
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ABSTRACT

The interruption of DNA binding is a gateway to the inhibition of unregulated expression of genes which can possibly lead to illnesses such as cancer and epilepsy. The goal of this research is to produce high affinity DNA binding molecules which could act to displace the binding of natural transcription factor thus reducing the over- or under-expression of various proteins. Literature shows that the interruption of DNA binding can be completed using both organic and inorganic means, such as by means of natural transcription factors like the NF-κB protein or with metal-ligand complexes in order to bind DNA. NF-κB, a protein that plays a vital role in cell growth and immune response, works as a transcription factor and binds DNA using a beta sheet loop region. Mimics of the NF-κB binding region will be made and analyzed via circular dichroism and fluorescence. Metal ligand complexes are able to bind to DNA through two different interactions: intercalation into DNA via the ligand portion and ion coordination with the negatively charged phosphate backbones of DNA. The analysis of the metal complex binding affinity will be done using UV-Vis fluorescence spectroscopy, circular dichroism (CD) studies, and gel electrophoresis assays. The overall purpose of this research is to identify the more efficient binder with the higher affinity for DNA binding between organic and inorganic molecules.

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Introduction

The disruption of DNA binding is a gateway to the inhibition of expression of harmful genes which can possibly lead to illnesses such as cancer and epilepsy and as a result, artificial control of gene expression has been of interest in the medical field.[6] The goal of this research is to achieve results, which could possibly lead to a better understanding of how DNA binds and eventually preventing binding in harmful strands. Literature shows that the interrupting of DNA binding can be done using both organic and inorganic means, such as using natural transcription factors like the NF-κB protein or using metals in complexes with ligand in order to bind DNA.[6] In this research, the difference in affinity for DNA binding between organic and inorganic compounds will be compared and the more efficient binder will be the main focus of research.

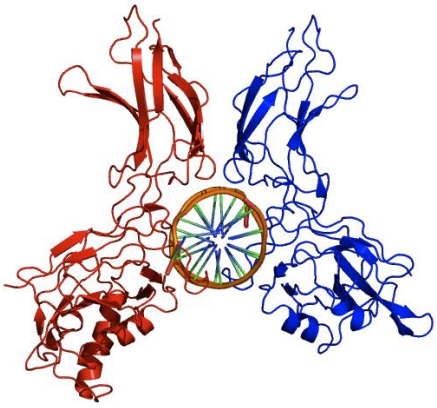

Figure 1. The binding of DNA via NF-κB. The figure depicts the two subunit in conjunction around the DNA while the β-hairpin region binds the DNA.[3]

NF-κB is an important protein that plays a vital role in cell growth and immune response.[3] Working as a transcription factor, the protein binds directly to DNA and helps control the levels of transcription, thus gene expression. When the regulation of NF-κB is flawed, links to illnesses such as carcinogenesis and epilepsy have been discovered.[7] The portion of the protein that makes the binding possible is the β-hairpin, or beta sheet loop region.[3] The binding of the β-hairpin can be mimicked so that the binding is similar to that of the original β-hairpin but its purpose would be to block the binding of harmful DNA. A mimic of the structured β-hairpin

found in the NF-κB will be used in binding studies such as fluorescence techniques like tryptophan quenching and could be completed in order to compare the binding affinity of the mimic to that of the original protein. The results of this experiment should analyze the ability of the mimic peptides to bind DNA as compared to NF-κB. Overall, if either mimic peptide is more successful in binding DNA than NF-κB, then the research could potentially be used in a clinical setting in order to prevent the overexpression of particular genes implicated in various diseases.

Metal ligand complexes are becoming well studied molecules in the disruption of DNA binding. In fact, this technique has already been used in cancer treatment in which drugs such as cisplatin, which contains platinum, binds and damages DNA in order to prevent cell division.[4] Metal ligand complexes are able to bind to DNA through two different interactions. The ligand portion has a planar heterocyclic structure and the aromatic portion is able to intercalate into the DNA, while the metal portion binds through ion coordination with the negatively charged phosphate backbones of DNA.[1] For the focus of this research, metals of the lanthanides series will be used as the metal with ligand complexes, because they have been a recent interest due to their cytotoxicity as compared to drugs like cisplatin which use metals such as platinum.[4] The analysis of the metal complexes' binding affinities will be completed using circular dichroism (CD) studies and gel electrophoresis assays in which the

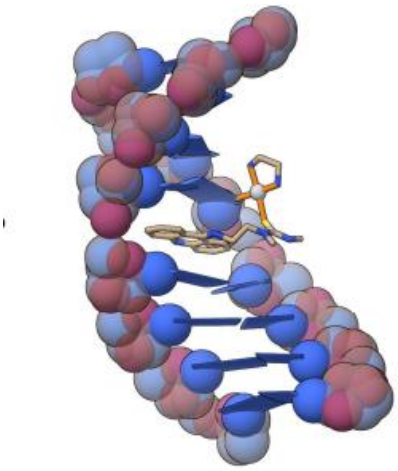

Figure 2. The binding of DNA via Metal-Ligand Complex. The figure depicts the ligand intercalating into the DNA, while the metal interacts with the phosphate backbone.[2]

expectation is that there will be a conformational change in the DNA as the metal-ligand complex binds the DNA. Multiple lanthanide metals will be used in order to determine the one with the highest binding affinity.

