

THE EFFECT OF POLYAMINES ON IMOTIF THERMAL STABLITY

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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of Sally McDonnell Barksdale Honors College

Oxford
May 2015

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ACKNOWLEDGMENTS

I would like to thank The Sally McDonnell Barksdale Honors College for the opportunity to pursue undergraduate research. I would also like to thank Dr. Randy Wadkins, my research advisor, for all of his help along the way. I appreciate the help from my second and third readers, Dr. Brad Jones, and Dr. Jason Ritchie. I would like to thank the Department of Chemistry and Biochemistry for the laboratory and resources. I would like to thank my parents for their continuous support since the very beginning. Without the help of all, this would not have been possible.

ABSTRACT

SHELBY CLAIRE LIDDELL: The Effect of Polyamines on I-motif Thermal Stability
(Under the direction of Randy Wadkins)

Polyamines are present inside cells at varying concentrations anywhere between 10 mM and 60 mM, and because of their molecular structure they contribute a positive charge to the interior of the cell. There has been previous evidence that the concentrations of polyamines have an effect on the stability of the DNA strands, which can affect the cellular processes. The particular DNA conformation studied is the i-motif, which is formed by intermolecular folding in cytosine rich strands. The i-motif studied is C20T, which is found in the promoter region of the oncogene, c-myc. Stabilization of c-myc can have an effect on the transcription of this oncogene. Because the formation of i-motifs is energetically unfavorable, scientists are studying molecules and ligands that can stabilize the i-motif making its formation more energetically favorable. Polyamines are hypothesized to stabilize the i-motif. The stability of the i-motif was measured by melting this single stranded DNA with varying concentrations of three different polyamines. The observed increased melting point of the i-motif signified an increased stability of the DNA i-motif structure.

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INTRODUCTION

Polyamines are ubiquitous aliphatic cations with low molecular weights (Fig. 1). Polyamines are synthesized in the cell enzymatically from the amino acid arginine. The three most abundant natural polyamines are spermine, spermidine, and putrescine. Under normal physiological conditions, the amino groups of polyamines are protonated, and thus they contribute positive charge to the inside of the cell (1). The intracellular level of polyamines and other ions, such as sodium and magnesium, contributing to the cytoplasm composition appears to be crucial to the integrity and normal functioning of the cell (2). Eukaryotic and Prokaryotic cells appear to highly regulate and control the levels of polyamines around a 10 mM to 60 mM range. Cells highly control and regulate their polyamine synthesis by the enzyme ornithine decarboxylase (ODC). When ODC expression is manipulated, the level of polyamines in the cell is affected. For example, treatment of cells with difluoromethylornithine- (DFMO), an inhibitor of ODC, results in a decrease in cellular polyamine levels (3).

Polyamines are involved in many vital cellular processes such as proper functioning of ion channels (4). Polyamines have also been shown to affect gene transcription at the transcriptional, post-transcriptional, and translational levels. When looking at a polyamine's role at the transcriptional level, Thomas et al., found that if ODC, a biosynthetic enzyme that controls the production of polyamines, is inhibited, transcription levels of the oncogenes *c-myc*, *c-fos* are decreased (5). After transfecting a plasmid coding for ODC overexpression into cells, ODC was

overexpressed, resulting in vigorous tumors (5). From this data, it was suggested that oncogenes possibly increase the transcription rate of ODC resulting in increased polyamine levels. Also it was inferred that, in turn, increased levels of polyamines in some way affect oncogene expression (5).

