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EFFECTS OF CROWDING AGENTS ON I-MOTIF DNA

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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the

requirements of the Sally McDonnell Barksdale Honors College

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ABSTRACT

Deoxyribonucleic acid (DNA) is a well-known double stranded, helical, biological molecule. In addition to its more commonly known structure, DNA can also form more complicated structures like G-quadruplexes and i-motifs (iM). The iMs are formed by cytosine rich DNA and are a four stranded structure that is typically looped around itself. The iM formation is typically pH-dependent and is favored in more acidic conditions; the pKa value is approximately 6.5. This pKa value allows for potential *in vivo* formation, since the cells have a pH of approximately 7.3. Due to this, iMs are thought to be powerful, innovative molecules for gene regulation and specific drug targeting and delivery mechanisms (1, 2, 3, 4, 11). In this thesis, the iM-forming DNA strand of AC01019.1 in various solutions of crowding agents, such as polyethylene glycol (PEG) and Dextran, were explored and tested (7). The purpose of this works was to determine the effects of the various concentrations of crowding agents and pH values on the formation of iM DNA. Throughout the research project, the data was measured and recorded using analytical tools such as a pH meter, a UV-Vis spectrometer, and a circular dichroism spectrometer. Software systems such as SpectraSuite, Olis, and Microsoft Excel were also used in displaying the collected data. All in all, the experiment was successful in showing how different concentrations of crowding agents affect iMs and their pH tolerance and the results show promise of the use of iMs in medicine.

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INTRODUCTION

Found in all living organisms, DNA is crucial to life as an essential biological molecule containing genetic information. From Fredrich Miescher's discovery to Watson and Crick's analysis of structure, the overviewing knowledge of DNA appears to be complete (8). However, evidence stating that there are non-Watson-Crick structures of DNA is now available. Previously only found *in vitro*, i-motifs (iM) are now being found and analyzed in human cells. These four stranded structures contain sequences containing multiple cytosines in a row. The iM combine duplex strands via intercalation of cytosine-cytosine base pairs, thus getting the name i-motif as seen in Figure 1 (9). The iM structure is stabilized by the hydrogen bonding between protonated cytosine bases (1, 2, 3, 7, 11). The role of iM DNA is thought to be related to gene expression, which is the ability of controlling what genes can or cannot be produced given certain cellular conditions. Importantly, iMs are believed to be involved in oncogene regulation, making them a potentially new target for cancer-fighting medications.

The iMs are typically formed in acidic to near neutral pHs, ranging from roughly 5.5 to 7 pH units; the approximate pKa value for i-motif DNA is 6.5 in dilute solutions. This pKa value close to neutral essentially allows for iMs to form *in vivo* since cells are approximately 7.3 pH units. This is in the absence of crowding agents that can help cause an upward shift in the pH range where the iM DNA can be formed. Crowding agents are capable of increasing the rate of enzymatic reactions, altering reaction products, protecting macromolecules from thermal denaturation, and accelerating protein folding. Typical crowding agents are polyethylene glycol (PEG), Dextran, or Ficoll that help mimic intracellular conditions (2, 7).

The goal of this thesis was to test the DNA strand AC017019.1 and determine if there is iM formation in solution and the effect of using molecular crowding agents on this iM formation