Overall, the goal of this research is to determine the binding affinity of the synthesized DNA binding molecules, with the goal that they will have a higher affinity and displace natural transcription factors. If successful, this research could be used in order to prevent the over- or under-expression of genes by these transcription factors.

Methods

Peptide Design

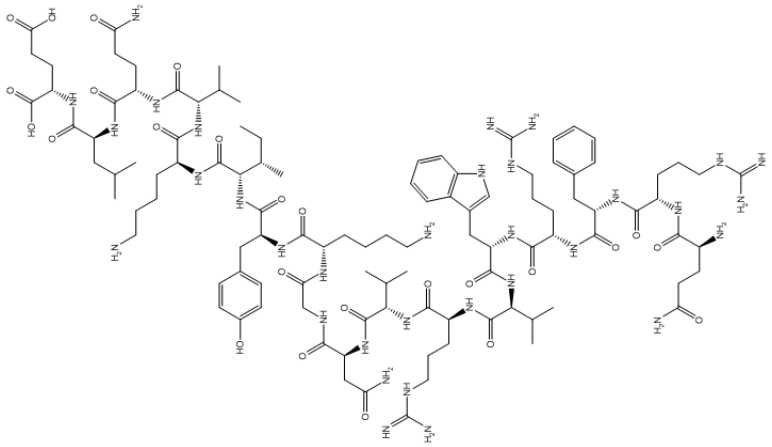

Figure 3. NF-κB Hybrid Structure. The sequence for the peptide mimic was based on the binding site of the original NF-κB protein and is as follows:

Gln-Arg-Phe-Arg-Trp-Val-Arg-Val-Asn-Gly-Lys-Tyr-Ile-Lys-Val-Gln-Leu-Glu

Metal-Ligand Complexes

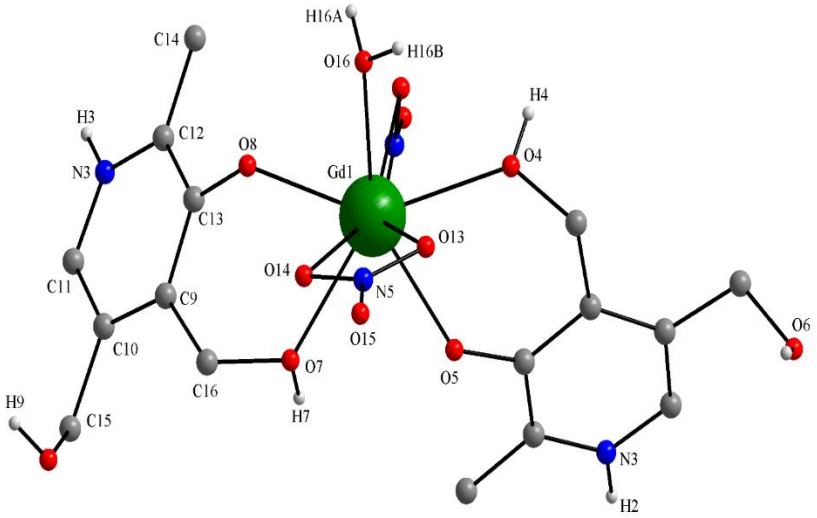

Figure 4. Gd-Ligand Complex. The molecular formula is as follows: [Gd III $(NO_3)_2$ (H_2O) $(pyr)_2$]. The other complexes (Tb, Dy, Er, and Ho) have the same structure with the central metal atom being varied for each.

Peptide Synthesis

For each peptide, CLEAR amide resin was employed to synthesize the peptide using a 0.1 mmol scale, with each amino acid coupled onto the resin with four equivalents of HBTU, a coupling reagent. Dimethylformamide was the solvent for all peptide synthesis. A 20% piperidine solution in DMF was utilized to deprotect the Fmoc protecting group from each amino acid. Coupling times of about 60 minutes and deprotection times of 20 minutes were employed. The order sequence was then run in a Protein Technologies PS3 peptide synthesizer coupling from the C terminal to the N terminal amino acid. The last eight amino acids were double coupled and an acetic anhydride capping solution was added to cap the N-terminal of the peptide.

