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The Study of NF- κ B Peptide Mimics and How Proteins Bind DNA

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

By
Allee Murray

Under the mentorship of Dr. Amanda Stewart

ABSTRACT

The protein complex nuclear factor kappa B (NF- κ B) is widely considered to be one of the most influential transcription factors when studying cellular functions. Peptide mimics of NF- κ B aim to inhibit DNA binding in order to displace the natural transcription factor, therefore inhibiting transcription and translation. In theory, NF- κ B is not the problem; the real problem lies in directing the synthesis and expression of harmful proteins. In conjunction with this, the project aims to study NF- κ B and its structure and function to determine what criteria are important for the binding of DNA in order to design a peptide that comes closer to this goal of producing a mimic of NF- κ B. To accomplish this, peptides were designed, synthesized, cleaved, dissolved, and purified in order to run mass spectrometry to determine whether the correct peptides were synthesized. Overall, if peptide mimics are more successful in binding DNA than NF- κ B, then the research could potentially be used in clinical settings in order to prevent the overexpression of particular genes implicated in various diseases.

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Introduction

The protein complex nuclear factor kappa B (NF- κ B) is widely considered to be one of the most influential and important transcription factors when studying cellular functions such as immune response, cell growth, and development.¹ A transcription factor is a protein that binds to specific DNA sequences in order to control transcriptional regulation. Typically, transcription factors are comprised of both repression and activation domains that are used to turn genes on or off on the DNA strand.² NF- κ B in particular is well-studied because the transcription factors encompassing it play a vital role in the regulation of the body's response to infection. The erroneous regulation of NF- κ B has been linked to carcinogenesis, epilepsy, and other serious neurological and neurodegenerative disorders.³ Understanding of the structure of NF- κ B and how it binds is necessary to understand the function. The NF- κ B proteins bind to DNA to regulate the transcriptional silencing of specific genes.

Specifically, NF- κ B typically binds to DNA with a looped region of amino acids off of a beta (β) sheet.¹ This binding region contains a β -hairpin, a β -sheet containing two β -strands that are linked to make up this looped region.

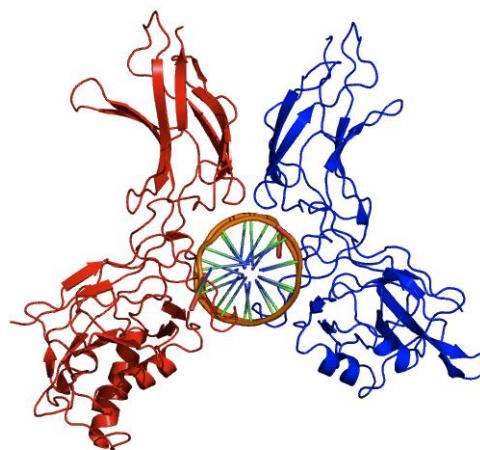


Figure 1. NF- κ B protein binding DNA. This shows both the p50 subunit and the p65 subunits of the NF- κ B protein and how it binds to helical DNA using a β -hairpin region.¹

In order for the peptide to truly be a mimic of NF- κ B, the structure and composition must be similar. Research has been done to determine the crystal structure of NF- κ B to study what truly causes the binding. From many previous studies, it has been determined that not only is the composition of DNA binding regions of proteins important, but the structure is also crucial for DNA binding. Although it is true that the mimic must be composed of similar amino acids, it must also have structural integrity.⁴ With the knowledge that the peptide binds using a β -hairpin region, the structure of synthesized peptide mimics will be verified in order to maintain the proper structure, even when amino acids are substituted.

In order to determine how to create mimics of NF- κ B that will displace the natural transcription factors, prior studies have to be considered to learn how NF- κ B binds and functions. In one study, it was acknowledged that NF- κ B is a highly regulated transcription complex. Comprised of subunits, the two most widely known are the p50 and p65 subunits.⁵ The two different subunits bind to the DNA differently, but there are significant similarities in the binding. Both subunits bind most notably using arginine, tyrosine, glutamine, histidine, and lysine, although these are located in different positions for the two subunits.¹ This knowledge is important when studying the mimics of NF- κ B, because intertwining high levels of these specific amino acids in the mimics should allow for a higher binding affinity.