(12). This particular strand was selected because previous studies indicate this DNA oligonucleotide could form iMs under neutral conditions (3,4). Based on recent literature, I hypothesized that mimicking cellular conditions by using greater concentrations of crowding agents like PEG or Dextran, would increase the likelihood of iM DNA presence in solution, even at elevated pHs.
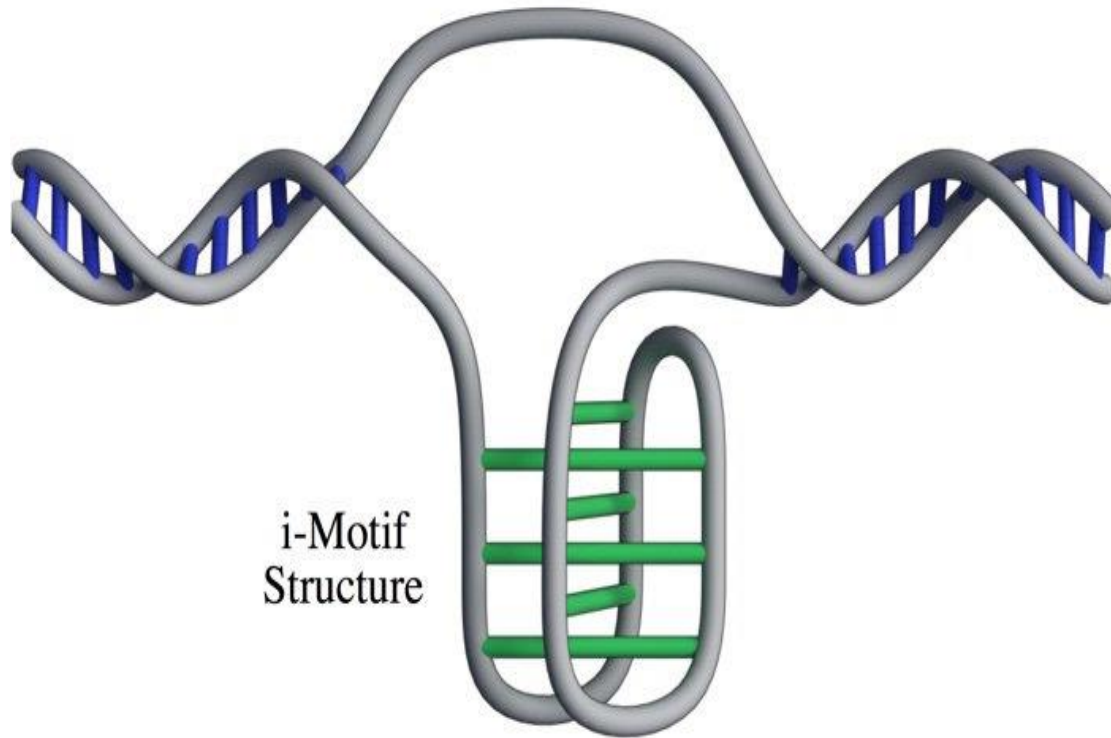
Figure 1: Structure of i-motif DNA. In this thesis, the DNA strand AC017019.1 was explored. Its DNA sequence is 5'- CCC CCC TCC CCC CCT CCC CCC TCC CCC C-3'. It is also found in the human Y chromosome (9, 12)

MATERIALS AND METHODS

To begin this experiment, a 30 mM sodium cacodylate ($C_2H_6AsNaO_2$) buffer solution was prepared using deionized water. From there, several solutions containing increasing amounts of polyethylene glycol-300 (PEG-300) were created including, 0, 5, 10, 20, 30, and 40% PEG-300. After the addition of the PEG-300, the pH of each solution needed to be adjusted; this was done via the use of a pH meter and 1 M HCl or NaOH solutions to increase and decrease acidity, respectively. The pH's were increased in increments of 0.2 pH units ranging from 5.2 to 8.2, the prime range of where iM DNA can be found. The theory behind how a pH meter works includes three different components: an internal electrode, a reference electrode, and a high input impedance meter (5). The pH is determined by the difference in concentration of H^+ ions between the reference buffer and the sample solution. It is important to adjust the pH values of the solutions AFTER making them rather than before; this is because the addition of the PEG-300 can alter the acidity/alkalinity of the solutions and yield a different pH value.