Cleavage

In order to cleavage the peptide from the resin on which it was synthesized, a cleavage reaction had to be conducted. To dry the resin, dichloromethane was used along with nitrogen gas to agitate the sample. The cleavage cocktail was composed of 95% trifluoroacetic acid (TFA), 2.5% tri-isopropalsilane (TIPS), and 2.5 water, and this was allowed to react with the peptide in a cleavage flask for two hours. The resulting solution was then drained into a round bottom flask which was then treated with air until enough TFA had evaporated and only a dime-sized amount remained. Then 20 mL of ether and 20 mL of water was added to the flask in order to allow the peptide to precipitate out of the solution. The final solution was then allowed to separate in a separatory funnel. The various layers were collected then frozen in liquid nitrogen, followed by three days on the lyophilizer, resulting in peptides in power form.

Dissolution

The dissolution of the peptide was necessary for the purification using chromatography. Various solutions were used in order to attempt dissolution. If unsuccessful, the samples were lyophilized, then dissolved in other solutions.

Purification

Using High-Performance Liquid Chromatography (HPLC), the peptide was purified using a peptide method which was composed of a gradient using first Solvent A

(95% HPLC Grade Water, 5% Acetonitrile, 0.1% trifluoroacetic acid), then Solvent B (95% Acetonitrile, 5% HPLC Grade Water, 0.1% trifluoroacetic acid), with increasing Solvent B from 0% to 40% over 25 minutes, regardless of the method. In order to analyze a peptide, 40 microliters was injected. The peaks were collected based on absorbance, and these peaks contained the peptides produced in the sythesis.

Mass Spectrometry

A Microflex MALDI-ToF MS (mass spectrometry) was performed in order to analyze the purified peptide collected from the HPLC. All fractions were analyzed with a matrix composed of α-cyano-4-hydroxycinnamic acid in standard solution (50% acetonitrile, 47.5% DI water, and 2.75% trifluoroacetic acid).

Circular Dichroism

A. NF-κB Hybrid

Circular dichroism was performed in order to analyze the secondary structure of the peptide mimics. The samples were prepared using 50µL of peptide with 200µL of 10 mM Na_2HPO_4, 100 mM NaCl, pH 7.2. Peptides were analyzed at temperature 25ºC with a wavelength range of 190-260nm.

B. Metal-Ligand Complexes

Circular dichroism titrations were performed in order to detect if there were changes in secondary structure of the 250 ng/µL calf thymus DNA upon metal complex

binding. The samples were prepared using 70 µL of calf thymus DNA with 200 µL of 10 mM Na_2HPO_4 pH 7.4. The metal-ligand complexes were titrated into the DNA using 1µL aliquots. All titrations were completed at 25ºC from 220-340nm.

UV-Vis Fluorescence Binding Study

A binding study was conducted using UV-vis fluorescence. Each sample was prepared with a consistent peptide concentration (8 µM), an increasing DNA concentration (0-60 µM), and 10 mM Na_2HPO_4, 100 mM NaCl, pH 7.3. Total sample volume was equal to 800 µL with 20 samples total. The parameters were the following: Slit width in 5, out 2.5, wavelength 300-500 nm. The excitation wavelength was set to 297 nm and the emission was 348 nm.

Results

NF-κB Hybrid Peptide Data

Mass Spectrometry

Using MALDI-ToF, mass spectrometry was completed in order to verify the peptide's mass after the synthesis, cleavage, and purification. The purification process involved desalting the peptide using a desalting column in order to remove any salts from the peptide. Mass spectrometry identified the peptide fraction which correlated to the theoretical mass. The data shown in Figure 5 shows a highly purified peptide with the experimental value of 2361.93. NF-κB Hybrid has a theoretical mass of 2360.25.

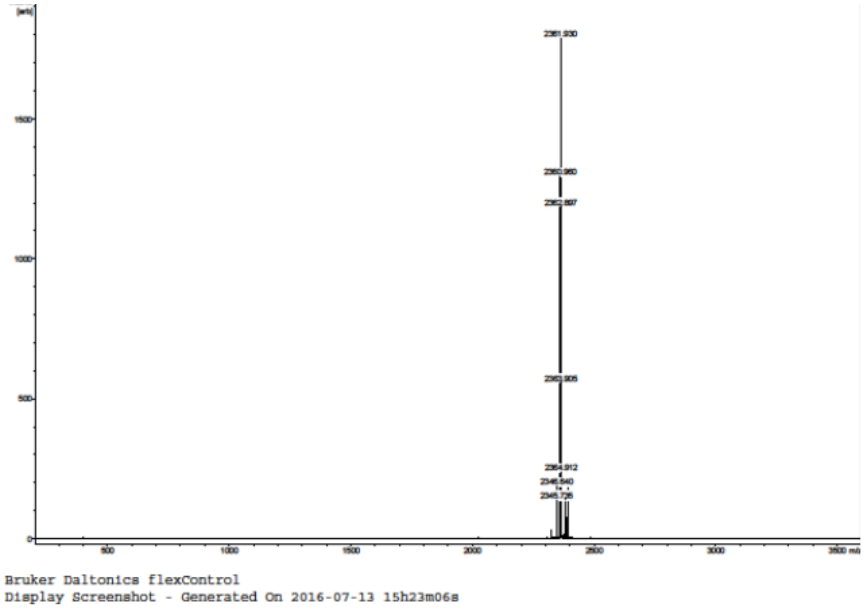

Figure 5. Mass spectrometry of NF-κB Hybrid Fractions 2, 3. The graph shows a high peak at 2361.93 and expected mass is 2360.25.