In an additional study, the variations of the dynamics of NF- κ B were studied. From this study, it was determined that different members of the same family of NF- κ B proteins bind different DNA sequences differently.⁶ This presents the high level of variation that comes with studying NF- κ B, as some versions may bind one form of DNA

better than another form. While this can make studying the protein more difficult, it can also present further research options. If one synthesized peptide type may not bind a specific DNA type well, there might be other applications for the synthesized peptide in other DNA applications in the future.

The overall goal is to inhibit DNA binding in order to displace the natural transcription factor and therefore inhibit transcription and translation. In theory, NF- κ B is not the problem; the real problem lies in directing the synthesis and expression of harmful proteins. In conjunction with this, this project aimed to study NF- κ B and its structure and function. From this, the criteria that are important for the binding of DNA were studied in order to design a peptide that comes closer to this goal of producing a mimic of NF- κ B and therefore inhibiting it. It is important to regulate gene expression and by successfully synthesizing and producing a peptide that does this is the beginning of accomplishing this objective.

Methods

Peptide Design

Table 1. NF- κ B Peptide Mimic Sequences

Mutant Peptide Sequence 1 ("Original")	Pro-Tyr-Leu-Gln-Ile-Arg-Phe-Arg-Val-Asn-Gly- Lys-Trp-Val-Lys-Pro-Gln-Val-Lys-Ile
NF- κ B Peptide Sequence 2 ("Hybrid")	Gln-Arg-Phe-Arg-Trp-Val-Arg-Val-Asn-Gly- Lys-Tyr-Ile-Lys-Val-Gln-Leu-Glu

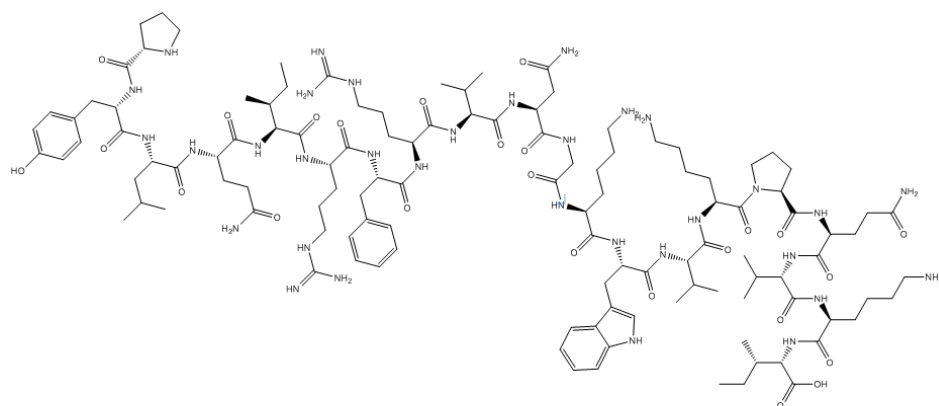


Figure 2. NF-κB peptide mimic (sequence 1)

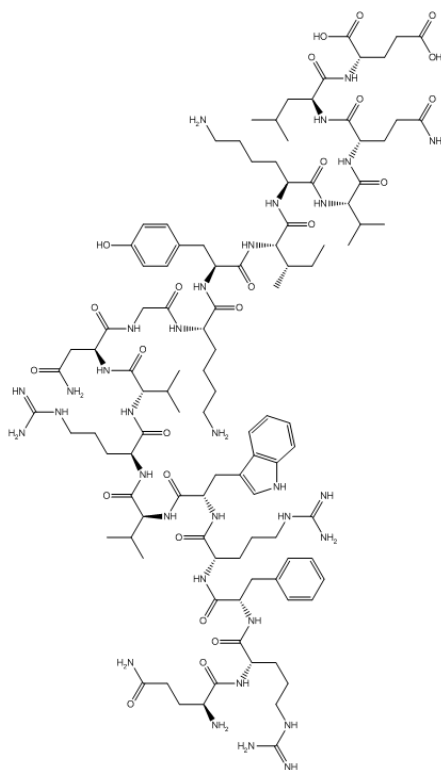


Figure 3. NF-κB peptide mimic (sequence 2)