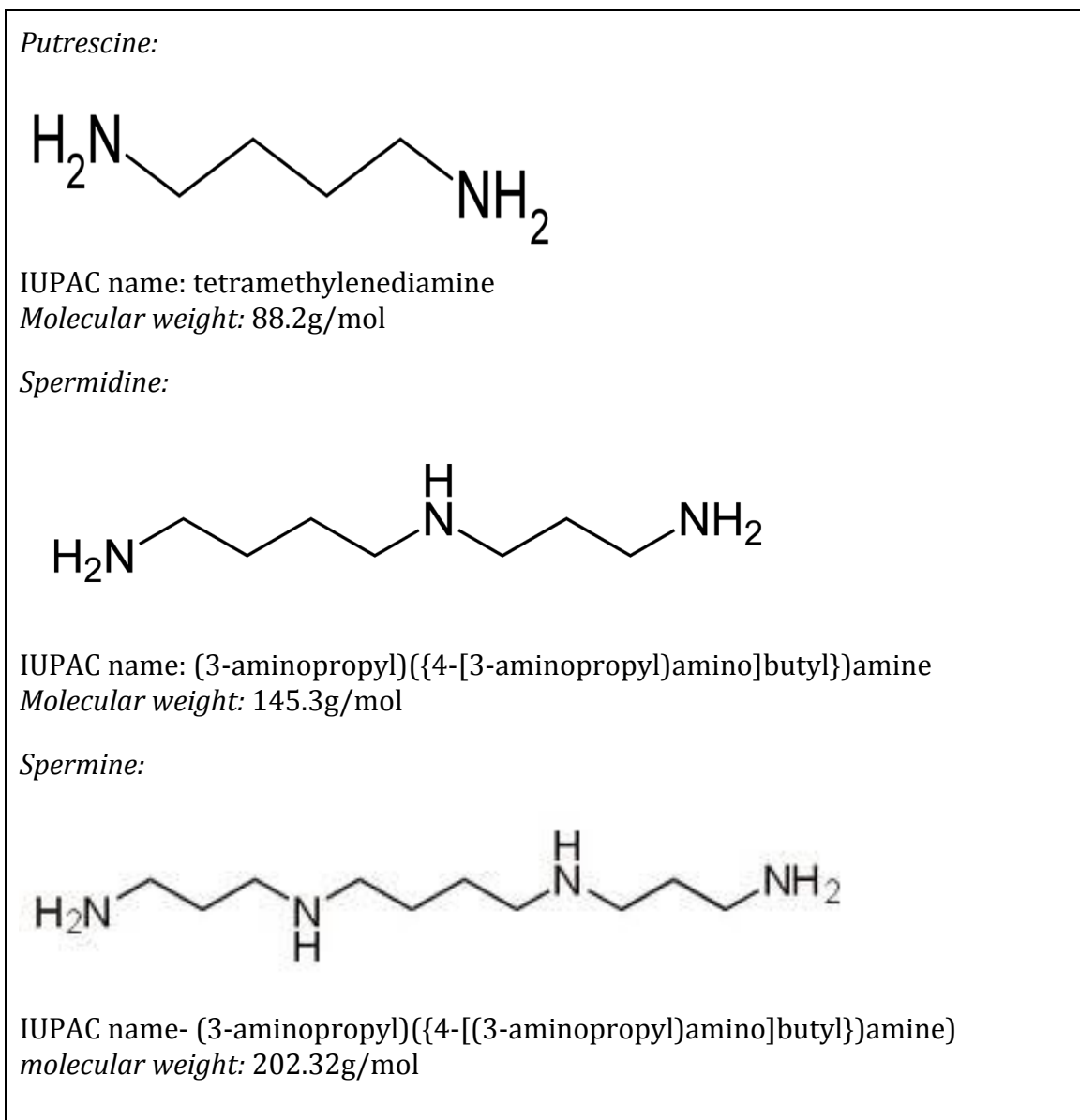


FIGURE 1 MOST ABUNDANT BIOLOGICAL POLYAMINES: The molecular structure, molecular weight, and the IUPAC name for putrescine, spermidine, and spermine.

For years polyamines have been studied and shown to affect the physical properties of nucleic acids. Polyamines have previously been studied for their effect on normal double strand Watson-Crick DNA. Spermine and also an unusual pentamine (caldopentamine) both affect the melting point of Watson-Crick DNA strands and can drive the B-form (right-handed helix) to Z- form (left-handed helix) structural transition (6). The distinct differences in B-DNA as opposed to Z-DNA can affect the sensitivity of DNA to proteins, carcinogens and various ligands (5). The separation of charges from the amino groups in polyamines plays a role in the effect the polyamine can have on Watson Crick strand DNA (6). The four-carbon chain in spermine appeared to affiliate with particular locations in the B-DNA major groove (6). In other previous studies, RNA tertiary structure stability was examined with diamines (2). In addition to the familiar Watson-Crick structure, DNA can adopt other unusual conformations. For example, single stranded DNA rich in the cytosine can adopt a four-stranded structure known as an i-motif (Fig. 2). I-motif structures are pH dependent and have low thermal stability. The pH dependence is shown in Figure 2. The protonation holds the nitrogenous bases together when the i-motif structure is folded. At higher pHs such as pH 8, the i-motif will exist as an unfolded structure. Replication and transcription are common times when DNA can exist as single stranded. As a single stranded molecule, the i-motif forms by the strand folding onto itself and it is held together by hydrogen bonds (Fig. 2). The single DNA strand used in this experiment is C20T, with the sequence,

5'TTCCCTACCCTCCCCACCCTAA3'.

