

INVESTIGATING THE INTERACTIONS OF AZACYANINES WITH
TRIPLEX NUCLEIC ACIDS

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

INVESTIGATING THE INTERACTIONS OF AZACYANINES WITH TRIPLEX NUCLEIC ACIDS

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Triple helical nucleic acid structures have gained substantial attention with the emergence of anti-sense and anti-gene strategies. In anti-gene strategies, the idea is to form a triple helical structure in the gene by binding a third nucleic acid sequence into the major groove of that gene. Therefore, the binding of transcription proteins, polymerases, *etc.* to that gene could be prevented to ultimately result in the inhibition of the transcription. One of the challenges in anti-gene strategies is the fact that the third strand's binding is weak for most triplex forming sequences and it dissociates from the stable duplex easily. One solution to get over this problem is to increase the stability of triplex structures by using small molecules. The design and synthesis of such triplex stabilizing small molecules have drawn considerable in the last decades due to the use of these molecules as therapeutic agents. Within this context, the importance of alkyl chain length and structure on the benzimidazole derivatives of Azacyanine molecules which tightly and selectively bind to specific triple helix structures have been revealed by the UV-vis, CD and Fluorescence spectroscopies via the thermal denaturation and titration studies. Furthermore, the effect of pH and salt concentration on polyd(A)·polyd(T)·polyd(T)-Azacyanine interactions have also been studied by CD spectroscopy. With viscosity measurements, the binding mode of the Azacyanines to polyd(A)·polyd(T)·polyd(T)- triplex have been determined.

Keywords: Azacyanine, Triple Helix Structures, Anti-Gene, Anti-Sense

ÖZ

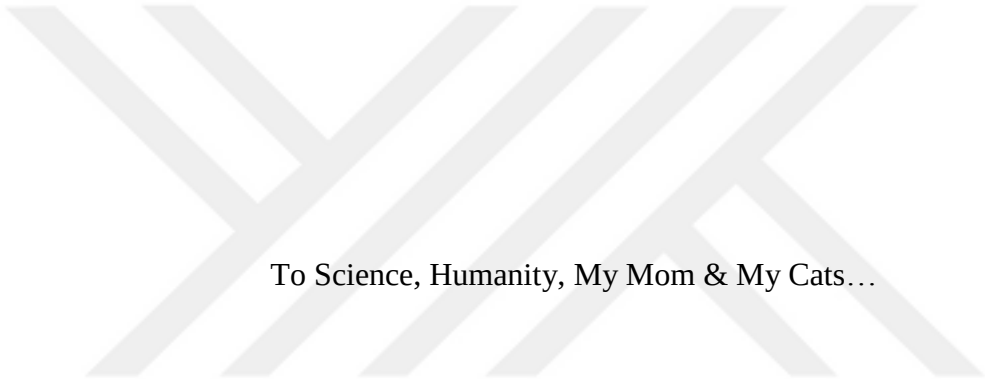
ÜÇLÜ NÜKLEİK ASİT YAPILARININ AZASIYANİNLERLE ETKİLEŞİMLERİNİN İNCELENMESİ

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Üçlü DNA sarmal yapıları anti-gen ve anti-sense stratejilerinin ortaya çıkması ile önem kazanmıştır. Anti-gen stratejilerinde temel hedef örneğin transkript olacak genin büyük oluğuna (major groove) üçüncü bir dizinin bağlanması ile üçlü sarmal yapı oluşturularak, örneğin transkripsiyonda görev alan proteinlerin, polimerazların vb. gene bağlanmalarını ve görevlerini yerine getirmelerini engellemektir. Anti-gen stratejilerindeki en temel sorunlardan biri oluşturulan üçlü sarmal yapıların yeterince sağlam olmaması ve üçüncü dizinin kolaylıkla ikili sarmaldan ayrılmasıdır. Bu sorunun bir çözümü üçlü sarmal yapıların sağlamlığını bu yapılara bağlanan küçük molekülleri kullanarak arttırmaktır. Bu çalışma kapsamında UV-vis, CD ve Floresans spektroskopileri kullanılarak belirli üçlü sarmal yapılarına kuvvetli ve seçici bir şekilde bağlandığı tespit edilen benzimidazol türevi Azasiyanin moleküllerinin polyd(A)·polyd(T)·polyd(T) yapısının kararlılığının arttırılmasında benzimidazol halka yapısındaki alkil zincirin uzunluğunun ve yapısının önemli bir rol oynadığı termal denatürasyon ve titrasyon çalışmaları ile açığa çıkartılmıştır. Ayrıca, proje kapsamında pH ve tuz derişiminin polyd(A)·polyd(T)·polyd(T)-Azasiyanin etkileşimleri üzerindeki etkisi CD spektroskopisi ile belirlenmiştir. Viskozite ölçümleri ile de Azasiyaninlerin polyd(A)·polyd(T)·polyd(T)-sarmalina bağlanma modu belirlenmiştir.

Anahtar Kelimeler: Azasiyanin, Üçlü Sarmal Yapılar, Anti-Gen, Anti-Sens



To Science, Humanity, My Mom & My Cats...

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LIST OF ABBREVIATIONS

ABBREVIATIONS

A_{260} = Absorbance at 260 nm; A_{336} = Absorbance at 336 nm

ATP = Adenosine triphosphate

Aza = Azacyanine

BPES = Buffered phosphate EDTA with sodium chloride

bp = Base pair

CD = Circular dichroism

DNA = Deoxyribonucleic acid

DMSO (C_2H_6OS) = Dimethyl sulphoxide

dsDNA = Double stranded deoxyribonucleic acid

EDTA = Ethylenediamine tetra acetic acid

HCl = Hydrochloric acid

Mg^{2+} = Magnesium

NMR = Nuclear magnetic resonance

Na_2HPO_4 = Disodium hydrogen phosphate

NaH_2PO_4 = Sodium dihydrogen phosphate

Na_2EDTA = Disodium EDTA

NaCl = Sodium chloride

NaOH = Sodium hydroxide

RNA = Ribonucleic acid

ssDNA = Single stranded deoxyribonucleic acid

T_m = Thermal denaturation temperature

TFO = Triplex forming oligonucleotide

UV-vis = Ultraviolet-visible



LIST OF SYMBOLS

SYMBOLS

$^{\circ}\text{C}$ = Centigrade degree

cm = Centimeter

ϵ = Epsilon (for extinction coefficient)

$^{\circ}$ = Degree

g = Gram

L = Liter

λ = Lambda

m = Mass of the chemical

μL = Microliter

μM = Micromolar

mL = Milliliter

mM = Millimolar

min = Minutes

M = Molar

nm = Nanometer

CHAPTER 1

INTRODUCTION

1.1 DNA Structure

The findings based on the research within the past 100 years demonstrate that every single chromosome of an organism contains linearly arranged genes located on particular locations. The leading work of Avery, McLeod and McCarty in 1944 states that genes are made of deoxyribonucleic acid (DNA) as their building blocks rather than proteins (Miller and Therman, 2001).

The pairing of DNA nucleobases was explained in 1953 by Watson and Crick with a double-helix model of DNA taking the advantage of X-ray crystallographic data of Wilkins and Franklin in which DNA comprises two twisted nucleotide strands. The strands of nucleotides bind through stacking interactions and hydrogen bonding with two hydrogen bonds for Adenine (A)- thymine (T) and three hydrogen bonds for guanine (G)- cytosine (C). According to the model suggested by Watson and Crick, the nucleotides have complementarity to each other on which the sequence of each nucleotides on each chromosome carries a particular piece of genetic information. According to Crick, successive triplets of bases in DNA encodes this information in order to transform it into the messenger RNA (mRNA) (Miller and Therman, 2001).

The developmental stages are decided based on the products of nucleotide sequences on the genome. The information to generate all the proteins needed for an organism is kept in DNA while many diverse roles essential for the development and functioning an organism are played by proteins. Regulation of biotic molecules namely receptors, transcription factors in signal transduction pathways and

controlling structural organization for metabolic reactions have been made by the help of proteins (Krebs et al. 2014).

Elucidating the double-helical structure of DNA with four linearly arranged nucleobases help to understand the significance and crucial function of DNA. All the essential information to develop an organism from both sperm and egg is encoded in DNA using four simple bits. Pathways such as regulation of gene expression, translation, DNA repair and replication have evolved around DNA (Sinden, 1994).

The physical preservation of all the information is facilitated using the structure of DNA as each hydrogen bond between the nucleobases is found inside the double helix, whereas the phosphate backbone occurs outside the helix. The unique structure of DNA can be recognized through studying the interactions of proteins with DNA which is processed in a sequence-independent manner. The probation of a sequence-specific protein (like repressor) of a DNA is done by the unwinding it, providing an acces to the genetic information for certain processes like replication and transcription (Sinden, 1994).

1.1.1 Double Helix

B-form DNA (known as Watson-Crick structure) is composed of two winding polynucleotide strands with a right-handed twist in which pentose sugar of 2-deoxyribose is present. Phosphate groups held the sugars together forming the phosphodiester bonds between the third and fifth carbon atoms of the contiguous sugar rings. The directional backbone of phosphodiester that is present in individual nucleotides connects the 5-phosphoryl group of one nucleotide to the 3-hydroxyl group of the next nucleotide.

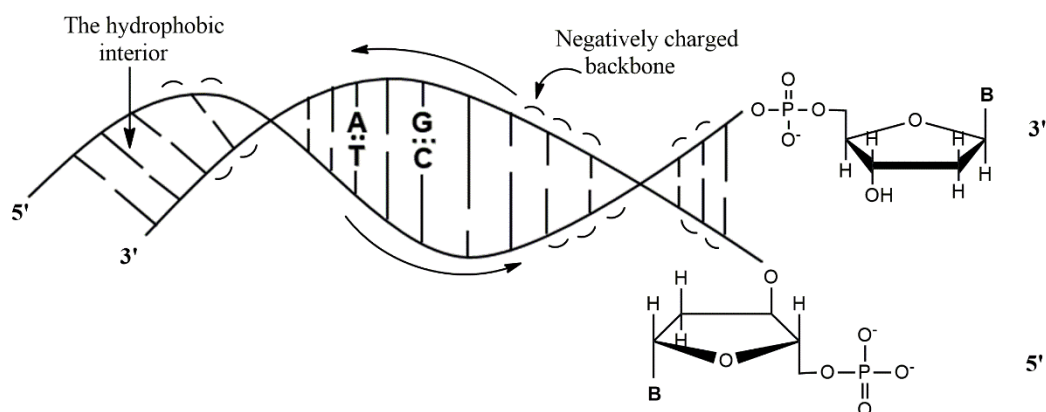


Figure 1.1. Structural features of DNA

(A = Adenine = purine, T = Thymine = pyrimidine (DNA only), G = Guanine = purine, C = Cytosine = pyrimidine, U = Uracil = pyrimidine (RNA only), AT/GC base pairs)

1.1.1.1 Antiparallel Strands

Two strands expand in antiparallel directions in DNA (Figure 1.1) in which the sequence of template strand binds to from the 5' end. Also, the sequence of the complementary strand binds to the 3' end as phosphate backbones of the two strands expands in the contrary directions. The double helix is held as a whole by the stacking interactions and hydrogen bonding between the complementary bases (Bloomfield, Crothers and Tinoco Jr., 1999).

1.1.1.2 Major Groove–Minor Groove

Two grooves are formed as minor and major groove by the winding of the two DNA strands (Figure 1.2) as the bases of DNA bind around the axis of helix with an angle about 120° instead of 180° . In the major groove, the two backbones are located apart from each other, whereas they are closer in the minor groove. The two grooves differ from each other in terms of depth, width and size. Accordingly,

the solvent engages in interactions with the major groove more than the minor one paving the way for proteins to experience numerous sequence-specific interactions with the DNA throughout the major groove since it carries the sequence information.

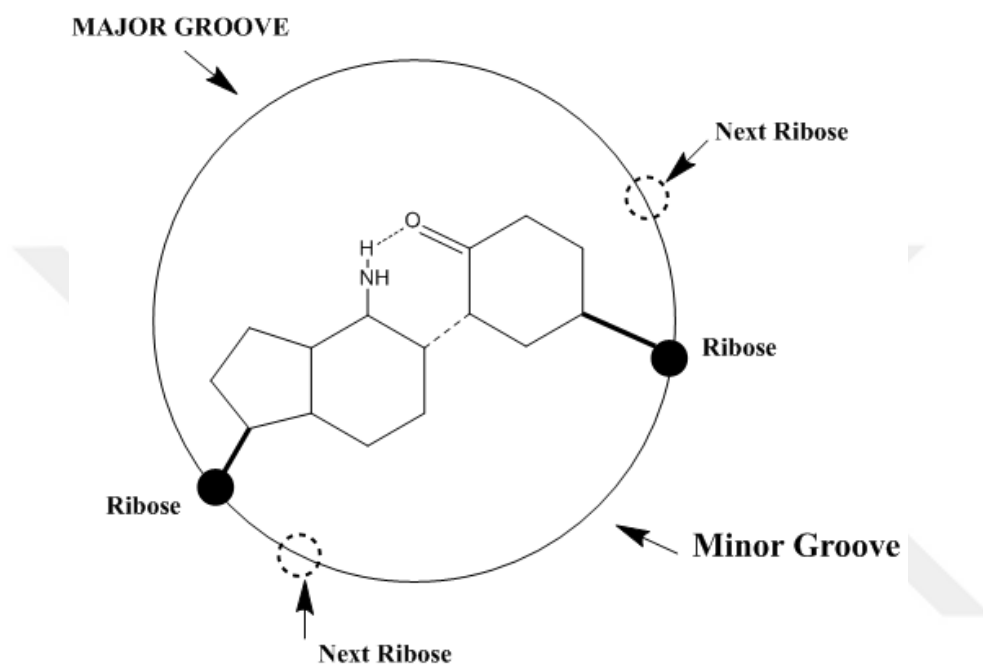


Figure 1.2. Major groove–minor groove

1.1.1.3 A-, B-, and Z-DNA

DNA occurs more commonly as the B-form conformation in cellular solutions as it is illustrated in Figure 1.1. However, the other structures that are formed to shape like double-helical DNA structures can possibly occur under different solution conditions and specific sequences. The A and B conformations can be distinguished based on how much their helices rise per turn. Even though they are both right-handed, the nucleobases are perpendicular to the helix-axis. Under dehydrating conditions, type A forms as the dominant conformation, while in the

left-handed Z-DNA, phosphate backbones of the two antiparallel DNA strands are held unevenly (Figure 1.3). On the G and C base-paired rich sequences, Z-DNA appears to be the most common structure (Gilbert, 2000).

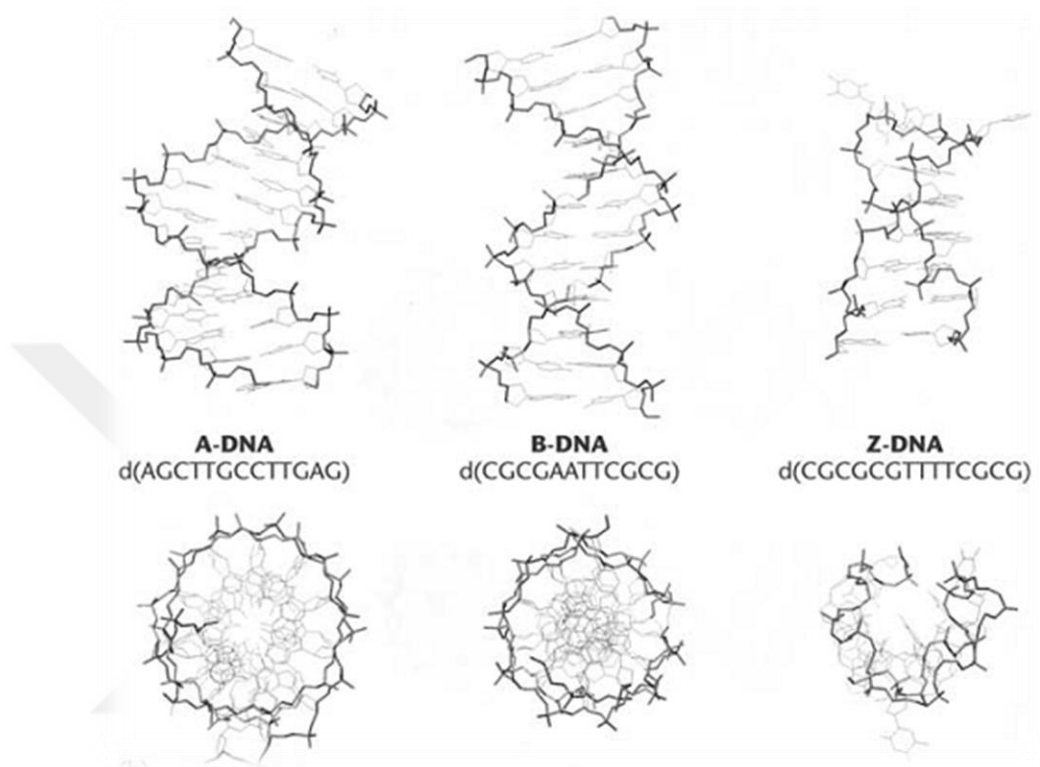


Figure 1.3. A-, B-and Z-DNA

As seen from the side of the helix (above), and looking down the helix axis (below) (Ussery, 2002).

1.1.2 Other DNA Conformations

Nucleic acids can also adopt various conformations other than the double helical conformations. There are multiple donor and acceptor sites for hydrogen bonding on DNA which enable DNA to adopt several conformations such as single-stranded hairpins, parallel-stranded DNA, triplexes, tetraplexes, G-quadruplexes,

and i-motif (Jain and Bhattacharya, 2010). These forms are believed to play a role in regulating gene expression and the onset of diseases (Moriyama et al).

Cruciform DNA (Holliday Junction), and triplex DNA are known to be involved in gene expression, replication, and recombination. Naturally occurring sequences can form triplex DNA and in human genome, there is one triplex DNA in every 50,000 base pairs. The formation of triplex structure can cause DNA double-strand breaks by affecting the progression of replication forks and these double-stranded breaks are used in genome editing as they stimulate short DNA insertions or deletions by homologous recombination. Also, triplex-forming oligonucleotides (TFOs) can bind to double-stranded DNA and inhibit transcription regulating the gene expression (Yamayoshi et al., 2017).

1.1.3 Triple Helix DNA Structures and Their Significance

The presence and the formation of the triple helices were first discovered by Gary Felsenfeld and Alex Rich in 1957 right after the discovery of the duplex B-form DNA by Watson and Crick. Felsenfeld and Rich revealed the structure of AU•U triplex in which the third poly(U) strand binds to the major groove of AU duplex through Hoogsteen base pairing (Felsenfeld and Rich, 1957). Within the same study, they also revealed the stabilization of the triple helical structure by cations like Mg^{2+} and Na^+ (Felsenfeld and Rich, 1957). Subsequently, many different triple helical structures with Hoogsteen and reverse-Hoogsteen base pairing were discovered both in in-vitro and in vivo studies (Mirkin et al., 1987; Hanvey et al., 1988; Wells et al., 1988; Blackburn et al., 2006). The most common types of these base-pairings observed in triplex structures are given in Figure 1.4.

The significance of triplex structures was underestimated until 1990s when a growing interest started upon the proposition of anti-gene strategies. As an instance, Claude H  l  ne (H  l  ne, 1991) suggested that the transcription of a gene could be impeded and that a given chromosome could be intercalated on a certain point by

the transformation of triplex-forming oligonucleotides (TFO) to the cell nuclei (Figure 1.5). Also, Hélène proposed that such anti-gene strategies could be used in triggering apoptosis (normal cell death) or in suppressing certain genes in cancer-cells. Hélène's proposal signified the possible importance of triplex forming oligonucleotides in therapy (Hélène, 1991; Jain et al. 2008).

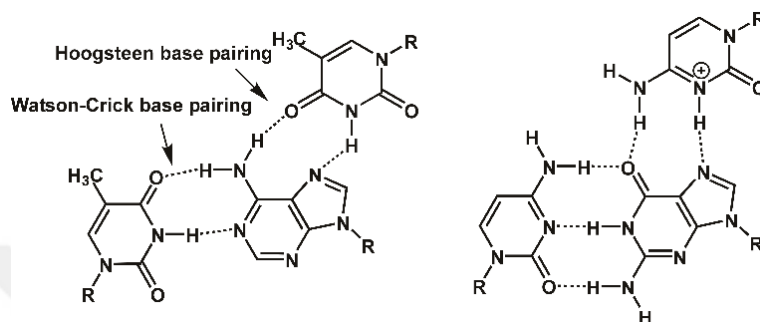


Figure 1.4. Chemical structures of base triplets.

T·A·T (at left) and C·G·C⁺ (at right) were shown. Hoogsteen and Watson- Crick base pairings are presented in Figure 1.4. The “R” represents the sugar group attached to the nucleic acid bases. The figure was taken from Persil Çetinkol, 2008a.

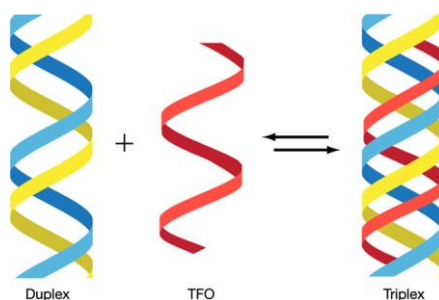


Figure 1.5. Constitution of triplex structure by TFO binding to duplex structure.

The Figure was taken from Jain et al.,2008.

On the other hand, the instability of triplex structures is considered as the main drawback of their possible usage in therapeutic anti-gene strategies. TFOs easily dissociate from the major groove of duplex because of the repulsion between

the negatively charged backbones of the double helix and the TFOs. As stated by Felsenfeld and Rich, ions like Mg^{2+} and Na^+ stabilize the triplex structures up to a point by neutralizing these negative charges (Felsenfeld and Rich, 1957; Maher, 1992; Maher, 1996; Plum, 1997).

1.1.4 H-DNA and Significance of H-DNA

It is known that non-canonical triplex structures form in vivo and cause genetic instabilities. Predominantly, these intramolecular structures that form by the disentangling of duplex structures and binding of the pyrimidine sequence to the major groove of the duplex structure by rotation, are called H-DNA (Figure 1.6). H-DNAs are usually found in the promoter regions of the genes imposing DNA polymerase functions resulting in many genetic diseases, such as. Friedreich's ataxia. (Wells, 1996; Wang and Vasquez 2004, Tang et al. 2011). Furthermore, H-DNA formable region of the promoter residing on human c-MYC gene is known to overlap with the region of failure on c-MYC gene, which causes lymphoma and leukemia (Mirkin and Frank-Kamenetskii, 1994; Jain et al, 2008).

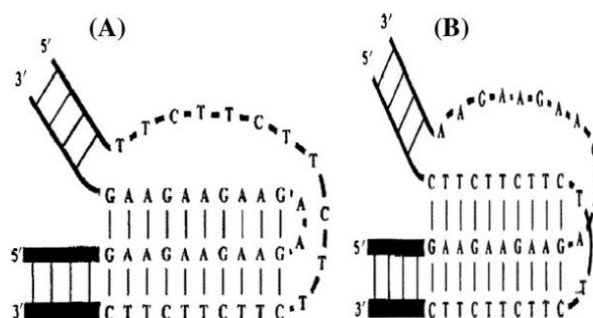


Figure 1.6. Schematic structures of (A) purine (Purine·Purine·Pyrimidine) and (B) pyrimidine (Pyrimidine·Purine·Pyrimidine) triplex H-DNA.

The picture was taken from Rajeswari (2012).

There are two classes of intramolecular triple helices in terms of the orientation of the third strand. In the first class, the third strand runs parallel to the duplex purine strand. These helices are generally formed by pyrimidine-rich oligonucleotides and described as T·AT and C⁺·GC triplets. In the second class, the third strand runs antiparallel to the duplex purine strand and generally is a purine-rich sequence. The second-class triplexes include G·GC and A·AT triplets. While the former requires cytosine protonation and is only stable at low pHs, the latter is pH independent. However, both of them require the presence of divalent metal ions, especially Mg²⁺ (Cassidy et al., 1994).

1.1.4.1 Friedreich's Ataxia

Friedreich's Ataxia is a genetic neurodegenerative disease caused by the formation of H-DNA and has an incidence rate of 1/50,000 in population. Normal people have 6 to 34 (GAA)_n·(TTC)_n repeat sequences while patients have between 6 and 1700. These repeat sequences are believed to cause the formation of H-DNA and inhibit FXN gene's transcription and the lower synthesis of mitochondrial frataxin. There isn't any FDA approved drugs known to treat Friedreich's Ataxia (Wells, 1996; Jain and Rajeswari, 2002; Wells, 2008, Rajeswari, 2012).

1.1.5 Triplex Formation by Using Single Stranded DNA

Promoting the DNA triplex formation by the association of a single-stranded-DNA with the double helical DNA via Hoogsteen or reversed Hoogsteen base pairing is another strategy for the modulation of gene expression (Sheng et al. 2013).

This single-stranded-DNA is called the triplex forming oligonucleotide (TFO) and can interfere with essential cellular processes such as transcription and translation (anti-gene and anti-sense strategies by being present on the major groove of the double helical DNA (Jain and Bhattacharya, 2010) and prevent the interactions of double helical DNA with other molecules such as proteins.

The existence of TFOs in biological systems instinctively is the advantage of this strategy while cellular uptake, sensitivity to nucleases, instability of the formed triplex, possibility of self-triplex formation (self-associating structure) and the poor discrimination of DNA from RNA are stated as its disadvantages. It is feasible to introduce chemical modifications such as threose nucleic acid (TNA), peptide nucleic acid (PNA) and locked nucleic acid (LNA) to overcome these disadvantages. Most DNA intercalators are also used to increase the stability of the triplex structure (Sheng et al. 2013).

1.1.6 DNA Stability

1.1.6.1 Hydrophobic Stacking, Hydrogen Bonding and Stability

The DNA double helix is stabilized by hydrophobic interactions resulting from the individual base pairs' stacking on top of each other in the nonpolar interior of the double helix (Figure 1.1 and Figure 1.2). The hydrogen bonds also contribute somewhat to the overall stability of the double helix but more importantly they contribute greatly to the specificity of the formation of the correct base pairing. An incorrect base pairing would not be as stable as the correct base pairing. The hydrogen bonds of the double helix ensure that the bases are paired correctly (Gilbert, 2000).

1.1.6.2 Melting (Denaturation) and Annealing (Renaturation)

One and the easiest way of measuring the stability of the double helix is performing thermal denaturation experiments. Generally, the double helix can be denatured by heating (melting). During denaturation, DNA loses its structure, and the two strands get separated. Most commonly, the melting temperature of a DNA double helix can be determined by monitoring the increase in absorbance at 260 nm via UV-vis spectroscopy. The increase in absorbance with increasing temperature is

called hyperchromicity. DNA denaturation is reversible which means when the DNA solution is cooled down slowly under appropriate conditions, the two strands reform the double helix with the correct base pairing. The process of denaturation and renaturation of the DNA strands to assure the formation of the proper secondary structures is called annealing (Gilbert, 2000).

1.1.7 Factors Increasing DNA Stability

1.1.7.1 Decreased Temperature and Increased GC content

DNA is more stable at the temperatures below its annealing point. The stability of the double helix is also affected by the GC content. The GC base pair has three hydrogen bonds and stacks better while the AT base pair has only two. For these reasons, GC-rich DNA sequences are more stable than AT-rich regions (Gilbert, 2000).

The temperature at which one-half of the absorbance changes is called the DNA melting temperature. The melting temperature indicates how tightly the DNA double helix strands are held together. The melting temperature increases in a direct proportion to the relative number of GC base pairs in the DNA. Likewise, the DNA molecules in which the two strands of the double helix, which are paired properly at each position, melt at higher temperatures than those with two strands which are not perfectly complementary to each other (Hardin et al., 2012).

1.1.7.2 Increased Salt Concentration (Ionic Strength)

The phosphates of the backbone with a negative charge, tend to repel each other. This repulsion between the negatively-charged backbones destabilizes the DNA double helix. Consequently, adequate ionic strength (high salt concentration) shields the negatively-charged phosphates, decreasing the repulsion, and as a result, stabilizes the double helix (Gilbert, 2000). However, denaturation which is the loss

of the natural three-dimensional structure of a macromolecule caused by agents such as heat, extreme pHs, urea, salt, and other chemicals usually leads to loss of biological activity (Hardin et al., 2012).

1.2 Targeting DNA Using Small Molecules

For the past forty years, structural-based drug designs (SBDD) have been performed in order to identify the lead compounds and develop powerful drug candidates. More specifically, proteins have been the target of the lead compounds rather than nucleic acids, but nucleic acids have also found application areas in order to treat viral diseases and cancer.

Along with the knowledge and advancements in nucleic acid chemistry, molecular biology and genetics, nucleic acids have become one of the main targets lately. They take a role in the genetic information storage, replication, transcription, and translation. Their complex structure, molecular mechanism and functions have been revealed and structural information about the numerous ligand-DNA and ligand-RNA complexes have been determined (Sheng et al. 2013).

There are mainly two types of interactions between these small molecules and DNA: Covalent and non-covalent interactions (Rehman et al. 2015).

1.2.1 Covalent Interactions

The covalent interactions are due to the chemical bond formation between the DNA & RNA and metal-containing or organic compounds. Cisplatin, a Pt-containing compound, is one of the most widely used and effective anticancer drugs approved by FDA but it targets both cancer and normal cells. The possible biological targets of this drug have been determined; its mechanism of actions have been elucidated and its new derivatives have been designed. It can interact with many components in the cell, and the result of the Pt-DNA interactions impedes many

downstream biological processes and takes the control of the cell. Major groove of Pt-DNA is compressed and minor groove is widened making double stranded DNA accessible for structure-specific recognition by proteins. Over 3000 analogues of this drug have been examined by clinical trials to overcome the side effects like toxicity, intrinsic or acquired resistance. Furthermore, novel drug delivery systems have been developed to process more efficient delivery to tumor cells (Sheng et al. 2013).

Organic compounds have been used in addition to the Pt compounds that bind covalently to DNA. Nitrogen mustard drugs, mitomycin C and psoralen are such compounds to name a few. Their modes of molecular recognition and interactions with DNA are different from Pt-containing compounds. Nitrogen mustard compounds are effective anticancer drugs which form non-specific cross linking (N7-dG/N7-dG inter-strand cross-linking) without bending the DNA and preventing the DNA duplex separation (Sheng et al. 2013).

Mitomycin C is a potential antitumor drug performing through guanine alkylation in the d(C-G).d(C-G) steps of the DNA duplex. Mitomycin ring is located in the minor groove where conformational perturbation occurs in the modified DNA structure by widening minor groove. The bulky modification does not alter the overall conformation of DNA (Sheng et al. 2013).

Psoralens, on the other hand, are photo-mutagenic and photochemotherapeutic agents used in the treatment of vitiligo, psoriasis, and other cutaneous diseases functioning through the inter-strand cross-linking with DNA and RNA and blocking the replication, transcription and other DNA activities. Its major synthetic derivative, HTM, is more soluble in water and extends over both major and minor grooves maintaining the overall conformation. The cross-linking, base stacking and hydrophobic interactions make the duplex more stable (Sheng et al. 2013).

Aflatoxin B1 (AFB) involves in mutation of p53 tumor suppressor gene and plays a role in the development of human liver cancer. It is oxidized to AFB-exo-8,9-epoxide by cytochrome p450. Upon its interaction with the duplex DNA, cationic

adduct is produced which hydrolyzes into AFB form amidopyrimidine adduct and intercalates the damaged nucleotides causing backbone torsion and overall duplex perturbation (Sheng et al. 2013).

The cross-link of these covalently binding molecules with DNA results in modifications and perturbations in DNA. Consequently, protein partners that are no longer able to bind to DNA and engage in, thus replication and translation are hindered during chemotherapy treatments. Due to the lower selectivity and higher toxicity, the resistance of these drugs is aimed to be enhanced and the molecular mechanisms are tried to be revealed to offer new molecular designs and synthesis (Sheng et al. 2013).

1.2.2 Non-covalent Interaction

The strategy of non-covalent interactions includes major and minor groove binding, intercalative binding, and targeting the DNA via multi-functionalized ligands (Rehman et al. 2015).

1.2.2.1 Minor Groove Binding

Minor groove binders such as polyamides or heterocyclic dications like DAPI, berenil, Hoechst 33258, netropsin, distamycin and pentamidine offer the highest sequence specificity on inhibition of the specific gene expression. While they prefer AT-rich regions, they can also bind to A/T, T/A, G/C and C/G pairs. They have great potential to treat diseases at DNA level with their antibacterial, antiprotozoal, antitumor and antiviral activities. Their shapes generally match the curvature of the minor groove via interactions like van der Waals and hydrogen bonding (Sheng et al. 2013).

Hoechst 33258 is clamped by the phosphate backbone of DNA in minor groove via van der Waals contacts while netropsin and distamycin form hydrogen

bonds with DNA. In 1:1 binding mode, the minor groove is compressed giving the access to other molecules binding to the major groove of the DNA duplex while in 1:2 mode, the major groove is compressed (Sheng et al. 2013).

Polyamides permeate through cell membranes, reach to chromatin, occupy the binding region of transcription factors, perturb the overall duplex conformation by bending the helix, constricting the major groove and perform sequence-specific recognition (Sheng et al. 2013).

DB921, on the other hand, strongly binds to the minor groove with the help of its shape that matches to the curvature of DNA, and interacts with the bases through water-mediated hydrogen binding (Sheng et al. 2013).

CD27 also has a planar crescent shape and forms bifurcated hydrogen bonds with specifically AATT sequences through widening the minor groove and forming van der Waals interactions (Sheng et al. 2013).

1.2.2.2 Major Groove Binders

The number of major groove binders is limited compared to the number of minor groove binders. They are capable of binding to the major groove due to their size and intrinsic distribution of hydrophobic and hydrophilic moieties. Neocarzinostatin, nogalamycin and several aminoglycoside (neomycin) conjugates are some of the major groove binders (Sheng et al. 2013).

Neocarzinostatin chromophore (NCS) forms a complex with a protein in its natural form and causes the DNA damage by hydrogen abstraction from DNA through diradical intermediate. It can bind to the minor groove or intercalate into DNA while it can also form a bulge-forming structure upon binding to DNA from the major groove, leading to the single-strand cleavage. On the other hand, neomycin binds to A-like conformations of nucleic acids (DNA and RNA) through the major groove and is able to stabilize triplex form while it is unable to bind to the major

grooves of B-form DNA. They have disadvantages such as limited cellular uptake and nuclear localization (Sheng et al. 2013).

1.2.2.3 Intercalators

Intercalators bind to the duplex via the hydrophobic, ionic, hydrogen-bonding and van der Waals interactions. Their basic, cationic or electrophilic functional groups maintain proper and better intercalation. They alter DNA functions by lengthening the DNA through intercalating planar molecules between the base pairs and causing the helix twist to distort. However, due to the low binding specificity except cryptolepine (5-methyl indolo [2,3b]-quinoline), intercalators generally cause toxicity. Parallel intercalation of the base pairs and asymmetric molecules is essential for specificity (Sheng et al. 2013).

While the intercalators such as adriamycin, daunomycin, daunorubicin, dactinomycin, echinomycin and ditercalinium are used as antitumor, antineoplastic, antimalarial, antibiotic, antifungal agents and chemotherapeutic agents, most of them are highly toxic and nonspecific. Hence, performing biochemical, biophysical, and structure-based research on DNA-drug complexes are important for the design of more specific and less toxic novel compounds (Sheng et al. 2013).

Daunomycin is an anthracycline-type planar molecule used in chemotherapy of leukemia. Planar heterocyclic moiety intercalates between the GC steps and increases the duplex stability, while the sugar moiety is located in the minor groove in each duplex unit. Daunomycin prefers the purine-pyrimidine step elongating, twisting DNA and increasing the duplex stability while reducing flexibility of phosphate groups at DNA backbone (Sheng et al. 2013).

Adriamycin is a derivative of daunomycin with an extra hydroxyl group on the C14 position used in the solid tumor treatment (Sheng et al. 2013).

Ditercalinium is a synthetic anticancer drug. It bis-intercalates into the two CpG steps of a short DNA sequence (CGCG)₂ and bends the minor groove. Hence,

it induces DNA repair in both prokaryotic and eukaryotic cells by activating the repair process until the cell's death (Sheng et al. 2013).

Echinomycin is a quinoxaline antibiotic agent able to bis-intercalate into the two CpG steps in duplexes. It interferes with replication and transcription by increasing the extension and rigidity of DNA. The increase in the entropy upon the intercalation of first group makes the intercalation of the second group much more straightforward and easier than the first one (Sheng et al. 2013).

Cryptolepine (5-methyl indolo [2,3b]-quinoline) is an antimalarial and cytotoxic drug and a potent topoisomerase II inhibitor. It intercalates preferentially into CpG-rich sequences (Sheng et al. 2013).

1.2.2.4 Multi-functionalized Ligands

Targeting DNA through multi-functionalized ligands is another possibility. Most of the covalently binding structures such as neomycin-hoechst 33258-pyrene contain a second interacting element. The design of small molecule ligands with multiple interacting elements improve the stability and specificity of the interaction (Sheng et al. 2013).

Pyrrolo[2.1-c][1,4]benzodiazepine-benzimidazole hybrid (PBD-BIMZ) has a significant anticancer activity and covalently linked to the guanine base at its exocyclic 2-amino group in a DNA duplex. It is oriented in the minor groove while its benzimidazole moiety is looking towards the 5'-end of the modified guanine (Sheng et al. 2013).

PBD-naphthalimide covalently interacts with a guanine within the major groove of DNA decamer while the naphthalimide moiety remains between the A-T and A-T base pair step (Sheng et al. 2013).

Several other minor groove binders or intercalating agents have been covalently linked with DNA at specific positions and this covalent binding can

greatly stabilize DNA-ligand interactions and alter duplex conformation (Sheng et al. 2013).