Circular Dichroism Analysis

Circular dichroism was completed on the peptide mimic, NF-κB Hybrid, in order to determine the secondary structure. NF-κB Hybrid has the desired structure of the NF-κB protein binding site, a β-sheet, as shown by the 210 nm minimum, and some random coil, as shown by the minimum at 195 nm. This secondary structure could be an indicator to the binding capability of NF-κB Hybrid. Previous research has shown that structured peptides bind single stranded DNA better that unstructured peptides.[8] This study will investigate if this is true for a dsDNA binding by transcription factors.

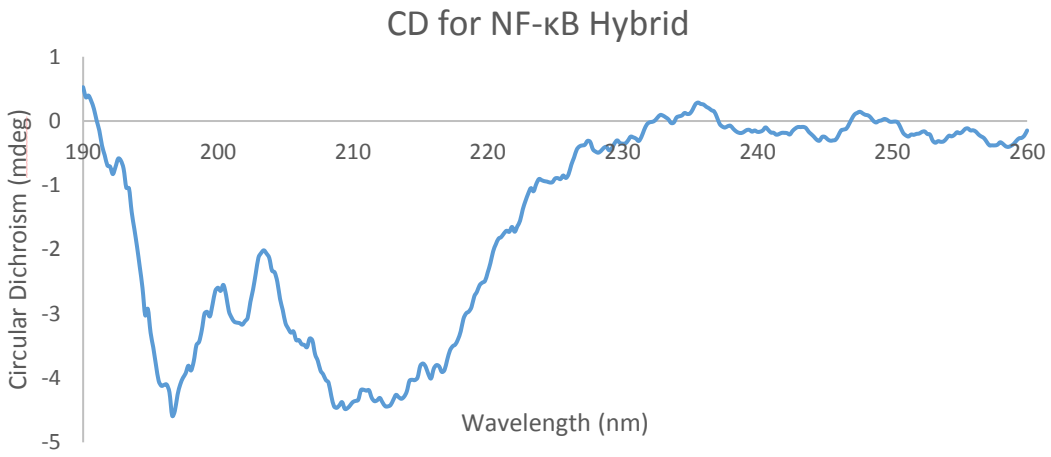

Figure 6. Circular dichroism spectrum of NF-κB Hybrid that shows two minima which indicate a β-sheet (210 nm) with random coil (195nm). The sample was analyzed at 25^{oC} from 190-260 nm in 10 mM Na_2HPO_4, 100 mM NaCl, and pH 7.2

Fluorescence DNA Binding Study

Fluorescence binding studies were completed in duplicate on NF-κB Hybrid using various DNA sequences. The concentrations of the peptide and DNA were determined using UV-vis (Table 1) and were used to calculate volumes for each sample analyzed. The peptide concentration was kept constant at 8 μM, while the DNA concentration increased from 0-60 μM (Tables 2, 3, and 4). When the peptide binds DNA, the spectra shows a decrease. As the DNA concentration increases, there is a greater shift in the spectra, as shown by Figures 7 and 8.

Table 1. Fluorescence stock stock solution concentrations in the three trials with NF-κB Hybrid using three different DNA sequences: κB, Unmethylated, and Methylated.

	Trial 1	**Trial 2**	**Trial 3**
Peptide Concentration	395 μM	339 μM	228 μM
DNA Concentration	685 μM	575 μM	732 μM
DNA Sequence	κB Sequence	Unmethylated	Methylated
	5'-GGAGTGTCCC-3' 3'-CCTCACAGGG-5'	5'-GTATCCGGATAC-3' 3'-CATAGGCCTATG-5'	5'-GTATC/Me-dC/GGATAC-3' 3'-CATAGGC/Me-dC/TATG-5'

Table 2. Fraction concentrations for the fluorescence binding study of NF-κB Hybrid and the κB DNA sequence. Each fraction was run in duplicate in the study.

Fluorescence Concentration Chart				
Sample	**Buffer (μL)**	**Peptide (8μM)**	**DNA (μM)**	**DNA (μL)**
1	779	21	0	0
2	777	21	2	2
3	773	21	5	6
4	767	21	10	12
5	761	21	15	18
6	756	21	20	23
7	750	21	25	29
8	744	21	30	35
9	732	21	40	47
10	709	21	60	70

Table 3. Fraction concentrations for the fluorescence binding study of NF-κB Hybrid and the Unmethylated DNA sequence. Each fraction was run in duplicate in the study.