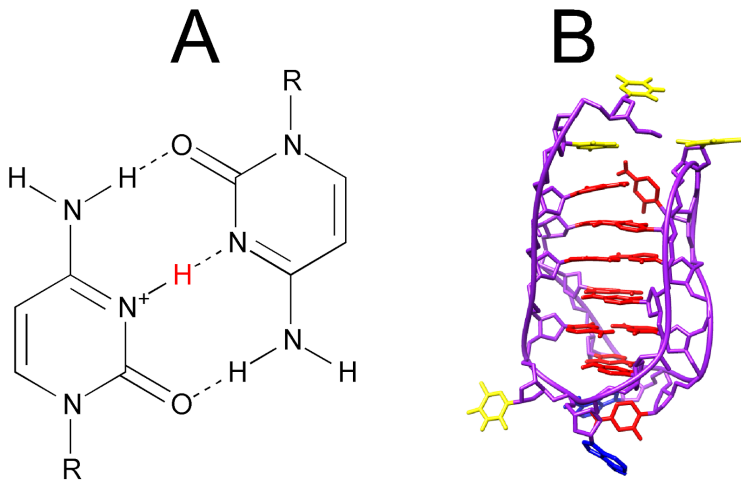


FIGURE 2 NITROGENOUS BASE PAIRING AND I-MOTIF SCHEMATIC: Part A of this figure displays a schematic of how the nitrogenous bases bond together when the strand of DNA folds on itself into an i-motif. The structure is stabilized by a shared proton, and thus is pH dependent. Part B of this figure portrays the i-motif structure in a 3D model.

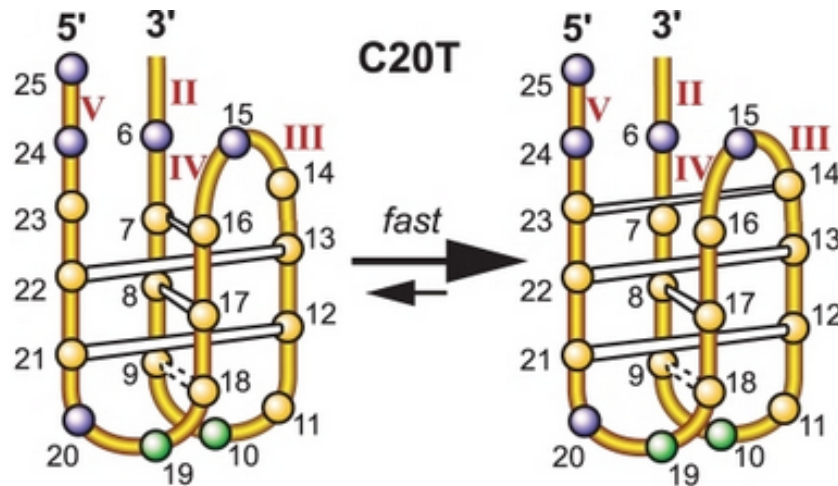


FIGURE 3: ALTERNATIVE INTERMOLECULAR BONDING IN C20T: A schematic displaying the carbons in the C20T i-motif structure. Both of these images portray possible different intermolecular bonding in the C20T i-motif. The equilibrium lies far to the right.

A rationale for studying the effect of polyamines on the thermal stability of i-motif structures is a question posed by a previous study in 2013 (2). Draper et al

performed a study comparing the putrescine interactions with magnesium cations in RNA tertiary structures of a bacterial cell (2). Four different bacterial RNA structures were studied, each with a tertiary structure that differed in ionic interaction. It was found that when putrescine ions were present along with magnesium ions the stabilizing effects of putrescine decreased, but stabilization of the RNA strand was seen when solely putrescine was present (2). The bacterial RNA that was used seemed to fold similarly to the C20T i-motif. So how could a C20T i-motif be stabilized with putrescine and the structurally similar polyamines: spermine and spermidine? Will the same effect from Draper et al., (2) be observed when using an i-motif structure as opposed to bacterial RNA structure? (2). The question posed is whether putrescine can stabilize i-motif structures without magnesium or potassium cations.

Another rationale for studying i-motifs and polyamines is the correlation studies have shown between polyamine levels and cancer (8,9). Rapidly growing and proliferating cells exhibit elevated levels of polyamines (8). MCF-7 breast cancer cells and Chinese hamster ovaries were studied and the importance of polyamines in the cell cycles was determined (9). The G1, G2 phases exhibited elevated levels of polyamines in MCF-7 breast cancer cells that were synchronized in G1 phase (9). It was found that a hormone, estradiol, increased the intracellular levels of polyamines (9). Quadruplex DNA, i-motifs, and polyamines have previously been associated with cancer in different ways. Guanine rich strands of DNA can easily form intermolecular structures called quadruplexes (7). These G rich strands, which are complementary to the C rich stranded i-motifs, have been

shown to exist in the promoter regions of cancer genes such as c-MYC, Bcl-2, VEGF and c-KIT (6).