Additionally, another crowding agent, Dextran-6000, was briefly investigated in a similar fashion. This was also utilized in a 30 mM solution of sodium cacodylate and deionized water. Previous research had shown that the Dextran solutions become cloudy and a caramel color with time; the reason behind this is unknown ranging from poor sample preparation to an unknown oxidation-reduction reaction occurring. Much like previous findings, this caramelizing, cloudy solution was also visualized in the trial solution I prepared. Further research into why this reaction was assigned to another student to investigate this, and the research pertaining to PEG-300 as a crowding agent was resumed; the Dextran was determined to contain a biological contaminant from natural sources that affected the expected chemical outcome.

After the PEG-300 solutions were created and had their respective pH's adjusted, the iM DNA samples could be analyzed. This was first done by using the known iM producing DNA strand AC017019.1, using 1000 μL of a buffer with given pH and PEG-300 amounts, and 4 μL of iM DNA sample, resulting in a 4 μM solution of iM DNA. Verification of the concentration was done by the use of a UV-Vis spectrometer and the software SpectraSuite at pH increments of 0.2 pH units. A UV-Vis spectrometer relies on the Beer-Lambert law where the absorbance, path length, molar extinction coefficient, and concentration are connected (6, 10). This equation as well as a block diagram can be seen Equation 1 and Figure 2. The absorbance was taken at 270 nm, where iM DNA will absorb the most light. With the application of the Beer-Lamber law, the concentrations were confirmed or adjusted via dilution and prepared for the circular dichroism (CD) spectrometer.

In order to visualize that there was in fact iM DNA present within the samples, a scan using CD spectroscopy and the software Olis was used. The idea behind this technique is the differential absorption of left and right circularly polarized light, where optically active chiral molecules will preferentially absorb one direction of the circularly polarized light. The difference in absorption of the left and right circularly polarized light can be measured and quantified (11). A diagram of how a CD spectrometer works can be seen in Figure 3. From the software, plots can be made displaying the spectral signature of iM DNA.
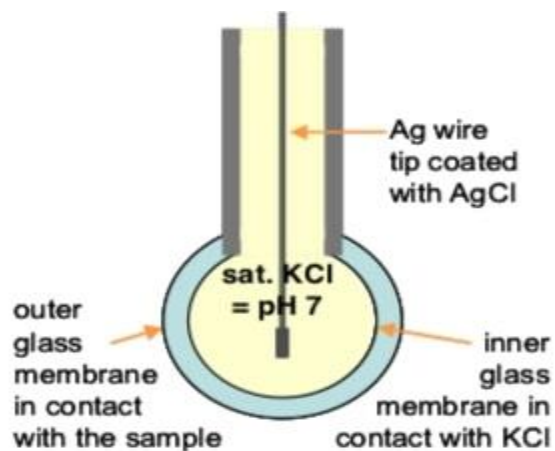
Figure 2: A schematic of how a pH meter works. The difference between the reference electrode and the measuring electrode yields the pH. (5)