1.3 Small Molecules Targeting Triplex DNA Structures

Triplex structures might also be stabilized through binding to small molecules, particularly intercalators (Escudé et al., 1998; Blackburn et al., 2006). Most of the intercalators are planar molecules which have a polycyclic aromatic ring and at least one positive charge. They have a crescent shape which suits to the shape of a base triplet. They stabilize the secondary structures by stacking between the nucleic acid bases. The binding mechanism of intercalators is driven via electrostatic, dipole-dipole, London dispersion and π - π interactions between the nucleic acid bases and the aromatic intercalator. The local helix structure unwinds to accommodate the small molecule binding (Persil Çetinkol, 2008a). Benzo[e]pyridoindole, coralyne, naphthylquinole, anthraquinones and acrydine derivatives (Figure 1.7) are some of the selectively and strongly binding intercalators to the triplex structures, that are also reclaimed within the scope of therapeutic drug development studies (Blackburn et al., 2006).

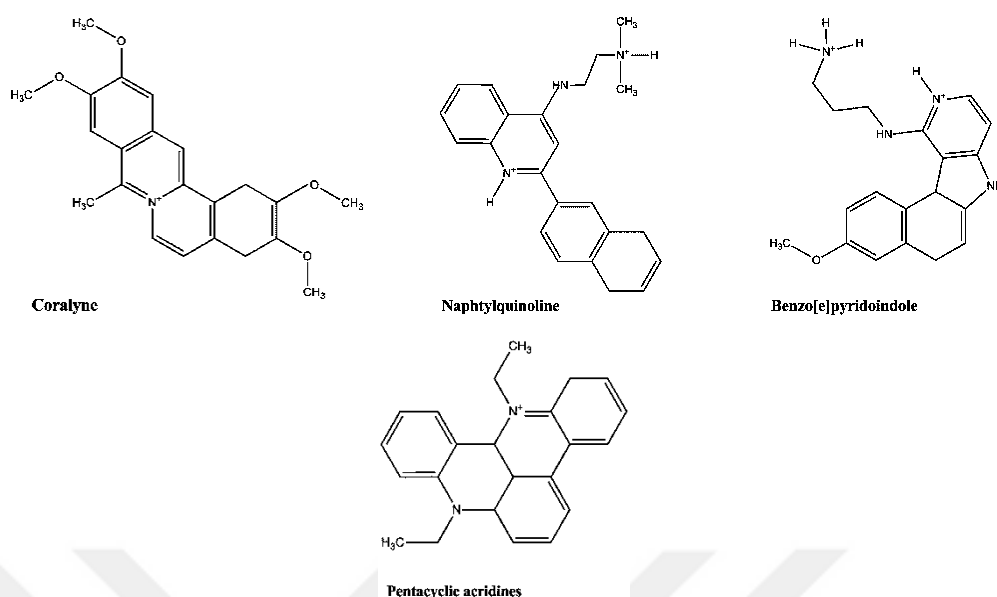


Figure 1.7 Chemical structures of molecules binding to triplex structures selectively.

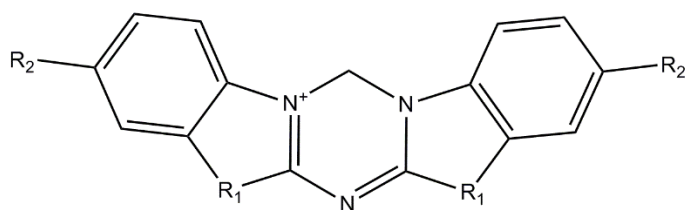
The figure was adapted from Blackburn et al. (2006).

The small molecules that intercalate in DNA and RNA are powerful agents for controlling nucleic acid structural transitions. Small molecule-induced alterations in topological and mechanical properties of DNA can affect the metabolism and growth of the cells (Afrin et al, 2017). Such alterations can interfere with diverse downstream processes and the activity of various important enzymes and proteins involved in maintaining the structure and function of the cell (Rahman et al., 2015).

The development and characterization of triplex stabilizing molecules is mainly focused on planar molecules with a crescent shape which mimics the shape of a base triplet. A number of small molecules that bind to and stabilize triplex DNA are given in Section 1.3.1- 1.3.5.

1.3.1 Azacyanines

Azacyanines (Figure 1.8), which have benzimidazole or benzothiazole ring structure are small molecules firstly synthesized by Kurth et al. (Haddadin et al., 2000; Huang et al., 2001).



Azamethyl: $R_1 = \text{NCH}_3$, $R_2 = \text{H}$
Azaethyl: $R_1 = \text{NCH}_2\text{CH}_3$, $R_2 = \text{H}$
Azapropyl: $R_1 = \text{NCH}_2\text{CH}_2\text{CH}_3$, $R_2 = \text{H}$
Azaisopropyl: $R_1 = \text{NCH}(\text{CH}_3)_2$, $R_2 = \text{H}$
Azabutyl: $R_1 = \text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$, $R_2 = \text{H}$
Azaisobutyl: $R_1 = \text{NC}(\text{CH}_3)_3$, $R_2 = \text{H}$
Aza4: $R_1 = \text{S}$, $R_2 = \text{H}$
Aza5: $R_1 = \text{S}$, $R_2 = \text{OCH}_3$

Figure 1.8. Structure of Azacyanines

Persil Çetinkol et. al. suggested that Azacyanines (Aza3 (named Azamethyl in this study), Aza4 and Aza5) can bind strongly to the purine nucleic acids. They conclude that Aza3, Aza4 and Aza5 molecules are too big to intercalate into the duplex and they rather intercalate into the non-canonical purine rich structures. In one of their studies, they reported the binding of Aza3 to polyr(A) sequences with binding affinity of $K_a = 3.8 \times 10^{-5} \text{ M}^{-1}$ (Persil Çetinkol and Hud, 2009). In another study, they reported the binding of Aza3 molecule to the quadruplex human telomeric sequences and duplex calf thymus DNA with $K_a = 1.28 \times 10^6 \text{ M}^{-1}$ and $K_a = 9.2 \times 10^3 \text{ M}^{-1}$, respectively (Persil Çetinkol et al., 2008b).

These studies revived the question of how Azacyanine molecules interact with DNA in vivo. Prof. Lobachev's group surveyed the ability of Azacyanines binding to GAA•TTC repeat sequences and their ability to stabilize the triplex

structures in vivo. They reported the chromosome arm loss in the yeast cells comprise GAA•TTC repeats in the presence of Azacyanines. They observed that the Azacyanines could halt the cell division in G2/M phase. More importantly, the effect of Azacyanines on these yeast cells turned out to be dose-dependent (Figure 1.9 and Figure 1.10) (Kim, 2009).

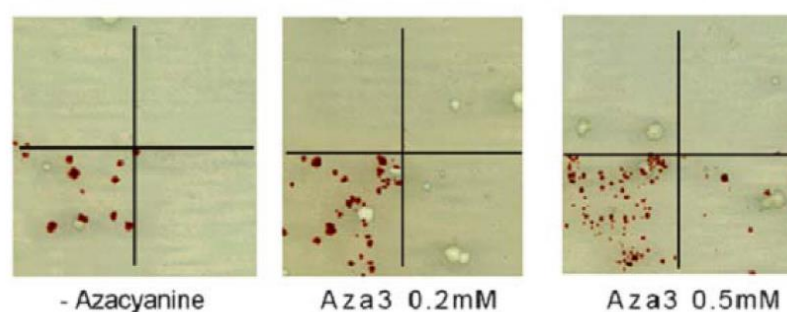


Figure 1.9. Results of chromosomal arm loss experiments.

The results of chromosomal arm loss experiments are shown. The figure was taken from Kim (2009).

Accordingly, Aza5 molecule has been proven to have more effect on yeast cells than the Aza3 and Aza4 molecules. Lobachev's group suggested that the observed effect might possibly be due to the binding of Azacyanines to the triplex DNA and damaging the DNA at these points.

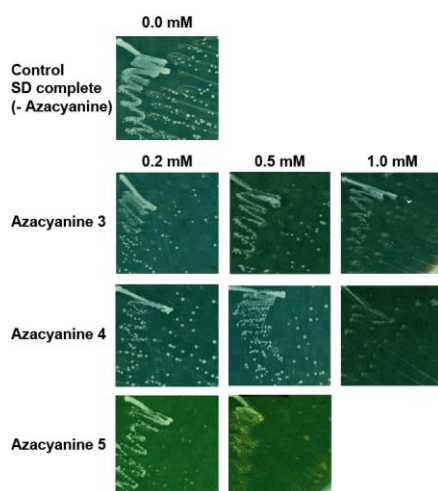


Figure 1.10. The effect of Aza3 on the yeast cells.

Azacyanines inhibits the cell growth in a dose-dependent manner. The Figure was taken from Kim (2009).

1.3.2 Neomycin

Neomycin is a major groove binder that stabilizes TAT triplex and the base-mixed DNA triplexes without affecting the DNA duplex. It has high selectivity to triplex DNA due to its shape which is complementary for the triplex Watson-Hoogsteen groove. Intercalator-neomycin conjugates have also been developed such as pyrene-neomycine and BQQ-neomycin (Sheng et al. 2013).

1.3.3 DODC

Carbocyanine dye 3,3'-diethyloxadibocyanine (DODC) was initially identified as a potential quadruplex selective binder through the database searching procedure using DOCK algorithm. However, experimentally it was found to be binding to the triplex DNA of polydA:[polydT]₂, almost with threefold affinity

compared to $d(G_{10}T_4G_{10})$, $d(AG_3(T_2AG_3)_3)$ and $[d(T_2G_{20}T_2)]_4$ quadruplexes (Ren and Chaires, 2000).

1.3.4 Thiazole Orange

Thiazole Orange (TO, 4-[3-methyl-2,3-dihydro(benzo-1,3-thiazole)-2-methylidene]quinolinium iodide) is one of the oldest synthetic cyanine dyes widely used in reticulocyte analysis, permeating live cell membranes and stain residual RNA in reticulocytes. It binds to dsDNA and ssDNA homopolymers relatively weakly. It also binds to G-quadruplex structures in very similar way to double or single-stranded DNA. On the other hand, TO binds very tightly to the intramolecular triplexes of poly(dG-dG)-poly(dC) and monomolecular G-quadruplex (G4 wires) DNA (Lubitz et al., 2010).

1.3.5 Coralyne

Coralyne (Figure 1.7) is a small crescent-shaped molecule that is known to preferentially intercalate DNA triplexes over duplexes and increase their thermal stability (Jain, Polak and Hud, 2003). It has a four fused six-membered ring resulting in a larger surface area than the tricyclic planar molecules typically capable of intercalating DNA and RNA duplexes (i.e. the acridines and phenanthridines).

It is an alkaloid drug with anti-leukemic properties. Its anti-leukemic property is likely to be related to its ability to intercalate DNA. In addition, it inhibits the activity of topoisomerase by stabilizing DNA-topoisomerase complexes (Joung, Çetinkol, Hud, Cheatham III, 2009).

Coralyne also binds to homo-adenine DNA (and RNA) oligonucleotides more tightly than it does to Watson-Crick DNA. It binds to polyd(A) with a stoichiometry of one coralyne per four adenine bases, and is released from polyd(A) cooperatively with the melting transition temperature of around 50 °C (Polak and

Hud, 2002). It promotes the formation of an antiparallel homo-adenine duplex at neutral pH. The helix of this duplex is compatible with flanking Watson-Crick helices. An antiparallel duplex can form between homo-adenine sequences in the presence of coralyne and this homo-adenine-coralyne structure can be incorporated into duplex structure that include Watson-Crick pairs. Thereby, coralyne binding can be used to alter nucleic acid secondary structure and drive the formation of non-Watson-Crick duplexes (Persil et al., 2004).

Coralyne molecule causes denaturation of $d(A)_{32} \cdot d(T)_{32}$ and polyd(A)·polyd(T) structures and enables the formation of an intercalated triple helix and a homo adenine self-structure. However, it does not interact with polyd(T) sequences (Polak and Hud, 2002; Jain, Polak and Hud, 2003). In the presence of the coralyne, polyd(A)·polyd(T) disproportionates at around 35°C, and the disproportionated strands form an intercalated polyd(A)·polyd(T)·polyd(T)-coralyne and polyd(A)-coralyne complexes. And, the polyd(A)·polyd(T)·polyd(T)-coralyne complex denatures at 82°C (Polak and Hud, 2002) later on. The interactions of coralyne with $d(A)_{16} \cdot d(T)_{16}$ was also investigated previously (Jain, Polak and Hud, 2003).

1.4 The Scope of the Study

The interactions of three Azacyanine molecules (Azacyanine 3, Azacyanine 4 and Azacyanine 5) with human telomeric DNA and poly(A) were previously studied (Persil Çetinkol et al., 2008a,b; Persil Çetinkol and Hud., 2009). Furthermore, the effect of Azacyanines and coralyne on chromosomal fragility mediated by triplex forming $(GAA)_n/(TTC)_n$ repeats in yeast cells was investigated by Kim (Kim, 2009). Kim's studies revealed the Azacyanines ability to induce $(GAA)_n/(TTC)_n$ mediated arm loss in a dose-dependent manner and chromosome

fragility in yeast cells. In addition, they observed that the replication of the yeast cells is halted at the G2 phase of the cell cycle in the presence of Azacyanines. They presumed that the effect of Azacyanines is due to their ability to stabilize triplex formation in GAA/TTC tracts and to trigger the double-stranded break formation. Surprisingly, coralyne which has been established as a selective triplex binder was not able to induce such an effect (Kim, 2009; Lee et al., 1993; Ren and Chaires, 1999). Previously, it has been shown that coralyne can bind to triplex DNA structures with such a high affinity able to disproportionate polyd(A)·polyd(T) duplex into intercalated polyd(A)·polyd(A)·polyd(T) triplex and polyd(A) self-structure (Polak and Hud, 2002; Jain, Polak and Hud, 2003).

The main goal of this study is to investigate the interactions of Aza3 molecule with the triplex forming d(A)₃₂·d(T)₃₂·d(T)₃₂, polyd(A)·polyd(T)·polyd(T) and DTR1 containing (GAA) repeat sequences to establish its binding ability to triplex structures. The interactions of Aza3 with d(A)₃₂·d(T)₃₂, polyd(A)·polyd(T) and (GAA) containing duplex structure of DD1 have also been investigated. The stabilization ability of Aza3 was determined via UV-vis and CD thermal denaturation experiments. The binding constant and the ratio of binding to polyd(A)·polyd(T)·polyd(T) were determined via fluorescence experiments and the mode of binding was determined via viscosity measurements. Although the interactions of Azacyanines with the triplex forming nucleic acids were characterized before (Persil Çetinkol and Hud, 2009), there isn't any in-vitro studies concerning the binding of Azacyanines to the triplex structures to the best of our knowledge.

Furthermore, the interactions of Aza3 derivatives (Azaethyl, Azapropyl, Azaisopropyl, Azabutyl and Azaisobutyl) with the polyd(A)·polyd(T)·polyd(T) triplex have been investigated to assess the effect of molecular structure, specifically the effect of alkyl chain length and branching on the benzimidazole ring, on triplex over duplex affinity and selectivity.

Finally, Aza4 and Aza5's interactions with polyd(A)·polyd(T)·polyd(T) were also examined, and the relationship between the binding ability of them to the triplex structures.

Overall, within this study, high selectivity and affinity of Azacyanine molecules to triplex structures and the effect of molecular structure on the binding abilities of these molecules, which could be the reason behind the cell division inhibition, is analyzed and the therapeutic potentials of these molecules are uncovered.

Our results clearly support that Azamethyl has a high selectivity towards polyd(A)·polyd(T)·polyd(T). Its binding affinity with polyd(A)·polyd(T) duplex is really low. It is able to induce the disproportionation of intercalated polyd(A)·polyd(T) duplex into intercalated polyd(A)·polyd(T)·polyd(T) triplex and polyd(A) self-structure. More importantly, the results presented here reveal that the binding affinity and selectivity of Azacyanines to polyd(A)·polyd(T)·polyd(T) decreases with an increase in the linear alkyl chain length and branching in the benzimidazole ring. We believe that revealing the modulation of DNA binding affinity and selectivity of a small molecule by changing the alkyl chain length and branching on the small molecule structure is a significant step towards rational drug design.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

Dimethyl sulfoxide (DMSO, C_2H_6OS), Na_2HPO_4 , NaH_2PO_4 , Na_2EDTA , $NaCl$, $NaOH$ and HCl used in sample preparation were purchased from Sigma-Aldrich (Europe) or TCI Chemicals (Europe) and were used without further purification. The examined oligonucleotides were purchased from Integrated DNA Technologies, (IDT, Europe) and the polynucleotides were purchased from Sigma-Aldrich (Europe). All the oligonucleotides and polynucleotides were used without further purification (Table 2.1).

Table 2.1 Nucleic acid sequences used in the experiments and their molar absorptivity constants

Conformation	Nucleic Acid	λ (nm)	ϵ (M ⁻¹ cm ⁻¹)	Monomeric Unit
Single-stranded (singlet) DNA	polyd(A)	257	8600 ^a	Nucleotide
	polyd(T)	264	8520 ^a	Nucleotide
	5'-TCGC GAAGAAGAAGAAGAA CGCT -3' (SS1)	260	240700 ^b	Nucleotide
	3'-AGCG CTT CTTCTT CTT CTT GCGA-5' (SS2)	260	200400 ^b	Nucleotide
	3'- GAAGAAGAAGAAGAA- 5' (SS3)	260	178200 ^b	Nucleotide
Double-stranded (duplex) DNA	d(A) ₃₂ .d(T) ₃₂	260	d(A) ₃₂ : 387400 ^b d(T) ₃₂ : 259800 ^b	Base pair
	polyd(A).polyd(T)	260	12000 ^a	Base pair
	DD1	260	(SS1): 240700 ^b (SS2): 200400 ^b	Base pair
	DD2	260	(SS2): 200400 ^b (SS3): 178200 ^b	Base pair
Triple-stranded (triplex) DNA	d(A) ₃₂ .[d(T) ₃₂] ₂	260	d(A) ₃₂ : 387400 ^b d(T) ₃₂ : 259800 ^b	Base triplet
	polyd(A).[polyd(T)] ₂	260	polyd(A).polyd(T): 12000 ^a	Base triplet
		264	polyd(T): 8520 ^a	
	DTR1	260	(SS1): 240700 ^b	Base triplet
			(SS2): 200400 ^b (SS3): 178200 ^b	

^a from Ragazzon et al., 2007 , ^b from Integrated DNA Technologies (IDT).

2.2 Sample Preparation

2.2.1 DNA Sample

In order to prepare stock solutions, all oligonucleotides and polynucleotides were dissolved in millipore water and their concentrations were calculated using their extinction coefficients provided by the supplier or reported in the literature (Table 2.1). Duplex and triplex structures were purchased as double-stranded (Sigma-Aldrich, Europe) or were prepared through appropriate mixing of the required nucleic acids (duplex and single-stranded) in desired equimolar ratios (equal mixing 1:1 for duplex structures and 1:2 mixing for triplex structure). To assure the proper formation of the secondary structures, all nucleic acid samples were annealed by heating at 95°C for 5 minutes in a water bath and then were left to cool down slowly to reach room temperature. The small molecules were added to the annealed samples about 30 minutes before the measurements. All the samples were prepared in 1X BPE buffer, pH=7.0 (6 mM Na₂HPO₄, 2 mM NaH₂PO₄, 1 mM Na₂EDTA) (conditions are chosen to be close to the in vivo conditions). Unless stated otherwise, the nucleic acid samples were prepared as 60.0 µM nucleotide, 30.0 µM base pair or base triplet.

2.2.2 Small Molecules

The Azacyanines were synthesized, purified and characterized according to a well-established previously-reported method (Haddadin et.al, 2000; Küçükakdağ Doğu, 2019). Their names, chemical formulas and experimentally found extinction coefficients are listed in Table 2.2. To prepare the stock solutions of Azacyanines, their solids were dissolved in DMSO and the concentrations were determined using the UV-vis absorbance at their λ_{max} values using the molar absorption coefficients. Generally, the added DMSO volume was between 0.0-10.0 µL to the 3500 µL sample, making the DMSO volume less than 0.3%.

Table 2.2 Azacyanines and their properties used in this study*.

Compound Name	Chemical Formula	Molar Absorptivity Constant
		(L.mol ⁻¹ .cm ⁻¹)-
Azamethyl	C ₁₇ H ₁₆ N ₅ I	ε=45500 (343 nm)
Azaethyl	C ₁₉ H ₂₀ N ₅ I	ε=44700 (343 nm)
Azapropyl	C ₂₁ H ₂₄ N ₅ I	ε=43300 (343 nm)
Azaisopropyl	C ₂₁ H ₂₄ N ₅ I	ε=45100 (343 nm)
Azabutyl	C ₂₃ H ₂₈ N ₅ I	ε=44000 (343 nm)
Azaisobutyl	C ₂₃ H ₂₈ N ₅ I	ε=44700 (343 nm)
Aza4	C ₁₅ H ₁₁ N ₃ S ₂ I	ε=30500 (387 nm)
Aza5	C ₁₇ H ₁₄ N ₃ O ₂ S ₂ I	ε=25150 (407 nm)

* Küçükakdağ Doğu, Ayça. 2019. “Targeting Human Telomeric DNA with Azacyanines”, Dissertation, Middle East Technical University.

2.2.2.1 Synthesis and Characterization of Azacyanines

The synthesis of the Azacyanine compounds had been reported previously by Huang et. al., 2001, Persil Cetinkol et. al., 2008a, Güloğlu, 2018, Tutuncu et. al., 2018 and Küçükakdağ, 2019. Here, the previously reported synthesis method was adopted.

2.2.2.1.1 Synthesis of Benzimidazole Derivatives

Briefly, diiodomethane was added to 1-R-1H-benzimidazole-2-ylamine dissolved in acetonitrile under reflux conditions under N₂ gas (Figure 2.1). The mixture was refluxed for 5 to 24 hours, depending on the starting material. After

reflux, the solution was cooled down and filtered. The precipitate was washed with dichloromethane (CH_2Cl_2) and acetone ($\text{C}_3\text{H}_6\text{O}$). The characterization of all the compounds investigated here using ^1H NMR, ^{13}C NMR, mass spectroscopy and elemental analysis were recently published in Tutuncu et. al. The 1-R-1H-benzimidazole-2-ylamine starting materials used in this study are given in Figure 2.2.

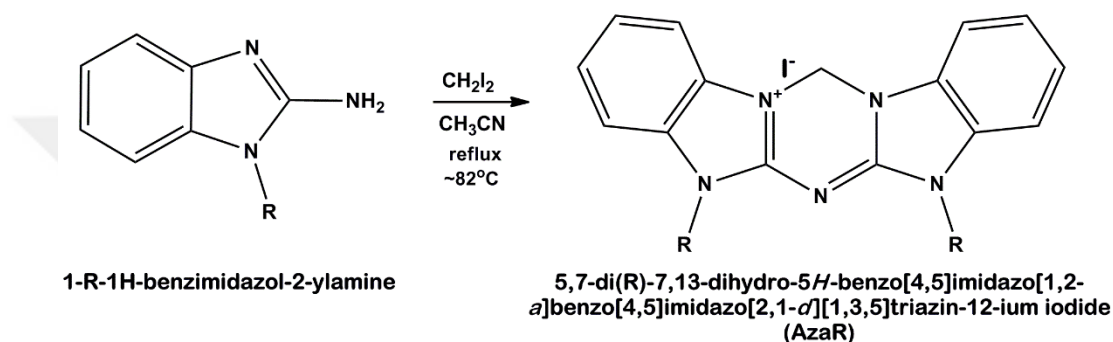


Figure 2.1. Synthesis of benzimidazole derivatives.

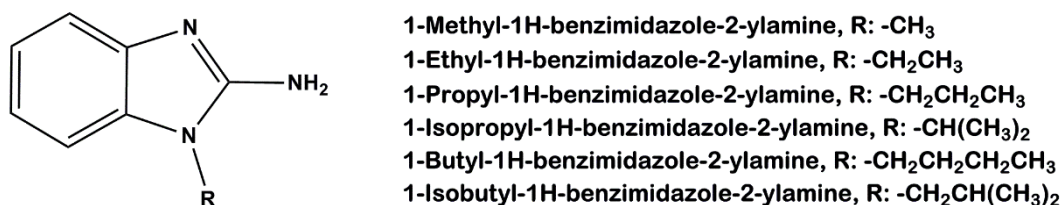


Figure 2.2. Structures of the starting materials used in benzimidazole derivatives synthesis.

2.2.2.1.2 Synthesis of Benzothiazole Derivatives

Briefly, diiodomethane was added to 2-amino-6R-benzothiazole dissolved in 2-(2-ethoxyethoxy)ethanol (diethylene glycol monoethyl ether) under reflux conditions under N₂ gas (Figure 2.3). The mixture was refluxed for about 20 minutes. After 20 minutes, the solution was cooled down, precipitated with ethyl acetate, refrigerated for 12 mins and filtered. The precipitate was washed with dichloromethane (CH₂Cl₂) and acetone (C₃H₆O). The compounds were characterized by using ¹H NMR, ¹³C NMR, mass spectroscopy and elemental analysis (Guloglu, 2018). The 2-amino-6R-benzothiazole starting materials used in this study are given in Figure 2.4.

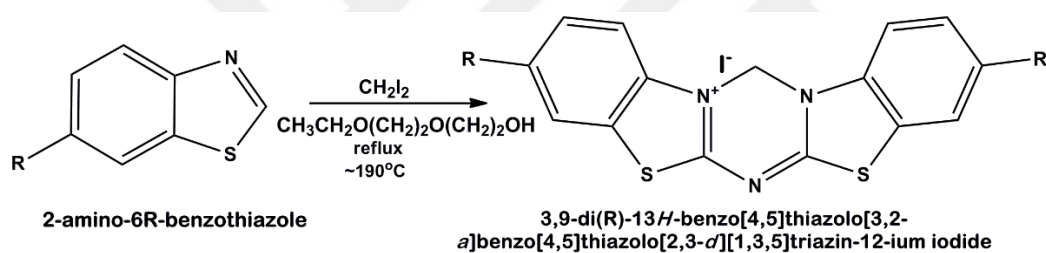


Figure 2.3. Synthesis of benzothiazole derivatives.

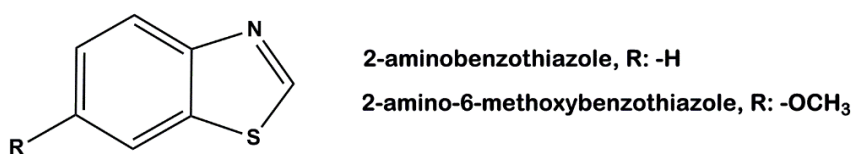


Figure 2.4. Structures of the starting materials used in benzothiazole derivatives synthesis.

2.3 UV-vis Spectroscopy Experiments

An Agilent Cary 8454 UV-vis diode array spectrophotometer equipped with an Agilent 89090A peltier and a Jasco V-730 spectrophotometer was used in UV-vis measurements. The samples were 60.0 μ M nucleotide, 30.0 μ M base pair or base triplet, respectively in 1X BPE buffer (pH 7.0) and 100 mM NaCl. The nucleic acid samples were prepared as 15.0 μ M in small molecule concentration. For thermal denaturation experiments, the samples were heated from 5°C to 95°C for oligonucleotides and were heated from 15°C to 95°C for polynucleotides with 1°C/minute heating rate and the UV-vis spectra were collected at 1°C increments between 190 nm and 800-1100 nm. We did not find it necessary to go down to 5°C since the polyd(A)·polyd(T)·polyd(T) triplex was stable at 15°C. The cooling traces were also collected under the same conditions. The baselines of UV-vis spectra were corrected using 1X BPE buffer with 100 mM NaCl solution as the blank. Thermal denaturation profiles were obtained by plotting the absorbance (at 260 nm or at 336 nm) versus the temperature. Melting temperatures were obtained from the first derivatives of the thermal denaturation profiles.

2.4 Circular Dichroism (CD) Spectroscopy Experiments

CD studies were performed within the service procurement of Bilkent-UNAM. The CD spectra of the samples were acquired on a JASCO J-815 circular dichroism spectropolarimeter equipped with a Peltier temperature control unit (PTC-423S/15). The CD spectra for all the samples were collected between 230 and 400 nm or 500 nm at 5°C at 200 nm/min scanning speed with the band width of 1 nm. We were not able to collect the spectra below 230 nm since the HT voltage was high below 230 nm. All the CD spectra were baseline-corrected using the buffer solution. CD melting profiles for polyd(A), polyd(T), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) in the absence and presence of Azamethyl were obtained by increasing the temperature from 5°C to 95°C with spectrum collection at each step of 5°C. The

samples were left to sit at that temperature for 2 minutes before data collection. For all the CD measurements, the data were obtained as an average of two measurements.

2.5 Fluorescence Spectroscopy Experiments

The fluorescence spectra of the samples were collected via Varian Cary Eclipse fluorescence spectrophotometer. The samples of Azacyanines were excited at 324 nm and the emission spectra were collected between 325 nm and 675 nm. Among the benzothizole samples, Aza4 was excited at 339 nm and its emission spectra were collected between 400 nm and 675 nm while Aza5 was excited at 419 nm and the emission spectra were collected between 420 nm and 700 nm. The excitation and emission slits were set as 2.5 nm /5.0 nm and the scan rate was adjusted at 300 nm/min.

2.6 Determination of the Binding Affinities via Fluorescence Spectroscopy

Briefly, the solution of a small molecule (Azamethyl or its derivative, 1.0 μM) was titrated with the solution of a concentrated nucleic acid (400.0 μM base triplet) containing the same amount of the small molecule (1.0 μM). The titration continued until the emission spectrum became constant. Then, the binding constants were obtained by fitting the Integrated Fluorescence Intensity data as a function of nucleic acid concentration using IGOR Pro software. The binding constant (association constant) was calculated with the Scatchard analysis (Persil Çetinkol et al., 2008).

2.7 Determination of Binding Ratio by Job Plot Analysis

The Job Plot analysis was carried out at room temperature using UV-vis and fluorescence spectroscopy techniques with the combined concentration of Azamethyl and polyd(A)·polyd(T)·polyd(T) at 15.0 μM . Briefly, a sample of polyd(A)·polyd(T)·polyd(T) containing 30.0 μM base triplet was placed in a cuvette and its spectrum profiles were recorded. Gradually, the polyd(A)·polyd(T)·polyd(T) solution in the cuvette was replaced by 15.0 μM Azamethyl solution to obtain new

samples with different polyd(A)·polyd(T)·polyd(T):Azamethyl ratios in a such way that the concentration of polyd(A)·polyd(T)·polyd(T) binding sites + Azamethyl was 15.0 μ M. For UV-vis measurements, the spectrum of each sample was collected and the Job plot profiles were obtained by drawing the the absorbance of the Azamethyl molecule at 345 nm with respect to the polyd(A)·polyd(T)·polyd(T):Azamethyl ratio. For fluorescence spectroscopy, the spectra of samples were collected and the Job plot profiles were obtained through drawing the underneath areas of the emmision profiles obtained through the excitation at 324 nm and emmison at 325 nm with respect to the polyd(A)·polyd(T)·polyd(T):Azamethyl ratio. The number of break points at the obtained profiles represents the number of binding modes, wheras the ratio of polyd(A)·polyd(T)·polyd(T):Azamethyl on the break points represents the binding ratio (Polak and Hud, 2002; Persil and Hud 2009).

2.8 Determination of the Binding Mode with Viscosity Measurements

Viscosity measurements were performed at the service procurement of Bilkent-UNAM using the cone/plate cylindrical rheometer (Anton Paar-Physica MCR 301, type: H-PTD200-no: 80534853) at 5°C. Shear stress was specified as 50 s⁻¹ while conical type was rotating at 50 mm radius and 1°C angle. Briefly, the Azacyanine was added in a manner of certain concentration onto the sample of polyd(A)·polyd(T)·polyd(T). Each measurement was carried in the course of 100 s and was repeated twice. The data were graphed using viscosity values vs. Azacyanine concentration.



CHAPTER 3

RESULTS AND DISCUSSION

Some of the findings in this study were published in the manuscript by Tütüncü et al. ,2018.

3.1 Investigation of the Interactions of Nucleic Acids with Azacyanines and Determination of their Thermal Denaturation Temperatures Using UV-vis Spectroscopy

The interactions of Azamethyl molecule between $d(A)_{32}$, $d(T)_{32}$, $d(A)_{32} \cdot d(T)_{32}$ double helix, $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ triple helix, polyd(A), polyd(T), polyd(A)·polyd(T) double helix and polyd(A)·polyd(T)·polyd(T) triple helix were investigated using UV-vis spectroscopy. The oligonucleotide or polynucleotide samples were prepared in the presence and absence of Azamethyl and their UV-vis spectra were collected between 5°C-95°C or 15°C-95°C for oligonucleotide samples and for polynucleotide samples, respectively.

3.1.1 Interactions of Azamethyl with $d(A)_{32}$, $d(T)_{32}$, $d(A)_{32} \cdot d(T)_{32}$ and $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$, UV-vis Spectroscopy

The interactions of Azamethyl with $d(A)_{32}$, $d(T)_{32}$, $d(A)_{32} \cdot d(T)_{32}$ double helix and $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ triple helix were investigated by comparing the UV-vis spectra of each sample at 5°C and 95°C. The obtained UV-vis spectra are given below (Figure 3.1- Figure 3.8). In each spectrum, the absorbance at 5°C represents the binding of Azamethyl to the corresponding nucleic acid structure. At 95°C, the secondary structure of the nucleic acid denatures and therefore, Azamethyl is released. Upon heating the oligonucleotides, there is always an increase in the

absorbance due to the change in the interactions of bases with light in the solution due to an increase in the flexibility. The increase in the secondary structure is generally higher during denaturation (Gilbert H. F., 2000).

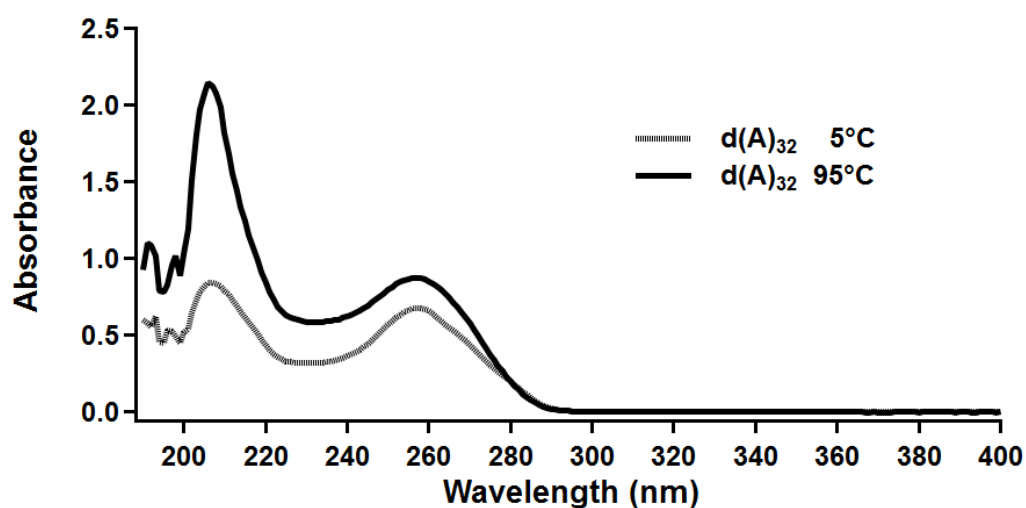


Figure 3.1. UV-vis spectra of d(A)₃₂ at 5°C and 95°C.

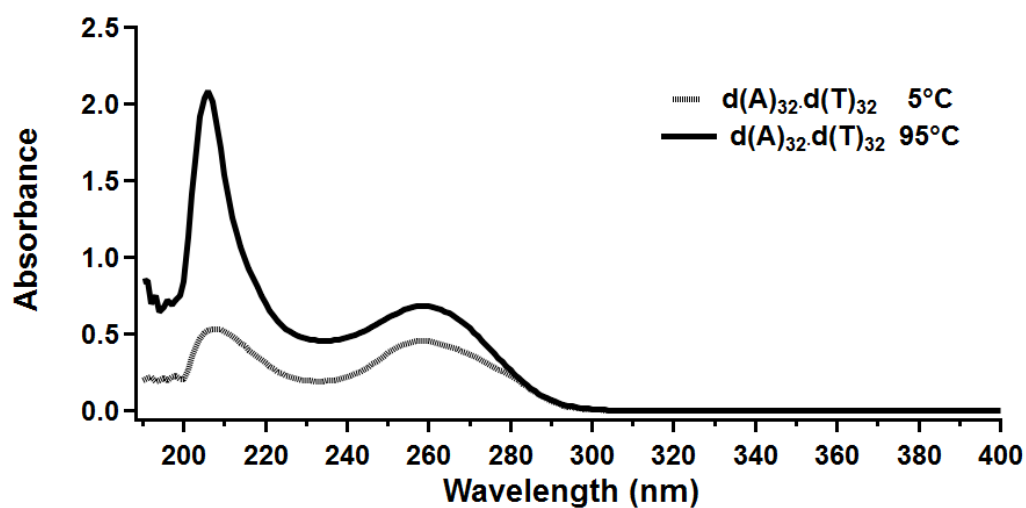


Figure 3.2. UV-vis spectra of d(A)₃₂·d(T)₃₂ at 5°C and 95°C.