Peptide Synthesis

The NF- κ B binding sequence (Table 1) was synthesized by solid phase peptide synthesis using a PS3 Peptide Technologies Peptide Synthesizer. All of the amino acids were coupled for 20 minutes before being deprotected in duplicate for 5 minutes using 20% piperidine. The solvent used throughout this process was DMF and the peptide sequence was activated using the activator HBTU. Finally, the peptide was capped using acetic anhydride. Once synthesis was complete, the peptide was capped and placed into a peptide flask. The peptide was then cleaved from the resin using a cleavage cocktail consisting of 95% TFA, 2.5% TIPS and 2.5% water. After complete cleavage, the peptide solution was evaporated until a very small volume remained before the peptide was precipitated with 20 mL of cold ether. This caused the solution to separate into layers. The water layer, which contained the synthesized peptide, was collected and lyophilized in order to turn the peptide solution into a powder.

Purification and Desalting

Once the peptide was lyophilized, the peptide had to be dissolved based on the structure and composition of the peptide. A large portion of peptide was dissolved in approximately five mL of liquid and filtered. Once filtered, high purification liquid chromatography was used to purify the peptide. To do this, a C-18 silica based reversed phase high performance liquid chromatography (HPLC) column and two solvents (A & B) were used. Solvent A is comprised of 95% HPLC water, 5% Acetonitrile and 0.1% TFA, while solvent B is comprised of 95% acetonitrile, 5% HPLC water and 0.1% TFA.

The column was washed with solvent A in order to equilibrate the column. The peptide was then injected at small amounts to determine elution time, before being washed by solvent B. Once the proper elution time was found, the peptide injection amount was increased and fractions were collected over the elution time. Once purified, the peptide was placed on the lyophilizer in order to turn the peptide solution into powder. Once lyophilized, a small amount of each fraction was dissolved into 50 μL of distilled water and placed on the MALDI-ToF plate. Once dry, one microliter of matrix was added on top. A Bruker Microflex MALDI-ToF mass spectrometer was then used to determine the mass of the peptide and whether the fraction contains the correct peptide and is purified correctly.

Once the peptide and its mass were verified, the peptide was desalted. To do this, 2 mg of the peptide was dissolved in 300 μL of distilled water. The desalting column was washed with 25 mL of distilled water (five column volumes). The 300 μL peptide sample was then added to the column and seven fractions were collected. A ten microliter portion of each fraction was then diluted to 700 μL total. UV-vis spectroscopy was used to determine absorbance of each sample at 280 nm. Like fractions were then combined and lyophilized and checked again with MALDI-ToF mass spectrometry to confirm mass.

Circular Dichroism (CD) Analysis

The structure of the peptide was determined using CD analysis. One milligram of peptide was dissolved in one mL of CD buffer (10 mM Na_2HPO_4). CD was done using a temperature interval measurement method in order to determine the secondary structure

of the peptide sample. This was done at 25 degrees measuring a wavelength range of 260-190 nm.

Combining and Annealing DNA

In order to combine the single-stranded DNA pieces, first the concentrations of each had to be determined. The concentrations of the single strand DNA sequences were determined and the two sequences were combined to produce double helix DNA. The DNA was annealed by combining and heating at 95°C for five minutes before being allowed to cool to room temperature. Once annealed, the absorbance was taken at 260 nm to determine the concentration of the DNA sample (table 2).

Fluorescence DNA Binding Study

To determine how the peptide samples bind DNA, a fluorescence binding study was executed. In this study, peptide concentration was held constant while the amount of DNA was increased (table 3). These varying samples were combined with fluorescence buffer (10 mM Na₂HPO₄, 100 mM NaCl) to make a solution of 700 µL total. A fluorescence spectrometer was then used to run the study, with each sample being run in duplicate for a total of 20 samples. These values were then averaged to get final results.

Results

Mass spectrometry

Once the peptide was effectively and correctly synthesized, cleaved, and purified, the peptide was desalted to remove the salts and to prepare the sample for further studies. Both peptide sequences studied were desalted at different times, but using the same desalting column. Once completed and absorbance acquired, like fractions were combined and lyophilized. To determine which fractions contained the correct peptide (not ionized versions), mass spectrometry was used. Peptide sequence 2 was correctly identified by its molecular weight (figure 4) in the fraction containing the most lyophilized peptide.