$$A = \varepsilon bc$$

Equation 1: The Beer-Lamber Law where A is absorbance and is unitless, ε is the molar extinction coefficient with units of $M^{-1}cm^{-1}$, b is the path length with units of cm, and c is concentration with units of M. (6, 10)
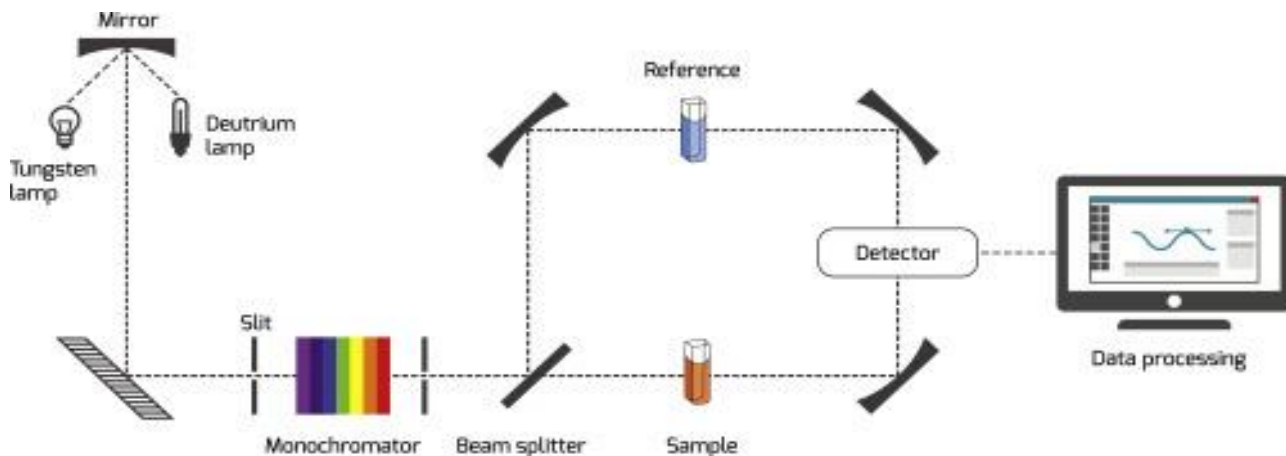
Figure 3: A block diagram of how a UV-Vis spectrometer works. By utilizing the Beer-Lambert Law, UV-Vis spectroscopy is useful in determining how much of compound there is and is often used as a detector in chromatography. (6,10)
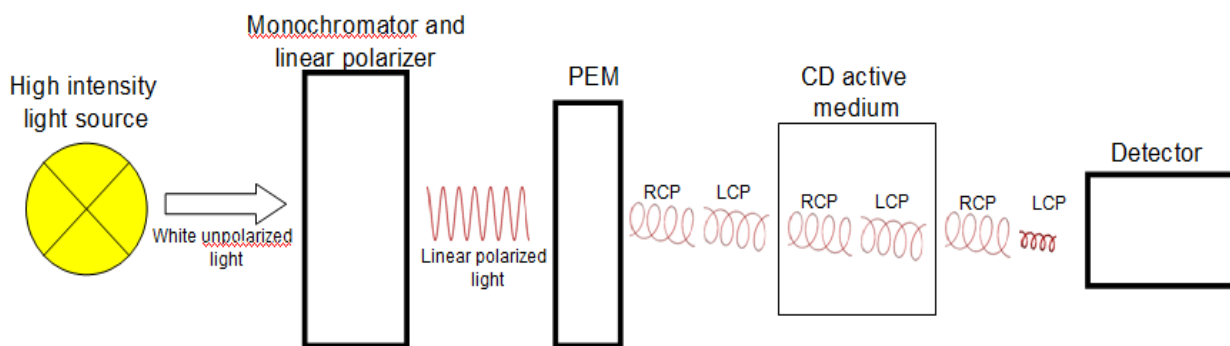

Figure 4: A block diagram of how a circular dichroism spectrometer works. Right circular polarized light (RCP) and left circular polarized light (LCP) is produced from the photoelastic modulator (PEM) which alters linear polarized light to circular polarized light. This is done by changing the direction of polarized light where optically active chiral molecules absorb one direction of rotating light. (11)