Fluorescence Concentration Chart				
Sample	**Buffer (µL)**	**Peptide (8µM)**	**DNA (µM)**	**DNA (µL)**
1	781	19	0	0
2	778	19	2	3
3	774	19	5	7
4	767	19	10	14
5	760	19	15	21
6	753	19	20	28
7	746	19	25	35
8	739	19	30	42
9	725	19	40	56
10	698	19	60	83

Table 4. Fraction concentrations for the fluorescence binding study of NF-κB Hybrid and the Methylated DNA sequence. Each fraction was run in duplicate in the study.

Fluorescence Concentration Chart				
Sample	**Buffer (µL)**	**Peptide (8µM)**	**DNA (µM)**	**DNA (µL)**
1	772	28	0	0
2	770	28	2	2
3	767	28	5	5
4	761	28	10	11
5	756	28	15	16
6	750	28	20	22
7	745	28	25	27
8	739	28	30	33
9	728	28	40	44
10	706	28	60	66

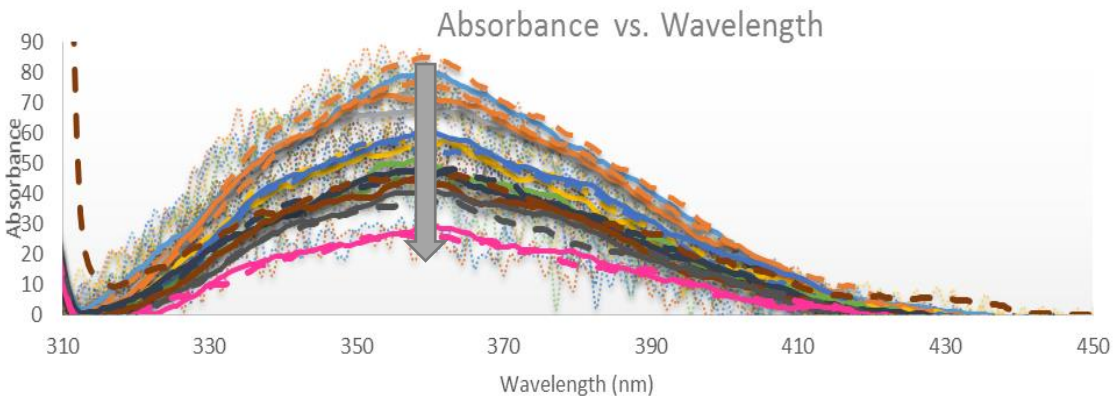

Figure 7. Fluorescence binding study which shows the binding activity between 8 μM NF-κB Hybrid and 0-60 μM kB DNA, sequence found in Table 1. The experiment was completed using 10 mM Na_2HPO_4, 100 mM NaCl, and pH 7.3.

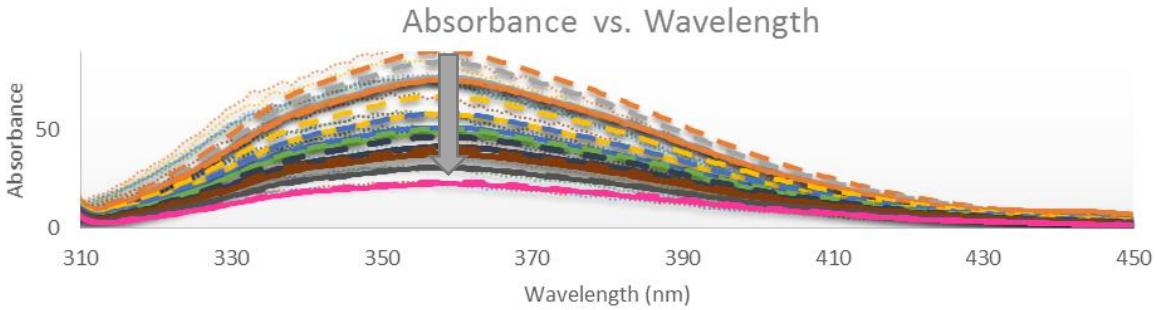

Figure 8. Fluorescence binding study which shows the binding activity between 8 μM NF-κB Hybrid and 0-60 μM Unmethylated DNA, sequence found in Table 1. The experiment was completed using 10 mM Na_2HPO_4, 100 mM NaCl, and pH 7.3.