The formation of these i-motif structures is energetically unfavorable, so scientists are trying to determine what stabilizes the i-motif structure. Some hypothesized stabilizing structures or molecules are proteins, various ligands and polyamines. This study is focusing on polyamines and if they stabilize the i-motif structure. The hypothesis tested in this thesis is that polyamines can bind to i-motif structures and stabilize them thermally. If such a process occurs in cells, it could be one mechanism by which polyamine expression affects oncogene product levels.

EXPERIMENTAL:

The three polyamines were studied separately by adding known concentrations to a DNA and buffer solution to observe the change in the melting point of the i-motif. Before thermal melting studies were conducted, the samples were heated to approximately 80 °C on a heating block followed by an absorbance scan at 80 °C and 25 °C to ensure the presence of DNA in the sample, evidenced by a peak around 260 nm that increased with temperature. The DNA C20T stock solutions were prepared using TE buffer pH at 8. The absorbance of the C20T stock sample was used to find the exact concentration of the stock DNA. Calculations were performed to determine the amount of DNA to add to the cuvette to ensure a 2.0 μM total concentration. The polyamine solutions were prepared as 1 Molar concentration stock solutions. The melting of i-motifs was conducted on a Cary 100 UV Visible spectrophotometer. The temperature range began at 25 °C and ended at

70 °C. The heating rate was 1.2 °C per minute and the temperature was held for 1.2 minutes at the new temperature before measuring absorbance. A Circular Dichroism spectra was also measured for the samples to determine if the i-motif was present after the polyamine was added (Fig. 8-9). The buffer used was a 20 mM Cacodylate buffer at pH 5.4. The first thermal-melt was the control without any polyamines, or simply DNA and buffer. Following this, melts using 0.5 mM and 1 mM polyamine concentration solutions were obtained, always using buffer and polyamine as a reference in the UV Vis. After the 0.5 mM and 1 mM polyamine concentrations were completed, the collected melting curves and melting points (T_m) acted as a starting point to determine if the melting point was affected by the addition of polyamines to the solutions. Concentrations of polyamine were increased until no further change in T_m was observed.

RESULTS

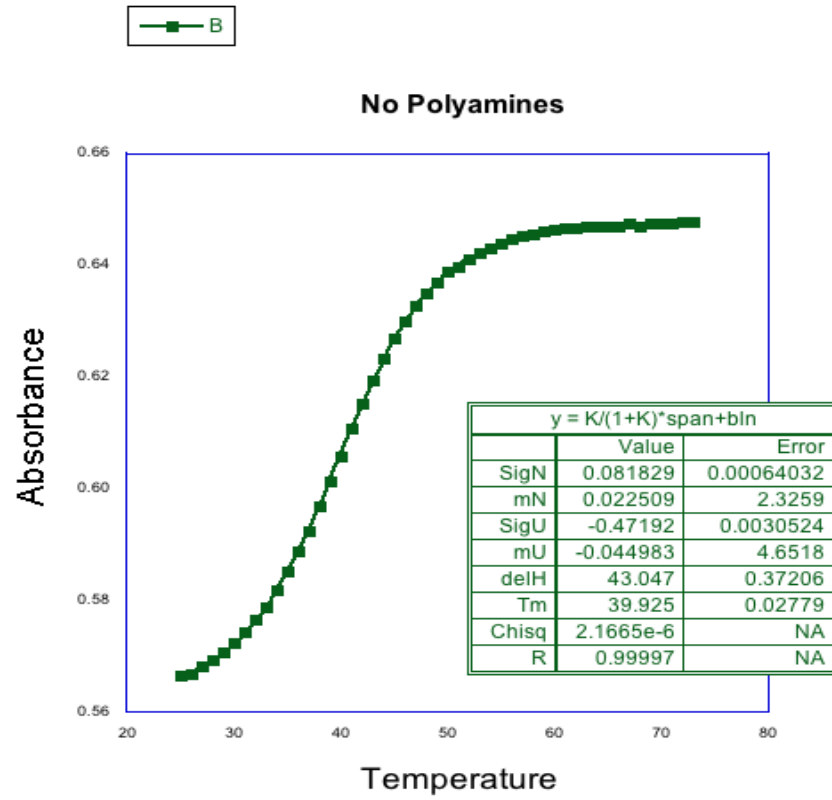


FIGURE 4 NO POLYAMINES MELTING CURVE: This melting curve is used as a control or reference and displays the melting point for the DNA i-motif structure in the absence of polyamines.