RESULTS

The results from the UV-Vis spectrometer pertaining to absorbance and concentration are crucial in the plots of the iM DNA. As absorbance and concentration are proportional to each other, these variables provided data used in the calibration of the iM plots produced by the CD spectrometer. By adjusting the spectra by their respective absorbance, the plots will be more accurate rather than assuming the same concentration values for all. Lastly, with the use of the CD spectrometer, iM DNA was confirmed within the samples. This could be seen by the signature peaks and valleys, characteristic of iM DNA presence. The data was analyzed and plotted for the sodium cacodylate solutions containing 0, 20, and 40% PEG-300 with the iM-forming DNA strand AC017019.1. As seen in the plots, iM DNA signatures typically formed between 220-310 nm with maximum and minimum peaks. These plots can be seen in Figures 5, 6, and 7.
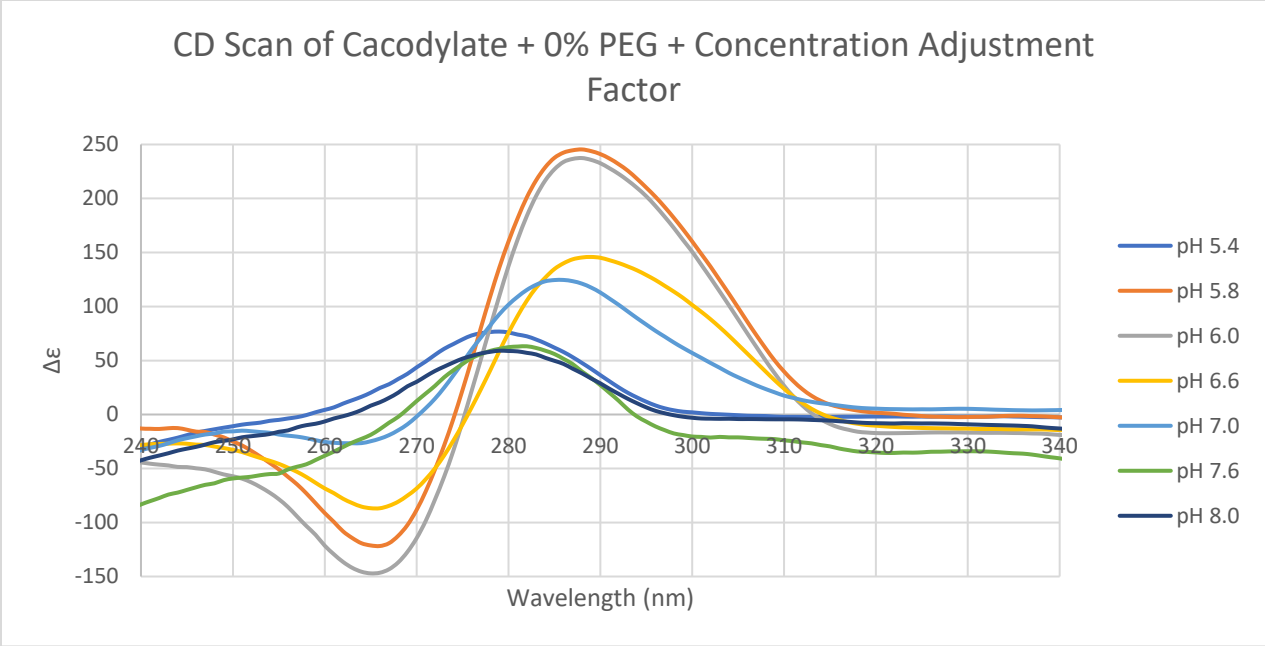
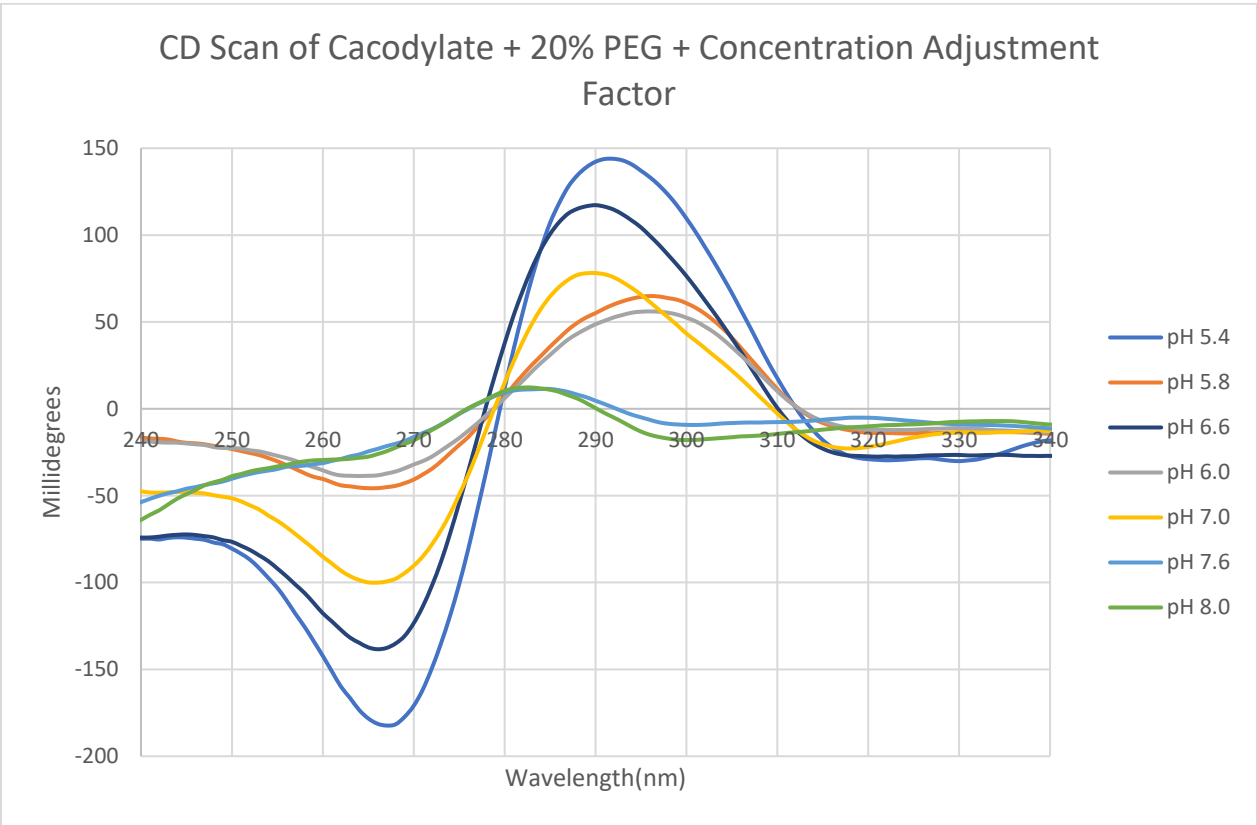Figure 5: The CD scan for 0% PEG-300 sodium cacodylate buffer containing the iM DNA