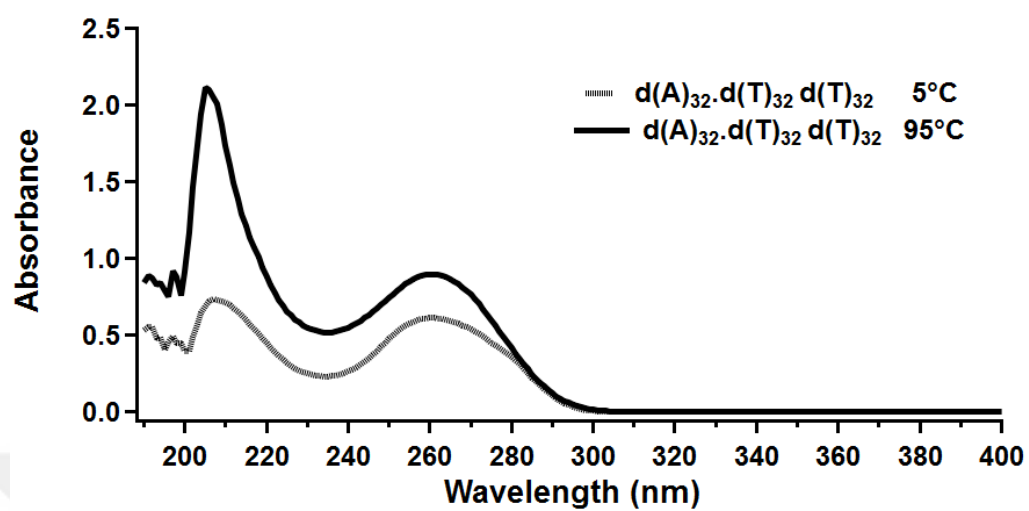


Figure 3.3. UV-vis spectra of $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ at 5°C and 95°C.

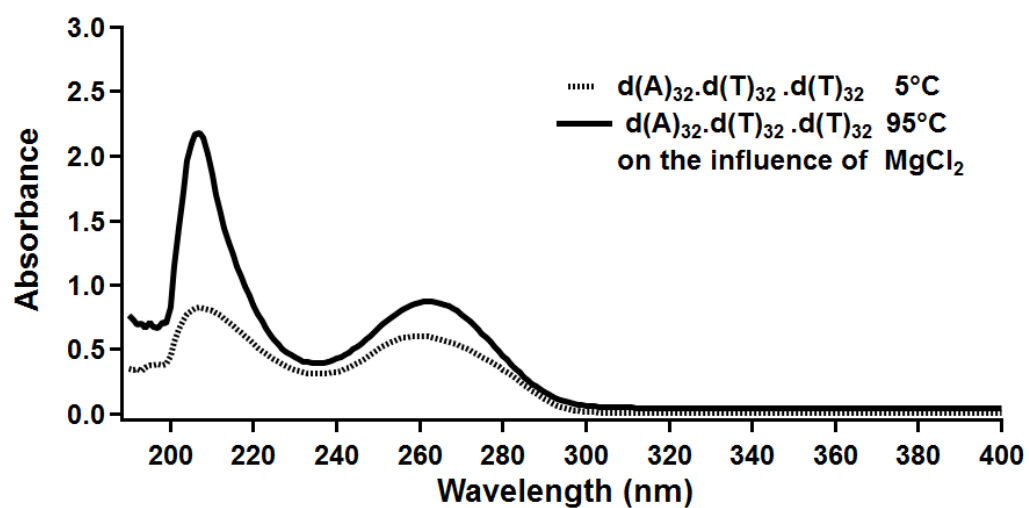


Figure 3.4. UV-vis spectra of $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ at 5°C and 95°C with 10 mM $MgCl_2$.

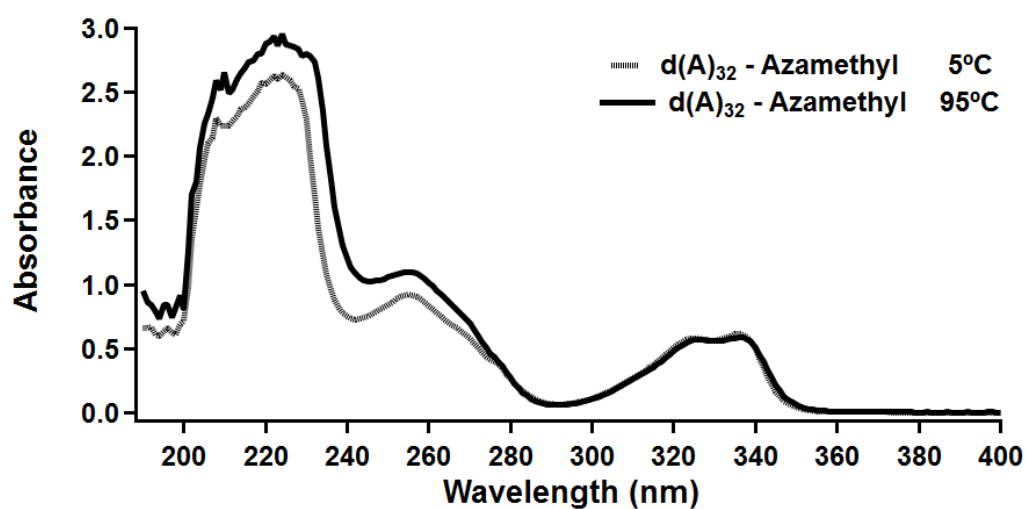


Figure 3.5. U V-Vis spectra of $d(A)_{32}$ -Azamethyl at 5°C and 95°C.

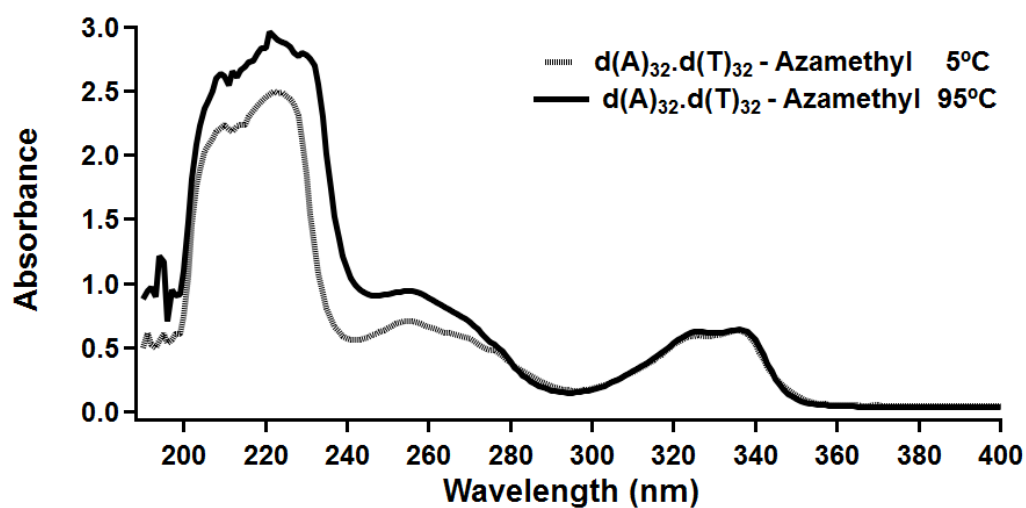


Figure 3.6. UV-vis spectra of $d(A)_{32} \cdot d(T)_{32}$ -Azamethyl at 5°C and 95°C.

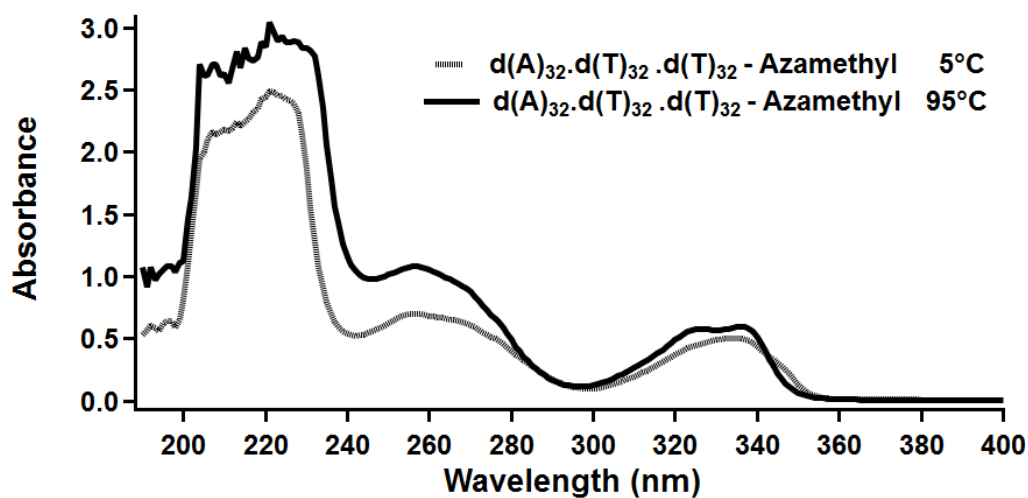


Figure 3.7. UV-vis spectra of $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ -Azamethyl at 5°C and 95°C.

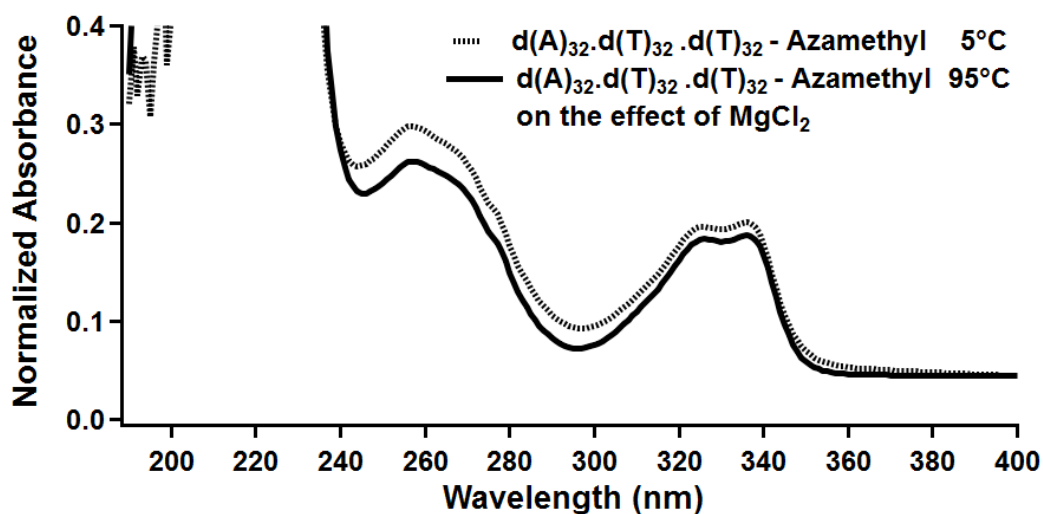


Figure 3.8. Normalized UV-vis spectra of $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ -Azamethyl at 5°C and 95°C with 10 mM $MgCl_2$.

When the UV-vis spectra of $d(A)_{32}$ (Figure 3.1), $d(A)_{32} \cdot d(T)_{32}$ double helix (Figure 3.2) and $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ triple helix (Figure 3.3) were compared to each other in the absence of Azamethyl at 5°C and 95°C, an increase was observed in the

UV-vis absorbance values between 230 nm and 300 nm with increasing temperature. This was an expected increase since the nucleic acid structures denature as the temperature rises and their interactions with light changes (Gilbert H. F., 2000). The same increase was observed also in the spectra of these nucleic acid structures in the presence of Azamethyl (Figure 3.5-Figure 3.7). On the other hand, it was observed that these samples differ from each other in terms of their UV-vis spectra between 300 nm and 360 nm where only Azamethyl absorbs the light. There was not a significant difference in the Azamethyl spectra between 5°C and 95°C in that wavelength region in the presence of d(A)₃₂ or d(A)₃₂·d(T)₃₂ (Figure 3.5 and Figure 3.6). At 5°C the nucleic acids fold to their compact structure probably because of adopting a secondary structure. At 95°C the folded nucleic acid structure denatures. Simultaneously, the small molecules in the solution associate with the folded structure at 5°C, and dissociate at 95°C. The similarity in the spectra of 5°C and 95°C, can be interpreted as the lack of interaction between Azamethyl and these nucleic acid structures, d(A)₃₂ or d(A)₃₂·d(T)₃₂. In contrast, the spectrum of d(A)₃₂·d(T)₃₂·d(T)₃₂ – Azamethyl between 300 nm and 360 nm at 5°C was shifted to a higher wavelength (known as the red shift) with a decline in its intensity compared to its spectrum at 95°C (Figure 3.7). These changes in the UV-vis spectrum is generally correlated with the π – π interactions of the small molecules with the nucleic acid bases and the intercalative binding mode (Bloomfield, Crothers and Tinoco Jr., 1999). The effect of additional salt on the stability of d(A)₃₂·d(T)₃₂·d(T)₃₂ triple helix was also investigated in the presence of additional 10.0 mM MgCl₂. As expected, there was an increase in the UV-vis absorbance values between 230 nm and 300 nm as the temperature increases. Moreover, a very slight blue shift was recorded in this region (Figure 3.4). The spectra of d(A)₃₂·d(T)₃₂·d(T)₃₂ – Azamethyl with 10.0 mM additional MgCl₂ was also collected at 5°C and 95°C (Figure 3.8). When the 300 nm and 360 nm regions of the spectra in Figure 3.7 and Figure 3.8 are compared, the additional salt seems to weaken the binding ability of Azamethyl. Neither a red shift nor hypochromic effect were observed in the presence of 10.0 mM MgCl₂.

3.1.2 Interactions of Azamethyl with polyd(A), polyd(T), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T), UV-vis Spectroscopy

The interactions of Azamethyl with the polyd(A), polyd(T), polyd(A)·polyd(T) double helix and polyd(A)·polyd(T)·polyd(T) triple helix were also investigated by comparing their UV-vis spectra collected in the absence and presence of Azamethyl at 15°C and 95 °C. The obtained UV-vis spectra are given in Figure 3.9-Figure 3.20. Since polynucleotides are more stable structures compared to oligonucleotides, starting thermal denaturations at 15°C was preferred rather than 5°C.

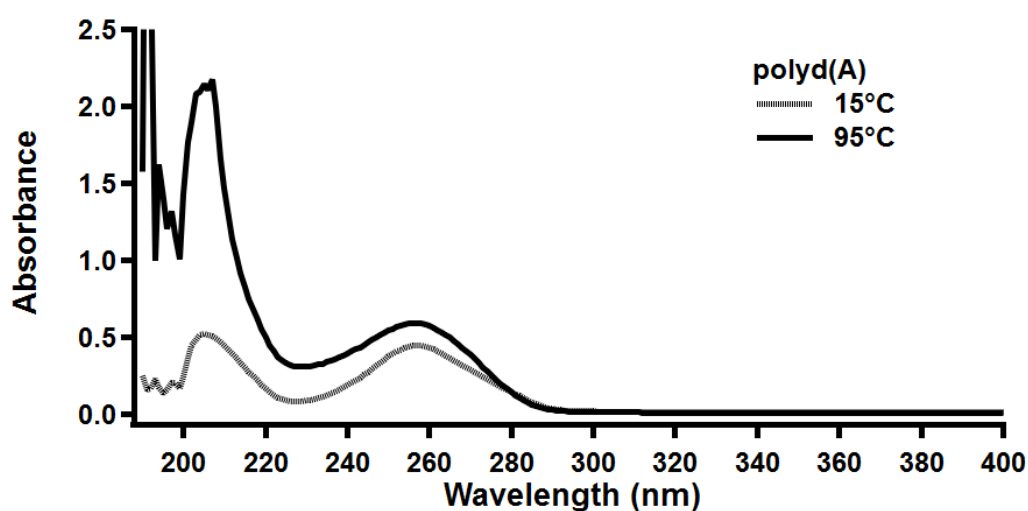


Figure 3.9. UV-vis spectra of polyd(A) at 15°C and 95°C.

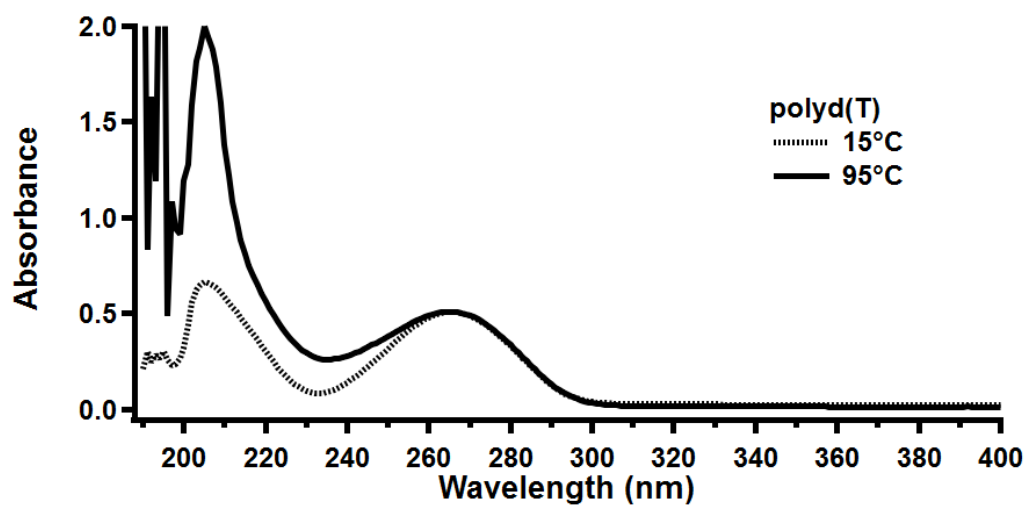


Figure 3.10. UV-vis spectra of polyd(T) at 15°C and 95°C.

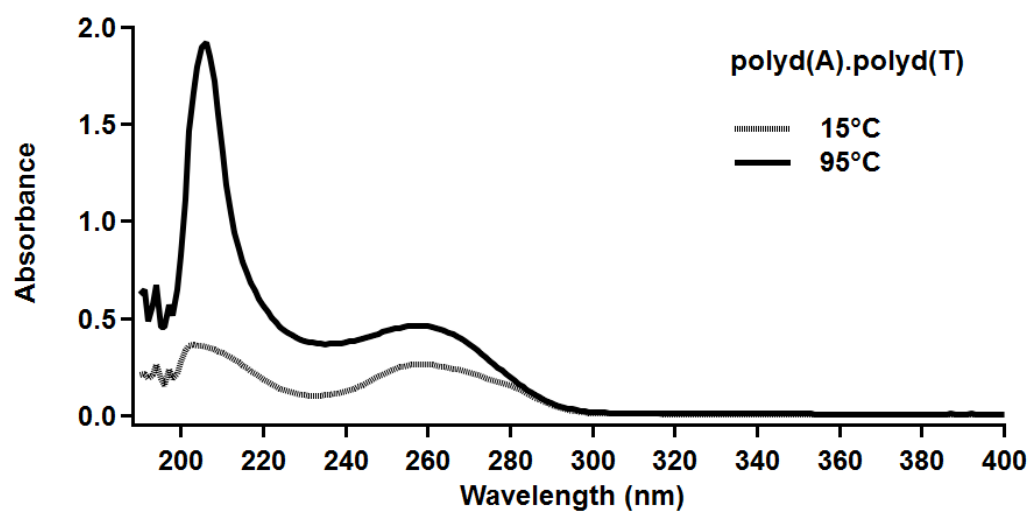


Figure 3.11. UV-vis spectra of polyd(A)·polyd(T) at 15°C and 95°C.

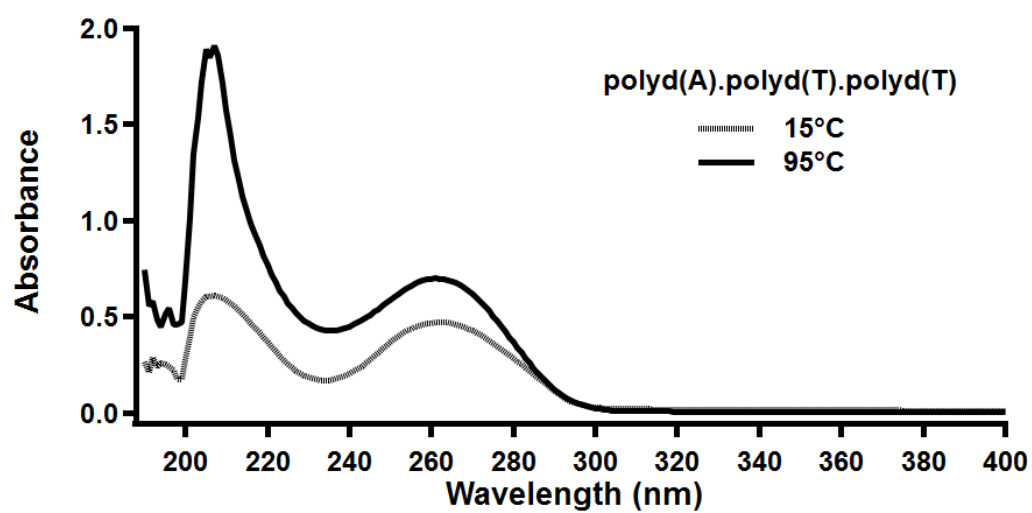


Figure 3.12. UV-vis spectra of polyd(A).polyd(T).polyd(T) at 15°C and 95°C.

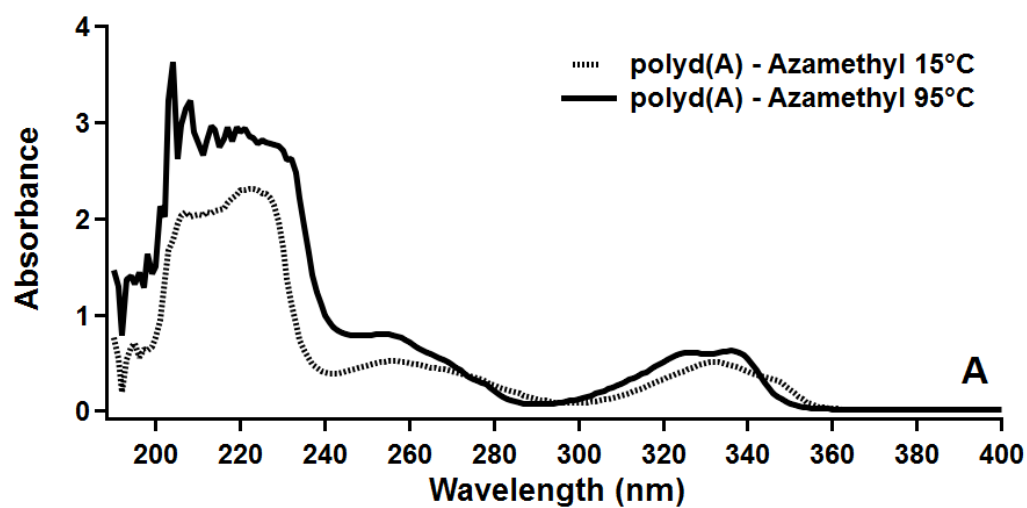


Figure 3.13. UV-vis spectra of polyd(A)-Azamethyl at 15°C and 95°C.

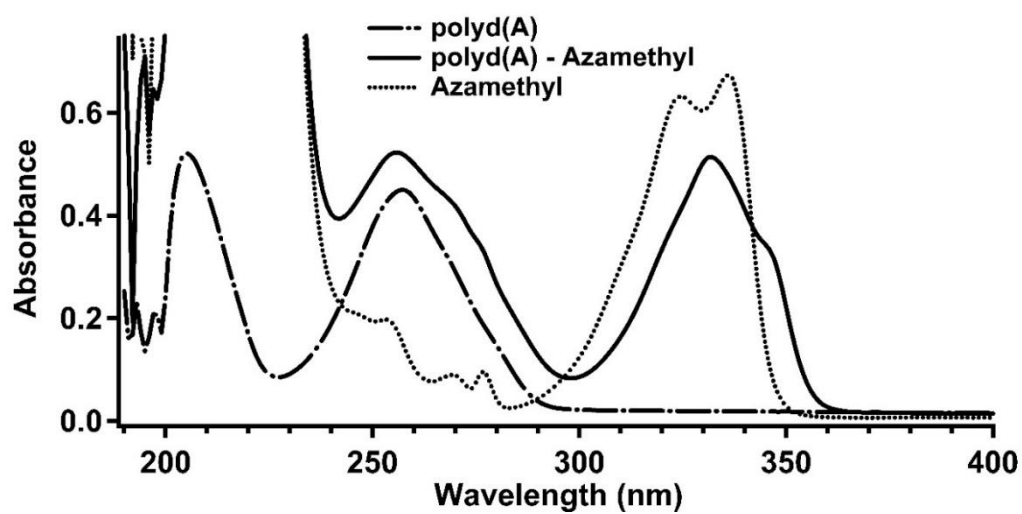


Figure 3.14. UV-vis spectra of polyd(A), polyd(A)-Azamethyl and Azamethyl at 15 °C.

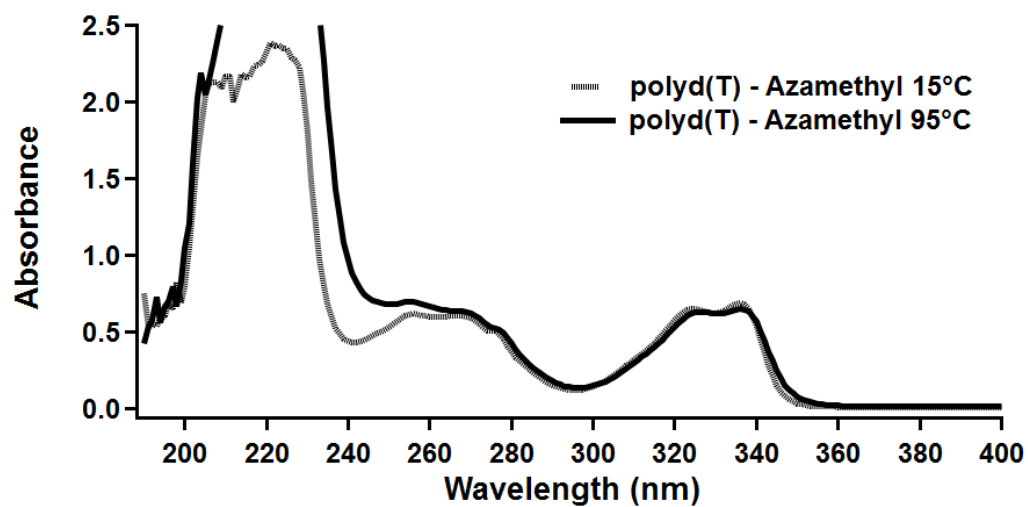


Figure 3.15. UV-vis spectra of polyd(T)-Azamethyl at 15°C and 95°C.

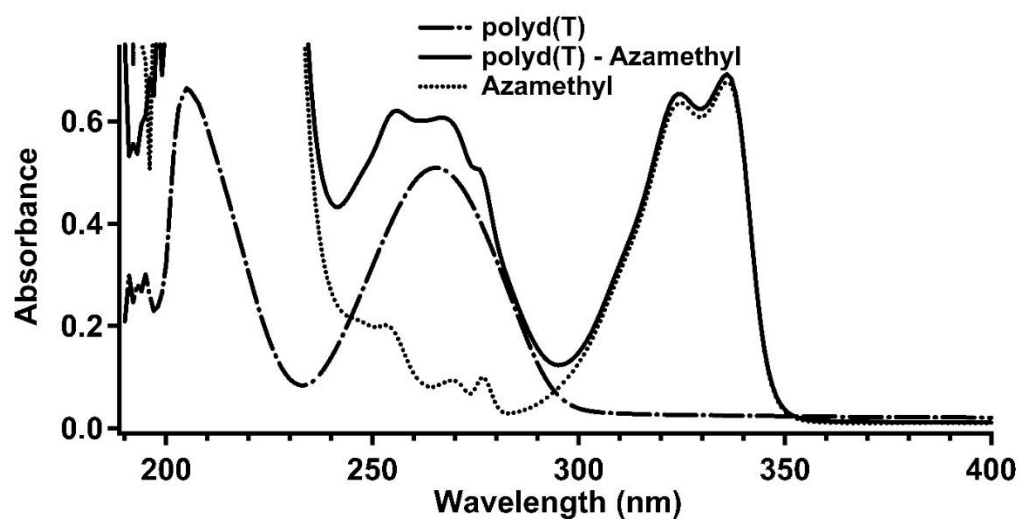


Figure 3.16. UV-vis spectra of polyd(T), polyd(T)-Azamethyl and Azamethyl at 15 °C.

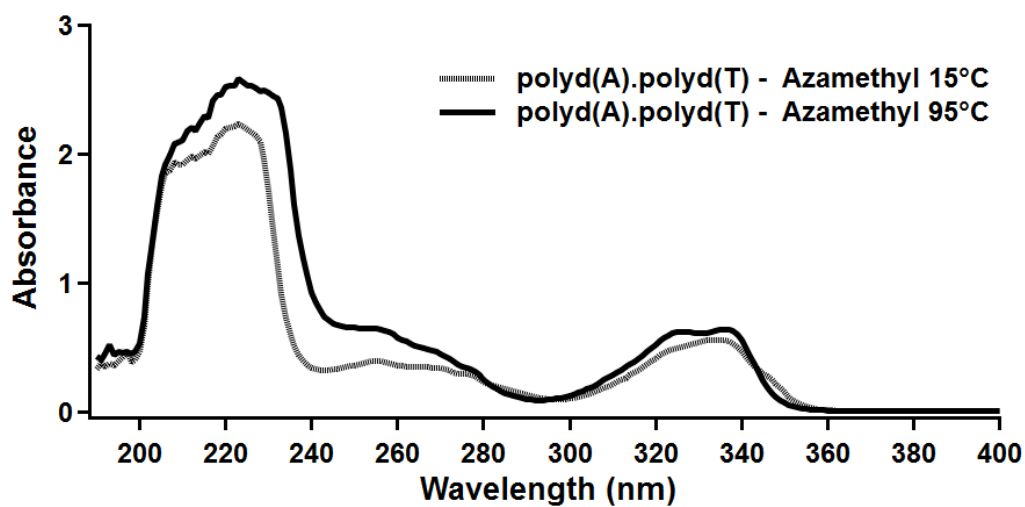


Figure 3.17. UV-vis spectra of polyd(A).polyd(T)-Azamethyl at 15°C and 95°C.

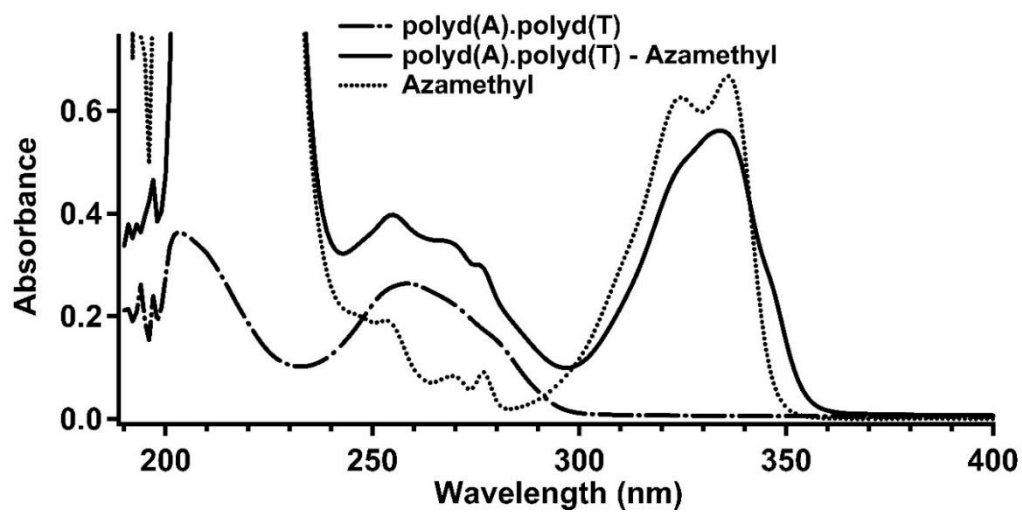


Figure 3.18. UV-vis spectra of polyd(A)·polyd(T), polyd(A)·polyd(T)–Azamethyl and Azamethyl at 15 °C.

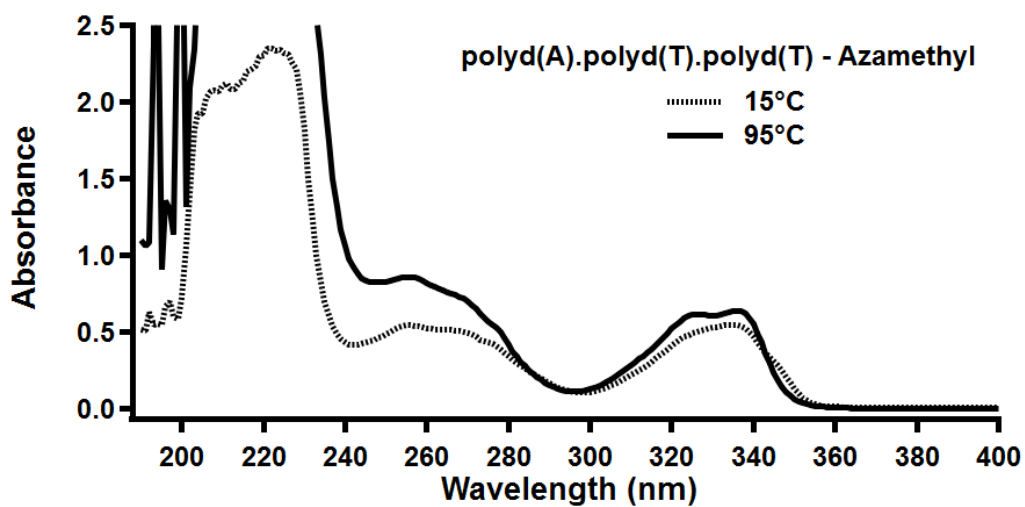


Figure 3.19. UV-vis spectra of polyd(A)·polyd(T)·polyd(T)-Azamethyl at 15°C and 95°C.

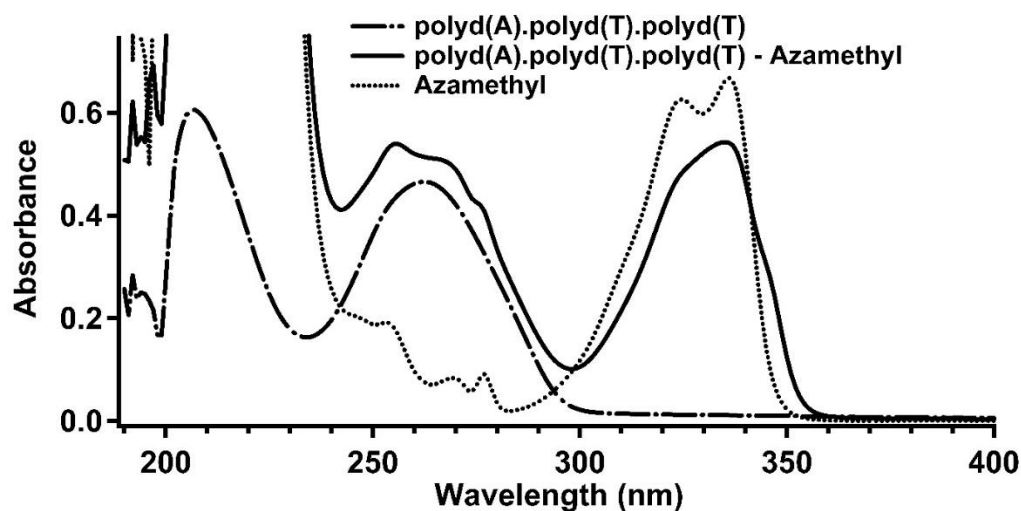


Figure 3.20. UV-vis spectra of polyd(A)·polyd(T)·polyd(T), polyd(A)·polyd(T)·polyd(T)–Azamethyl and Azamethyl at 15 °C.

When the UV-vis spectra for polyd(A) (Figure 3.9), polyd(A)·polyd(T) (Figure 3.11) and polyd(A)·polyd(T)·polyd(T) (Figure 3.12) were compared in the absence of Azamethyl at 15°C and 95°C, an increase in the UV-vis absorbance (hyperchromic effect) value between 230 nm and 300 nm was observed due to the unstacking of bases upon denaturation. As stated above, with increasing temperature, the nucleic acid denatures and absorbs light better. The same increase was not observed in the case of polyd(T)’s spectra since the pyrimidin T bases do not stack like purine bases and have a free conformation even at low temperatures (Figure 3.10) (Bloomfield, Crothers and Tinoco Jr., 1999).

A red shift and hypochromic effect were observed in the UV-vis spectra of Azamethyl in the presence of polyd(A) (Figure 3.13), polyd(A)·polyd(T) (Figure 3.17), and polyd(A)·polyd(T)·polyd(T) (Figure 3.19) at longer wavelengths indicating the binding of Azamethyl to these structures. The changes were more obvious in the comparison graphs of nucleic acid, nucleic acid-Azamethyl and Azamethyl given in Figure 3.14 (for polyd(A)), Figure 3.18 (for polyd(A)·polyd(T)) and Figure 3.20 (for polyd(A)·polyd(T)·polyd(T)). On the contrary, almost no change was observed in the presence of polyd(T) indicating the lack of affinity

between Azamethyl and polyd(T) (Figure 3.16). The spectra of polyd(T) at 15°C and 95°C were also similar to some extent. There was only a slight blue shift (shift to lower wavelengths) upon heating (Figure 3.15). In previous experiments with coralyne, it was also observed that there was no interaction between coralyne and polyd(T) (Polak and Hud, 2002; Jain, Polak and Hud, 2003).

3.1.3 Determination of the Thermal Denaturation Temperatures by Using UV-vis Spectroscopy

The interactions of Azamethyl molecule with the d(A)₃₂, d(T)₃₂, d(A)₃₂·d(T)₃₂ double helix, d(A)₃₂·d(T)₃₂·d(T)₃₂ triple helix, polyd(A), polyd(T), polyd(A)·polyd(T) double helix and polyd(A)·polyd(T)·polyd(T) triple helix were investigated through the UV-vis thermal denaturation experiments in the presence and absence of Azamethyl. The main goal of these studies was to determine the stabilization ability of Azamethyl on these secondary structures upon binding. The thermal denaturation profiles presented in Figure 3.21-Figure 3.27 were obtained by monitoring the absorbance changes at either 260 nm (maximum absorption wavelength of DNA bases) or 336 nm (maximum absorption wavelength of Azamethyl).