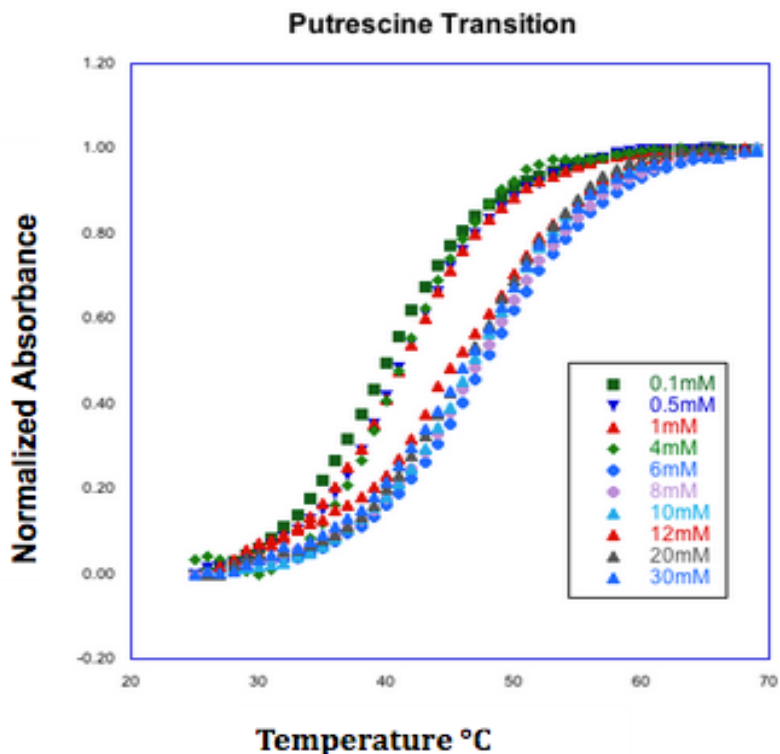


FIGURE 5 PUTRESCINE MELTING CURVE TRANSITION: The transition graph for putrescine displaying the increasing concentrations (left to right) corresponding with increased melting temperatures.

TABLE 1: PUTRESCINE MELTING TEMPERATURES: Collection of melting points and corresponding concentrations for putrescine.

Concentration of Polyamine (mM)	Melting Temperature (°C)	Error from Curve Fit
0.0	39.9	± 0.03
0.5	41.5	± 0.07
1.0	41.3	± 0.04
4.0	41.4	± 0.08
6.0	48.0	± 0.04
8.0	47.5	± 0.13
10.0	47.0	± 0.05
12.0	46.1	± 0.12
20.0	46.6	± 0.07
30.0	46.5	± 0.06

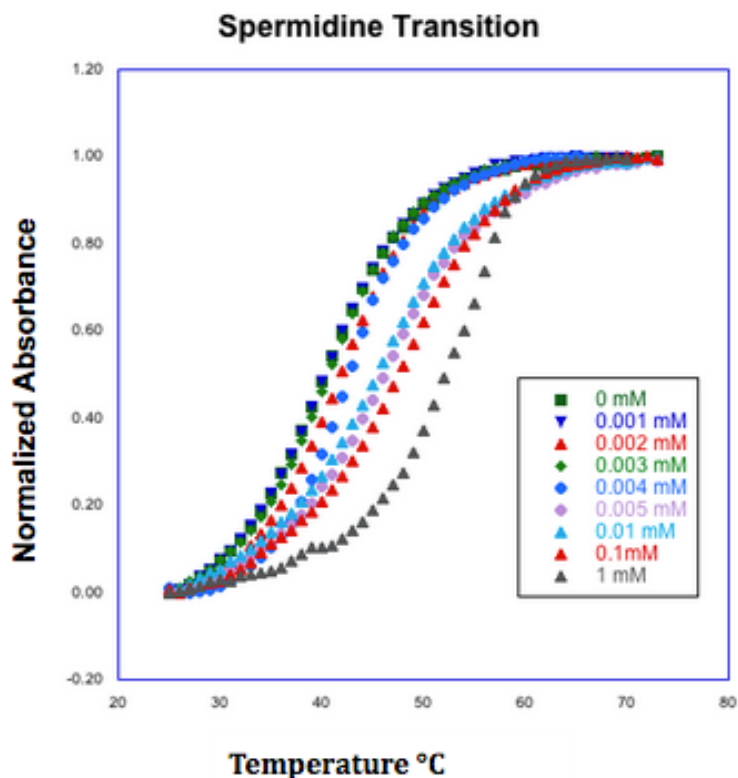


FIGURE 6 SPERMIDINE MELTING CURVE TRANSITION: The transition graph for Spermidine displaying the increasing concentrations (left to right) corresponding with increased melting temperatures.

TABLE 2: SPERMIDINE MELTING TEMPERATURES: Collection of melting point values and corresponding concentrations for spermidine.