Figure 6: The CD scan for 20% PEG-300 sodium cacodylate buffer containing the iM DNA
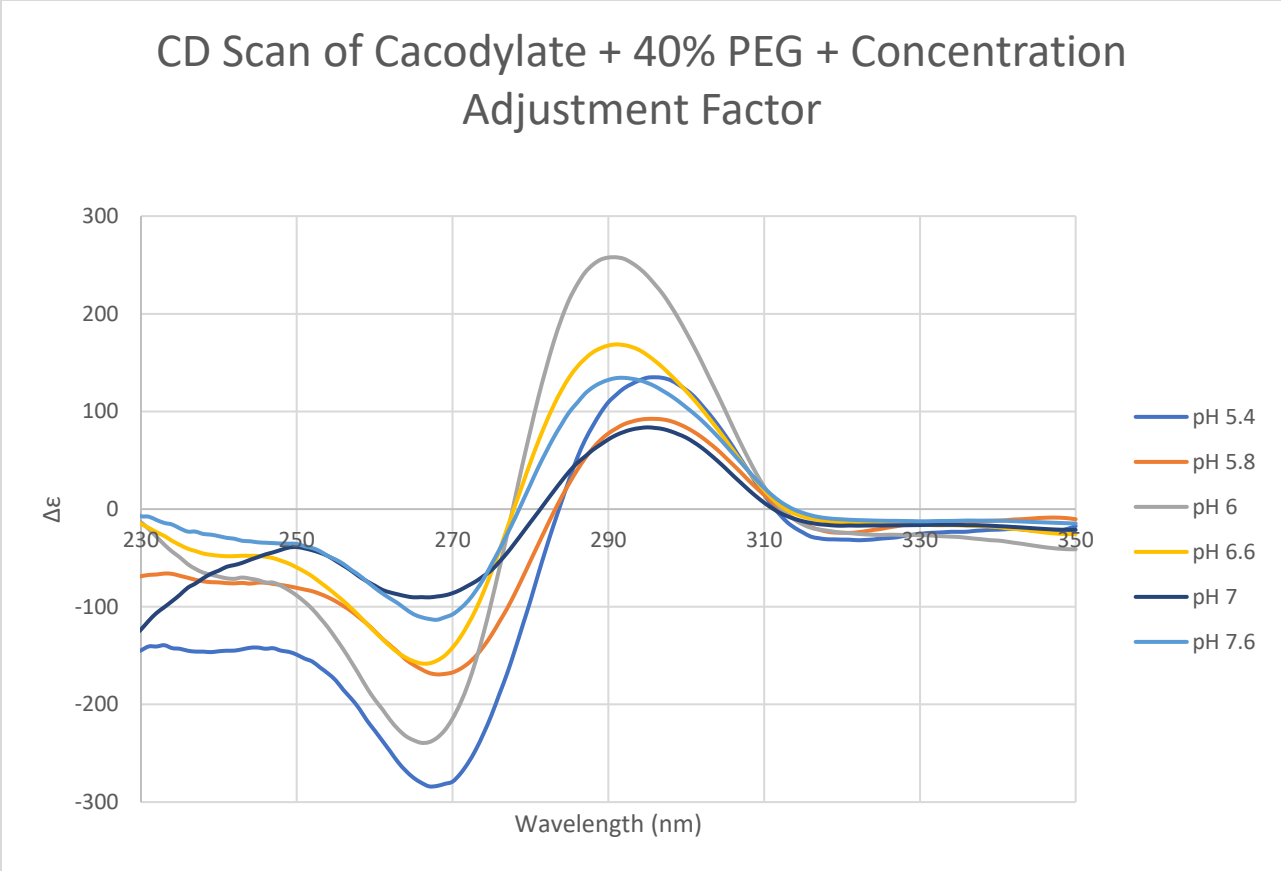
Figure 7: The CD scan for 40% PEG-300 sodium cacodylate buffer containing the iM DNA