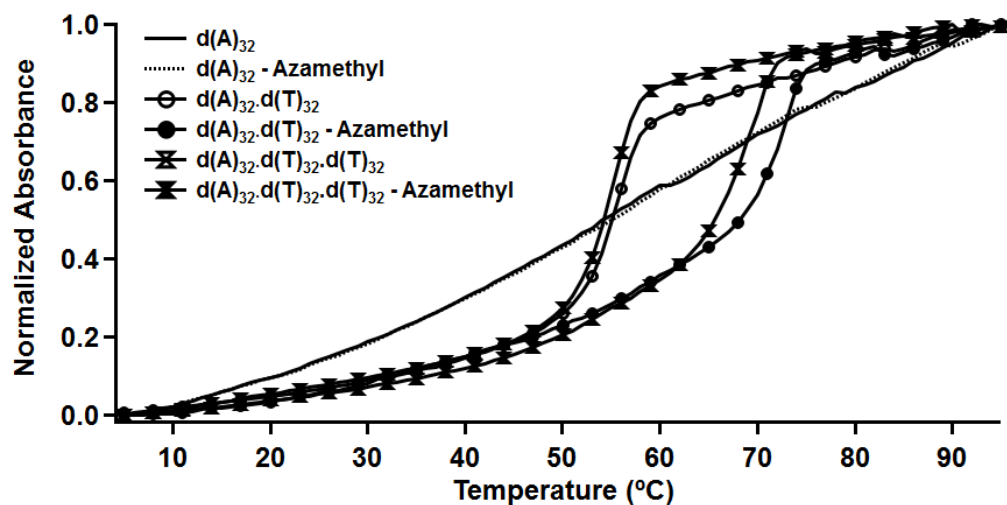


Figure 3.21. Thermal denaturation profiles of the $d(A)_{32}$, $d(A)_{32} \cdot d(T)_{32}$ and $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$

The profiles were obtained using the UV-vis absorbance values at 260 nm in the absence and presence of Azamethyl.

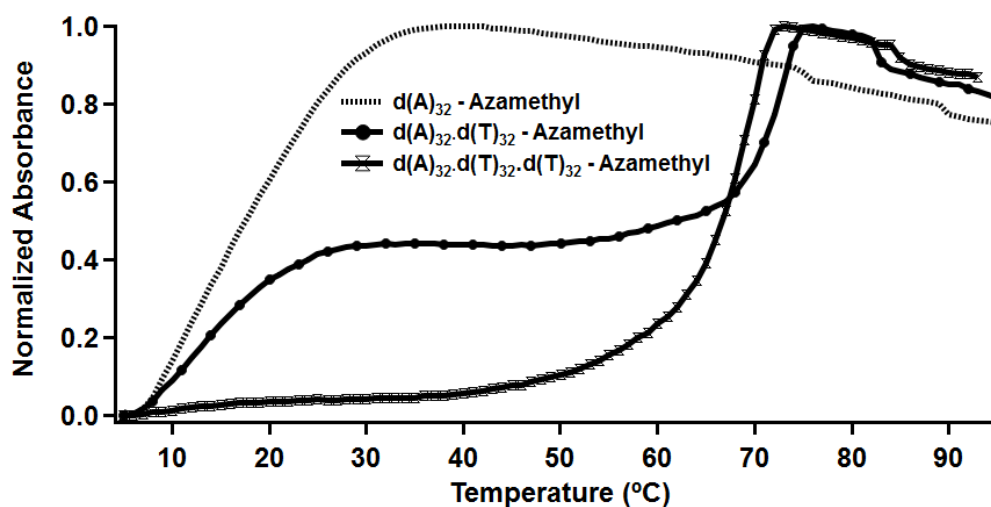


Figure 3.22. Thermal denaturation profiles of the $d(A)_{32}$, $d(A)_{32} \cdot d(T)_{32}$ and $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$

The profiles were obtained using the UV-vis absorbance values at 336 nm in the presence of Azamethyl.

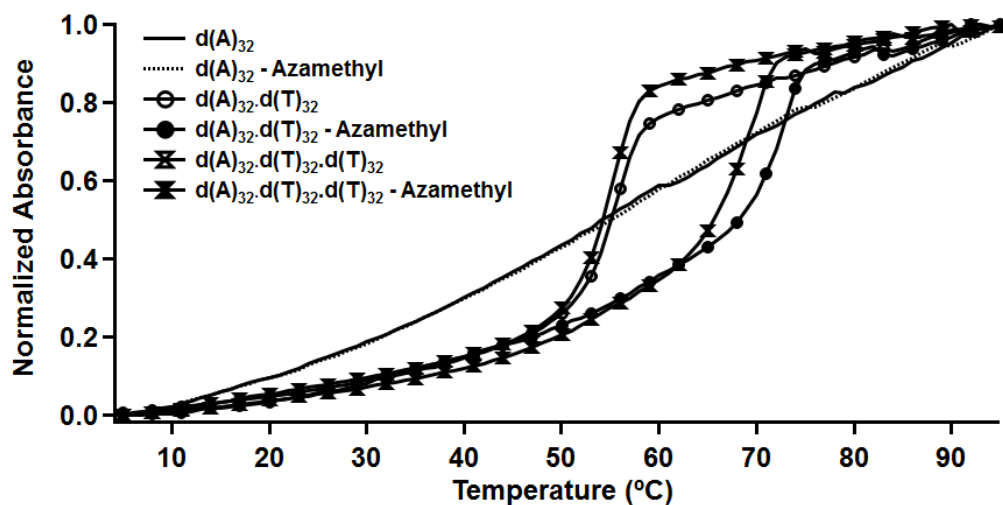


Figure 3.23. Thermal denaturation profiles of the $d(A)_{32}$, $d(A)_{32} \cdot d(T)_{32}$ and $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$

The profiles were obtained using the UV-vis absorbance values at 260 nm in the absence and presence of Azamethyl.

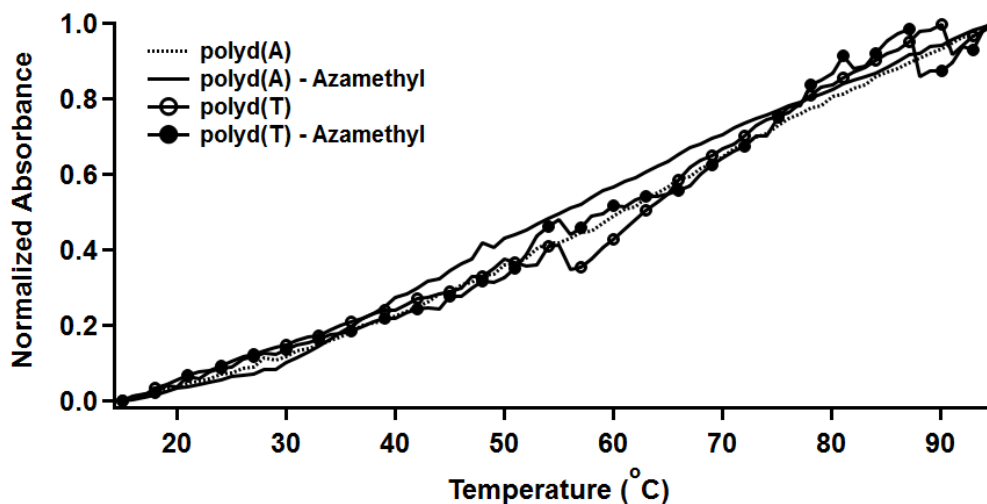


Figure 3.24. Thermal denaturation profiles of the polyd(A) and polyd(T)

The profiles were obtained using the UV-vis absorbance values at 260 nm in the absence and presence of Azamethyl.

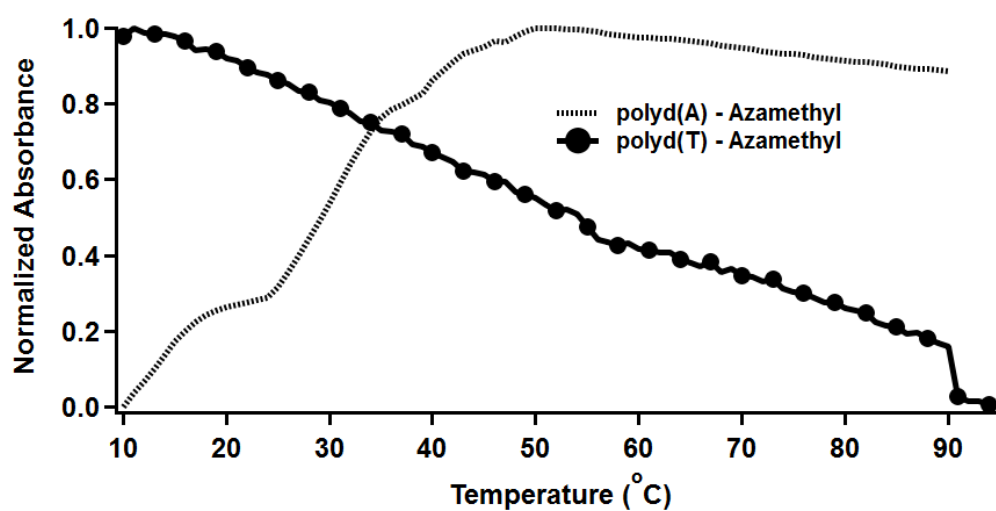


Figure 3.25. Thermal denaturation profiles of the polyd(A) and polyd(T)

The profiles were obtained using the UV-vis absorbance values at 336 nm in the presence of Azamethyl.

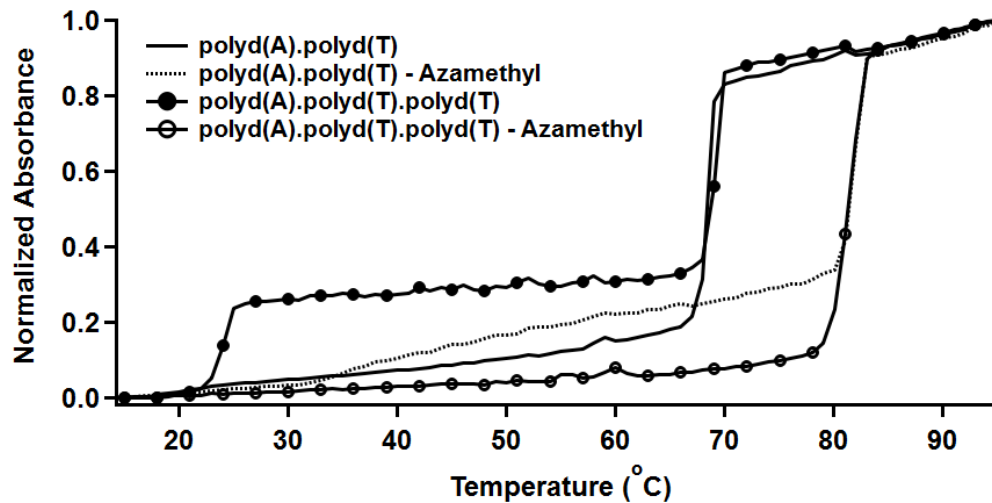


Figure 3.26. Thermal denaturation profiles of polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T)

The profiles were obtained using the UV-vis absorbance values at 260 nm in the absence and presence of Azamethyl.

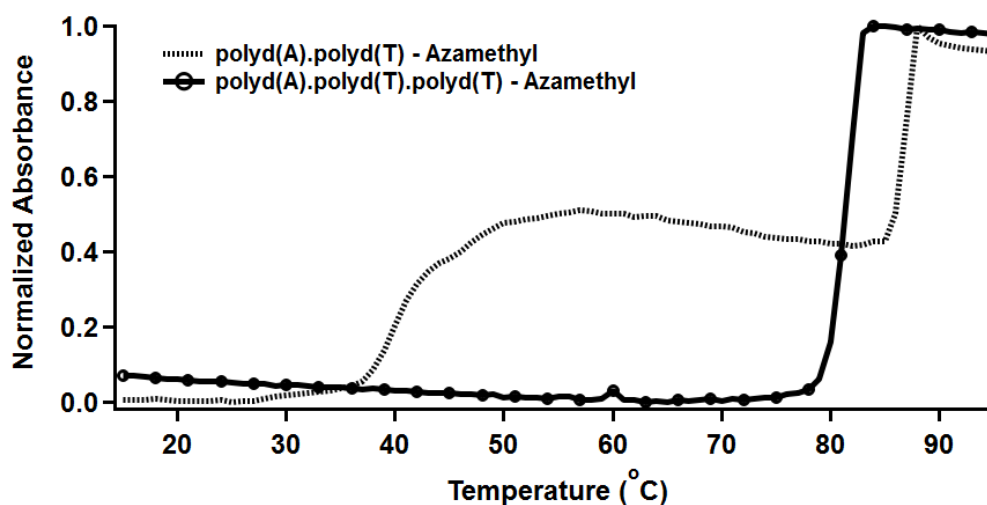


Figure 3.27. Thermal denaturation profiles of polyd(A)·poly(T) and polyd(A)·poly(T)·poly(T)

The profiles were obtained using the UV-vis absorbance values at 336 nm in the presence of Azamethyl.

The thermal denaturation profiles of $d(A)_{32}$, $d(A)_{32} \cdot d(T)_{32}$ and $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ given in Figure 3.21 demonstrates the presence of the secondary structures, as evident from the sudden rise in absorbance both for $d(A)_{32} \cdot d(T)_{32}$ and $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$. The derivation of the thermal denaturation curves revealed a thermal denaturation temperature (T_m) of 56°C for both structures (Table 3.1). For triplex structures, normally the observation of two transition temperatures, from triplex to duplex (3→2) and from duplex to single-stranded (2→1) was expected. Accordingly, seeing only a single transition for the triplex structure implies that it was unstable under these conditions. The observed thermal denaturation at 56°C belongs to the denaturation of the duplex structure (2→1). On the other hand, $d(A)_{32}$ was not able to form any secondary structure as there was only a gradual increase in its absorbance was observed upon an increase in the temperature (Figure 3.21). The $d(A)_{32}$'s thermal denaturation profile was not affected even in the presence of Azamethyl. Nevertheless, the secondary structures of $d(A)_{32} \cdot d(T)_{32}$ and

$d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ were stabilized upon Azamethyl binding as the sudden increase in the absorbance was observed at higher temperatures. The T_m value obtained by monitoring the absorbance change at 260 nm was $\sim 72^\circ\text{C}$, for both $d(A)_{32} \cdot d(T)_{32}$ and $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ in the presence of Azamethyl (Table 3.1). The thermal denaturation profiles were also obtained by monitoring the change at 336 nm with respect to temperatures for the samples with Azamethyl (Figure 3.22 and Table 3.2). The thermal denaturation profiles of $d(A)_{32}$, $d(A)_{32} \cdot d(T)_{32}$ and $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ displayed sudden changes in absorbance in the presence of Azamethyl indicating the denaturation with increasing temperature. The T_m values for $d(A)_{32} \cdot d(T)_{32}$ and $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ in the presence of Azamethyl which were obtained by monitoring the absorbance change at 336 nm was in an agreement with the T_m values obtained by monitoring the absorbance change at 260 nm. Conversely, a T_m value of $\sim 17^\circ\text{C}$ was observed for $d(A)_{32}$ -Azamethyl complex, which was not observed in the thermal denaturation profiles obtained at 260 nm (Table 3.1 and Table 3.2). Figure 3.24 and Figure 3.25 display the thermal denaturation profiles for polyd(A) and polyd(T) in the absence and presence of Azamethyl obtained by monitoring the absorbance changes at 260 nm and 336 nm, respectively. Neither polyd(A) nor polyd(T) displayed a secondary structure formation in the absence of Azamethyl. In the presence of Azamethyl however, no secondary structure formation was recorded at 260 nm. However, a secondary structure formation with a T_m of 30°C was observed for polyd(A)-Azamethyl by monitoring the absorbance change at 336 nm (Figure 3.25 and Table 3.2). For polyd(A).polyd(T) and polyd(A).polyd(T).polyd(T), a secondary structure formation was observed both in the absence and presence of Azamethyl (Figure 3.26). When Azamethyl was not present, the thermal denaturation temperature of $\sim 68^\circ\text{C}$ was observed both for polyd(A).polyd(T) and polyd(A).polyd(T).polyd(T) which belong to duplex to single-stranded transition ($2 \rightarrow 1$). In polyd(A).polyd(T).polyd(T), additional thermal denaturation temperature at 24°C was observed for the ($3 \rightarrow 2$) transition. Upon the addition of Azamethyl, only a single transition was observed at 82°C for both polyd(A).polyd(T) and polyd(A).polyd(T).polyd(T) (Table 3.1). On the other hand,

two sudden changes in absorbance were recorded for polyd(A).polyd(T) with only a single transition for polyd(A).polyd(T).polyd(T) when the change in the absorbance at 336 nm versus temperature was monitored (Figure 3.27). The presence of the two transitions indicates two different binding modes or secondary structures in polyd(A).polyd(T) upon Azamethyl binding.

All the T_m s obtained are listed in Table 3.1 and Table 3.2. For each nucleic acid structure, the T_m is specified in the presence and absence of Azamethyl by taking the first derivatives of the thermal denaturation profiles.

Table 3.1 UV-vis thermal denaturation temperatures, 260 nm.

	UV-vis T_m 's (°C)			
	1. Heating	1. Cooling	2. Heating	2. Cooling
d(A) ₃₂	-	-	-	-
d(A) ₃₂ - Azamethyl	-	-	-	-
d(A) ₃₂ ·d(T) ₃₂	56	54	55	54
d(A) ₃₂ ·d(T) ₃₂ - Azamethyl	73	57	73	57
d(A) ₃₂ ·d(T) ₃₂ ·d(T) ₃₂	56	54	55	54
d(A) ₃₂ ·d(T) ₃₂ ·d(T) ₃₂ - Azamethyl	72	57	71	57
polyd(A)	-	-	-	-
polyd(A) - Azamethyl	-	-	-	-
polyd(T)	-	-	-	-
polyd(T) - Azamethyl	-	-	-	-
polyd(A)·polyd(T)	68	67	68	67
polyd(A)·polyd(T) - Azamethyl	82	69	82	69
polyd(A)·polyd(T)·polyd(T)	24, 69	21, 68	24, 69	18, 67
polyd(A)·polyd(T)·polyd(T) - Azamethyl	82	69	82	69

Table 3.2 UV-vis thermal denaturation temperatures, 336 nm.

	UV-vis T_m 's (°C)			
	1. Heating	1. Cooling	2. Heating	2. Cooling
d(A) ₃₂ - Azamethyl	17	20	18	17
d(A) ₃₂ ·d(T) ₃₂ - Azamethyl	73	14, 57	73	14, 57
d(A) ₃₂ ·d(T) ₃₂ ·d(T) ₃₂ - Azamethyl	72	57	71	57
polyd(A) - Azamethyl	30	30	30	30
polyd(T) - Azamethyl	-	-	-	-
polyd(A)·polyd(T) - Azamethyl	35,82	28,69	28,82	28,69
polyd(A)·polyd(T)·polyd(T) - Azamethyl	82	69	82	69

Overall, when the absorbance changes at 260 nm were analyzed (Table 3.1) as expected, no T_m was obtained for the single-stranded d(A)₃₂, polyd(A) and polyd(T) in the absence of Azamethyl. For these structures, no T_m was observed also in the presence of Azamethyl through monitoring the absorbance change at 260 nm. However, in the case of d(A)₃₂-Azamethyl and polyd(A)-Azamethyl samples, T_m s of 17°C and 30°C were obtained respectively by monitoring the change in absorbance at 336 nm (Table 3.2). No T_m was obtained for polyd(T)-Azamethyl sample even by monitoring the changes in the absorbance at 336 nm. This indicates the lack of the tendency of Azamethyl to interact with polyd(T), supporting the results obtained in section 2.1.3. Since there was no interaction between Azamethyl and polyd(T), it was concluded that Azamethyl would not interact with the shorter d(T)₃₂ therefore, the interactions between d(T)₃₂ and Azamethyl were not investigated.

The T_m of d(A)₃₂·d(T)₃₂ duplex was found to be 56°C in the absence of Azamethyl. In the presence of Azamethyl, the T_m of d(A)₃₂·d(T)₃₂ duplex increased to 73°C (at both 260 and 336 nm), indicating the formation of a more stable structure upon Azamethyl binding.

On the other hand, the formation of $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ triple helix was not observed via thermal denaturation. In this sample, only the presence of T_m of 56°C was confirmed which is attributed to the double helix denaturation (2→1) ((Gondeau, Maurizot and Durand, 1998), (Debin, Malvy and Svinarchuk, 1997)). The stability of the triple helix was tried to be increased by adding 10 mM $MgCl_2$ to the sample, but again no T_m was observed. For polyd(A)·polyd(T)·polyd(T), the T_m was determined to be 24°C (at which triple helix denatures, (3→2)). Accordingly, under these conditions, it was not expected for $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ sample to form a stable triple helix above 5°C. Again, In the presence of Azamethyl, only one T_m at 72°C for 260 nm and 336 nm was observed for $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$. Both the T_m of $d(A)_{32} \cdot d(T)_{32}$ and $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ at 72°C were thought to belong to the $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ -Azamethyl complex rather than $d(A)_{32} \cdot d(T)_{32}$ -Azamethyl complex which will be explained later on. Briefly, our CD and competition dialysis (Güloğlu, 2018) suggested that Azamethyl has a selective affinity to $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ and polyd(A)·polyd(T)·polyd(T) triple structures compared to their corresponding double helical structures. Similar to coralyne, Azamethyl might be denaturing the double helix enabling the formation of an intercalated triple helix and a homo adenine self-structure. It is known that coralyne molecule causes such denaturation of the $d(A)_{32} \cdot d(T)_{32}$ and polyd(A)·polyd(T) structures (Polak and Hud, 2002; Jain, Polak and Hud, 2003). From another point of view, the evaluation of the T_m values at 336 nm (where the Azamethyl absorbs) revealed a thermal denaturation value of around 17°C in $d(A)_{32}$ -Azamethyl sample, indicating a possible interaction and formation of a secondary structure upon Azamethyl binding to $d(A)_{32}$. The interactions of $d(A)_{32}$ with Azamethyl were further investigated using CD spectroscopy.

When the obtained thermal denaturation values for polynucleic acid structures were examined using the absorbance values at 260 nm, no thermal denaturation value was observed for polyd(A), polyd(T), polyd(A)-Azamethyl and polyd(T)-Azamethyl samples. Since the polyd(A) and polyd(T) are single-stranded structures, they are not expected to have thermal denaturation values. The absence

of thermal denaturation values at 260 nm for polyd(A)-Azamethyl and polyd(T)-Azamethyl complexes indicates the lack of interactions between Azamethyl and polyd(A) or polyd(T). On the other hand, the evaluation of the thermal denaturations at 336 nm revealed a T_m of 30°C in polyd(A)-Azamethyl sample. This value indicates the formation of a secondary structure in polyd(A) in the presence of Azamethyl. In so doing, further CD investigations revealed the presence of interactions between polyd(A) and Azamethyl. The denaturation at about 17°C was observed in the sample of d(A)₃₂-Azamethyl. However, there was a difference between the heating and the cooling profiles supporting the presence of an unstable structure.

The T_m obtained for the polyd(A)·polyd(T) was around 68°C. Moreover, in the sample of polyd(A)·polyd(T)·polyd(T), it was observed that the triple helix structure was denaturing around 24°C and after that as expected, the double helix was denaturing around 68°C (actually specified as 69°C but the value is thought as an experimental error since the T_m obtained for the polyd(A)·polyd(T) was around 68°C). This value is in an acceptable agreement with the aforementioned value in a previous study by Polak and Hud (2002). An increase in the thermal denaturation values was observed when Azamethyl was added to both samples. Values of 35°C and 82°C were observed in the sample of polyd(A)·polyd(T)-Azamethyl at 336 nm in the first heating and 28°C and 82°C in the second heating. Also, a T_m value of 82°C at both 260 and 336 nm was observed for polyd(A)·polyd(T)·polyd(T)-Azamethyl in the first heating. It could be perceived mistakenly that these temperatures represent the stabilization of the double helix (from 68°C to 82°C) by Azamethyl. However, we believe 82°C belongs to the triple helical structure. The main reason behind this is our Circular Dichroism and competition dialysis data (Güloğlu, 2018). Additionally, the same kind of transitions were previously reported for coralyne. (Polak and Hud, 2002). The results obtained by Polak et al. are summarized in Table 3.3.

Without Azamethyl binding, the T_m of polyd(A)·polyd(T) was around 68°C. And, the T_m of polyd(A)·polyd(T)·polyd(T) were around 24°C (from triple helix to duplex) and around 68°C (from duplex to single strands). These temperature values are in a good agreement with the aforementioned values for polyd(A)·polyd(T) and

polyd(A)·polyd(T)·polyd(T) in the absence of coralyne (Table 3.3). An increase in the thermal denaturation values was observed when Azamethyl was added to both samples. The values of 35°C and 82°C in the first heating and 28°C and 82°C in the second heating were observed in the sample of polyd(A)·polyd(T)-Azamethyl. The appearance of 35°C might be surprising. Yet, it was also consistent with the observation reported for coralyne sample by Polak and Hud (2002). They observed that in the presence of the coralyne, polyd(A)·polyd(T) disproportionated at around 35°C, and the disproportionated strands were forming an intercalated polyd(A)·polyd(T)·polyd(T)-coralyne and polyd(A)-coralyne complexes. And, the polyd(A)·polyd(T)·polyd(T)-coralyne complex denaturated at 82°C (Table 3.3) later on. The denaturation profiles for polynucleotides in the presence of Azamethyl were very similar to the coralyne system (Polak and Hud, 2002). Consequently, the Azamethyl is also believed to disproportionate polyd(A)·polyd(T) due to its high affinity to polyd(A)·polyd(T)·polyd(T). Further CD (in our study) and the competition dialysis (Güloğlu, 2018) experiments also support our UV-vis findings of Azamethyl's higher affinity to polyd(A)·polyd(T)·polyd(T) compared to polyd(A)·polyd(T), such that the observed 82°C probably belongs to polyd(A)·polyd(T)·polyd(T)-Azamethyl complex.

Table 3.3 Thermal denaturation temperatures obtained in the study of Polak and Hud (2002).

	T_m (°C)
polyd(A)	-
polyd(A) - Coralyne	47
polyd(A)·polyd(T)	68
polyd(A)·polyd(T) - Coralyne	35 (duplex), 47 (polyd(A) self-structure), 82 (triplex)
polyd(A)·polyd(T)·polyd(T)	Not specified directly, but on the graphics on the Figure 7, it was observed that the thermal denaturation of the triple helix is around 24°C for 60.0 µM nucleic acid concentration. Also, for duplex, it is 68°C.
polyd(A)·polyd(T)·polyd(T) - Coralyne	82

3.2 Investigation of the Interactions of Azamethyl with the Nucleic Acids and Determination of the Thermal Denaturation Temperatures Using CD Spectroscopy

Azamethyl's interaction with the d(A)₃₂, d(T)₃₂, d(A)₃₂·d(T)₃₂ double helix, d(A)₃₂·d(T)₃₂·d(T)₃₂ triple helix, polyd(A), polyd(T), polyd(A)·polyd(T) double helix and polyd(A)·polyd(T)·polyd(T) triple helix were investigated also with CD. In order to assess the presence/absence of interactions, CD spectra of the nucleic acids were collected at 5°C in the presence and absence of Azamethyl. The corresponding CD spectra are given in Section 3.2.1, 3.2.2 and 3.2.3.

3.2.1 Interactions of Azamethyl with $d(A)_{32}$, $d(T)_{32}$, $d(A)_{32} \cdot d(T)_{32}$ and $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$, CD Spectroscopy

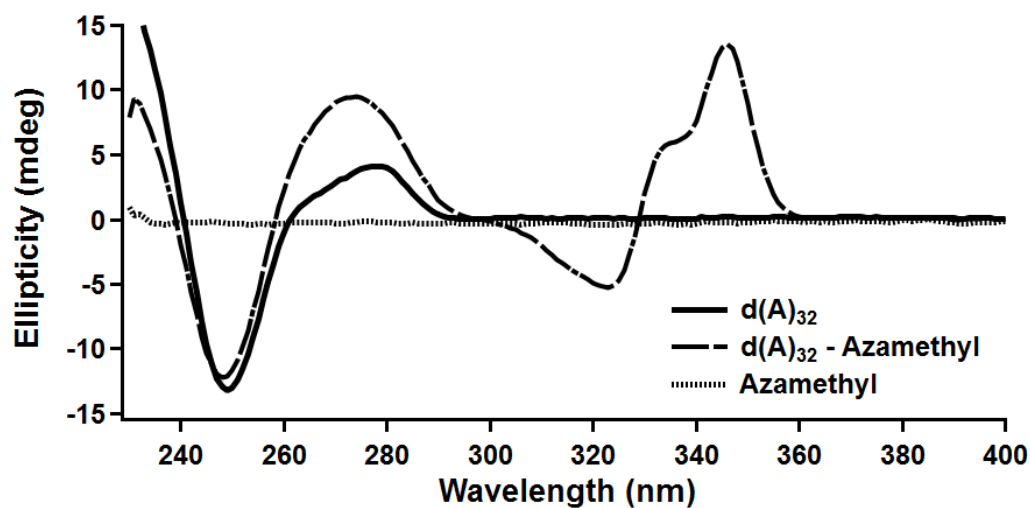


Figure 3.28. CD spectra of $d(A)_{32}$, $d(A)_{32}$ -Azamethyl and Azamethyl

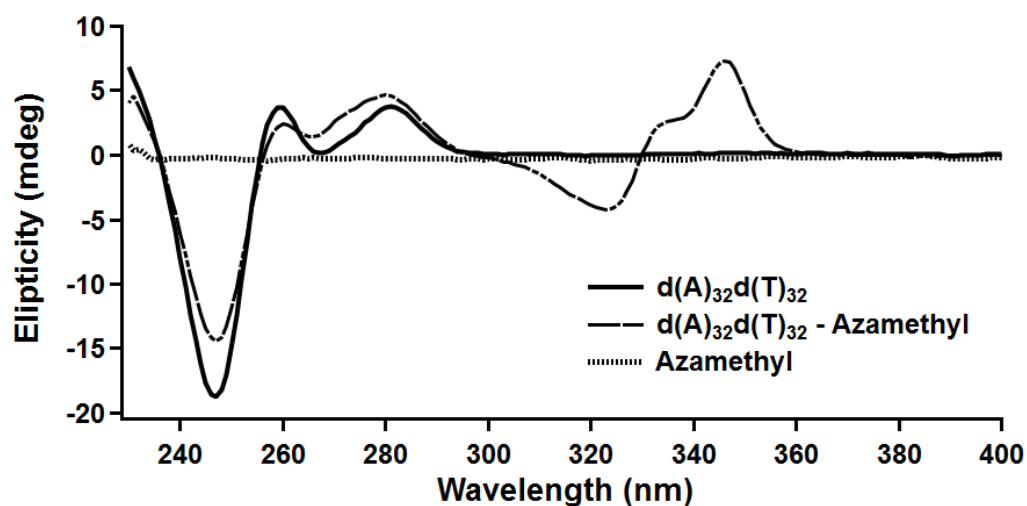


Figure 3.29. CD spectra of $d(A)_{32} \cdot d(T)_{32}$, $d(A)_{32} \cdot d(T)_{32}$ -Azamethyl and Azamethyl

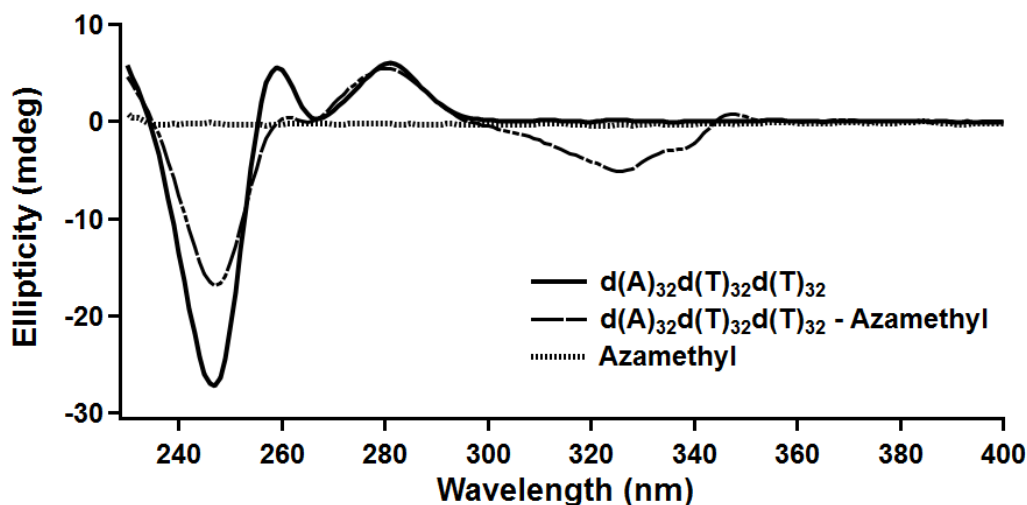


Figure 3.30. CD spectra of $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$, $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ -Azamethyl and Azamethyl

Azamethyl is an achiral molecule, so it does not give rise to any CD bands. On the contrary, the nucleic acids are chiral molecules due to asymmetric backbone sugars leading CD bands (Vorlickova et al. 2012). Even though the characteristics of spectra depend on the sequence and the helical structure, they usually have a negative peak around 250 nm and a positive peak around 275 nm (Bloomfield, Crothers and Tinoco Jr., 1999).

The CD spectra of $d(A)_{32}$, $d(T)_{32}$, $d(A)_{32} \cdot d(T)_{32}$ and $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ in the absence of Azamethyl were very similar to the CD spectra of these nucleic acids reported previously in the literature (Polak and Hud, 2002; Jain, Polak and Hud, 2003). In the presence of Azamethyl, the changes were observed between 230 to 300 nm (the region where both nucleic acids and Azamethyl have absorbance) and between 300 to 360 nm (induced peaks, the region where only Azamethyl has absorbance). The induced peak formation is considered as the sign of π - π stacking interactions between Azamethyl and the nucleic acid bases. In other words, it is the clearest sign of interaction between Azamethyl and the specified nucleic acid where chirality is induced in Azamethyl upon its interaction with the nucleic acid bases. Yet, it is obvious that the changes and the formation of the induced CD bands are

dependent on the nucleic acid structure. Azamethyl containing d(A)₃₂ (Figure 3.28) and d(A)₃₂·d(T)₃₂ (Figure 3.29) samples have both negative and positive peaks at around 320 nm and 345 nm, respectively. The absolute value of the intensity of the positive peak was higher (ellipticity= 13.6 mdeg) compared to that of the negative peak (ellipticity = -5.2 mdeg) in d(A)₃₂-Azamethyl sample. In the d(A)₃₂·d(T)₃₂-Azamethyl sample, the positive (ellipticity=7.3 mdeg) and negative (ellipticity=-4.3 mdeg) induced peaks are nearly in equal intensity, while in d(A)₃₂·d(T)₃₂·d(T)₃₂-Azamethyl, the absolute intensity of the negative induced peak (ellipticity = -5.2) is greater than the absolute intensity of the positive peak (ellipticity = 0.8) (Figure 3.30). The similar observation was reported by Polak and Hud (2002) for coralyne. As mentioned above, the interactions of Azamethyl with the d(T)₃₂ were not investigated as Azamethyl did not display any interaction in the presence of polyd(T) based on the UV-vis and CD (Section 3.1.1) studies.