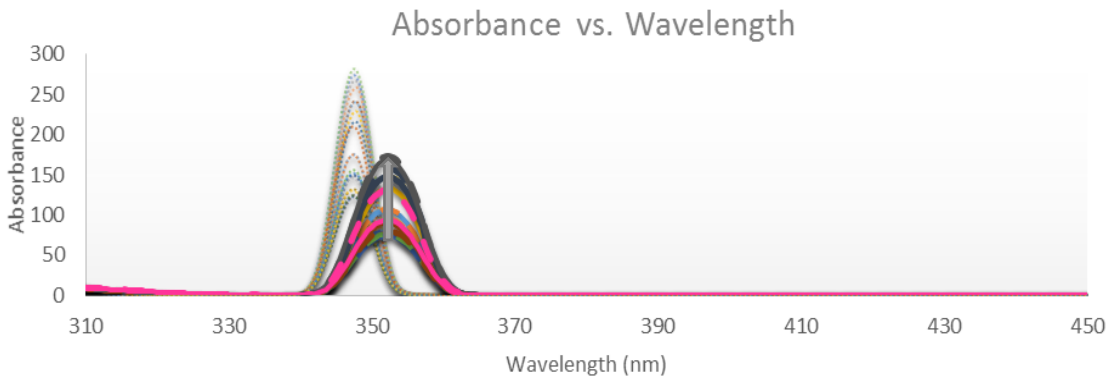

Figure 9. Fluorescence binding study which shows the binding activity between 8 μM NF-κB Hybrid and 0-60 μM Methylated DNA, sequence found in Table 1. The experiment was completed using 10 mM Na_2HPO_4, 100 mM NaCl, and pH 7.3.

Metal-Ligand Complexes Data

The metal-ligand complexes were analyzed using circular dichroism in order to identify binding between the metal-ligand complex and calf thymus DNA. When binding occurred, there was an apparent shift down in the spectra showing a change in the structure of the DNA. The initial shift in CD spectra indicated the binding affinity of each metal-ligand complex.

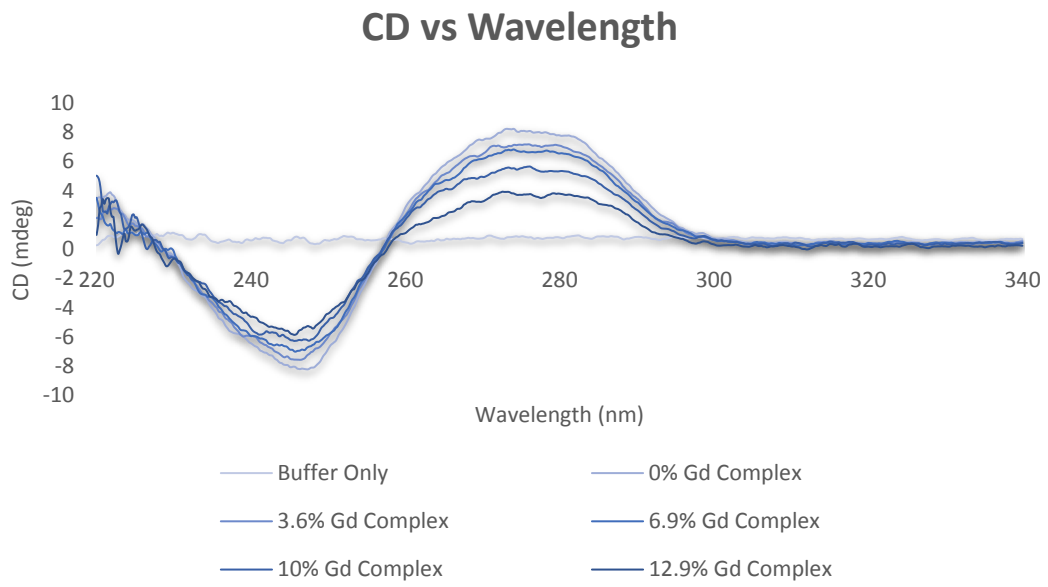

Figure 10. CD Titration of 3 mM Gd-Ligand Complex in 250 ng/µL Calf Thymus DNA showing a decrease in the DNA signal, which indicates binding activity.