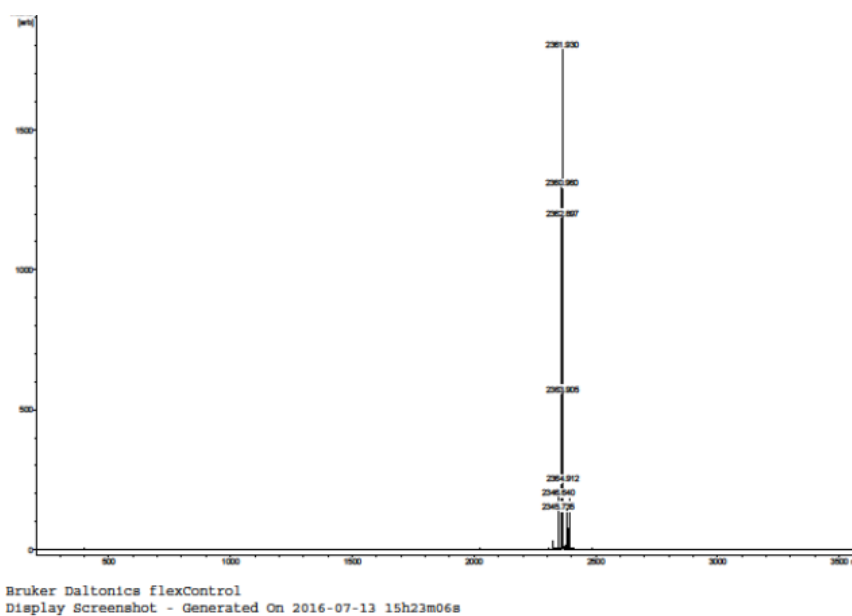


Figure 4. The mass spectrometry graph confirming the mass of peptide sequence 2. The graph shows a high peak at 2361.93 and expected weight is 2360.25.

Peptide sequence 1 was separated into several fractions. When these fractions were analyzed using mass spectrometry, the correct peptide mass (2511.03) was identified in fraction two which contained the least peptide, while fraction 1 which contained the most peptide when analyzed, had a mass of 2534.66 (figure 5, 6). The difference between the theoretical molecular weight of this peptide and the peptide fraction with the most volume of desalted peptide is approximately 23 units different. Although not completely confirmed, it is believed to be a sodium atom attached to the peptide. From previous studies done by our research lab⁷, it has been determined that there are higher levels of sodium in our water. For some reason, high levels of peptide sequence 1 after being desalted using deionized water from our lab seem to contain a change in molecular weight of approximately 23. This difference in molecular weight is currently being studied to determine the causes and what can be done to prevent this irrelevant binding in the future.

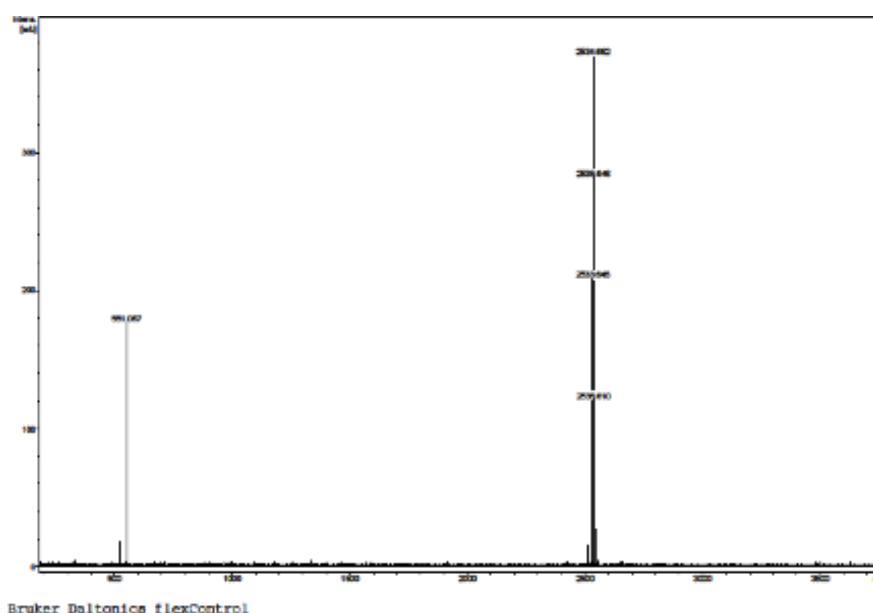


Figure 5. The mass spectrometry graph displaying fraction two with a molecular weight of 2534.66, approximately 23 different than the theoretical.