3.2.2 Interactions of Azamethyl with polyd(A), polyd(T), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T), CD Spectroscopy

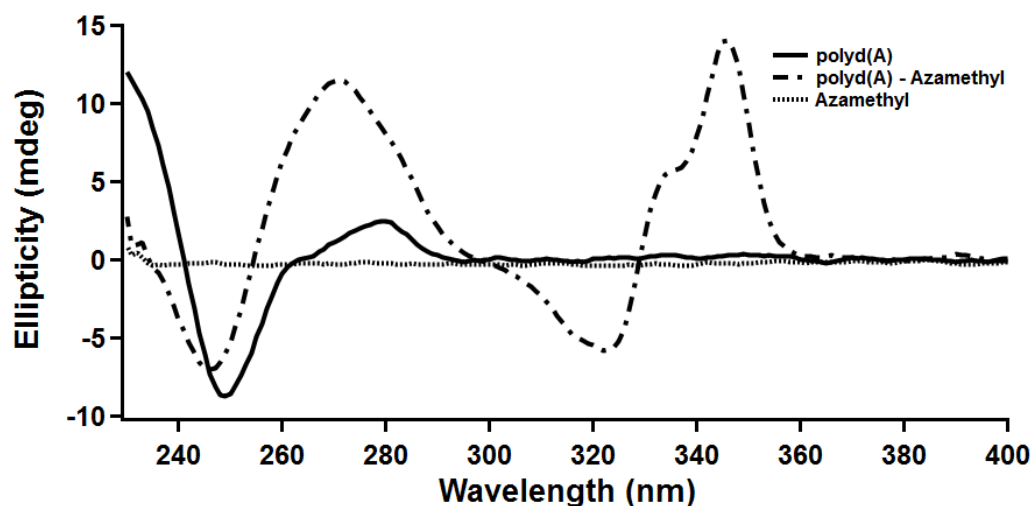


Figure 3.31. CD spectra of polyd(A), polyd(A)-Azamethyl and Azamethyl

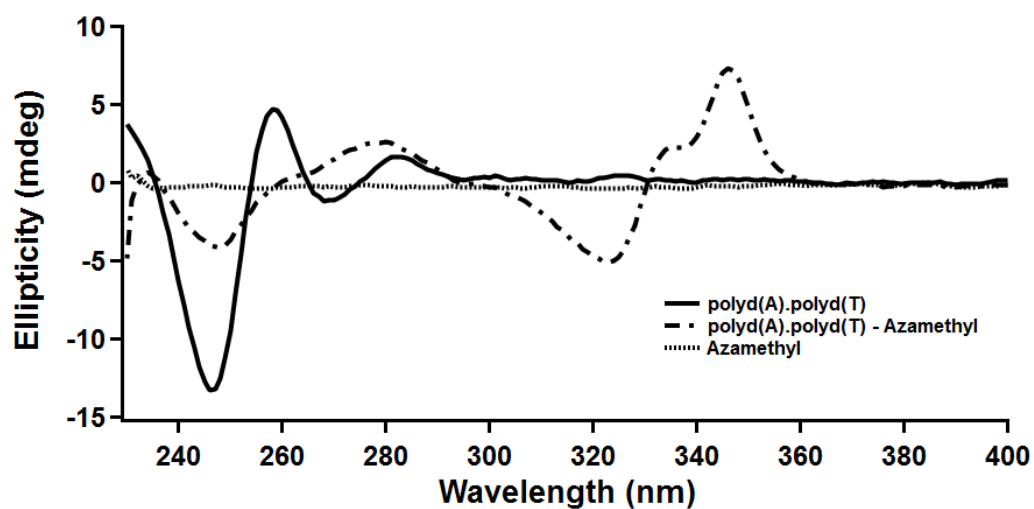


Figure 3.32. CD spectra of polyd(A)·polyd(T), polyd(A)·polyd(T)-Azamethyl and Azamethyl

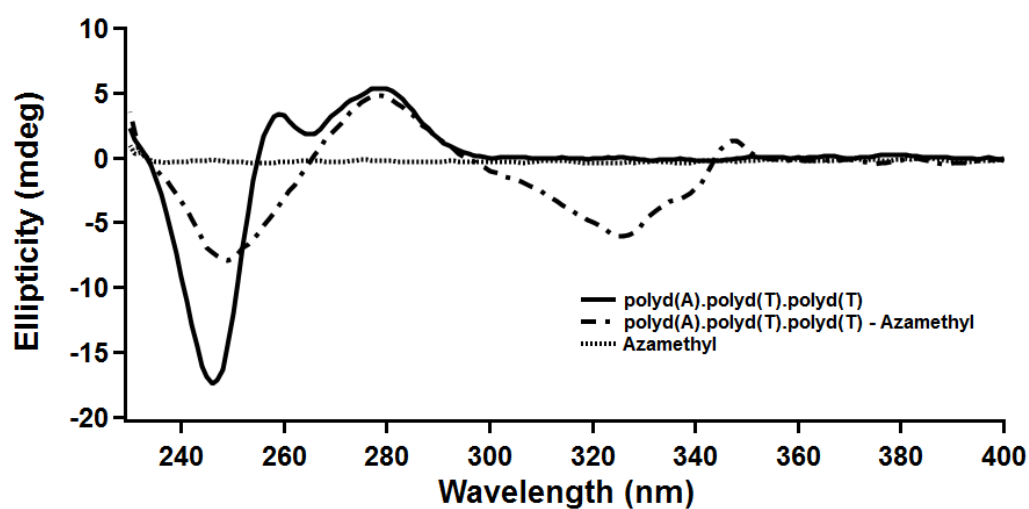


Figure 3.33. CD spectra of polyd(A)·polyd(T)·polyd(T), polyd(A)·polyd(T)·polyd(T)-Azamethyl and Azamethyl

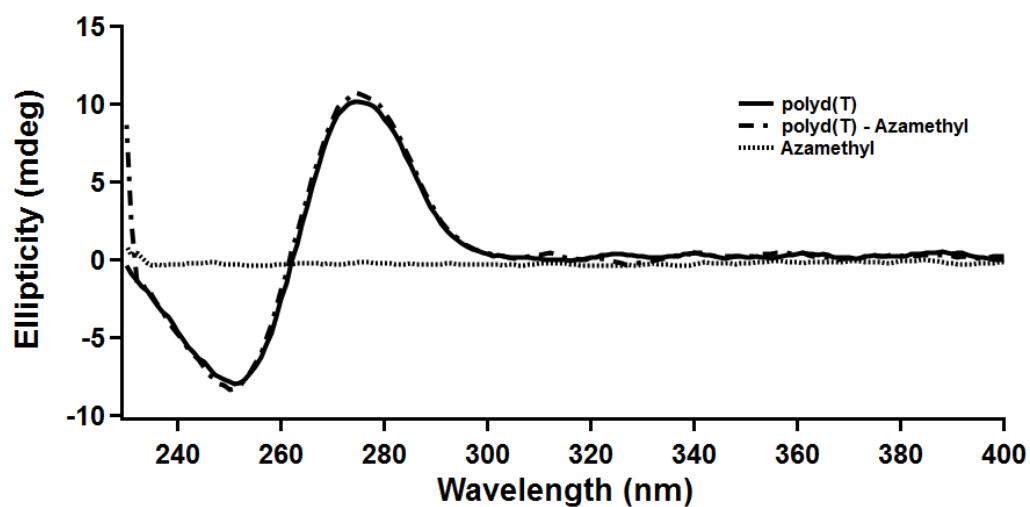


Figure 3.34. CD spectra of polyd(T), polyd(T)-Azamethyl and Azamethyl

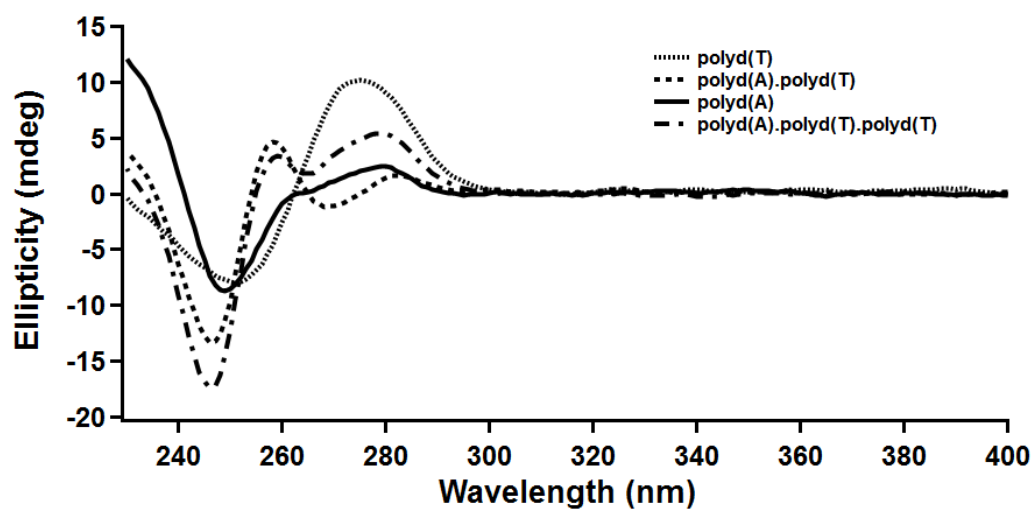


Figure 3.35. CD spectra of polyd(T), polyd(A), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) in the absence of Azamethyl.

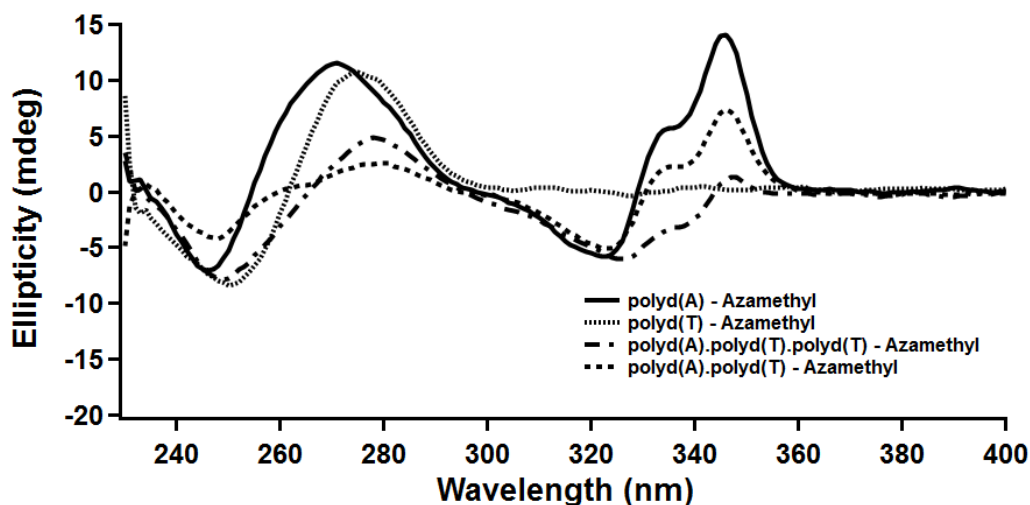


Figure 3.36. CD spectra of polyd(T), polyd(A), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) in the presence of Azamethyl.

When the interactions of Azamethyl with the polyd(A), polyd(T), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) were examined, it was observed that Azamethyl did not interact with polyd(T). The CD spectra of polyd(T) were nearly the same in the absence and presence of Azamethyl. Neither an induced peak formation nor a change was observed in the CD spectra (Figure 3.34). Furthermore, polyd(A) (Figure 3.31), polyd(A)·polyd(T) (Figure 3.32) and polyd(A)·polyd(T)·polyd(T) (Figure 3.33) also interacted with the Azamethyl similar to $d(A)_{32}$, $d(A)_{32} \cdot d(T)_{32}$ and $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$, respectively. A negative peak around 250 nm and a positive one around 280 nm were observed in polyd(A)'s spectrum (Figure 3.31). After the Azacyanine binding, both of the peaks exhibited a blue shift (about 9.01094 mdeg) and an increase in the intensity. The intensity of the peak at 250 nm increased from -8.5 mdeg to -0.160 mdeg and the intensity of the peak at 280 nm increased from 2.8 mdeg to 12.5 mdeg upon the Azamethyl binding and the shoulder at around 260 nm disappeared. There was an induced peak formation between 300 nm and 360 nm upon the Azamethyl binding. The absolute magnitude of the intensity of the negative peak (centered around 320 nm) was

smaller than that of the positive peak (centered around 345 nm). It should be noted that the positive peak also had a shoulder at 249 nm.

In the CD spectra of polyd(A)·polyd(T), a negative peak around 245 nm and two positive peaks around 260 and 280 nm were observed. After the Azacyanine binding, the intensity of the peak at 245 nm and 280 nm increased, while the intensity of the peak at 260 nm decreased. Again, the formation of the induced peaks with a local minimum at 320 nm and maximum with 345 nm was observed (Figure 3.32).

A negative peak around 245 nm and two positive peaks around 260 and 280 nm were observed in polyd(A)·polyd(T)·polyd(T)'s spectrum. However, the intensity of the peak at 245 nm increased from -18.0 mdeg to -7.4 mdeg and red shifted about 4 nm after the Azacyanine binding. The peak at 260 nm disappeared and induced peaks with minimum and maximum intensities at 325 nm and 350 nm appeared, respectively (Figure 3.32). The peak area of the positive band was very small compared to the negative peak.

Overall, in the presence of Azamethyl, there was a significant change in the CD spectra of polyd(A), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) between 230 nm and 300 nm in addition to the induced bands between 300 nm and 360 nm (Figure 3.35, Figure 3.36), whereas no change was observed in the spectrum of polyd(T) upon Azamethyl binding.

3.2.3 Determination of Thermal Denaturation Temperatures via CD Spectroscopy

The thermal denaturation profiles for d(A)₃₂, d(A)₃₂·d(T)₃₂ and d(A)₃₂·d(T)₃₂·d(T)₃₂, polyd(A), polyd(T), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) were also determined by CD spectroscopy in the absence and presence of Azamethyl (Figure 3.37 - Figure 3.50).

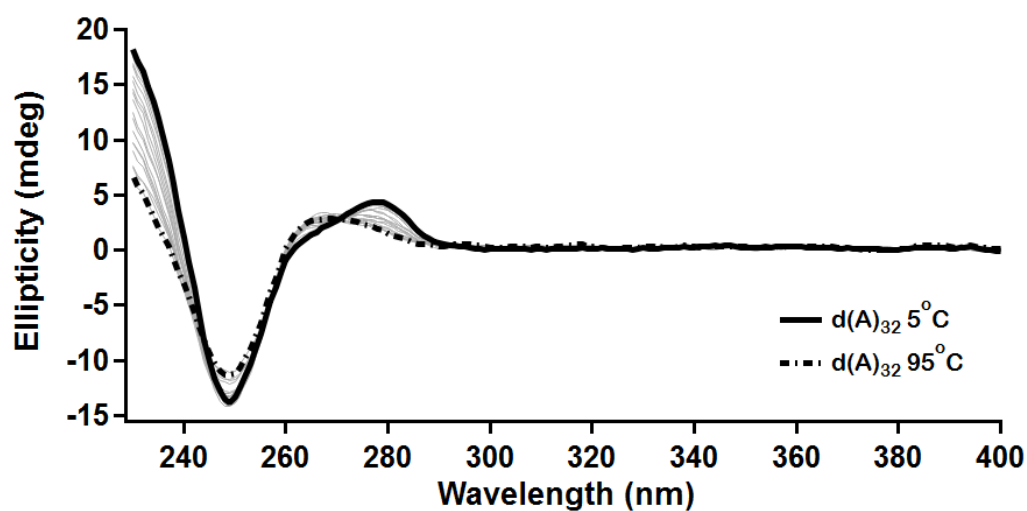


Figure 3.37. CD spectra of $d(A)_{32}$ between 5°C and 95°C

In order to make the change in the secondary structure with changing temperature more obvious, the spectra at 5°C and 95°C were drawn in bold straight line and dotted line respectively.

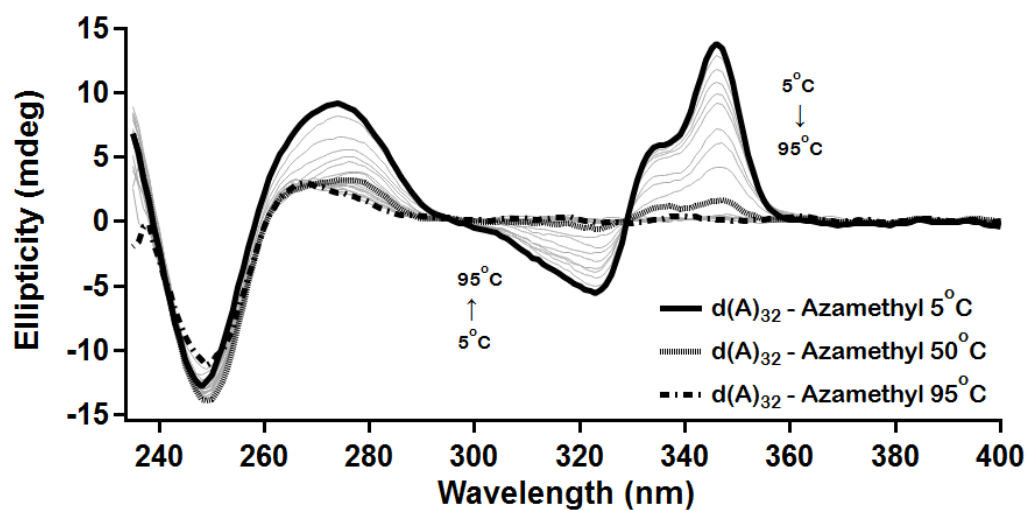


Figure 3.38. CD spectra of $d(A)_{32}$ -Azamethyl between 5°C and 95°C

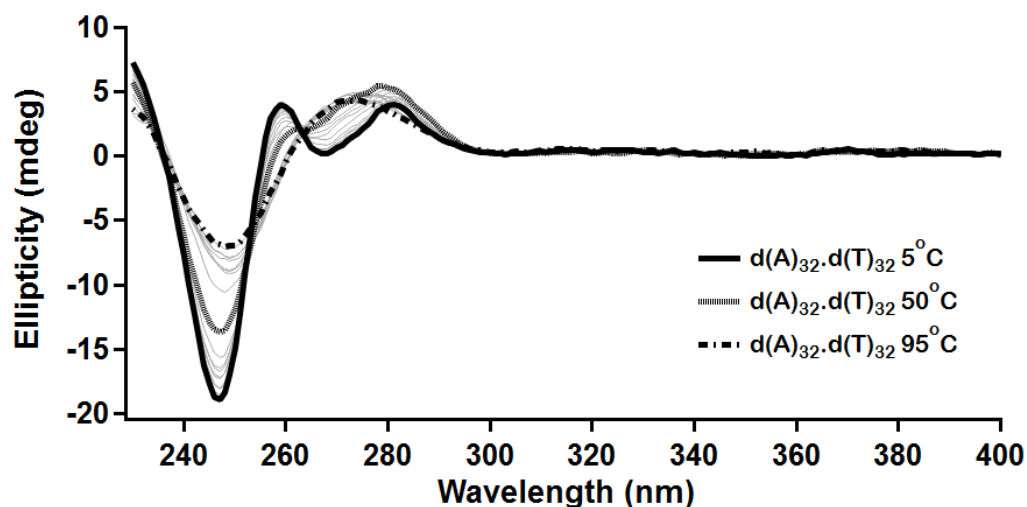


Figure 3.39. CD spectra of $d(A)_{32} \cdot d(T)_{32}$ between 5°C and 95°C

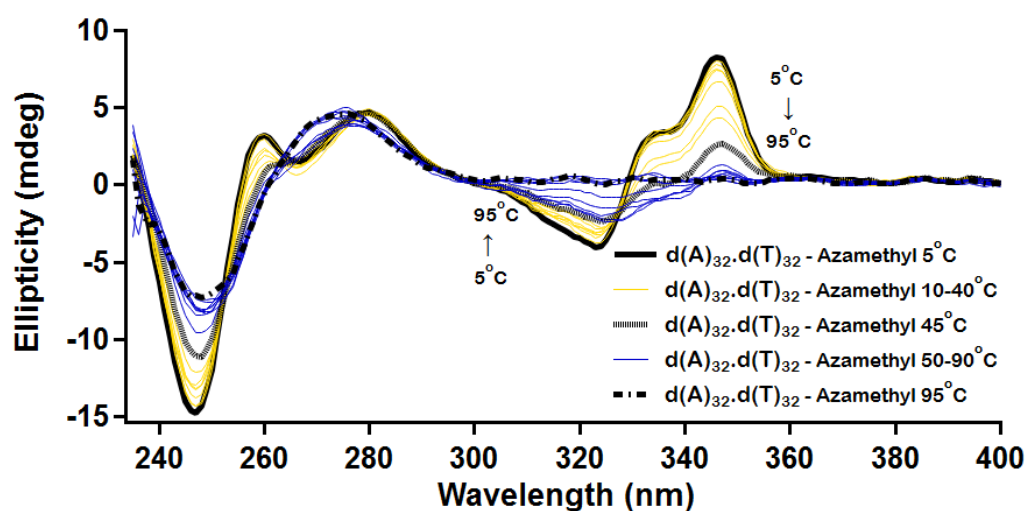


Figure 3.40. CD spectra of $d(A)_{32} \cdot d(T)_{32}$ -Azamethyl between 5°C and 95°C

The in-between spectra were drawn in purple and yellow according to the temperatures in which the secondary structure appeared to be different. With the rising temperature, the intensity of the peak at 250 nm decreased, while the peaks at 260 and 285 nm shifted to 275 nm and overlapped to be as one peak with the peak-shifting starting after 45°C. The induced peaks at 325 and 345 nm decreased as well. After 90°C, the induced peaks disappeared totally.

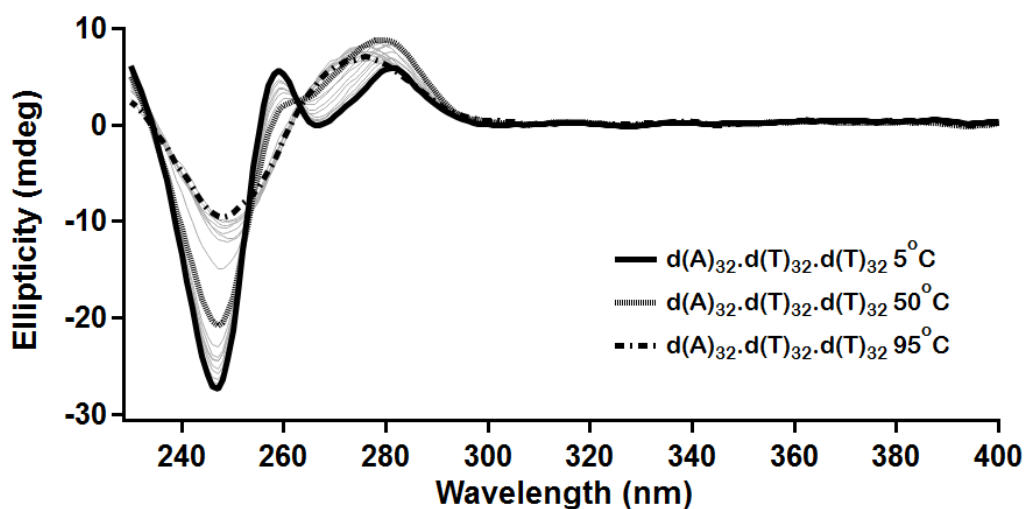


Figure 3.41. CD spectra of $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ between 5°C and 95°C

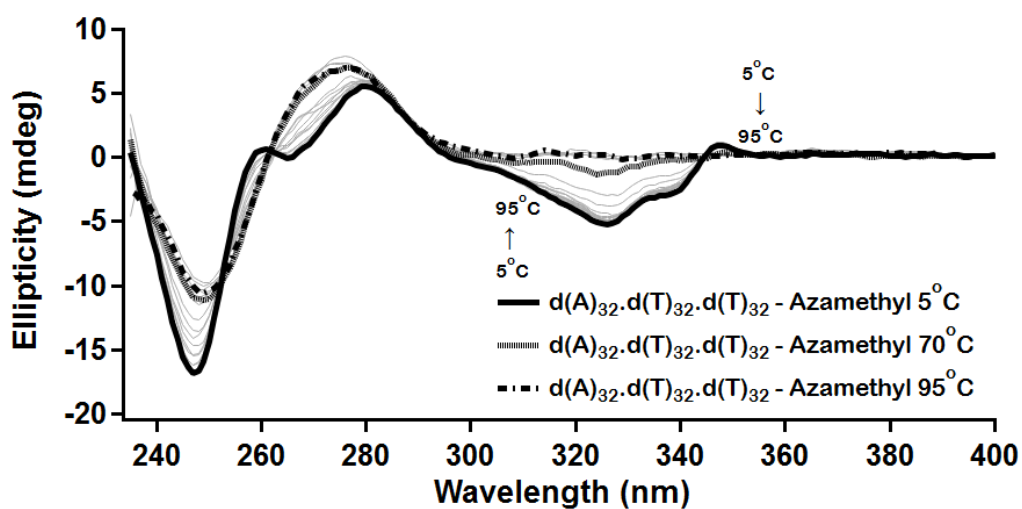


Figure 3.42. CD spectra of $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ -Azamethyl between 5°C and 95°C
With the rising temperature, the intensity of the peak decreased at 245 nm, while the intensities at 260 and 285 nms shifted to 275 nm to overlap as one peak with the peak-shifting at 45°C. The induced peaks at 325 and 345 nm also decreased. After 70°C, the induced peaks disappeared totally.

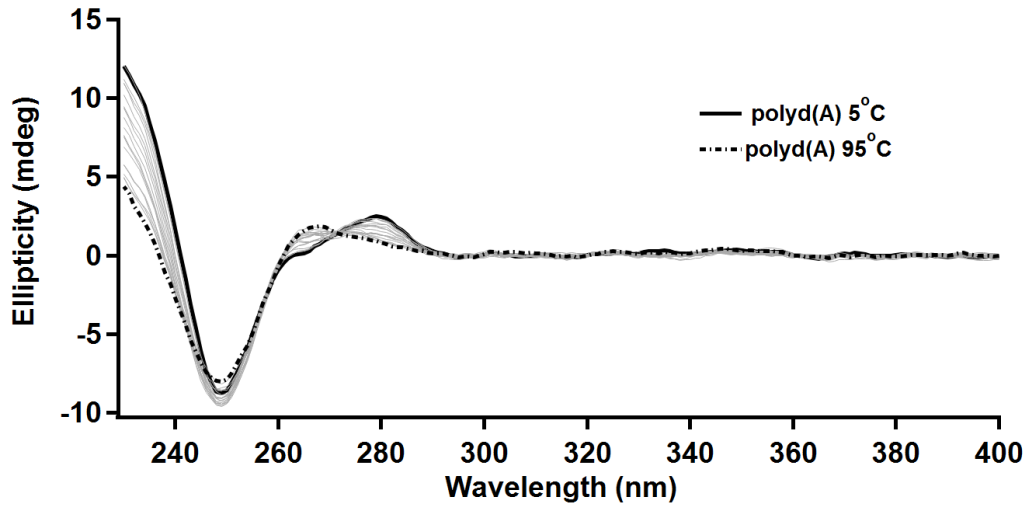


Figure 3.43. CD spectra of polyd(A) between 5°C and 95°C

With the rising temperature, the intensity of the peak at 250 nm declined, while the intensity of the peak at 280 nm shifted to 265 nm.

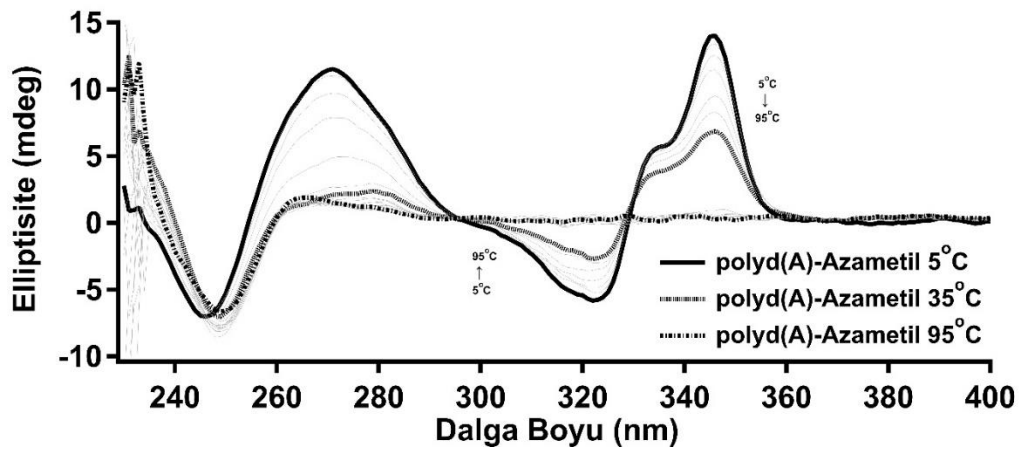


Figure 3.44. CD spectra of polyd(A)-Azamethyl between 5°C and 95°C

As the temperature rose, the intensity of peak at 245 nm decreased while the peak at 270 nm shifted to 265 nm. The shifting started after 35°C in this case. The intensities of the induced peaks at 325 and 345 nm decreased as well. After 35°C, the induced peaks disappeared completely.

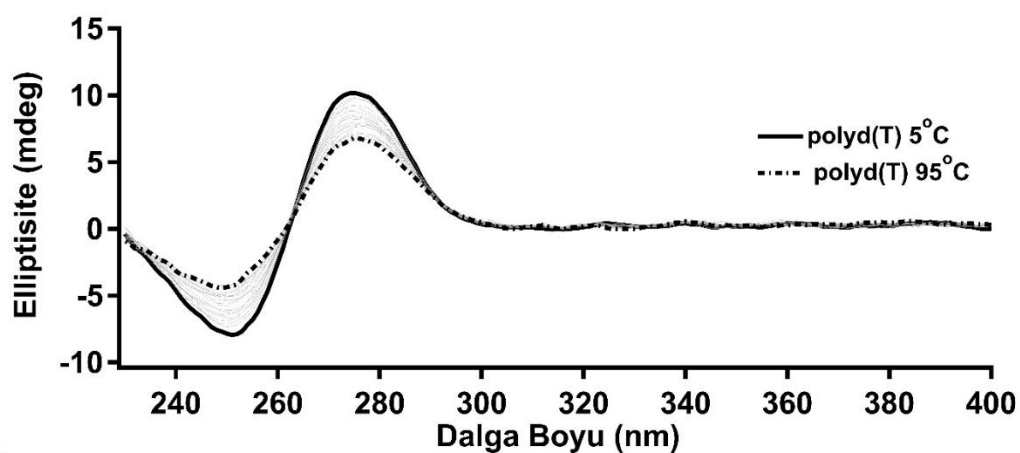


Figure 3.45. CD spectra of polyd(T) between 5°C and 95°C

With the rise in temperature, the intensity of the peaks at 250 and 275 nm decreased.

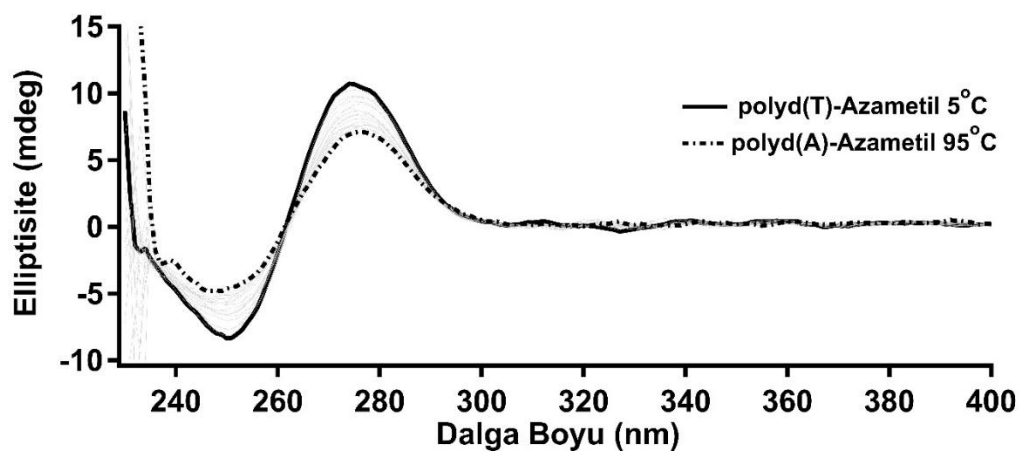


Figure 3.46. CD spectra of polyd(T)-Azamethyl between 5°C and 95°C

The rise in the temperature led to a fall in the intensities of the peaks at 250 and 275 nm. No induced peak formation was observed upon the Azacyanine binding.

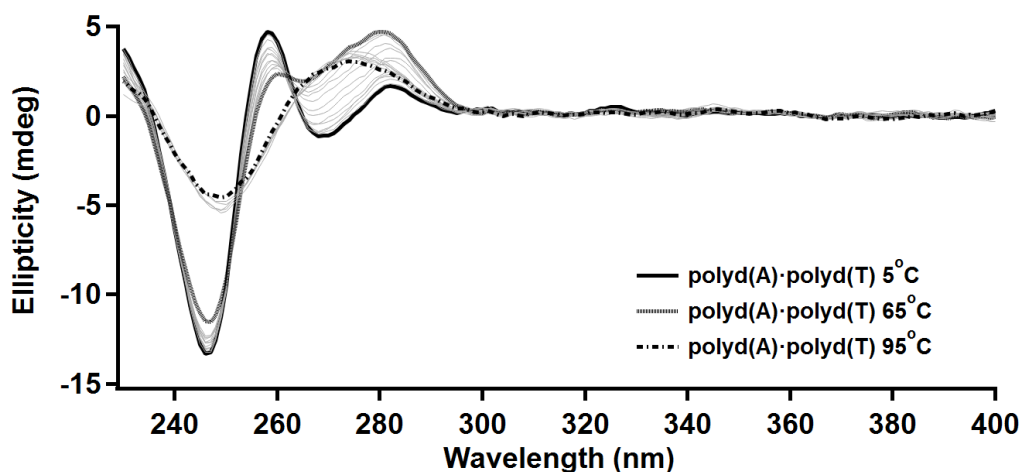


Figure 3.47. CD spectra of polyd(A)·polyd(T) between 5°C and 95°C

With the rise in temperature, the intensity of the peak at 245 nm decreased, while the peaks at 260 and 285 nm shifted to 275 nm to overlap as one peak. The peak-shifting started after 65°C.

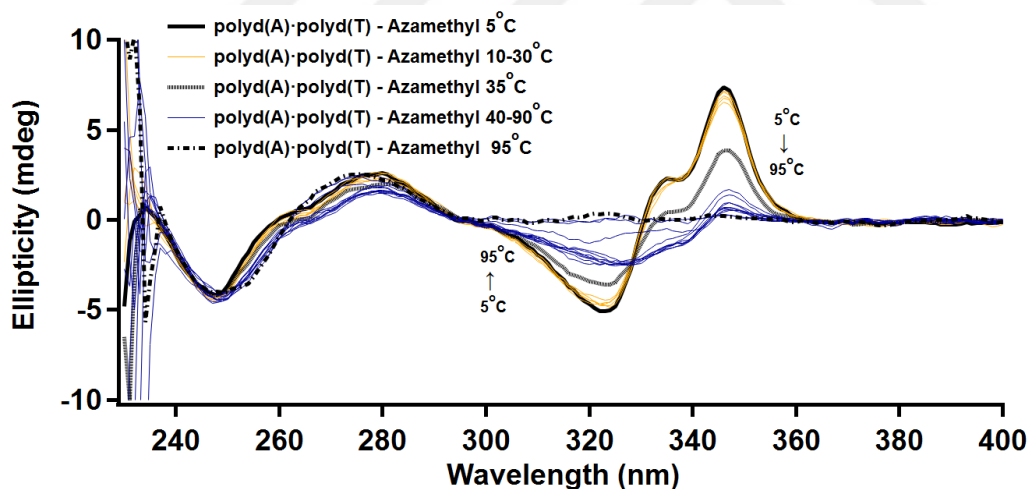


Figure 3.48. CD spectra of polyd(A)·polyd(T)-Azamethyl between 5°C and 95°C

The rise in temperature caused the intensity of the induced peaks at 325 and 345 nm to decrease. The change in the secondary structure started after 35°C. After 90°C, the induced peaks disappeared totally.

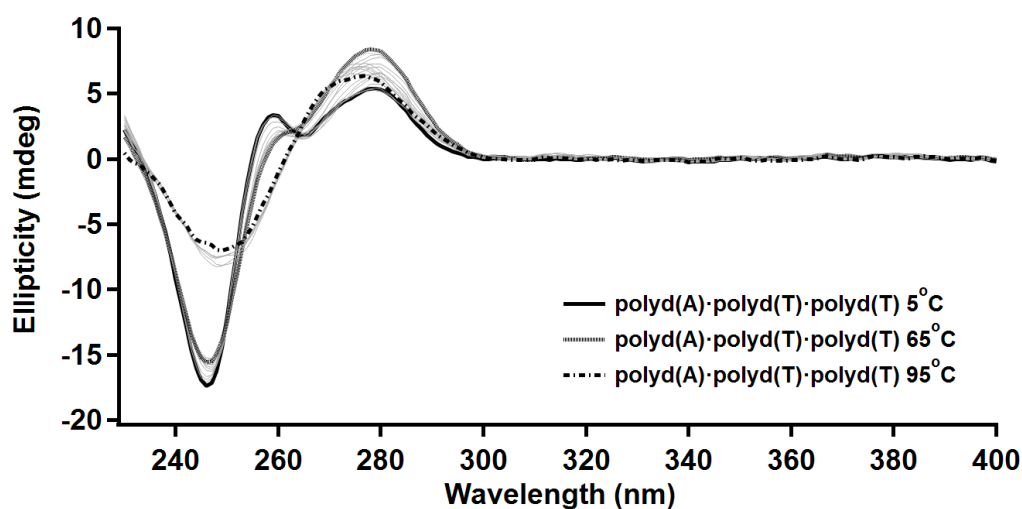


Figure 3.49. CD spectra of polyd(A)·poly(T)·poly(T) between 5°C and 95°C
 With the rise in temperature, the peak intensity at 250 nm decreased, while the peaks at 260 and 280 nm shifted to 275 nm to overlap as one peak. The shifting started after 65°C.

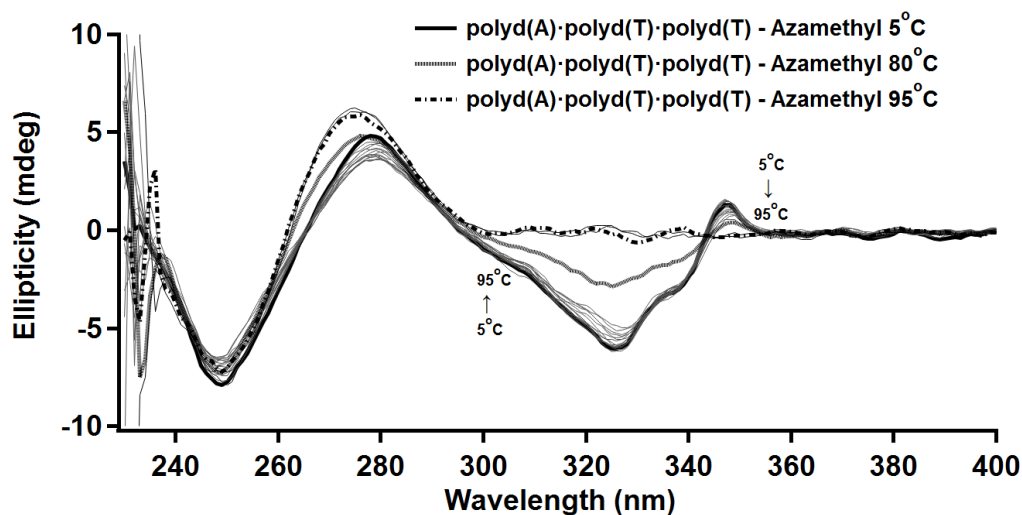


Figure 3.50. CD spectra of polyd(A)·poly(T)·poly(T)-Azamethyl between 5°C and 95°C

The rise in temperature led to a decline in the peak intensity at 250 nm, while the peak at 285 nm shifted to 275 nm. The intensity of the induced peaks at 325 and 345 nm also decreased. After 80°C, the induced peaks disappeared totally.