DISCUSSION

From the findings of the plots, iM DNA presence is apparent throughout all of the sampled of 0, 20, and 40% PEG-300 solutions; the signature bands help in the determination of the iM. One of the findings for this thesis is that the acidic conditions affect the iM and the relative size of the peaks. The more acidic the pH, regardless of the molecular crowding agent concentration, the stronger the signal and the larger the peak of the iM. This is likely because the of the more acidic favoring conditions of the iM DNA due to the pKa values of approximately 6.5. In addition, the PEG-300 crowding agent impacts the pH tolerance of the iM DNA. With increasing concentration of molecular crowding agents, iM presence was visualized in neutral solutions. This increased stability supports the for *in vivo* experiments pertaining to gene regulation and expression.

Another aspect that is seen within the plots includes a shift in the wavelength for the iMs. At acidic pHs such as 5.4 and 5.8 and low crowding agent concentration, the iM maximum peak height is consistently between the 280 and 290 nm. However, as the concentration of crowding agent increases, these acidic iMs have a red shift towards 300 nm. This was reproduced throughout the experiment and other students in the lab performing comparable research using different iM-forming DNA strands have yielded similar results. This likely eliminates the possibility of error. Conformational changes occurring within the iM DNA structure are a possibility for this phenomenon. Further exploration into what is occurring within these iM molecules will determine what is actually occurring. This may become a finding that can assist in oncogene targeting in cancer research. All in all, the findings of this thesis in using iM DNA sequence AC017019.1 and molecular crowding agents for *in vivo* use are promising; however, the red shift in the acidic pHs requires further investigation.

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