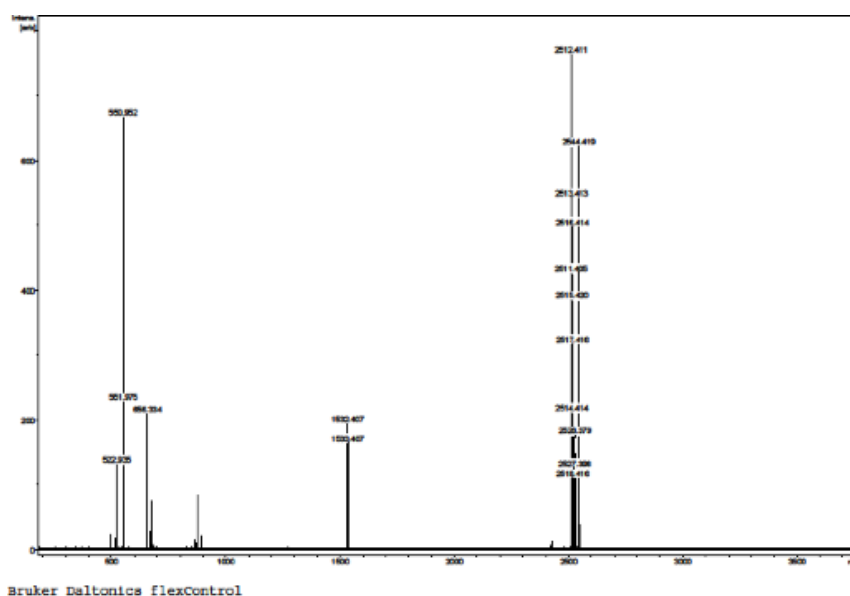


Figure 6. The mass spectrometry graph confirming the mass of peptide sequence 1. The graph shows a high peak at 2512.41 and expected weight is 2511.03.

Circular Dichroism Analysis

Once mass spectrometry was completed, circular dichroism (CD) was completed on both peptide sequences. Because peptide sequence 1 had issues with molecular weight, it was decided that CD analysis would be done on both fractions. When analyzing the sequences using CD, we hope to see a spectrum that confirms a beta sheet region within the sequence. With peptide sequence 1, the results of fraction 1 (peptide with higher molecular weight) confirmed random coil within the peptide sequence, based on its minimum peak at 195 nm, which is characteristic of a random coil peptide structure (figure 7). It is known that structure is very important to function, therefore the peptide sequence being in a random coil configuration points to the possibility that it will not bind to DNA as effectively as the NF- κ B protein. Fraction 2 in peptide sequence 1

seemed to have slightly better results, with a configuration that looks much more like a beta sheet, although not fully (figure 8). While this is great for research purposes, it also means that the fraction containing the least amount of peptide once desalted has the best results out of the two, which is not good when wanting to use the more structured peptide for further studies.