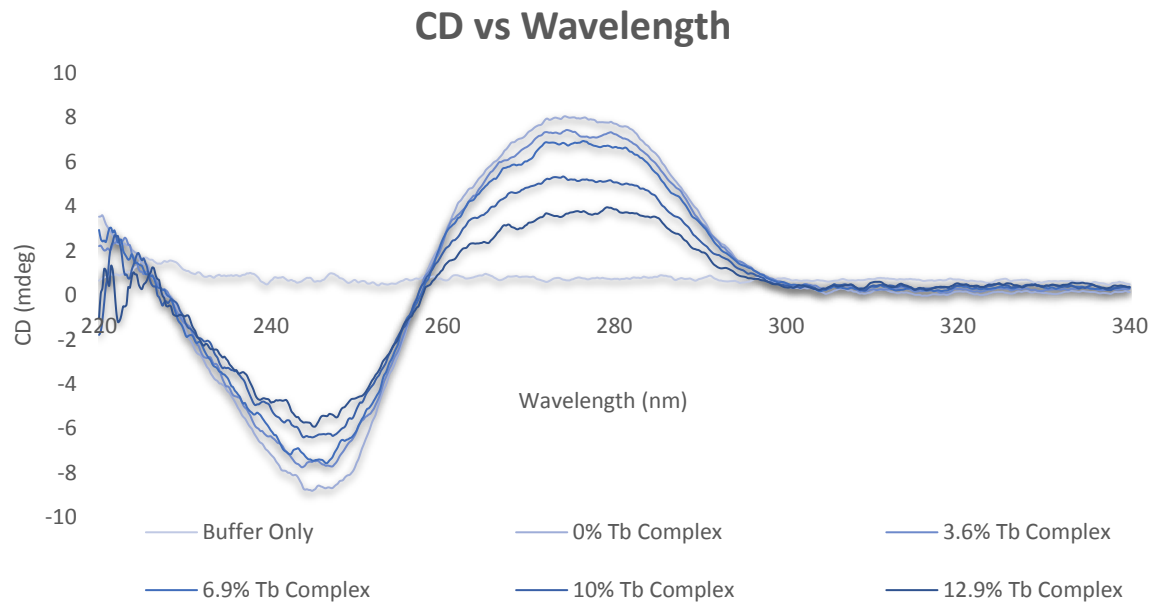

Figure 11. CD Titration of 3 mM Tb-Ligand Complex in 250 ng/µL Calf Thymus DNA showing a decrease in the DNA signal, which indicates binding activity.

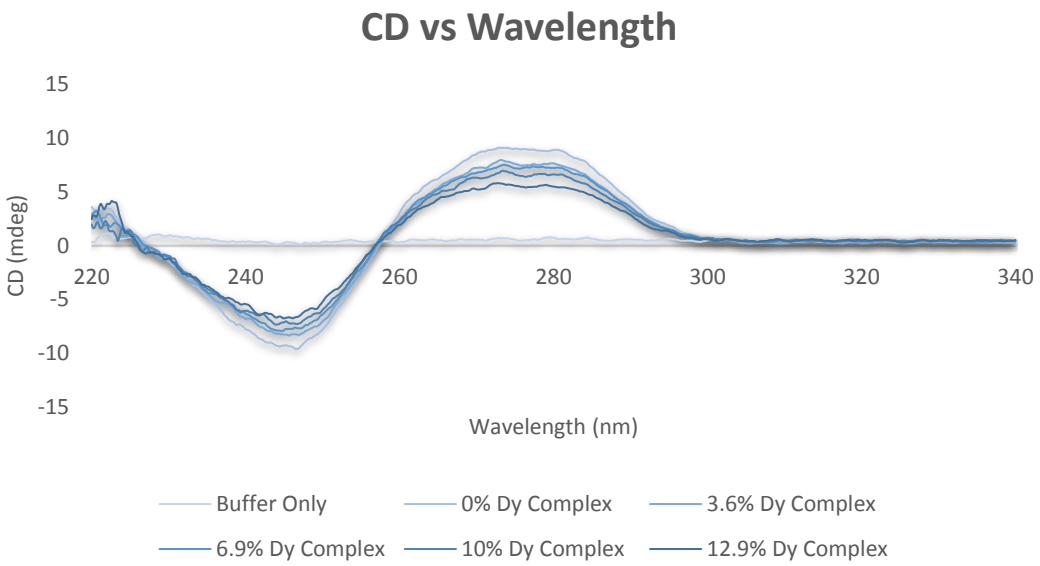

Figure 12. CD Titration of 3 mM Dy-Ligand Complex in 250 ng/µL Calf Thymus DNA showing a decrease in the DNA signal, which indicates binding activity.