The ellipticity change in the CD spectra of $d(A)_{32}$, $d(A)_{32} \cdot d(T)_{32}$ and $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$, polyd(A), polyd(T), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) in the presence and absence of Azamethyl (Figure 3.37–Figure 3.50) at 247, 260, 274, 324 and 347 nms with the increasing temperature were monitored and the thermal denaturation temperatures (T_m) were recorded (Table 3.4).

The thermal denaturation experiments of $d(A)_{32}$, $d(A)_{32} \cdot d(T)_{32}$ and $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$, polyd(A), polyd(T), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) were carried out in the absence and presence of Azamethyl via CD spectroscopy to monitor the changes in their secondary structure in the presence of Azamethyl. Another reason for these experiments was to shed light onto the possible disproportionation of polyd(A)·polyd(T) in the presence of Azamethyl.

The thermal denaturation temperatures obtained by CD spectroscopy were in a good agreement with those obtained by UV-vis spectroscopy.

When the temperature changes of $d(A)_{32}$, polyd(A) and polyd(T) were examined, as expected, neither the secondary structure nor the thermal denaturation temperature were obtained (Figure 3.37, Figure 3.43 and Figure 3.45). No secondary structure formation was observed for polyd(T) in the presence of Azamethyl as well (Figure 3.46). On the other hand, $d(A)_{32}$ and polyd(A) formed the secondary structures in the presence of Azamethyl with the T_m of 17.5°C and 30°C, respectively (Figure 3.38, Figure 3.44 and Table 3.4). The obtained results were in good agreement with the T_m values achieved by monitoring the UV-vis absorbance at 336 nm in $d(A)_{32}$ -Azamethyl and polyd(A)-Azamethyl samples.

Table 3.4 Thermal denaturation temperatures acquired from the CD spectra.

	T_m (°C)				
	247 nm	260 nm	274 nm	324 nm	347 nm
$d(A)_{32}$	-	-	-	-	-
$d(A)_{32} \cdot d(T)_{32}$	52.5	-	-	-	-
$d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$	52.5	52.5	-	-	-
$d(A)_{32}$ - Azamethyl	-	-	17.5	-	-
$d(A)_{32} \cdot d(T)_{32}$ - Azamethyl	-	-	32.5, 67.5	67.5	35, 67.5
$d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ - Azamethyl	-	-	67.5	67.5	
Polyd(A)	-	-	-	-	-
Polyd(T)	-	-	-	-	-
Polyd(A)·polyd(T)	67.5	67.5	-	-	-
Polyd(A)·polyd(T)·polyd(T)	67.5	25, 67.5	-	-	-
Polyd(A) - Azamethyl	-	25	25	30	30
Polyd(T) - Azamethyl	-	-	-	-	-
Polyd(A)·polyd(T) - Azamethyl	-	-	-	35, 80	35
Polyd(A)·polyd(T)·polyd(T) - Azamethyl	-	-	80	80	-

In the absence of Azamethyl, $d(A)_{32} \cdot d(T)_{32}$ denatures at 52.5 °C (Figure 3.39, Table 3.4). In the presence of Azamethyl, two thermal denaturations at about 35 and 67.5°C were observed for $d(A)_{32} \cdot d(T)_{32}$ (Figure 3.40, Table 3.4). The T_m of 52.5°C belongs to the single-stranded transition (2→1) of the duplex. This value obtained by UV-vis for this duplex transition was 56°C. In $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ in the absence of

Azamethyl, again only a single transition was observed at 52.5°C that belongs the duplex to single-stranded (2→1) transition. As mentioned before, $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ is not able to form a stable triplex by itself (Figure 3.41, Table 6). In the presence of Azamethyl, again only a single transition was observed at 67.5°C (Figure 3.42, Table 3.4). Given the similarities between the coralyne and the Azamethyl interactions and the higher affinity of Azamethyl to triplex structures over duplex structures, 67.5°C is believed to be belonging to intercalated triplex structure. Therefore, it could be concluded that the Azamethyl is able to disproportionate $d(A)_{32} \cdot d(T)_{32}$ duplex at 35°C, resulting in the formation of the triplex structure similar to coralyne (Jain, Polak and Hud, 2003).

In the absence of Azamethyl, polyd(A)·polyd(T) was found to be denaturing (2→1) at 67.5°C (Figure 3.47 and Table 3.4). In the presence of Azamethyl, polyd(A)·polyd(T) denatured around 35°C and 80°C (Figure 3.48, Table 3.4). Polyd(A)·polyd(T)·polyd(T) triple helix denatured at 25°C (3→2) and at 67.5°C (2→1). A T_m of 80°C was observed in the polyd(A)·polyd(T)·polyd(T)-Azamethyl sample (Figure 3.49, Figure 3.50 and Table 3.4). Likewise, it is believed that the Azamethyl can denature the polyd(A)·polyd(T) structure. The denaturation can be observed by comparing the CD spectra of polyd(A)·polyd(T) with respect to temperature (Figure 3.48). At 5°C, two induced CD bands including one negative band (between 300 nm and 330 nm) and one positive band (between 330 nm and 360 nm) were observed in the polyd(A)·polyd(T)-Azamethyl sample in which the positive one was quite larger than the negative one. With the slight increase in the temperature, the positive peak's intensity decreased after 35°C, whereas the negative one shifted towards the right and its intensity decreased slightly. After 35°C, the shape of the induced band became nearly identical with the induced peak observed for polyd(A)·polyd(T)·polyd(T)-Azamethyl (Figure 3.50). The same transition can also be observed in the spectrum of $d(A)_{32} \cdot d(T)_{32}$ -Azamethyl (Figure 3.40).

As a result, the greater similarity of the Azamethyl to coralyne was disclosed. As specified in the research of Polak and Hud (2002), coralyne does not interact with the polyd(T) and neither does Azamethyl. On the other hand, they both interact with

polyd(A) and cause the formation of the self-secondary structure. Azamethyl is also believed to denature polyd(A)·polyd(T) and induce the formation of the intercalated triplex of polyd(A)·polyd(T)·polyd(T)-Azamethyl structure that denatures at 80°C. Azamethyl also found to be greatly stabilizing the polyd(A)·polyd(T)·polyd(T) structure where its T_m increased from 24°C to 82°C in the presence of Azamethyl.

3.3 The Effect of Environmental Factors (pH and Salt Concentration) on the Interactions of polyd(A)·polyd(T)·polyd(T)-Azamethyl

3.3.1 pH Effect

The effect of pH on the interactions of Azamethyl with the triple helix of polyd(A)·polyd(T)·polyd(T) was investigated via CD spectroscopy. Moreover, the same experiment was conducted on Azamethyl by preparing a series of Azamethyl samples at different pH values (Figure 3.51).

Figure 3.51 depicts the effect of pH on Azamethyl. As shown, the CD spectrum of the Azamethyl solution did not undergo any change with changes in the pH.

Figure 3.52 displays the effect of pH on polyd(A)·polyd(T)·polyd(T)-Azamethyl. As can be seen, no significant change was observed in the CD spectra between pH=7 and pH=3. At pH less than 3, the density of the induced peak between 300 nm and 360 nm notably decreased revealing a change in the interactions of polyd(A)·polyd(T)·polyd(T) with Azamethyl. There were also slight changes in the CD spectrum of polyd(A)·polyd(T)·polyd(T)-Azamethyl between 230 nm and 300 nm indicating that the DNA structure was slightly affected by the pH changes.

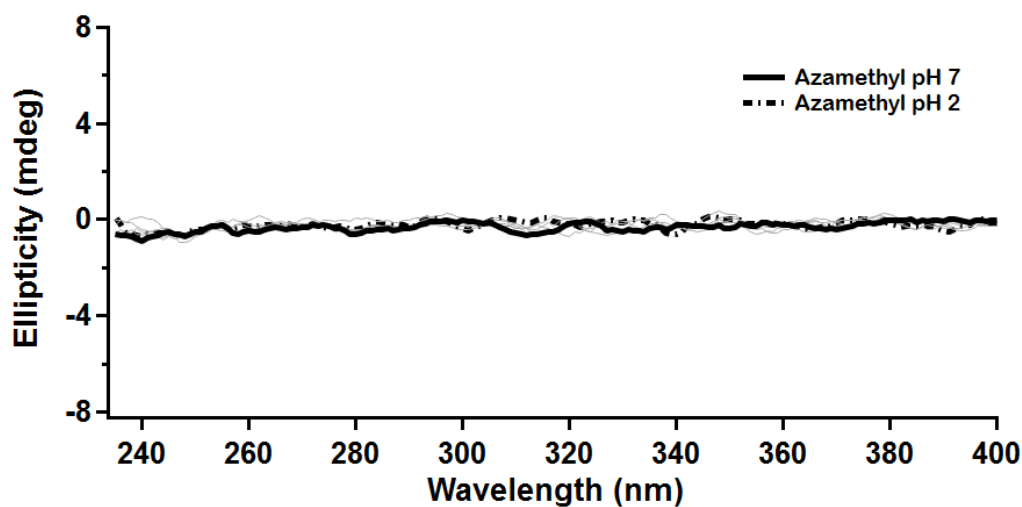


Figure 3.51. CD spectra of Azamethyl in different pH's.

CD spectra of the Azamethyl samples with pH values of 2, 3, 4, 5, 6 and 7 at 5°C. The CD spectra of the samples with the lowest pH value of 2 and the highest of 7 were drawn in bold lines.

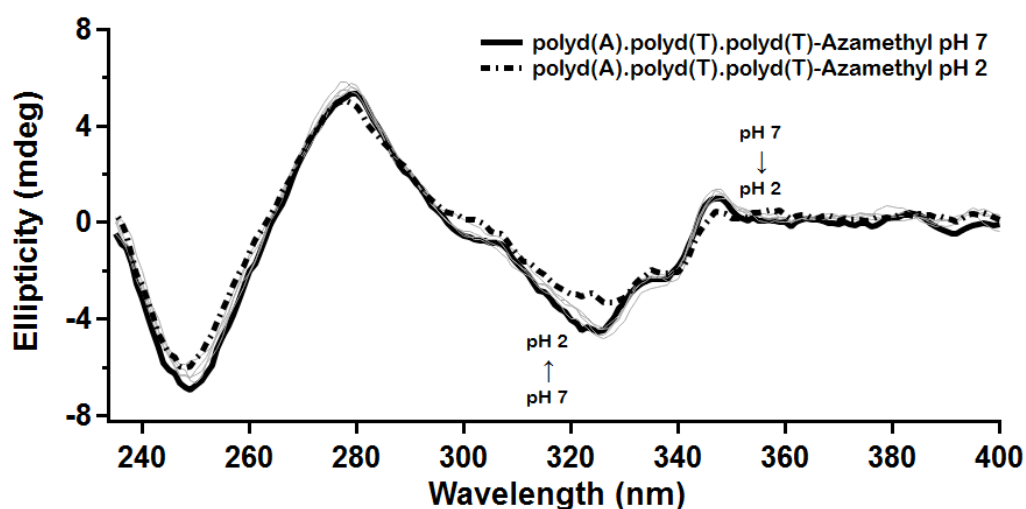


Figure 3.52. CD spectra of polyd(A)·polyd(T)·polyd(T)-Azamethyl in different pH's.

The CD spectra of the polyd(A)·polyd(T)·polyd(T)-Azamethyl samples with pH values of 2, 3, 4, 5, 6 and 7 at 5°C. The CD spectra of the samples having the lowest (pH=2) and the highest (pH=7) pHs were drawn in bold lines.

3.3.2 The Effect of NaCl Concentration

To investigate the effect of NaCl concentration on the interactions of polyd(A)·polyd(T)·polyd(T) with Azamethyl, the samples of polyd(A)·polyd(T)·polyd(T)-Azamethyl and Azamethyl (as a control) containing different concentrations of NaCl (100 mM, 150 mM, 200 mM, 300 mM, 400 mM and 500 mM) were prepared and their CD spectra were collected. As displayed in Figure 3.53, increasing the NaCl concentration had no remarkable effect on the CD response of Azamethyl solution.

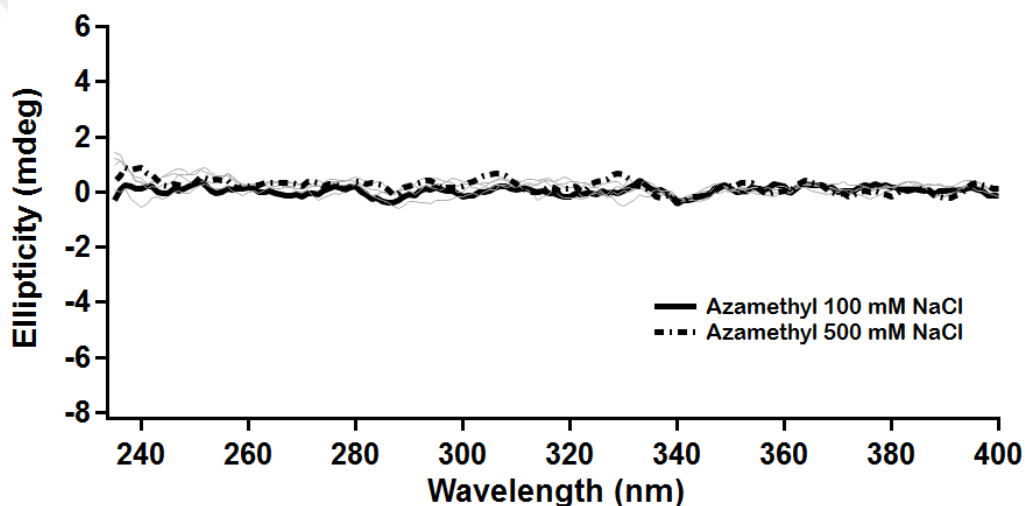


Figure 3.53. CD spectra of Azamethyl samples having different NaCl concentration

The CD spectra of Azamethyl samples with different NaCl concentrations (100 mM, 150 mM, 200 mM, 300 mM, 400 mM and 500 mM). The CD spectra of the samples having the lowest [NaCl] =100 mM and the highest [NaCl] = 500 mM were drawn using bold lines.

When the CD spectra of the polyd(A)·polyd(T)·polyd(T)-Azamethyl samples at different [NaCl] were compared, as expected, the interactions of polyd(A)·polyd(T)·polyd(T)-Azamethyl were negatively affected by the [NaCl] (Figure 3.54). With an increase in [NaCl], the intensity of the induced peak decreased revealing a decrease in polyd(A)·polyd(T)·polyd(T)-Azamethyl interactions. The main reason behind this observation was the neutralization of the negatively-charged DNA backbone with increasing NaCl concentration followed by less affinity of the DNA to the positively-charged Azamethyl. There was no significant change in the polyd(A)·polyd(T)·polyd(T)-Azamethyl spectrum between 230 nm and 300 nm indicating that increasing concentration of NaCl did not really alter the DNA structure.

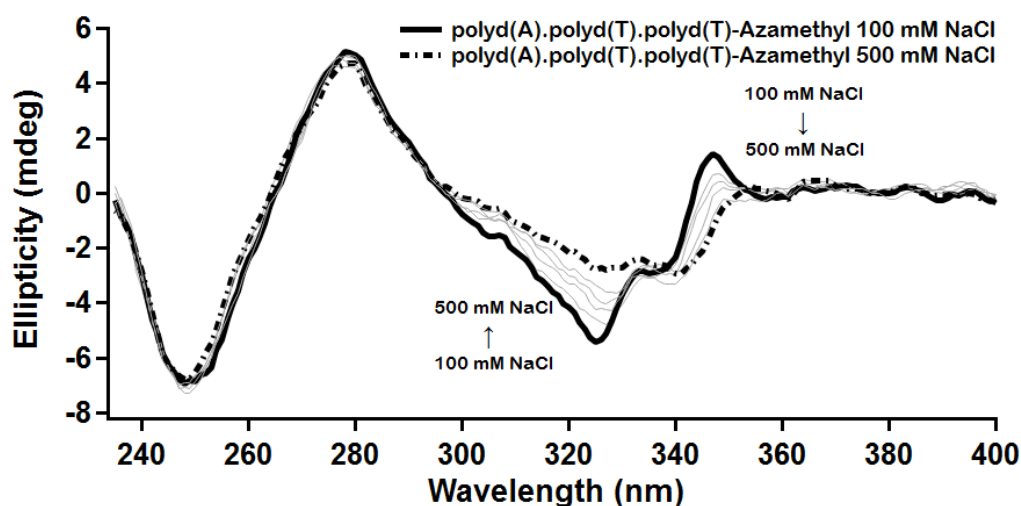


Figure 3.54. CD spectra of polyd(A)·polyd(T)·polyd(T)-Azamethyl samples having different NaCl concentration.

The CD spectra of the polyd(A)·polyd(T)·polyd(T)-Azamethyl samples at 5°C which have NaCl concentrations of 100 mM, 150 mM, 200 mM, 300 mM, 400 mM and 500 mM. The CD spectra of the samples having the lowest [NaCl] = 100 mM and the highest [NaCl] = 500 mM were drawn in bold lines in terms of explicitness.

3.4 Determination of the Binding Ratio of Azamethyl to polyd(A)·polyd(T)·polyd(T)

The binding ratio of Azamethyl to polyd(A)·polyd(T)·polyd(T) was determined by Job Plot analysis using UV-vis spectroscopy. In Job Plot analysis, the total Azamethyl and the binding site concentration were held constant, whereas their ratio was gradually changed (Figure 3.55). When the absorbance change at 345 nm was plotted with respect to $[\text{Azamethyl}]/([\text{Azamethyl}]+[\text{Base Triplet}/4])$, a break point was observed at the binding ratio of approximately 0.5 (Figure 3.56). This is consistent with the nearest neighbour exclusion principle observed for the intercalating molecules (Bloomfield, Crothers and Tinoco Jr., 1999).

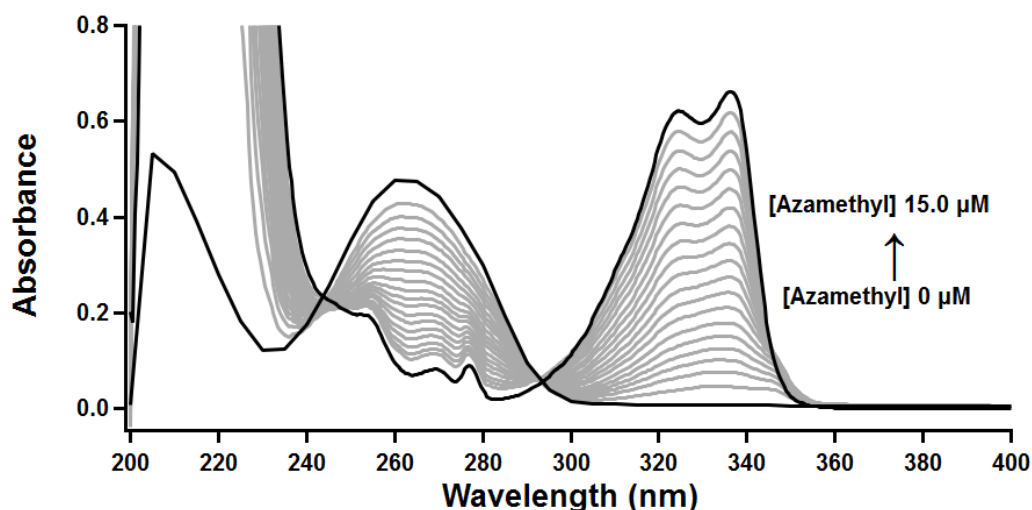


Figure 3.55. UV-vis spectra collected for Job Plot Analysis

The spectra of polyd(A)·polyd(T)·polyd(T) (Azamethyl-free) and Azamethyl (polyd(A)·polyd(T)·polyd(T)–free) were drawn in black lines for simplification.

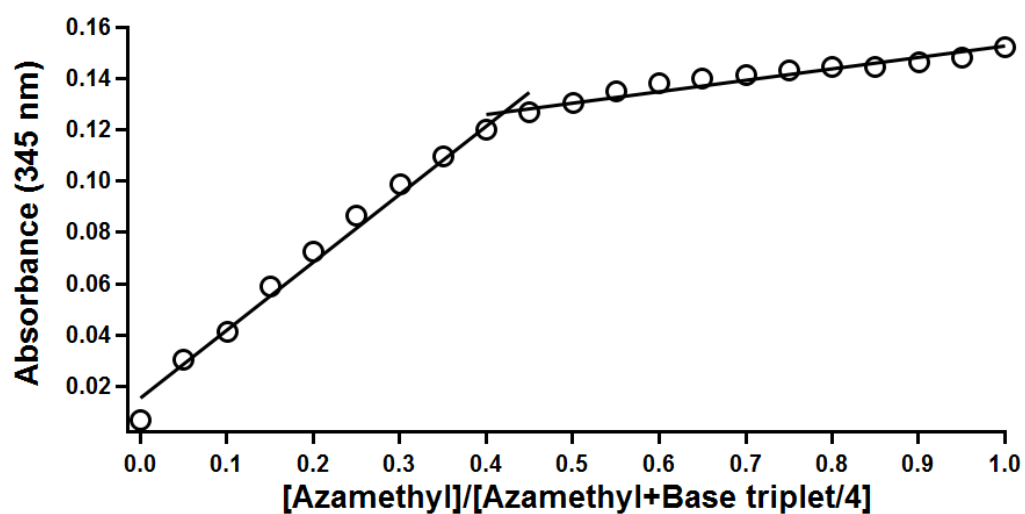


Figure 3.56. UV-vis Job Plot analyses of polyd(A)·polyd(T)·polyd(T)-Azamethyl.

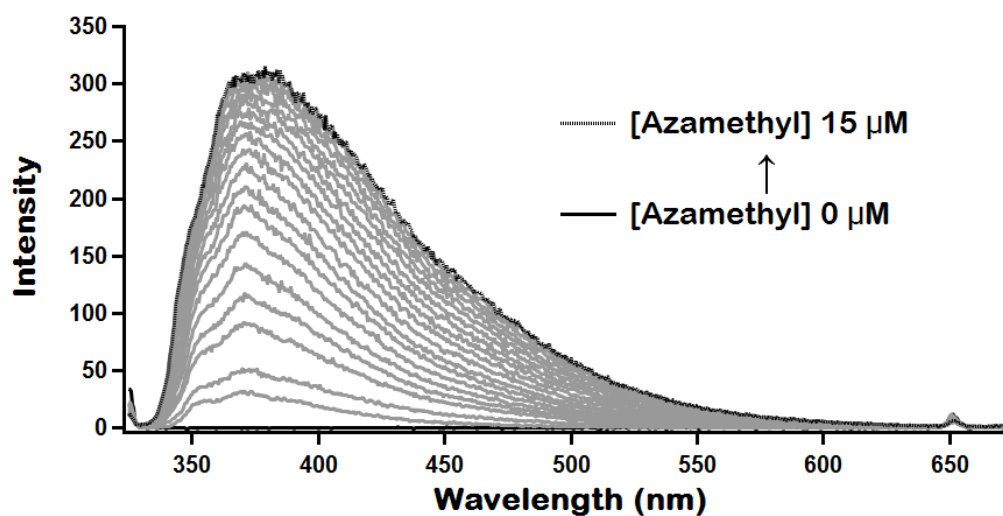


Figure 3.57. Florescence Job Plot analyses of polyd(A)·polyd(T)·polyd(T)-Azamethyl.

The spectra of polyd(A)·polyd(T)·polyd(T) (Azamethyl-free) and Azamethyl (polyd(A)·polyd(T)·polyd(T)-free) were drawn in black lines for simplification.

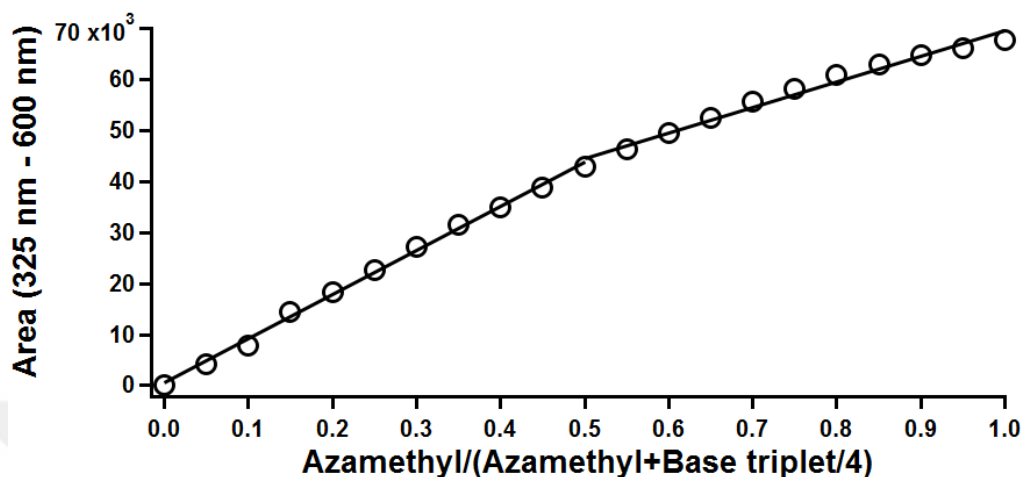


Figure 3.58. Florescence spectra collected for Job Plot analysis.

The UV-vis analysis experiments were repeated using florescence spectroscopy in order to get a much clear change in the spectrum profile. The collected florescence spectra at different Azamethyl:DNA ratios are given in Figure 3.57 and the obtained Job Plot results through taking the integrals of the area under the emission spectra are shown in Figure 3.58. The florescence measurements were in a good agreement with the UV-vis results, such that one Azamethyl binds to two base triplets.

3.5 Investigation of the Interactions of Azamethyl with the (GAA)_n Oligonucleotides by UV-vis Spectroscopy and Determination of the Thermal Denaturation Temperatures

The role of triplex formation through GAA repeats in Friedreich's ataxia (FRDA) was well-established (Grabczyk and Usdin, 2000) and here, the interactions of Azamethyl with the triplex sequences containing GAA repeats called DTR1 were investigated. DTR1 is a parallel triplex unlike polyd(A)·polyd(T)·polyd(T) triplex.

Basically, the thermal denaturation studies of the DTR1 triplex in the absence and presence of Azamethyl were carried out under the same conditions. The sequences that DTR1 consists of are given below:

5'-TCGC GAAGAAGAAGAAGAA CGTC-3' (SS1)

3'-AGCG CTT CTTCTT CTT CTT GCGA-5' (SS2)

3'-GAAGAAGAAGAAGAA-5' (SS3)

DTR1 triplex was previously studied by Rajeswari (2012) and the T_m of 56.5°C and 72°C were reported for DTR1 triplex (2→1) and the corresponding DD1 duplex (3→2), respectively. These studies have been conducted in the presence of 10 mM Sodiumcacodylate, 150 mM NaCl and 10 mM MgCl₂ at pH 7.4 but in the scope of this thesis, to compare to DTR1's behavior to d(A)₃₂·d(T)₃₂·d(T)₃₂ and/or polyd(A)·polyd(T)·polyd(T), the experiments were carried out in 1X BPE buffer with 100 mM NaCl at pH 7.0. When the change of the absorbance at 260 nm was monitored with respect to temperature, just a single T_m value was obtained at 67°C. This temperature was thought to be belonging to the duplex denaturation (2→1, Figure 3.59). When the salt concentration increased under the same conditions (to be consistent with the conditions of a previously reported study in the literature) (Rajeswari et al, 2012), a single melting temperature was achieved at 72°C again. The absorbance spectra at 5°C and 95°C of DTR1 in 100 mM NaCl and 185 mM NaCl can be seen in Figure 3.60 and Figure 3.61, respectively. When the thermal denaturation is repeated in the presence of Azamethyl under 100 mM NaCl, Aza3 slightly stabilized the DTR1 resulting in an increase in the T_m value from 68°C to 69°C. Yet, this 1°C change is also within our experimental error.

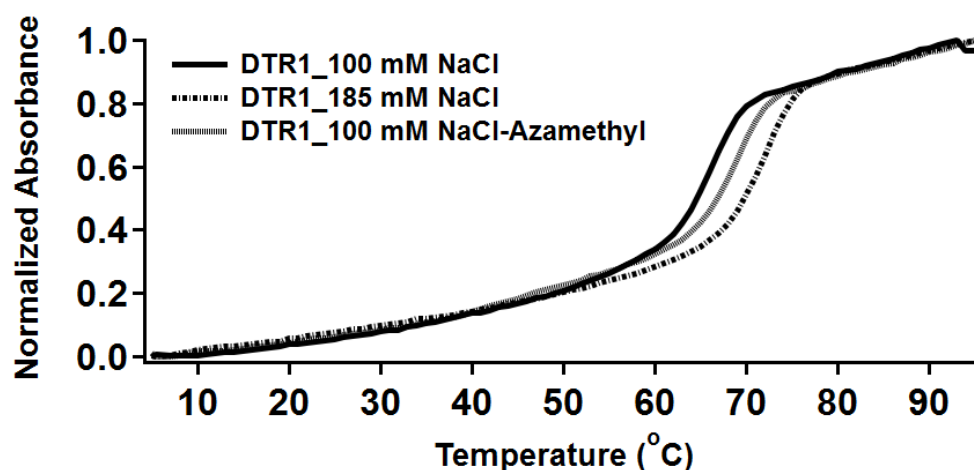


Figure 3.59. Thermal denaturation profiles of DTR1, 260 nm.

Thermal denaturation profiles acquired by monitoring the absorbance change of DTR1 triplex at 260 nm with the changing temperature. **DTR1_100 mM NaCl:** DTR1, 1X BPE, 100 mM NaCl, pH = 7.0. **DTR1_185 mM NaCl:** DTR1, 1X BPE, 185 mM NaCl, pH = 7.0. **DTR1_100 mM NaCl-Azamethyl:** DTR1-Azamethyl, 1X BPE, 100 mM NaCl, pH = 7.0.

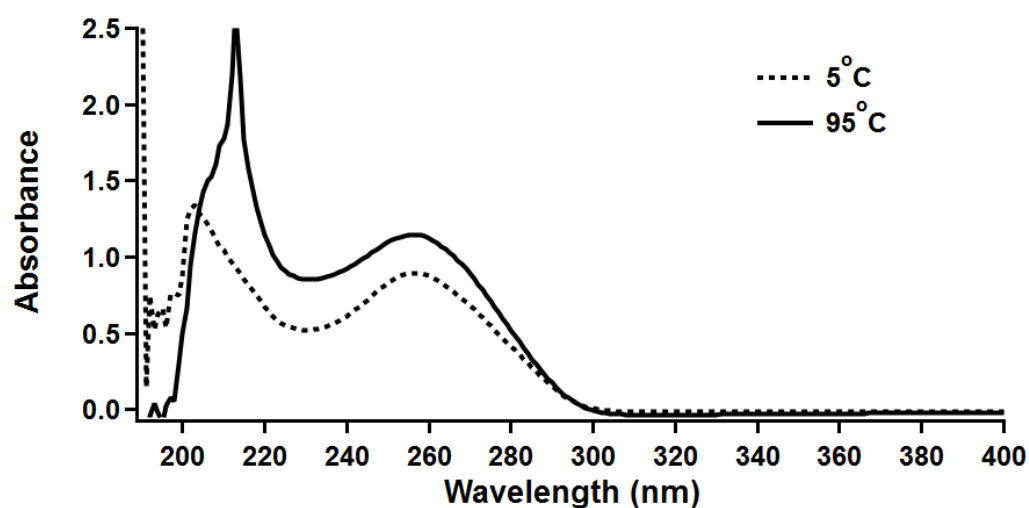


Figure 3.60. UV-vis spectra of DTR1 in 100 mM NaCl at 5°C and 95°C.

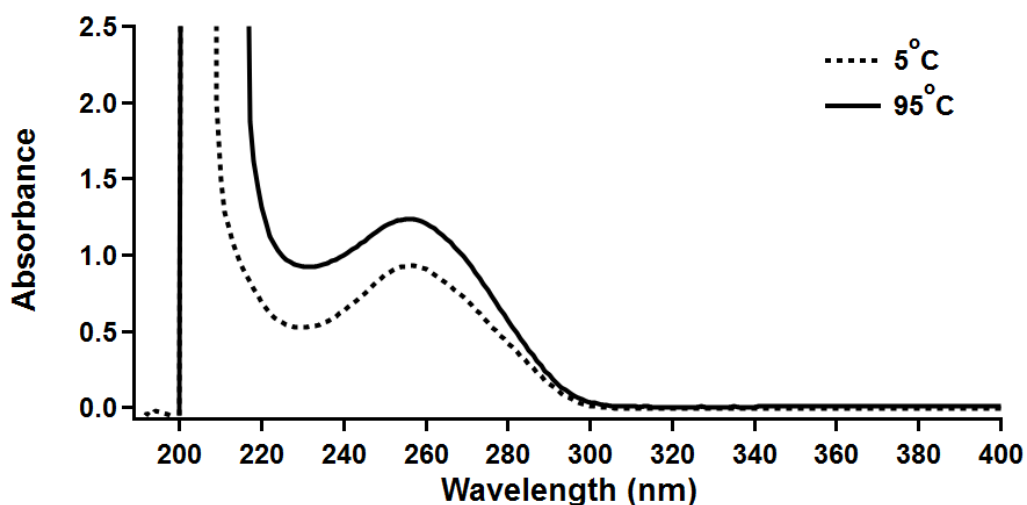


Figure 3.61. UV-vis spectra of DTR1 in 185 mM NaCl at 5°C and 95°C.

Moreover, two T_m values at 41°C and 68.5°C were obtained in the DTR1-Azamethyl sample by monitoring the absorbance change with respect to temperature at 324 nm or 326 nm (Figure 3.62). It is concluded that the T_m at 41°C belongs to triplex while the T_m at 68.5°C belongs to the duplex structure. Based on the UV-vis results of polyd(A)·polyd(T)·polyd(T) sample, it can be thought that the changes in the absorbance wavelengths of the drugs correspond to the denaturation of the triplex structure. Here, Azamethyl is thought to be facilitating the formation of a stable triplex structure that denatures at 41°C. When the UV-vis spectra of DTR1-Azamethyl were compared at 15°C and 95°C, a hypochromic effect and a red shift were observed in the bounded state (Figure 3.63) which can be taken as a sign of the interaction between DTR1 and Azamethyl.

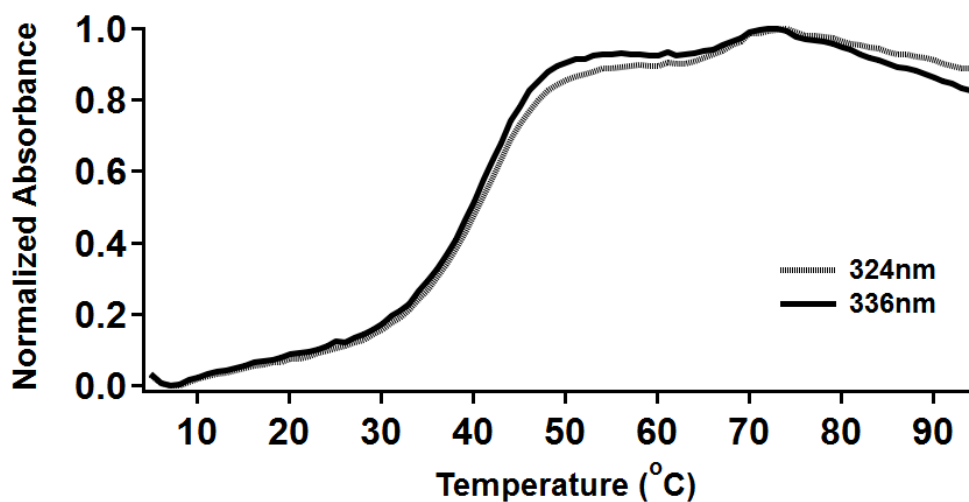


Figure 3.62. Thermal denaturation profiles of the DTR1-Azamethyl obtained by monitoring the change in UV-vis absorbance at 334 nm and 336 nm.

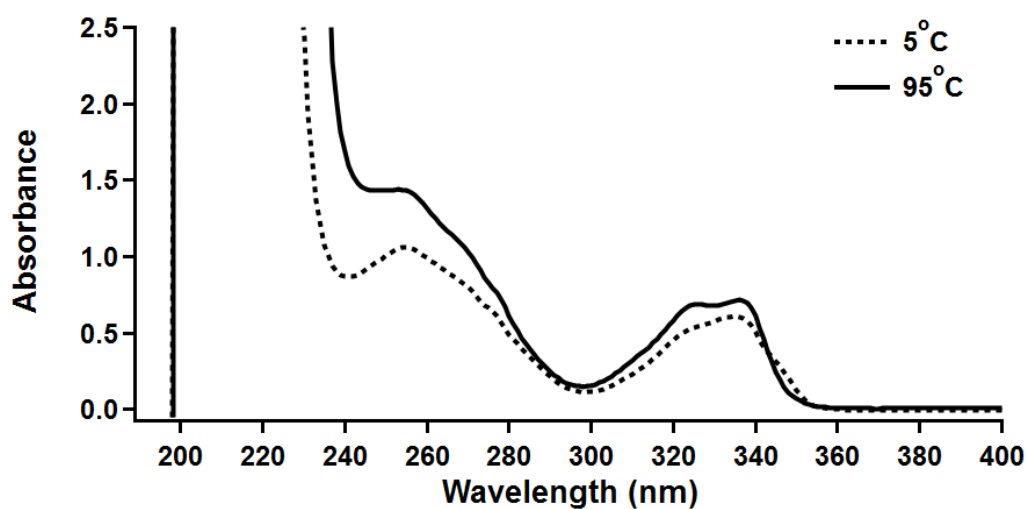


Figure 3.63. UV-vis spectra of DTR1-Azamethyl at 5°C and 95°C.