Concentration of Polyamine (mM)	Melting Temperature (°C)	Error from Curve Fit
0.000	39.9	±0.02
0.001	40.1	±0.03
0.002	41.8	±0.03
0.003	40.5	±0.04
0.004	42.5	±0.05
0.005	45.9	±0.06
0.010	45.4	±0.07
0.100	47.7	±0.13
1.000	52.5	±0.19

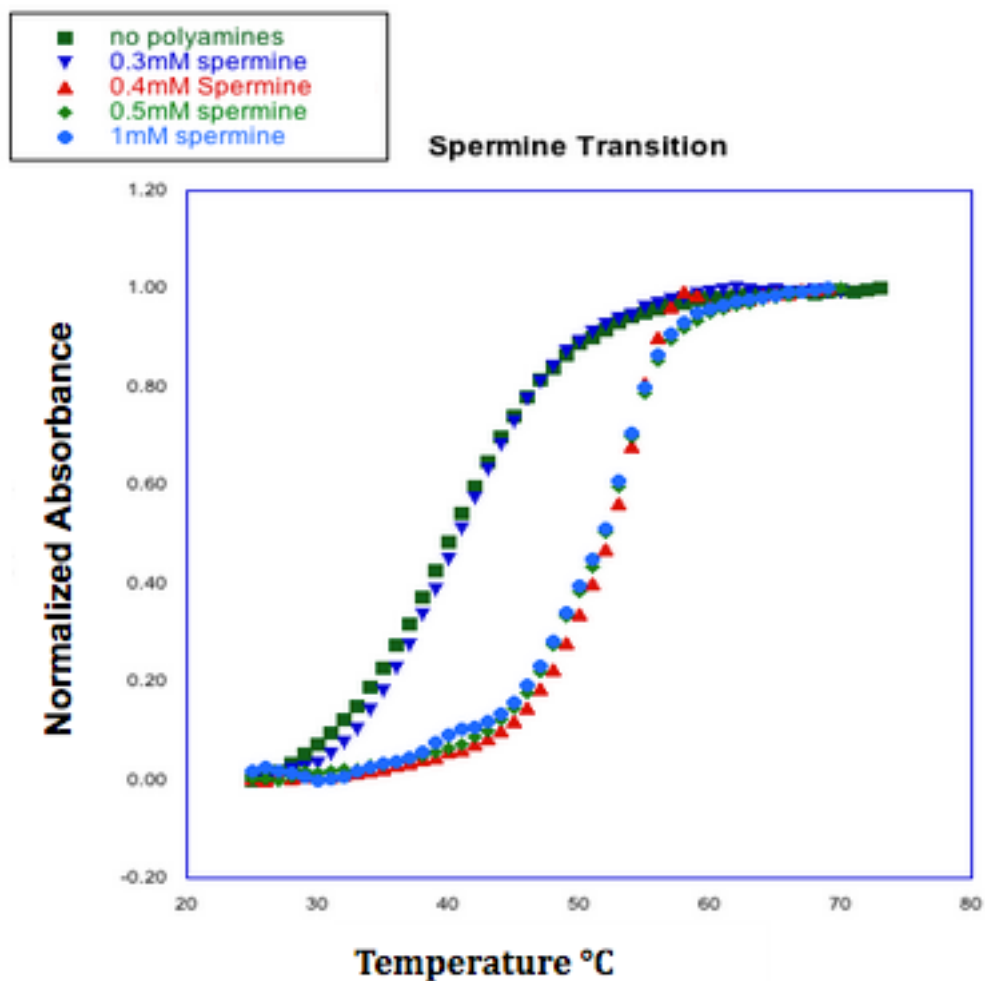


FIGURE 7 SPERMINE MELTING CURVE TRANSITION: The transition graph for spermine displaying the increasing concentrations (left to right) corresponding with increased melting temperatures.

TABLE 3: SPERMINE MELTING TEMPERATURES: Collection of melting point values and corresponding concentrations for spermine.

Concentration of Polyamine (mM)	Melting Temperature (°C)	Error from Curve Fit
0.0	39.9	± 0.03
0.3	40.7	± 0.04
0.4	52.4	± 0.10
0.5	51.6	± 0.12
1.0	51.5	± 0.15