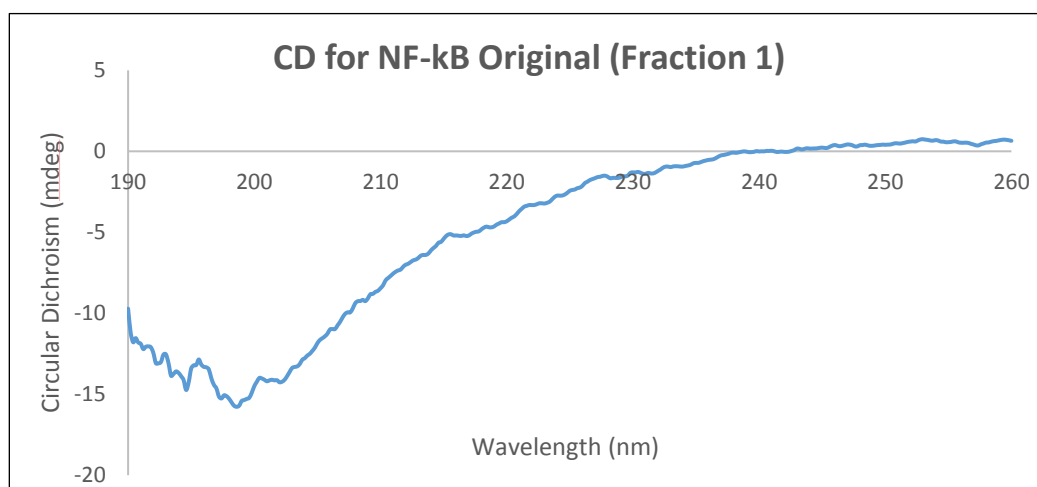


Figure 7. Circular dichroism spectra of peptide sequence 1 (fraction 1) that shows evidence of random coil, with no real β -sheet indicated.

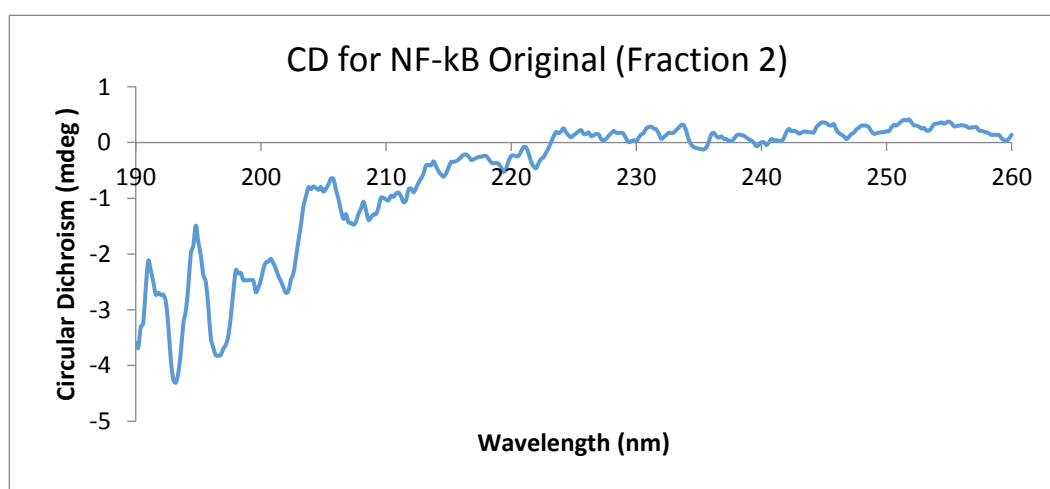


Figure 8. Circular dichroism spectra of peptide sequence 1 (fraction 2) that indicates random coil, with some β -sheet activity.

Peptide sequence 2, on the other hand, had good results when analyzed with CD. The CD analysis spectra showed that the peptide was in a beta sheet configuration, which is very similar to the NF- κ B protein (figure 9). As it is known how important structure is to binding, this led to the conclusion that peptide sequence 2 could present more consistent and successful binding patterns when binding studies were completed.

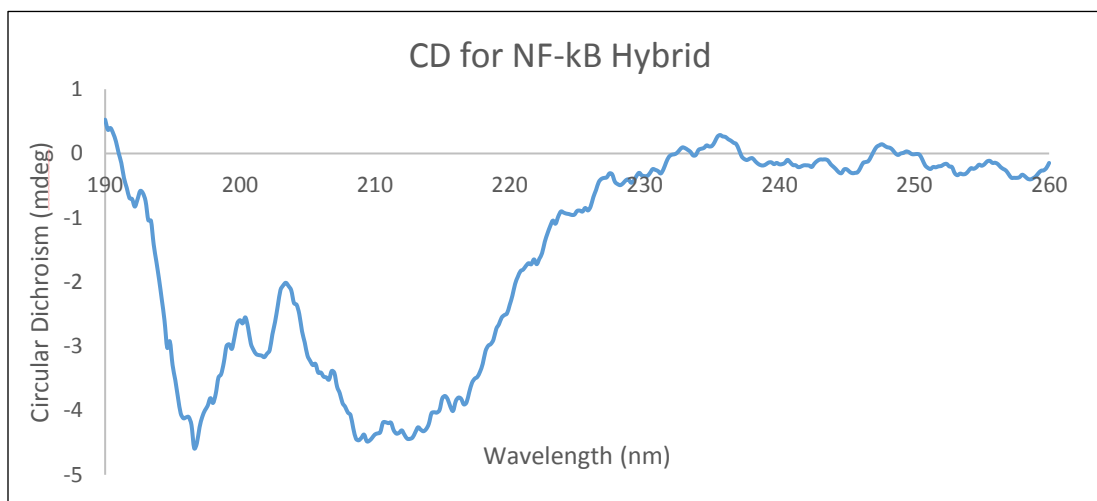


Figure 9. Circular dichroism spectra of peptide sequence 2 that shows two minima, one minima consistent with a beta sheet (minimum at 210-215) and one indicating some random coil.