The thermal denaturation studies of SS1, SS2, SS3 sequences and DD1 (SS1·SS2) duplex were also carried out in the presence and absence of Azamethyl as controls. The melting profiles of these sequences at 260 nm and the comparisons of their spectra at 5°C and 95°C are given in the coming sections.

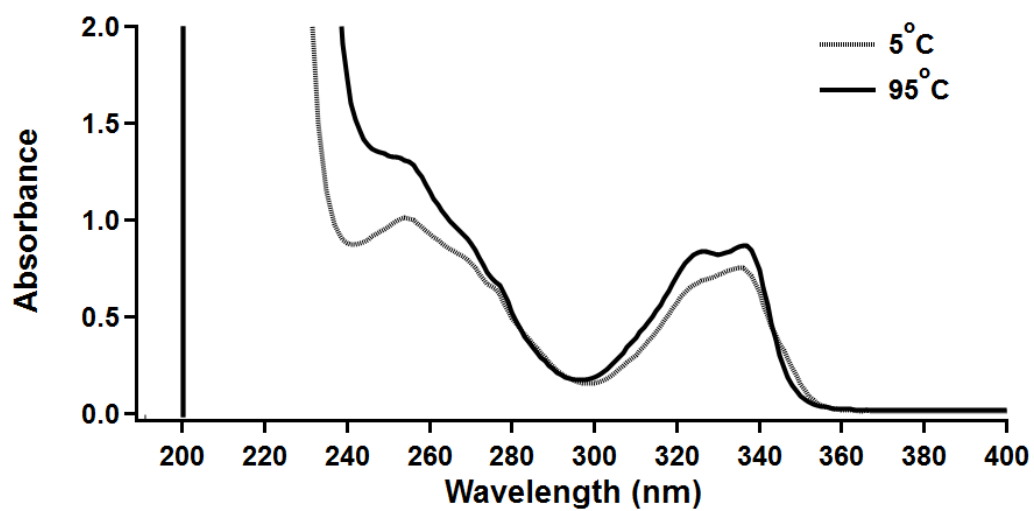


Figure 3.64. UV-vis spectra of SS1-Azamethyl at 5°C and 95°C.

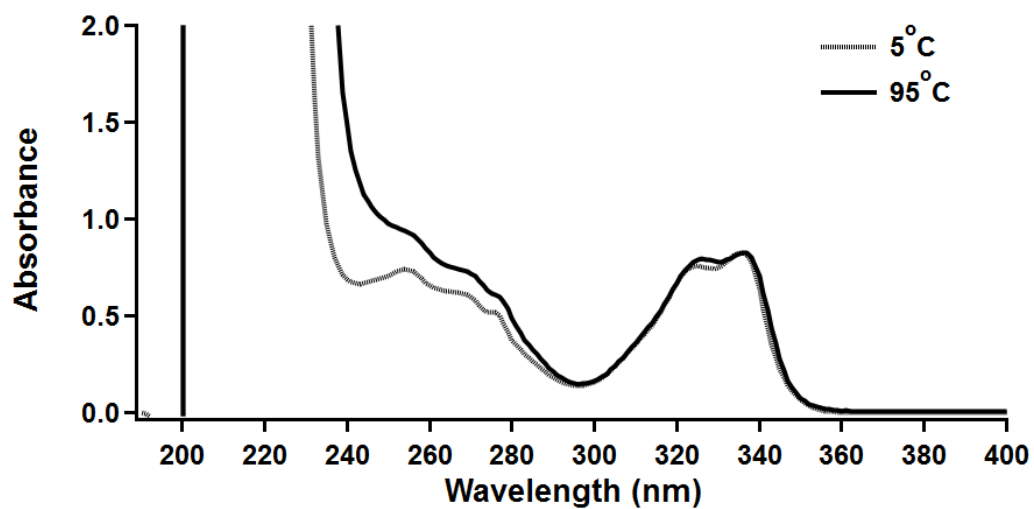


Figure 3.65. UV-vis spectra of SS2-Azamethyl at 5°C and 95°C.

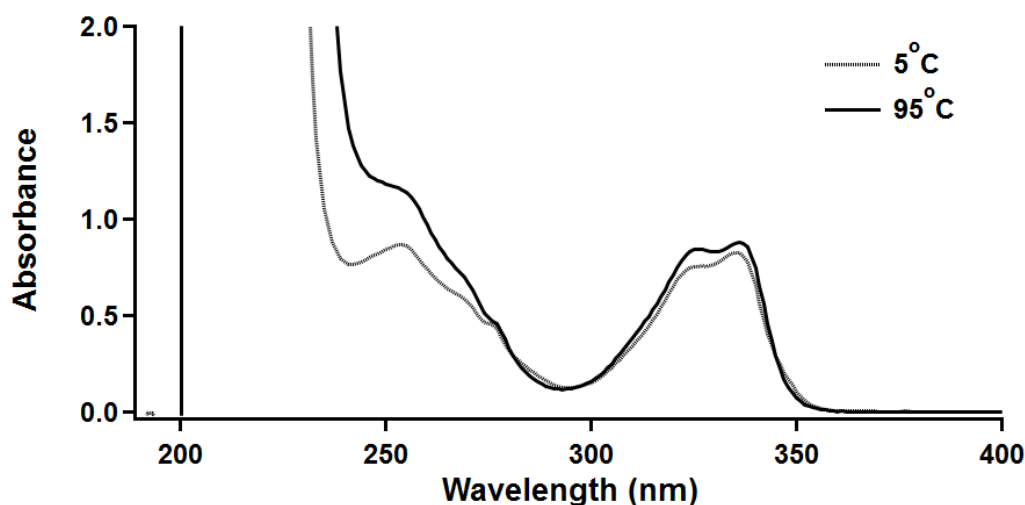


Figure 3.66. UV-vis spectra of SS3-Azamethyl at 5°C and 95°C.

When the UV-vis spectra of SS1 (Figure 3.64), SS2 (Figure 3.65) and SS3 (Figure 3.66) were compared in the presence of Azamethyl at 5°C and 95°C, a red shift and a hypochromic effect were observed in the SS1-Azamethyl and SS3-Azamethyl complexes. This hypochromic effect was higher in the presence of GAA-rich SS1 sequence. No secondary structure formation was observed in the thermal denaturation profiles of these sequences (by using the absorbance change at 260 nm) (Figure 3.67), and no T_m was recorded upon taking their first derivatives (Table 3.5).

On the other hand, according to the absorbance changes at 336 nm, both SS1 and SS3 were found to be interacting with Azamethyl. By differentiating the change of absorbances at 336 nm with temperature, a T_m value of 30°C for SS1-Azamethyl and 15°C for SS3-Azamethyl were obtained. No thermal denaturation temperature was obtained for SS2-Azamethyl complex. This is not an incidental result since it is apparent that Azamethyl interacts with the sequences rich in adenine and does not interact with the thymine-rich sequences like d(T)₃₂ and polyd(T). Here, SS1 and SS3 are adenine-rich sequences, whereas SS2 is a thymine-rich sequence. Yet, it was not clear why SS1-Azamethyl complex had a higher thermal denaturation temperature than SS3-Azamethyl complex since SS1 and SS3 had the same number

of GAA sequences. The additional flanking sequences of SS1 might be increasing the stability of SS1-Azamethyl complex. The first heating thermal denaturation profiles of these sequences are given at 336 nm (Figures Figure 3.68- Figure 3.70, respectively).

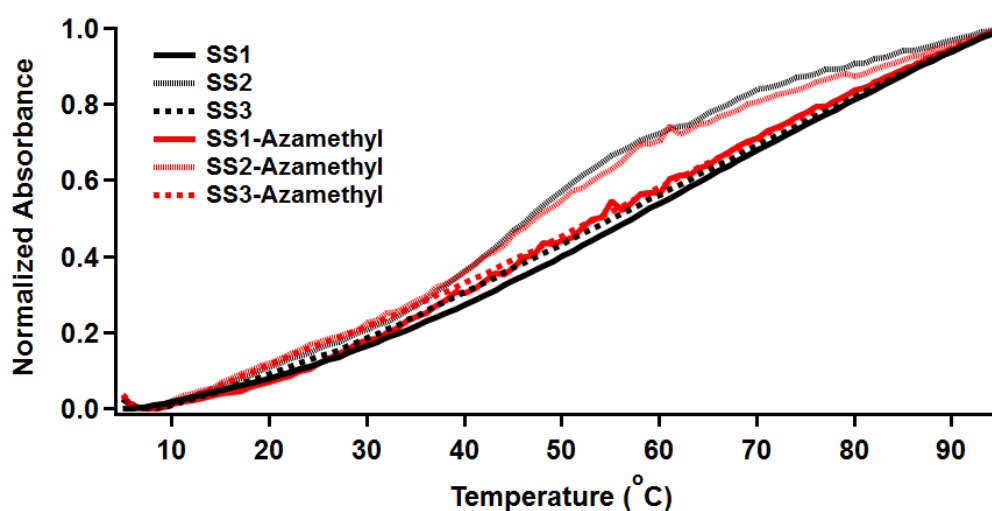


Figure 3.67. Thermal denaturation profiles of SS1, SS2 and SS3 in the presence and absence of Azamethyl obtained by monitoring the absorbance change at 260 nm.

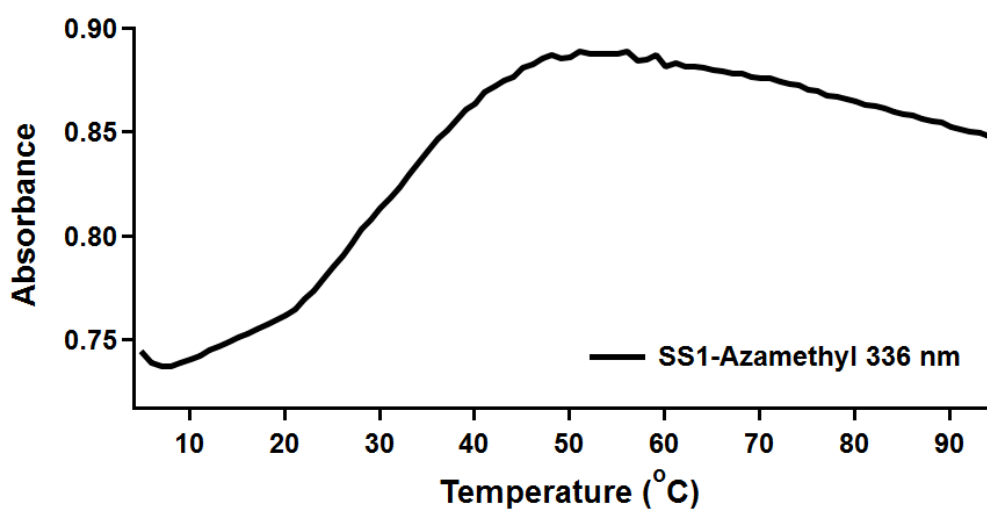


Figure 3.68. Thermal denaturation profile of SS1-Azamethyl, 336 nm.

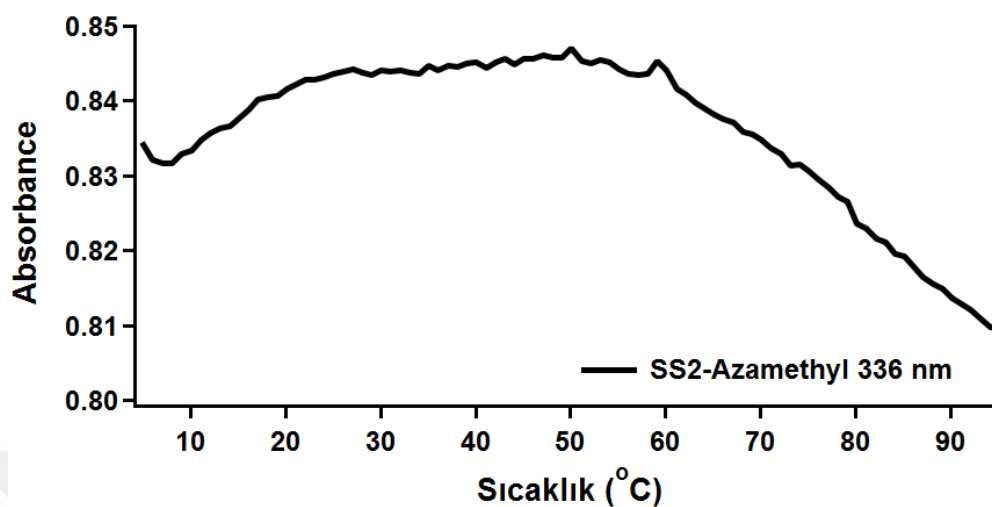


Figure 3.69. Thermal denaturation profile of SS2-Azamethyl, 336 nm.

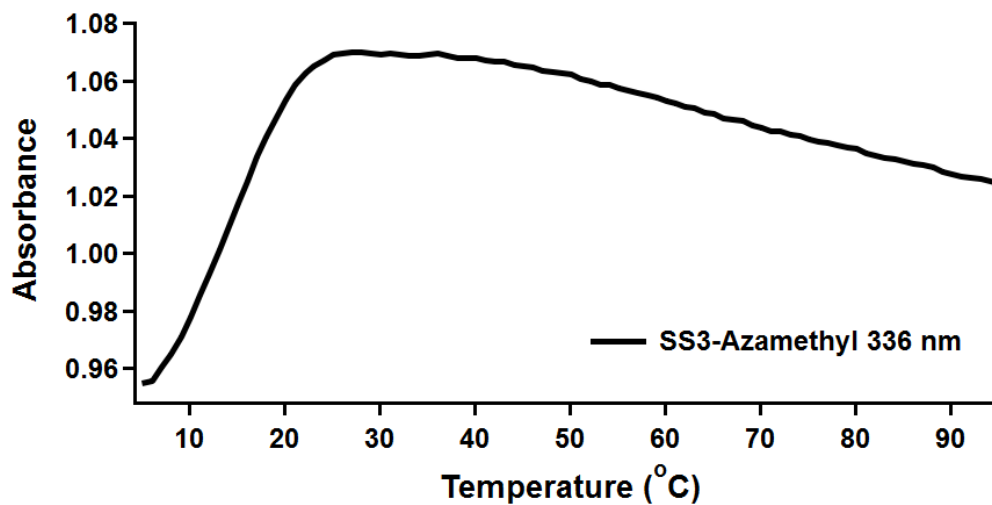


Figure 3.70. Thermal denaturation profile of SS3-Azamethyl, 336 nm.

The interactions of Azamethyl with the DD1 duplex was also investigated. Neither hypochromic effect nor red shift were observed when the UV-vis spectra of DD1-Azamethyl were compared at 5°C and 95°C (Figure 3.71). Also, no difference

was observed between the thermal denaturation profiles of DD1 and DD1-Azamethyl (Figure 3.72). All these results suggest that the Azamethyl has no interaction with DD1 duplex.

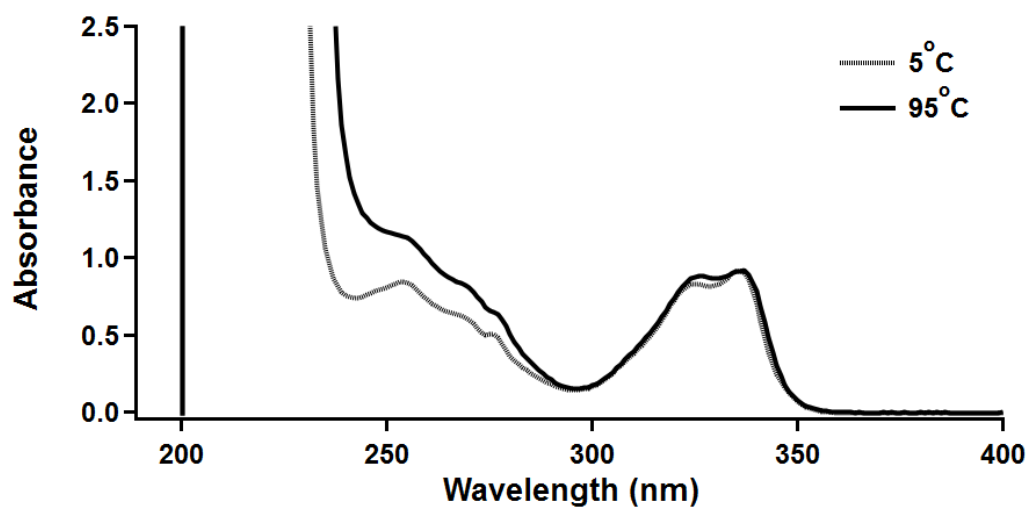


Figure 3.71. UV-vis spectra of DD1-Azamethyl at 5°C and 95°C

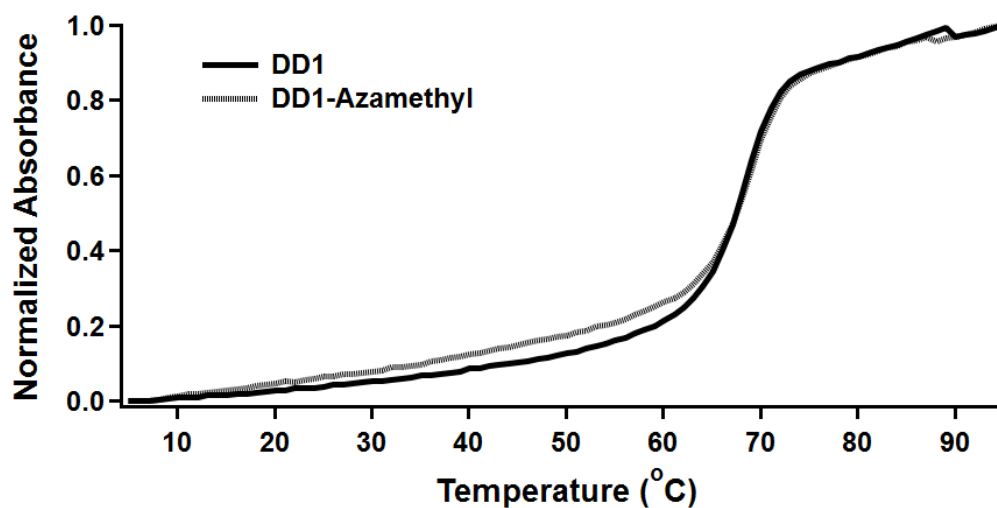


Figure 3.72. Thermal denaturation profiles of DD1 and DD1-Azamethyl, 260 nm.

The thermal denaturation temperatures derived from the thermal denaturation profiles are tabulated in Table 3.5.

Table 3.5 Thermal denaturation temperatures of DTR1 sequences, UV-vis, 260nm.

	T_m (°C)			
	1. Heating	1. Cooling	2. Heating	2. Cooling
SS1	-	-	-	-
SS2	-	-	-	-
SS3	-	-	-	-
DD1(SS1·SS2)	69	66	68	66
DTR1 (SS1·SS2·SS3)	68	65	68	66
DTR1 (SS1·SS2·SS3) (185 mM NaCl)	72	69	72	69
SS1-Azamethyl	-	-	-	-
SS2-Azamethyl	-	-	-	-
SS3-Azamethyl	-	-	-	-
DD1(SS1·SS2)-Azamethyl	69	67	69	67
DTR1 (SS1·SS2·SS3)-Azamethyl	69	66	69	66

To sum up, the survey of the interactions of Azamethyl with (GAA)_n oligonucleotides revealed the binding of Azamethyl to triplex structure of DTR1. DTR1-Azamethyl complex and this triplex structure had a T_m of about 40°C. DTR1 was not able to form a stable triplex by itself under these conditions and the only structure observed was the duplex structure with the T_m of 69°C. The comparison of the spectra of DTR1 at 5°C and 95°C verified the thermal denaturation results such that a hypochromic effect and a red shift were observed. These changes indicate the interaction between the DTR1 and Azamethyl. All these studies revealed that the Azamethyl had the ability to stabilize both parallel and anti-parallel triplex sequences. polyd(A)·polyd(T)·polyd(T) was representing the anti-parallel triplex structures having one purine and two pyrimidine chains while DTR1 was representing the parallel triplex structures having two purines and one pyrimidine chains. All these results demonstrate that Azamethyl can be a potential molecule for anti-gene strategies due to its ability to stabilize the triple helix structures.

On the other hand, Azamethyl does not interact with either DD1 or SS2. Yet, it interacts with SS1 and SS3 probably due to the presence of adenine nucleotides in these strands. Azamethyl is known to interact with homoadenine sequences (Çetinkol and Hud, 2009; Jain, Polak and Hud, 2003; Polak and Hud, 2002). The interaction of Azamethyl with the d(A)₃₂ and polyd(A) sequences was also confirmed during this study.

3.6 Investigation of the interactions of Azamethyl with the (GAA)_n oligonucleotides by CD spectroscopy

To verify the results of our UV-vis studies, the interactions of Azamethyl with the DTR1, DD2, SS1, SS2 and SS3 were also investigated via CD spectroscopy. First, the CD spectra of these sequences in the absence and presence of Azamethyl were collected at 5°C. The results are given in Figure 3.73 -Figure 3.80.

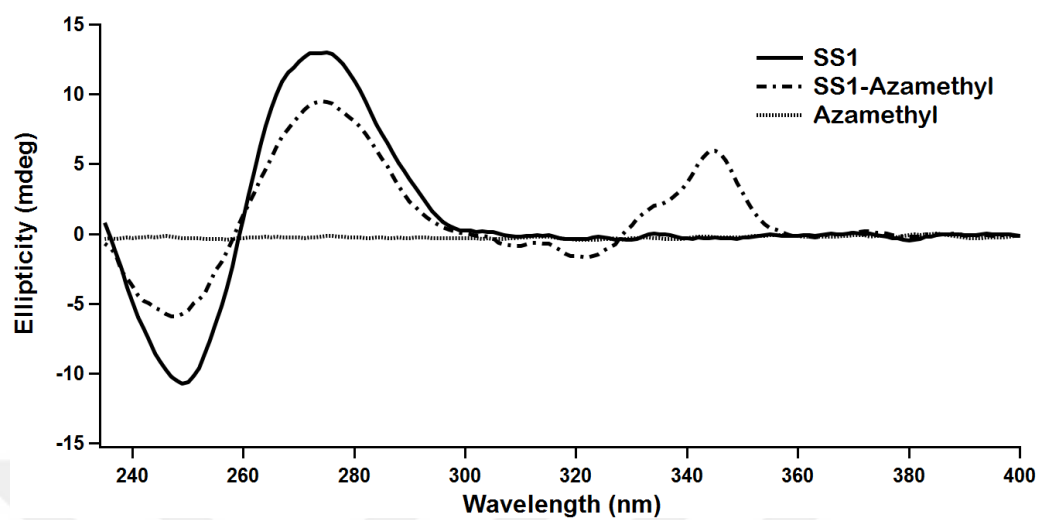


Figure 3.73. CD spectra of SS1, SS1-Azamethyl and Azamethyl at 5°C.

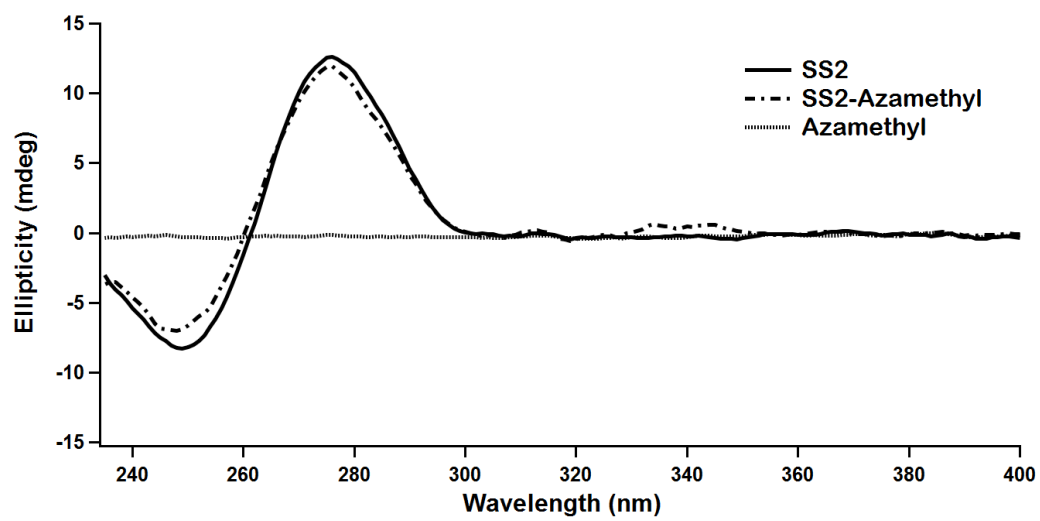


Figure 3.74. CD spectra of SS2, SS2-Azamethyl and Azamethyl at 5°C.

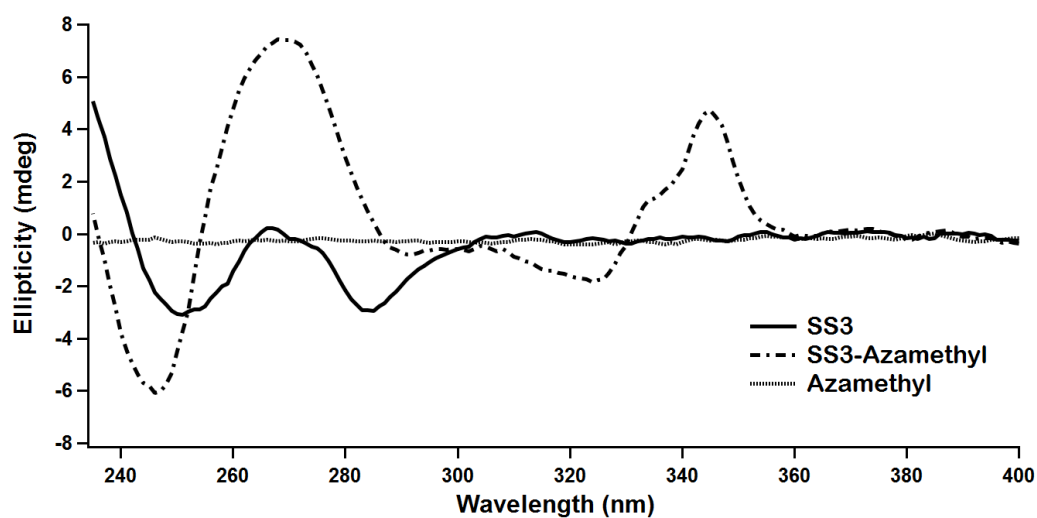


Figure 3.75. CD spectra of SS3, SS3-Azamethyl and Azamethyl at 5°C.

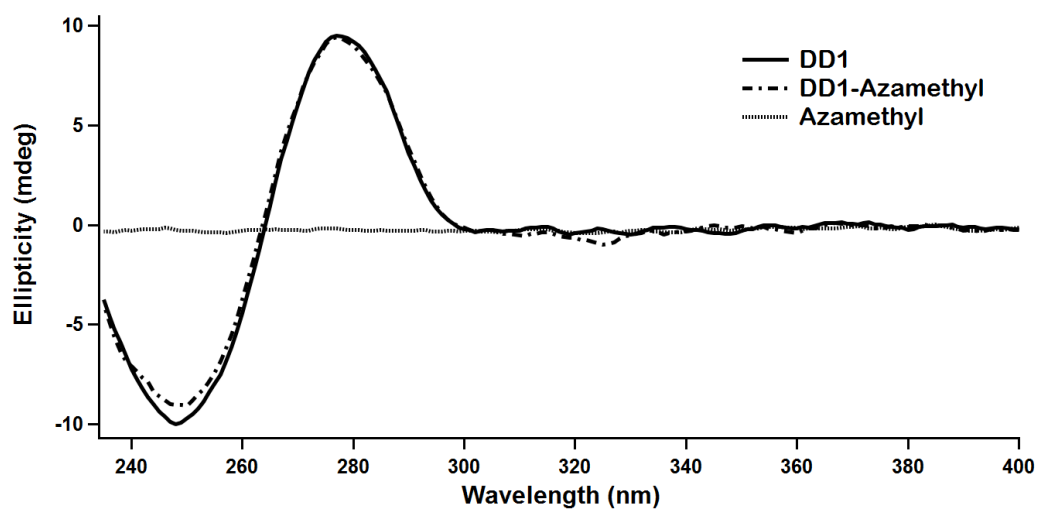


Figure 3.76. CD spectra of DD1, DD1-Azamethyl and Azamethyl at 5°C.

Upon Azacyanine binding, almost no change is visible for DD1.

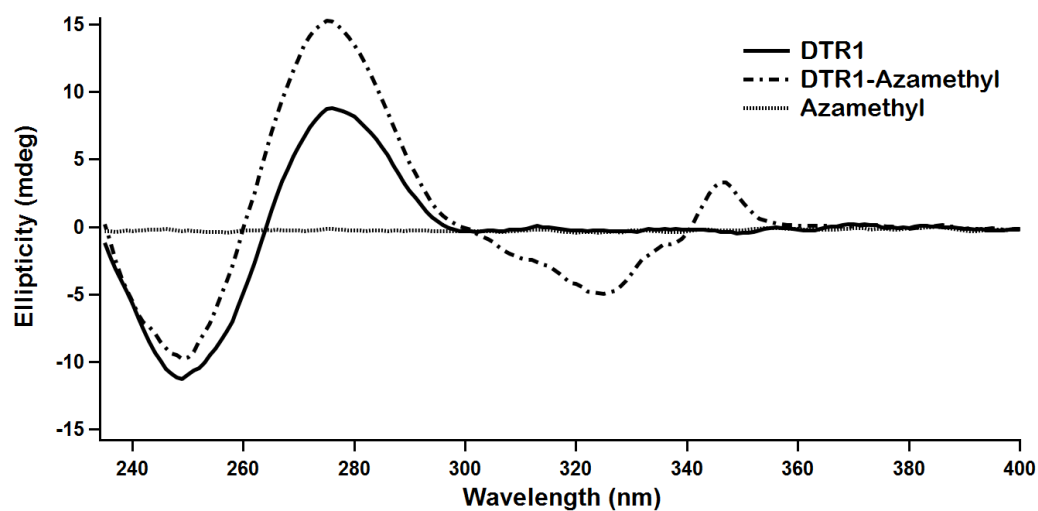


Figure 3.77. CD spectra of DTR1, DTR1-Azamethyl and Azamethyl at 5°C.

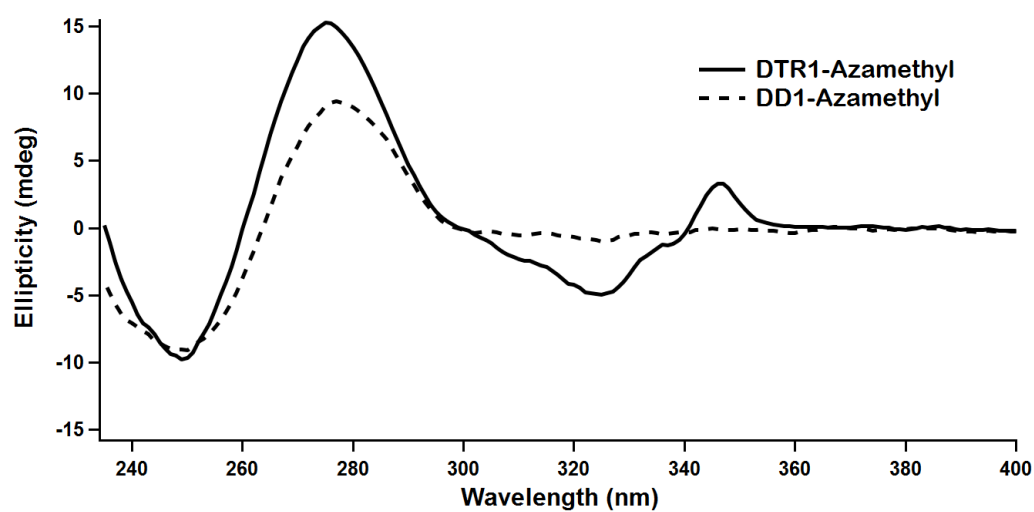


Figure 3.78. CD spectra of DTR1-Azamethyl and DD1-Azamethyl together at 5°C.

Figure 3.73 displays that there is an interaction between SS1 and Azamethyl. The induced band indicates the formation of the secondary structure of SS1-Azamethyl complex. The peaks below 300 nm show the change in the structure of SS1 upon Azamethyl binding. Here, while the intensity of the peak at 250 nm increases, the intensity of the positive peak at 275 nm decreases. The same result was obtained for SS3 (Figure 3.75) and DTR1 (Figure 3.77). On the other hand, there was almost no change in the spectrum of SS2 and DD1 upon addition of Azamethyl indicating that there is no interaction between the SS2 and Azamethyl as well as DD1 and Azamethyl (Figure 3.74, Figure 3.76).

Figure 3.79 and Figure 3.80 represent the CD spectra of all the sequences in the absence and presence of Azamethyl, respectively.

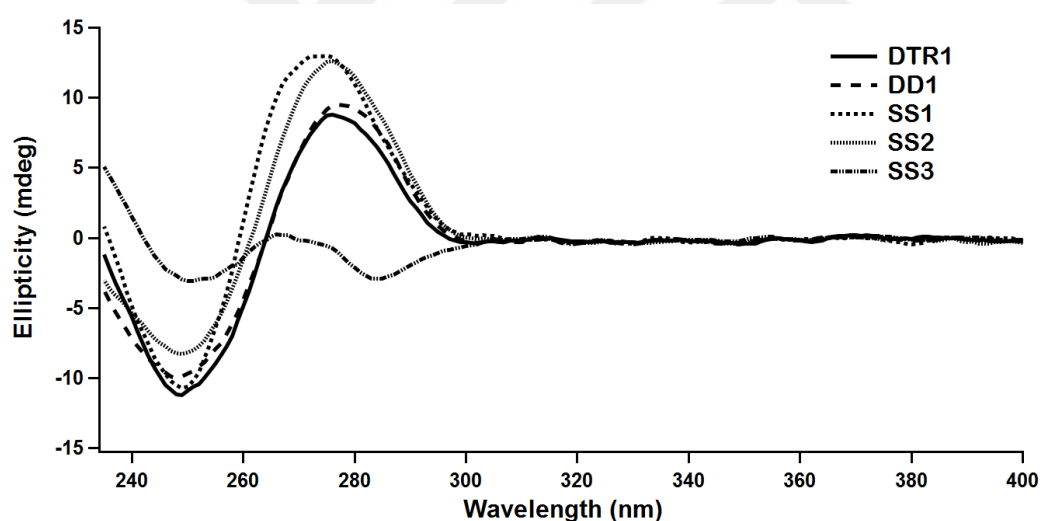


Figure 3.79. CD spectra of all sequences combined at 5°C.

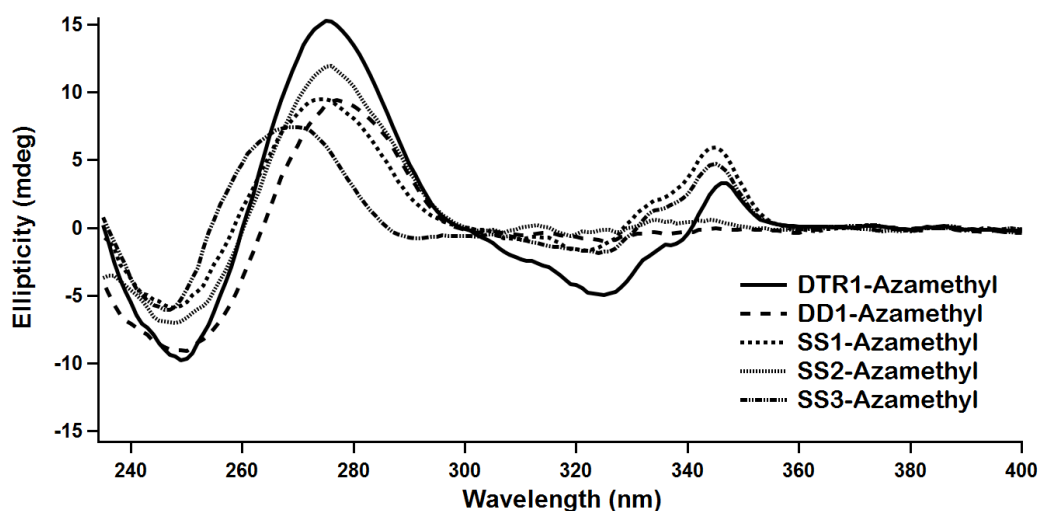


Figure 3.80. CD spectra of all sequences combined in the presence of Azamethyl at 5°C.

The acquired CD spectra confirms the UV-vis data regarding the interactions of Azamethyl with (GAA)_n oligonucleotides. The single chain of SS2 (Figure 3.74) and duplex of DD1 (Figure 3.78) do not interact with the Azamethyl and their spectra in the presence and absence of Azamethyl are almost the same. On the other hand, Azamethyl interacts with SS1 (Figure 3.73), SS3 (Figure 3.75) and DTR1 (Figure 3.77) and an induced peak is observed in their spectra between 300 to 360 nm. In the CD spectra of SS1-Azamethyl and SS3-Azamethyl, the intensity of the positive peak between 340 nm and 360 nm was greater than the absolute intensity of the negative induced peak while in the CD spectra of DTR-Azamethyl, the absolute intensity of the negative peak was greater than the intensity of the positive peak. Overall, the CD experiments are in perfect agreement with the results of UV-vis experiments, such that the DTR1, SS1 and SS3 sequences interacted with Azamethyl while DD2 and SS2 sequences did not.