CD Spectra of C20T and polyamines

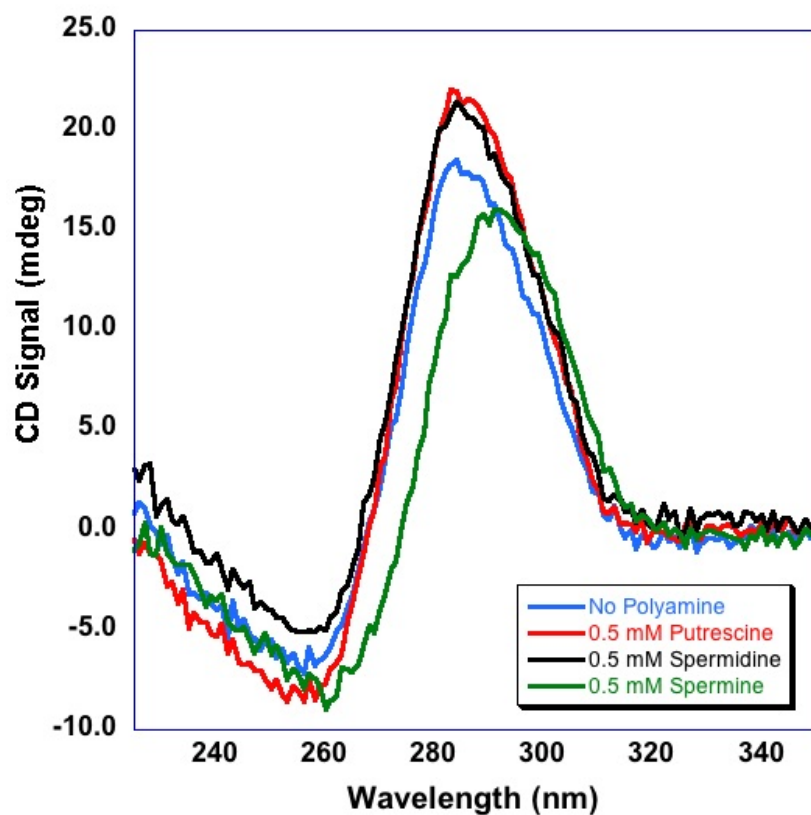


FIGURE 8: CD SPECTRA OF C20T AND POLYAMINES: Circular Dichroism spectra of the C20T i-motif structure followed by scans 0.5 mM concentration of each polyamine.

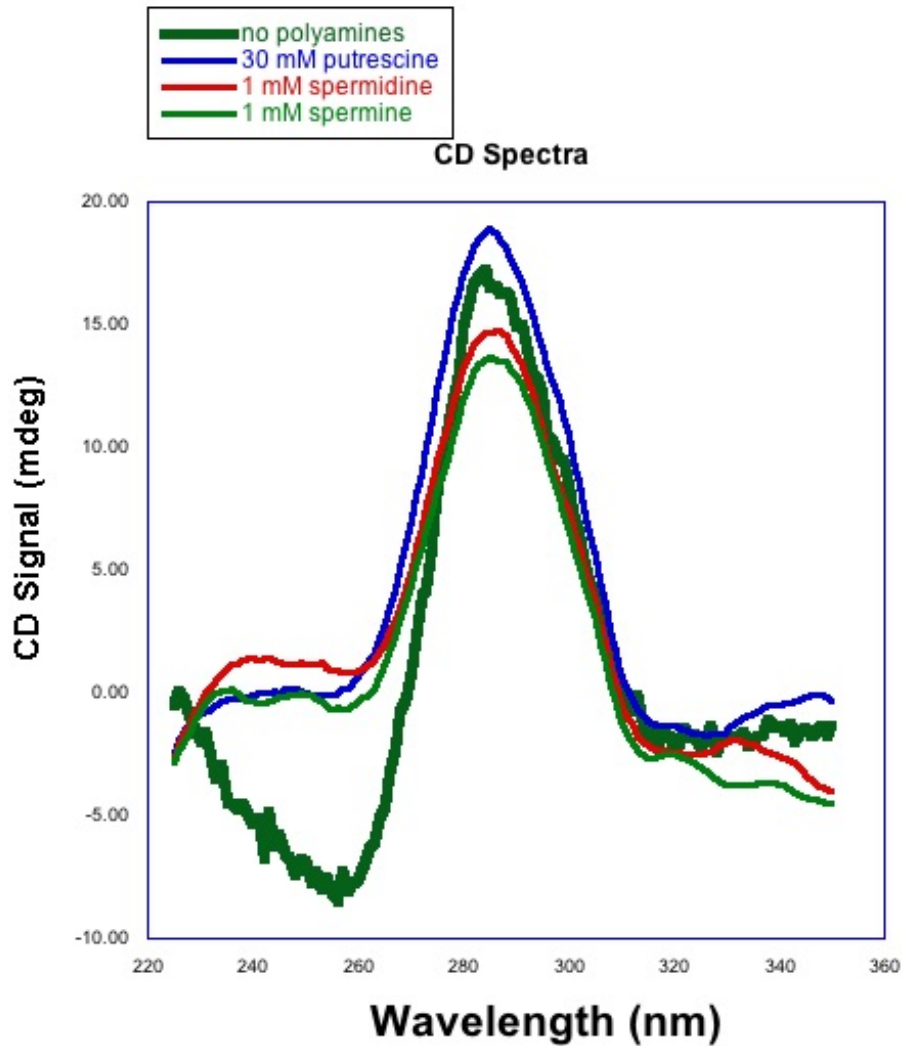


FIGURE 9: CD SPECTRA OF C20T AND MAXIMUM POLYAMINE CONCENTRATIONS: Circular Dichroism scans of 30mM putrescine, 1 mM spermine, and 1mM spermidine with the C20T i-motif.