Fluorescence DNA Binding Study

Fluorescence studies were then completed to determine which peptides bind DNA more successfully. To do this, first the peptide sample is dissolved into fluorescence buffer. Then, the concentrations of peptide and DNA (table 2) were used to complete calculations to determine the amounts of DNA and peptide needed to make fractions with increasing amounts of DNA (table 3). The fluorescence study was completed two different times, with each fraction being done in duplicate. If the peptide truly does bind

DNA, then as the amount of DNA increases, the peptide should bind the DNA more and therefore the graph should show less fluorescence of peptide due to tryptophan fluorescence being quenched by the DNA binding. The spectra created from the results of the binding study for peptide sequence 2 shows strong evidence that this sequence successfully binds DNA (figure 10, 11).

Table 2. Table displaying values for both peptide and DNA concentrations, along with the sequence of the DNA used in the fluorescence binding studies.

Peptide Concentration	395 μ M
DNA Concentration	685 μ M
DNA Sequence	5'-GGAGTGTCCC-3' 3'-CCTCACAGGG-5'

Table 3. Table displaying the values of peptide and DNA concentrations for each of the fractions used in the fluorescence binding studies.

Fluorescence Concentration Chart				
Sample	Buffer (μ L)	Peptide (8 μ M)	DNA (μ M)	DNA (μ L)
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

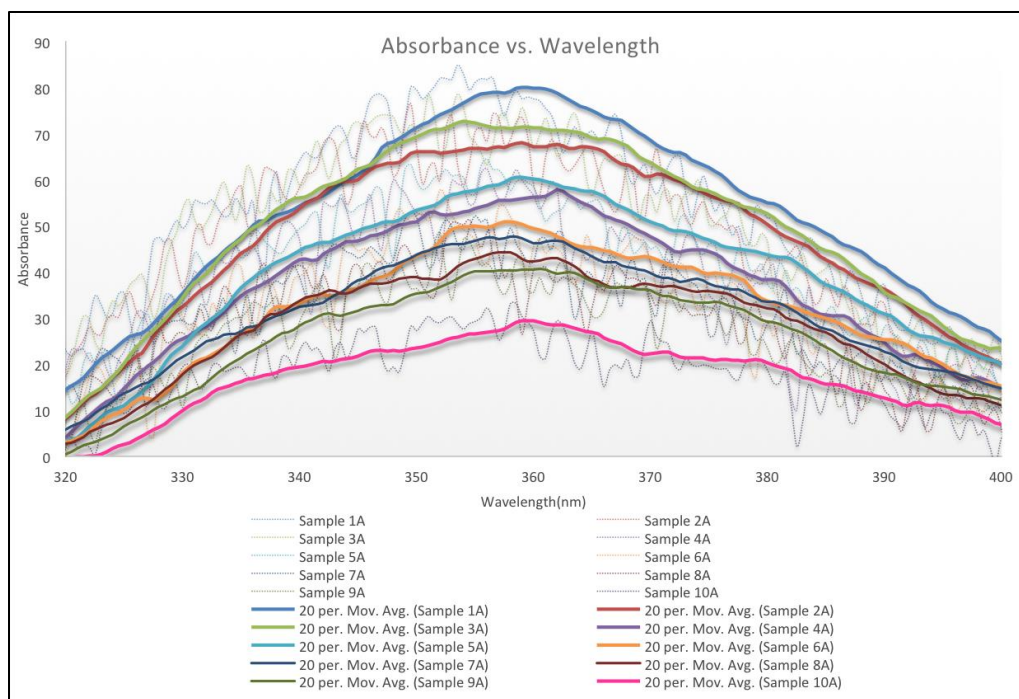


Figure 10. Tryptophan fluorescence quenching of peptide sequence 2 (trial 1) analyzed using $8\mu\text{M}$ peptide and 0 to $50\mu\text{M}$ DNA.