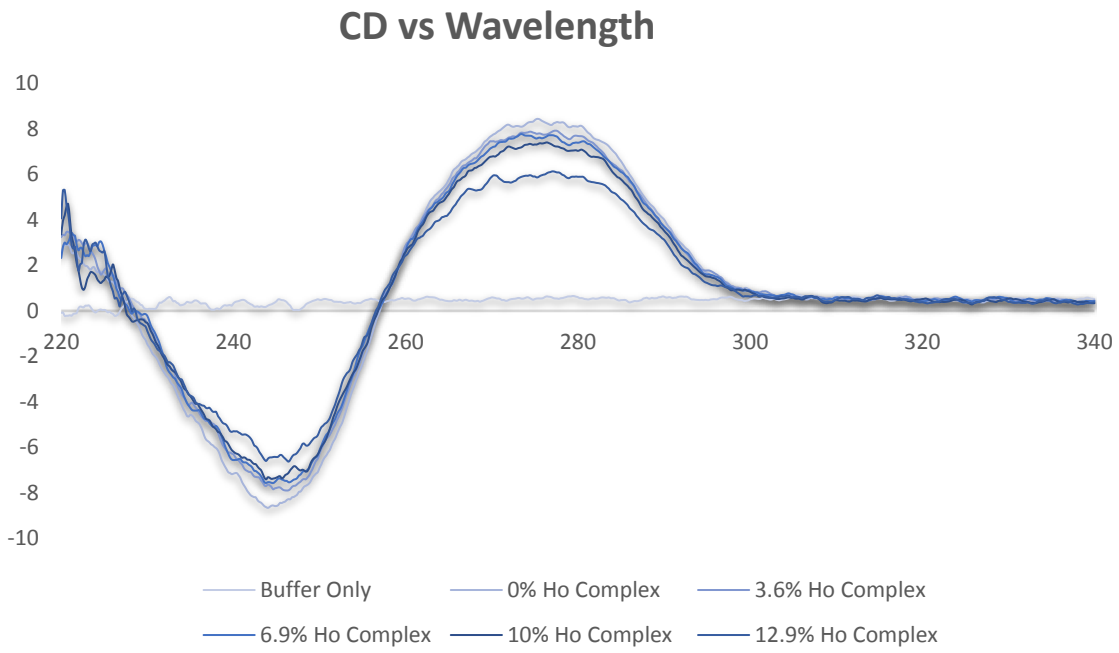

Figure 13. CD Titration of 3 mM Ho-Ligand Complex in 250 ng/µL Calf Thymus DNA showing a decrease in the DNA signal, which indicates binding activity.

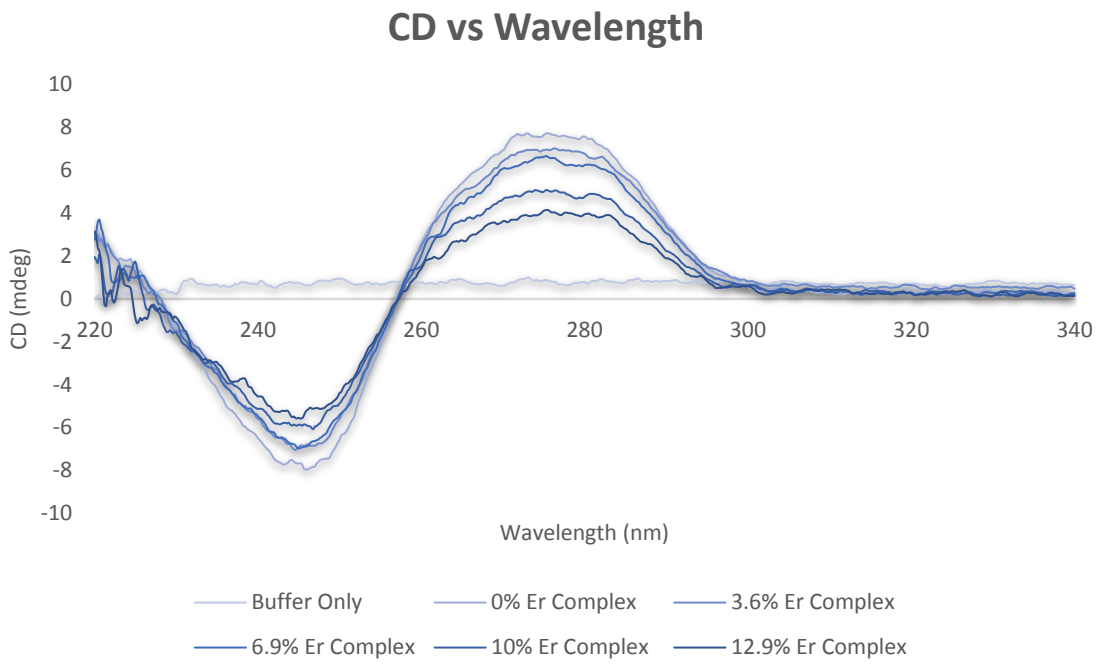

Figure 13. CD Titration of 3 mM Er-Ligand Complex in 250 ng/µL Calf Thymus DNA showing a decrease in the DNA signal, which indicates binding activity.

Discussion and Conclusion

Binding studies are an important method in which DNA binding molecules can be analyzed. The binding studies conducted within this research have shown that various DNA binding molecules can be analyzed using different methods and provide fruitful results. The fluorescence binding study shows that the NF-κB Hybrid is successful at binding the κB sequence and a methylated DNA sequence as well. The CD titration analysis showed that the lanthanide metal-ligand complexes successfully bind calf thymus DNA.

There is an abundance of future work which can be completed with this data. To start, more NF-κB mimics could be synthesized with different binding sequences in order to determine if there are more peptide mimics which can bind selectively. Additionally, the dissociation constant, K_d, can be found for the peptide mimics in order to be compared to the known K_d of the NF-κB protein. The metal-ligand complexes could be further analyzed by completing a gel electrophoresis assay. In the assays, the desired result will be a decrease in the amount of relaxed and supercoiled DNA in comparison to the control as the metal-ligand complex binds. Overall, this research explores the possibility that altering the expression of genes with organic and inorganic compounds can one day leads to a decrease in some diseases.

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