DISCUSSION:

Overall, the addition of polyamines to the DNA i-motif solution resulted in an increased melting point. As expected using the same sequenced strand of DNA when performing thermal scans with spermidine, putrescine, and spermine, the melting points should reach a common peak temperature. This was proven when

spermine and spermidine reached approximately 51 °C and putrescine reached a maximum temperature of approximately 47 °C.

The transition from folded to unfolded state was observed in each melting curve obtained. Increasing temperatures causes the i-motif to unfold from the helical structure to single stranded DNA. The T_m , found from fitting the data on Kaleidagraph software, is the temperature at which this transition occurs. An increase in T_m shows an increase in the stability of the i-motif structure because the more heat required to denature the strand, the more stable the strand. Looking at the results, putrescine appeared to bind the loosest. A higher concentration of putrescine was needed to see an effect on the T_m . This could be a result of putrescine's molecular structure. When compared to spermine and spermidine, it is smaller having only four carbons as opposed to spermine with ten carbon atoms and spermidine with seven carbon atoms (Fig. 1). Spermidine appeared to bind the tightest to the i-motif because a change in melting temperature was seen with a much smaller concentration (Fig. 6). An interesting point could be maybe spermidine "fits" the best in the i-motif groove. The length of the groove is approximately 14 to 19 Angstroms long. The length of one spermidine molecule is approximately 8 Angstroms. An explanation for the tight binding of spermidine to the i-motif could be that 2 spermidine molecules (16 Angstroms) "fit" best into the groove of the i-motif. Because the backbone of the i-motif is negatively charged from the phosphate groups and the polyamines exist as positive cations, these structures are electrostatically attracted.

A CD spectra of the control, containing no DNA just buffer and polyamine, was obtained followed by three scans after adding 0.5 mM of spermine, spermidine and putrescine separately (Fig. 8). Figure 8 appears to prove the existence of the i-motif structure in the presence of 0.5 mM of each polyamine. Previous scans in the lab have given the expected CD scan of the i-motif structure. The positive peak at approximately 290 nm and the negative peak at approximately 260 nm signify a normal CD spectrum of the i-motif structure. Following the completed melting studies, a CD spectrum was performed, this time using the highest concentration of each polyamine that was needed to reach the peak T_m . Interestingly this spectrum lacked the negative peak around 260 nm (Fig. 9). To rule out experimental error, the spectra was performed three times on various days. The spectra still appeared to be inconsistent with the known i-motif spectra previously obtained (Fig. 8-9). But because the positive peak was there and had not shifted, it could be deduced that the polyamines possibly had an effect on the structural integrity of the i-motif structure. This could pose further research to determine exactly how the integrity of the i-motif structure is disturbed or disrupted by polyamine concentrations higher than 0.5 mM.

FUTURE STUDIES:

Following this study, future scientists could observe if the stability of the C20T i-motif structure is seen with the addition of magnesium ions and potassium ions. The inverse relationship of the stability of Watson Crick DNA was observed when magnesium ions were present with putrescine (2). The stability of the i-motif

could be studied when polyamines are present with other naturally occurring ions in the cell to see if the i-motif favors the stabilization by magnesium and potassium ions as opposed to polyamine ions.

Another further research project could be to determine if the addition of polyamines affects the pH dependence of the i-motif structure. Because at pH 5.4 the i-motif is in a folded state due to hydrogen protonation of the base pairs (Fig. 2), maybe polyamines can stabilize the structure and the i-motif could still be folded at pH's higher than 5.4.

CONCLUSION:

In conclusion, an increase in stability, seen through an increase in T_m , was observed when each polyamine was added to the i-motif solution. The amount of polyamines used to see a change in melting temperature fell at physiologically relevant conditions, which are not higher than 60 mM. Putrescine appeared to bind the loosest to the i-motif structure as seen by the significantly higher concentration needed to see a change in the melting temperature. Spermidine appeared to be the polyamine that bound the most tightly because the increase in T_m was observed at a much smaller concentration (Table 2). This could be due to both the length in Angstroms of the i-motif groove and spermidine. Because an increase in stability is seen this can lead to further research and studies to determine how transcriptional products of the c-myc gene are affected when stabilized by polyamines. This data could also be one step further to determine how and why cancerous tumor cells exhibit higher levels of polyamines than normal functioning cells.

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