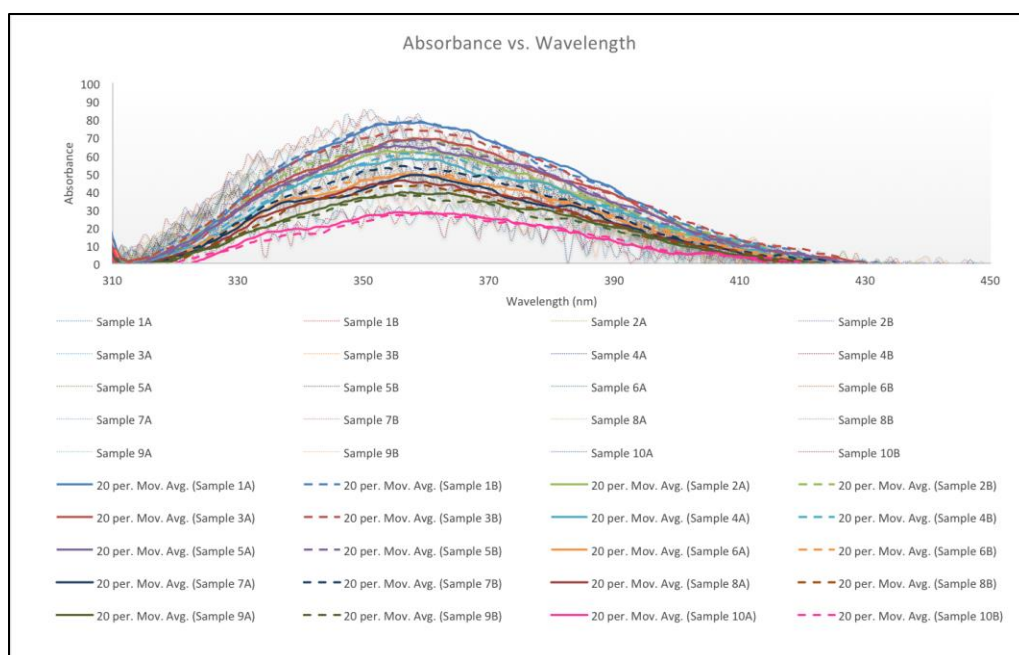


Figure 11. Fluorescence binding spectra of peptide sequence 2 (trial 2), presenting similar results as trial 1

Fluorescence binding studies have not been completed yet for peptide sequence 1, due to insolubility issues in the fluorescence buffer. In the future, the goal is to complete this study for this sequence in order to fully compare the two sequences. From this point, it is expected that peptide sequence 1 will bind DNA less successfully than peptide sequence 2, based on prior knowledge gained from this study, although these suspected results cannot be verified fully until binding studies are completed.

Discussion & Conclusion

It is known that when analyzing a peptide's DNA binding affinity, structure is very important. Through experimenting with two different peptide mimics of NF- κ B, this known fact has been reiterated. Peptide sequence 2 has presented good evidence that structure does contribute to binding of DNA. One reason peptide sequence 2 is so much more structured than peptide sequence 1 is due to its combination of amino acids. Although both peptide sequences are comprised of many of the same amino acids, the order in which the amino acids are coupled in the sequence is very important. In peptide sequence 1, more side chain interactions between amino acids such as arginine and phenylalanine and lysine and glutamine cause more random coil, while in peptide sequence 2, lysine and tryptophan interact causing a more structured peptide. Although DNA binding studies have not yet been completed for peptide sequence 1, it is suspected that once completed the results will show better DNA binding with peptide sequence 2, due to sequence 2 being more structured than sequence 1.

Future work could be done on this project to further our knowledge of NF- κ B peptide mimics and DNA binding. Once DNA binding has been completed for peptide sequence 1, further analysis of these peptides and others will be studied. The binding of these peptides to the kB DNA sequence will be compared to the binding to different DNA sequences to determine if different sequences are selective in binding. Additionally, other DNA binding molecules will be examined in order to further analyze selectivity for specific sequences. Finally, other peptide sequences will be synthesized, purified, and examined in order to further enhance the knowledge of how proteins bind to DNA.

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