3.7 Investigation of the Interactions of Azamethyl Derivatives with polyd(A), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) by UV-vis and CD Spectroscopies, Determination of Their Thermal Denaturation Temperatures

In order to determine the effect of molecules' structure on the binding ability of Azacyanines to polyd(A), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T), the interactions of Azaethyl, Azapropyl, Azaisopropyl, Azabutyl and Azaisobutyl with these nucleic acid structures were also examined via UV-vis and CD spectroscopy techniques. Our goal was to understand the role of alkyl chain length and the branching in the benzimidazole ring system on the binding ability of these compounds. Since Azamethyl had no interactions with polyd(T), the interactions of these derivatives with the polyd(T) was not further investigated.

3.7.1 Determination of the Interactions of Azamethyl Derivatives with polyd(A), polyd(A)·Polyd(T) and polyd(A)·polyd(T)·polyd(T) by UV-vis Spectroscopy

The UV-vis spectra of the complex of each derivative with polyd(A), polyd(A)·polyd(T) duplex and polyd(A)·polyd(T)·polyd(T) triplex were collected and compared at 15°C and 95°C. As stated above, the small molecule is expected to bind to the secondary structure at 15°C and to be free at 95°C.

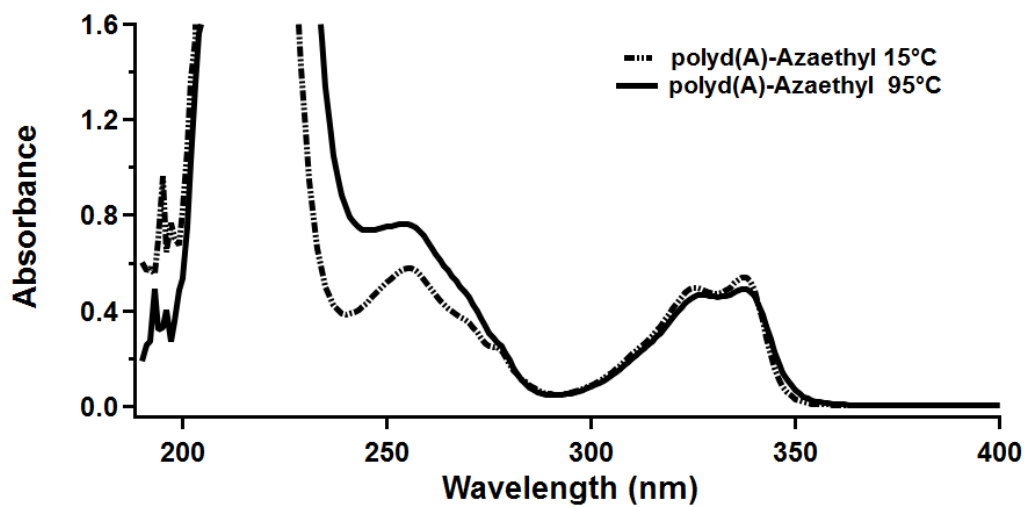


Figure 3.81. UV-vis spectra of polyd(A)-Azaethyl at 15°C and 95°C.

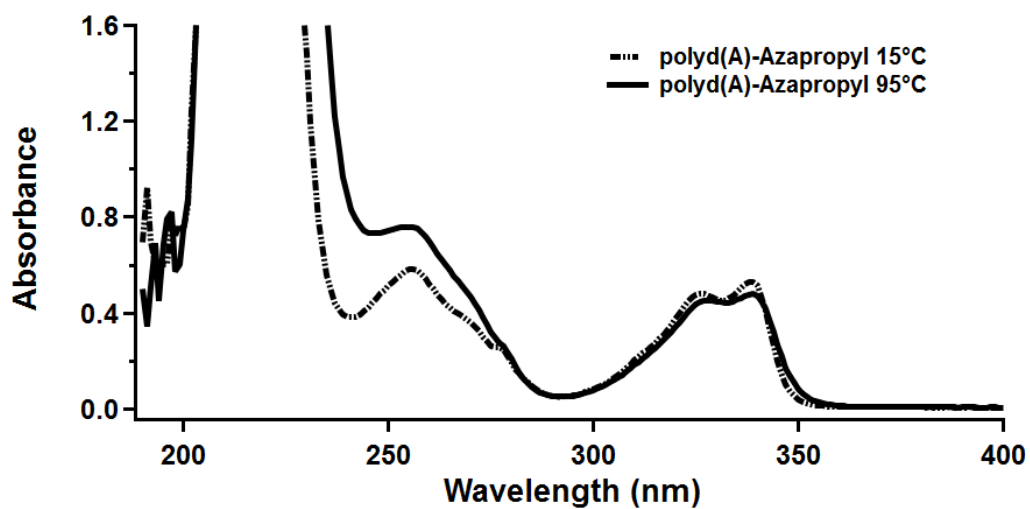


Figure 3.82. UV-vis spectra of polyd(A)-Azapropyl at 15°C and 95°C.

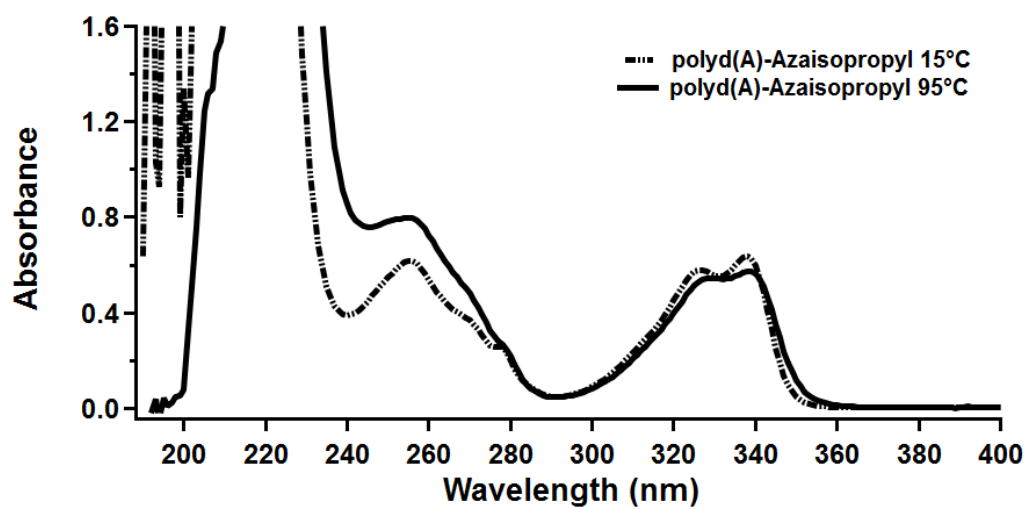


Figure 3.83 UV-vis spectra of polyd(A)-Azaisopropyl at 15°C and 95°C.

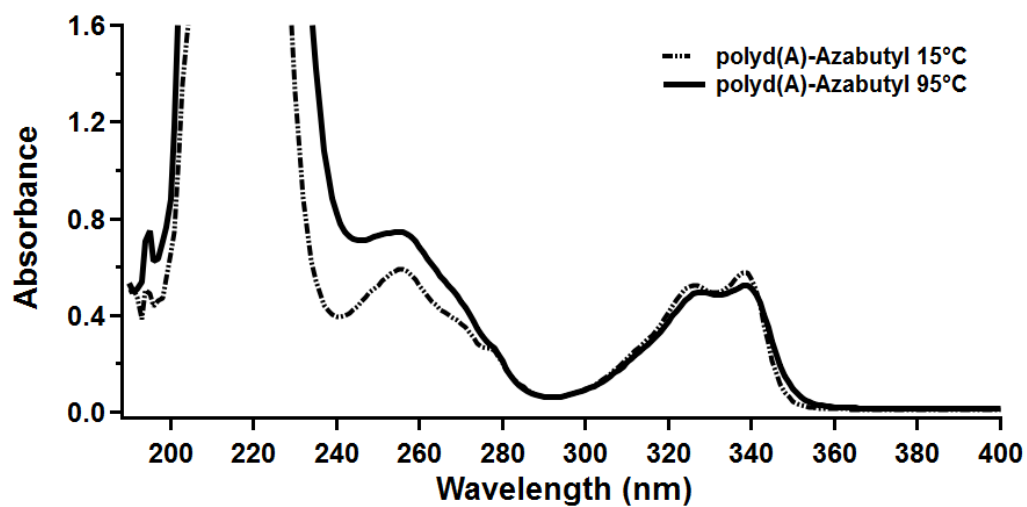


Figure 3.84. UV-vis spectra of polyd(A)-Azabutyl at 15°C and 95°C.

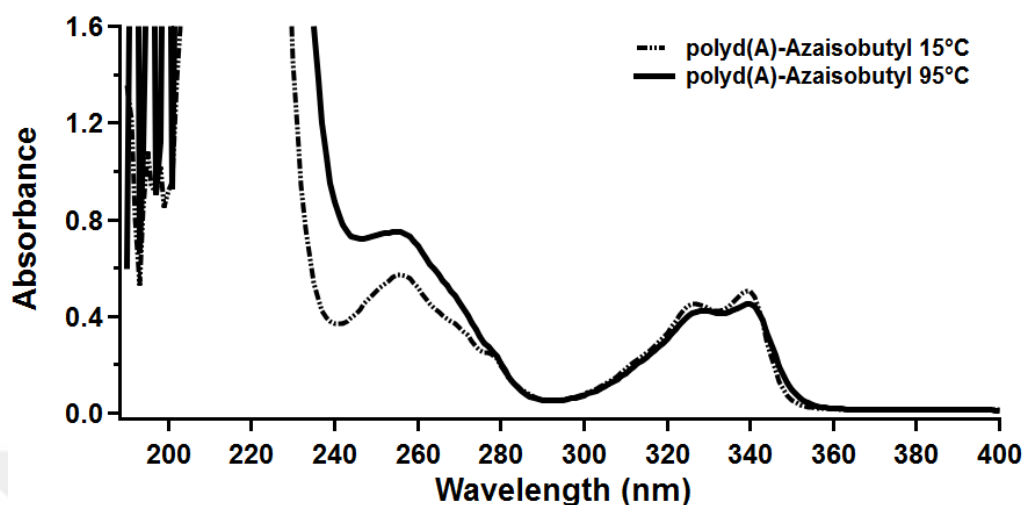


Figure 3.85. UV-vis spectra of polyd(A)-Azaisobutyl at 15°C and 95°C.

When the UV-vis spectra of polyd(A)-Azaethyl (Figure 3.81), polyd(A)-Azapropyl (Figure 3.82), polyd(A)-Azaisopropyl (Figure 3.83), polyd(A)-Azabutyl (Figure 3.84) and polyd(A)-Azaisobutyl (Figure 3.85) were examined, neither a hypochromic effect nor a red shift were observed in their spectra. On the contrary, a very slight blue shift along with an increase in the absorbance (hyperchromic effect) were observed in these spectra indicating that Azaethyl, Azapropyl, Azaisopropyl, Azabutyl and Azaisobutyl had no interaction with polyd(A) unlike Azamethyl.

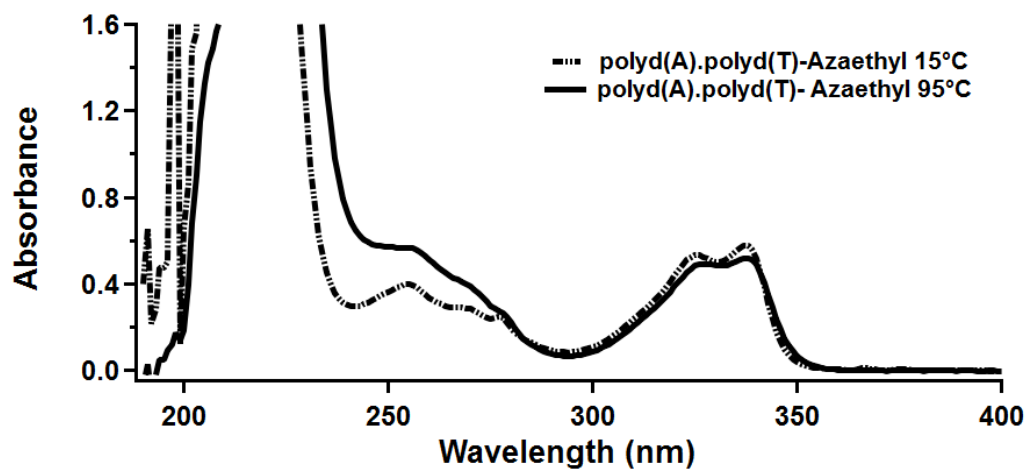


Figure 3.86. UV-vis spectra of polyd(A).polyd(T)-Azaethyl at 15°C and 95°C.

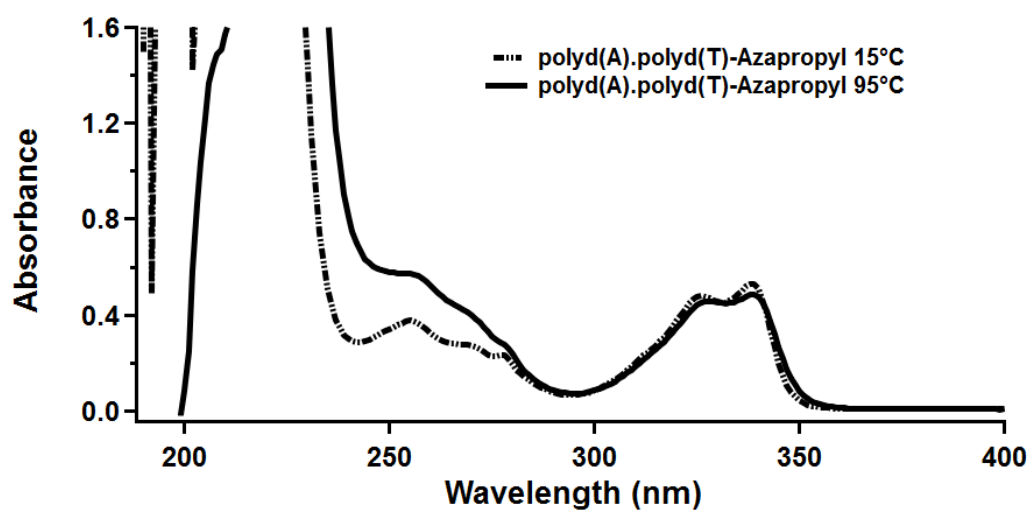


Figure 3.87. UV-vis spectra of polyd(A).polyd(T)-Azapropyl at 15°C and 95°C.

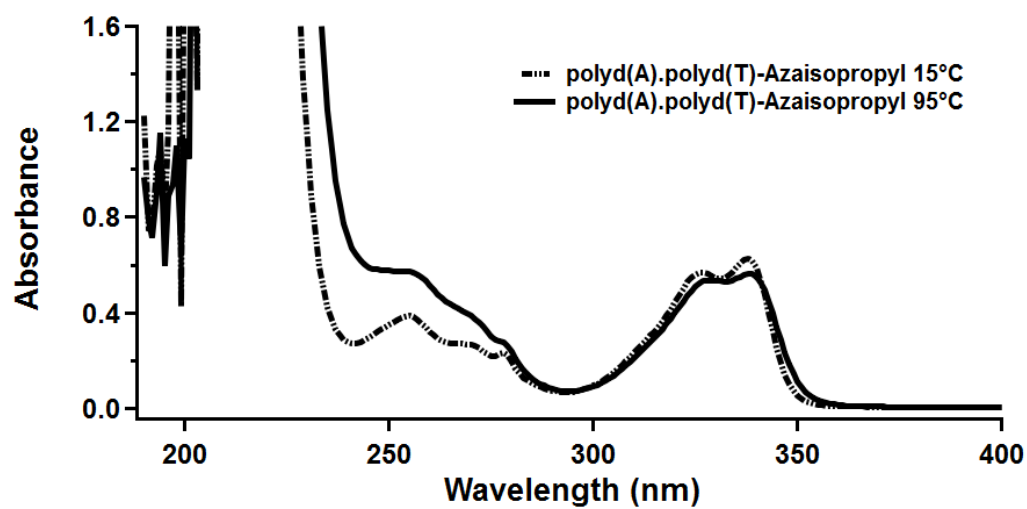


Figure 3.88. UV-vis spectra of polyd(A)·polyd(T)-Azaisopropyl at 15°C and 95°C.

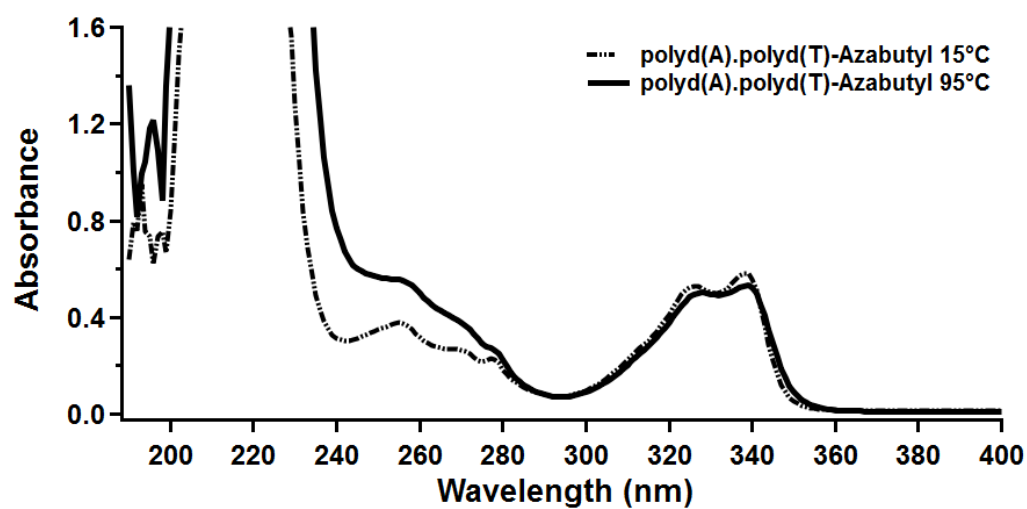


Figure 3.89. UV-vis spectra of polyd(A)·polyd(T)-Azabutyl at 15°C and 95°C.

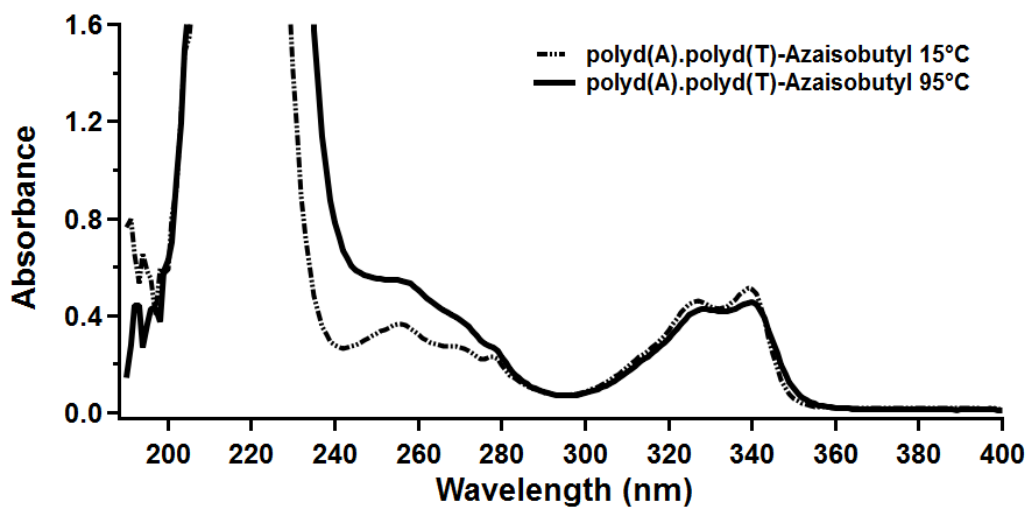


Figure 3.90. UV-vis spectra of polyd(A)·polyd(T)-Azaisobutyl at 15°C and 95°C.

When the UV-vis spectra of the complexes of Azaethyl (Figure 3.86), Azapropyl (Figure 3.87), Azaisopropyl (Figure 3.88), Azabutyl (Figure 3.89) and Azaisobutyl (Figure 3.90) with the polyd(A)·polyd(T) were examined, neither a red shift nor a hypochromic effect were observed indicating the lack of interactions between Azaethyl, Azapropyl, Azaisopropyl, Azabutyl or Azaisobutyl with polyd(A)·polyd(T) unlike Azamethyl.

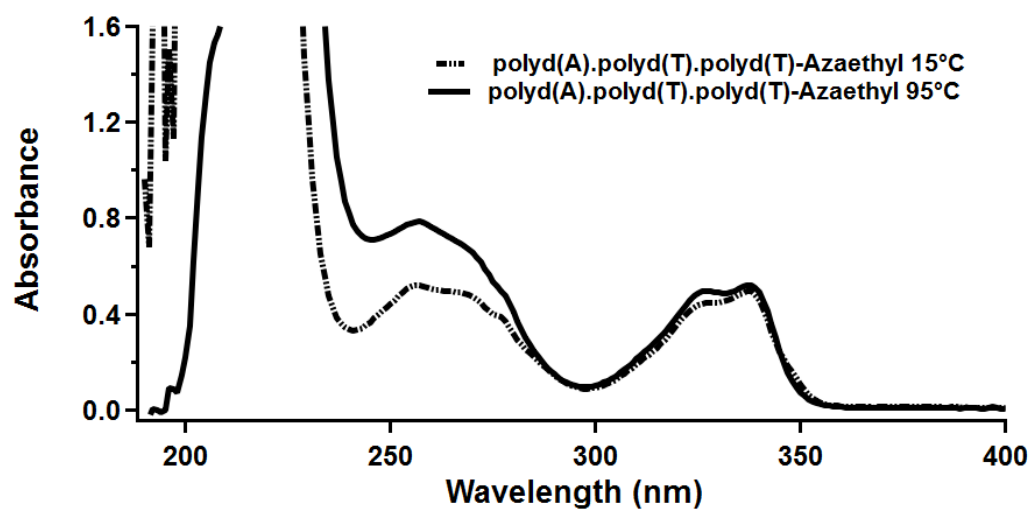


Figure 3.91. UV-vis spectra of polyd(A)·polyd(T)·polyd(T)-Azaethyl at 15°C and 95°C.

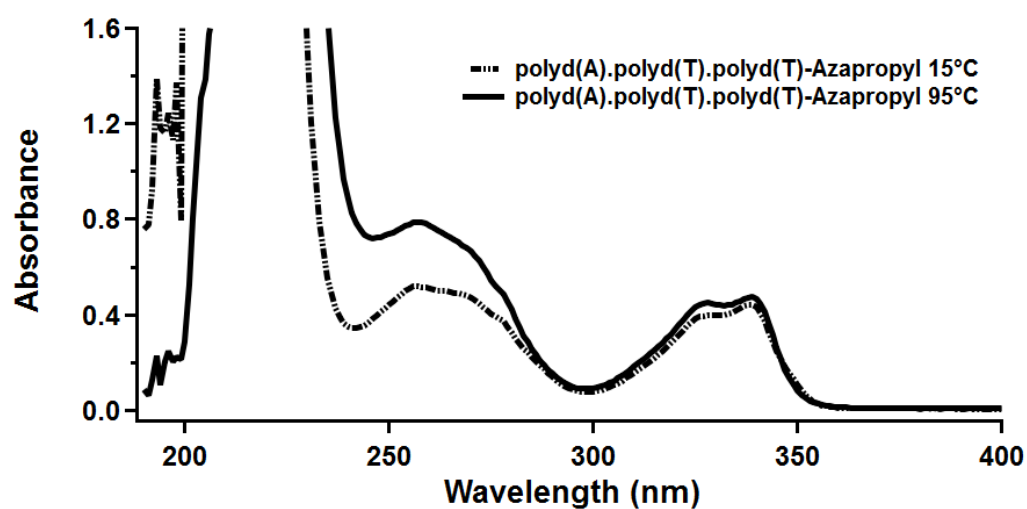


Figure 3.92. UV-vis spectra of polyd(A)·polyd(T)·polyd(T)-Azapropyl at 15°C and 95°C.

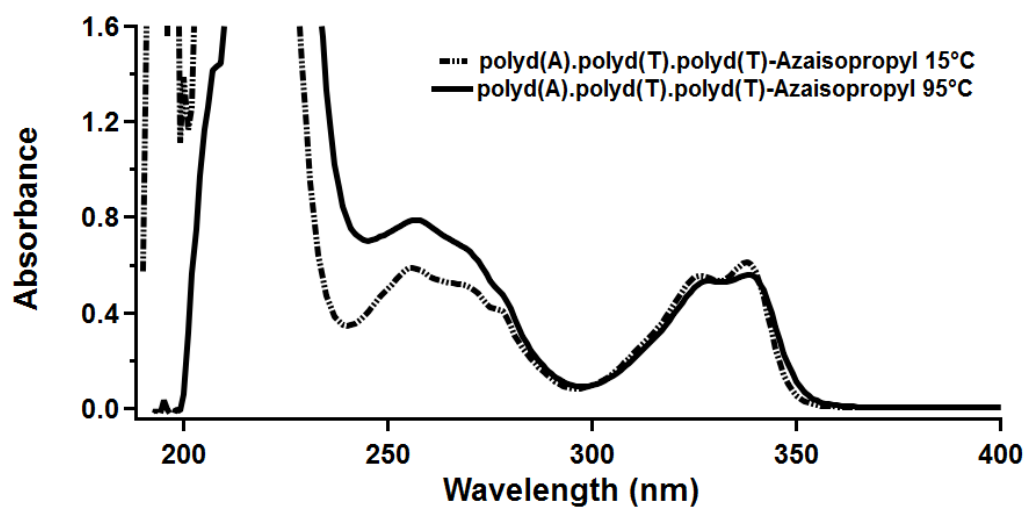


Figure 3.93. UV-vis spectra of polyd(A)·polyd(T)·polyd(T)-Azaisopropyl at 15°C and 95°C.

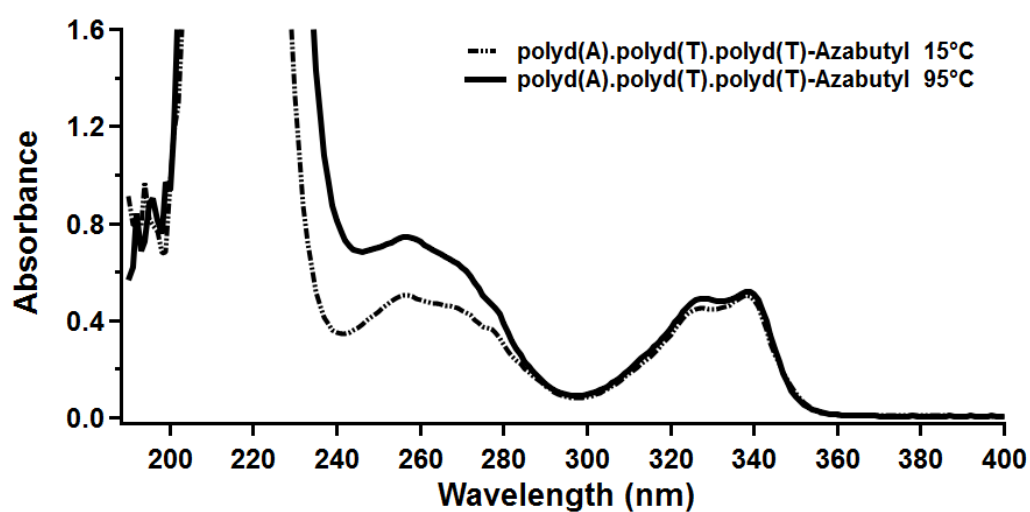


Figure 3.94. UV-vis spectra of polyd(A)·polyd(T)·polyd(T)-Azabutyl at 15°C and 95°C.

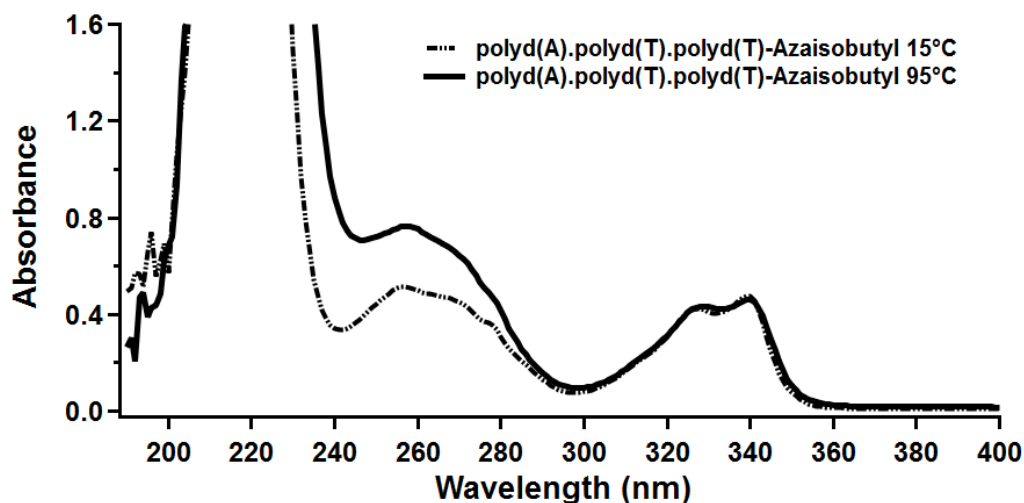


Figure 3.95. UV-vis spectra of polyd(A)·polyd(T)·polyd(T)-Azaisobutyl at 15°C and 95°C.

When the UV-vis spectra of the complexes of Azaethyl (Figure 3.91), Azapropyl (Figure 3.92), Azaisopropyl (Figure 3.93), Azabutyl (Figure 3.94) and Azaisobutyl (Figure 3.95) with polyd(A)·polyd(T)·polyd(T) were examined, a red shift and an hypochromic effect were observed in the longer UV-vis absorbance wavelengths between 300 and 360 nm. These changes indicate interactions between these molecules and polyd(A)·polyd(T)·polyd(T). Yet, it was noted that the observed red shift and the hypochromic effect were less than the observed red shift and hypochromic effect observed upon the binding of Azamethyl to polyd(A)·polyd(T)·polyd(T) (Figure 3.19).

Overall, by just examining the UV-vis spectra of the samples, it can be inferred that the Azamethyl derivatives have no interactions with and polyd(A) and polyd(A)·polyd(T) duplex, whereas they interact with polyd(A)·polyd(T)·polyd(T).

3.7.2 Determination of Thermal Denaturation Temperatures of the Complexes of Azamethyl Derivatives with polyd(A), polyd(T), polyd(A)·polyd(T) or polyd(A)·polyd(T)·polyd(T) by UV-vis Spectroscopy

The thermal denaturation experiments for the complexes of Azaethyl, Azapropyl, Azaisopropyl, Azabutyl and Azaisobutyl with polyd(A), polyd(A)·polyd(T) duplex or polyd(A)·polyd(T)·polyd(T) triplex were also carried out by monitoring the UV-vis absorbance changes at 260 nm and 336 nm with changing temperature.

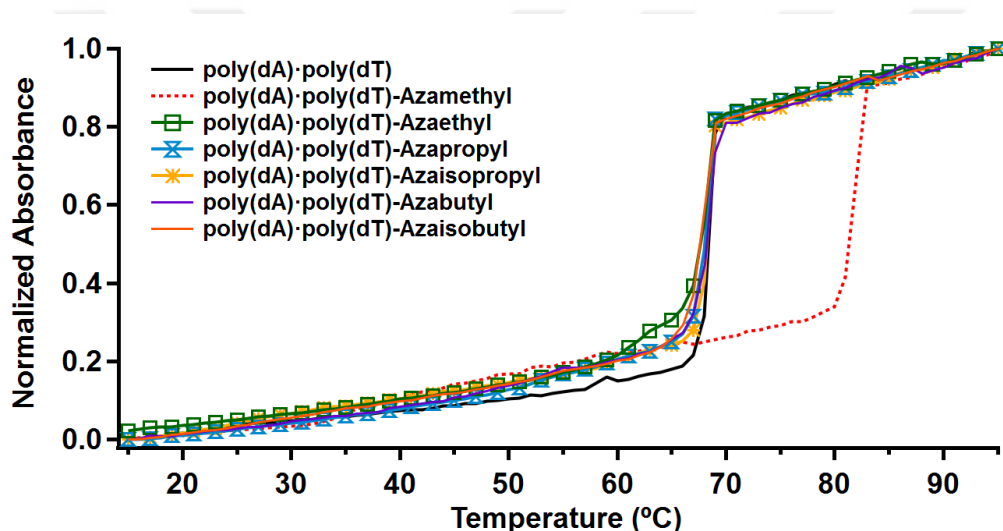


Figure 3.96. Polyd(A)·poly(T)-Azacyanine thermal denaturation profiles, 260 nm.

Thermal denaturation profiles of polyd(A)·poly(T) in the presence and absence of Azacyanines obtained by monitoring UV-vis absorbance at 260 nm with varying temperature.

The normalized thermal denaturation profiles of polyd(A)·polyd(T) duplex and polyd(A)·polyd(T)·polyd(T) triplex at 260 nm in the presence and absence of Azamethyl and its derivatives are displayed in Figure 3.96. And, the thermal denaturation temperatures obtained from the derivation of thermal denaturation profiles are presented in Table 3.6 and Table 3.7. The thermal denaturation values and profiles for the Azamethyl complexes (Section 3.1.3) are also given in Figure 3.96, Table 3.6 and Table 3.7 to assess the effect of the alkyl chain length more clearly.

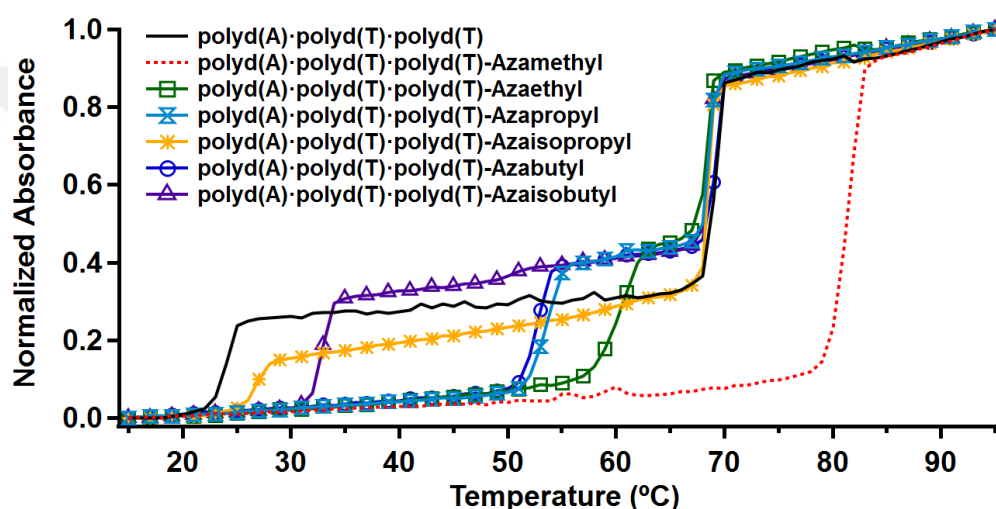


Figure 3.97. Polyd(A)·poly(T)·poly(T)-Azacyanine thermal denaturation profiles , 260 nm.

Thermal denaturation profiles of polyd(A)·poly(T)·poly(T) in the presence and absence of Azacyanines obtained by monitoring the UV-vis absorbance at 260 nm with varying temperature.

Table 3.6 Thermal denaturation temperatures for Azacyanine-polynucleic acid complexes, UV-vis, 260 nm.

	T_m (°C)			
	1. Heating	1. Cooling	2. Heating	2. Cooling
Polyd(A)	-	-	-	-
Polyd(T)	-	-	-	-
Polyd(A)·polyd(T)	68	67	68	67
Polyd(A)·polyd(T)·polyd(T)	24, 69	21, 68	24, 69	18, 67
Polyd(A)-Azamethyl	-	-	-	-
Polyd(T)- Azamethyl	-	-	-	-
Polyd(A)·polyd(T)- Azamethyl	82	69	82	69
Polyd(A)·polyd(T)·polyd(T)- Azamethyl	82	69	82	69
Polyd(A)-Azaethyl	-	-	-	-
Polyd(A)·polyd(T)- Azaethyl	69	67	68	67
Polyd(A)·polyd(T)·polyd(T)- Azaethyl	61, 68	59, 67	61, 68	59, 67
Polyd(A)-Azapropyl	-	-	-	-
Polyd(A)·polyd(T)- Azapropyl	68	67	68	66
Polyd(A)·polyd(T)·polyd(T)-Azapropyl	54, 69	52, 67	54, 68	52, 67
Polyd(A)-Azaisopropyl	-	-	-	-
Polyd(A)·polyd(T)- Azaisopropyl	68	67	68	67
Polyd(A)·polyd(T)·polyd(T)- Azaisopropyl	27, 69	25, 67	26, 68	24, 67
Polyd(A)-Azabutyl	-	-	-	-
Polyd(A)·polyd(T)-Azabutyl	68	67	68	67
Polyd(A)·polyd(T)·polyd(T)-Azabutyl	53, 69	51, 68	53, 69	51, 68
Polyd(A)-Azaisobutyl	-	-	-	-
Polyd(A)·polyd(T)-Azaisobutyl	68	66	68	66
Polyd(A)·polyd(T)·polyd(T)- Azaisobutyl	33, 69	31, 67	33, 68	30, 67

Table 3.7 Thermal denaturation temperatures for Azacyanine-polynucleic acid complexes, UV-vis, 336 nm.

	T _m (°C)			
	1. Heating	1. Cooling	2. Heating	2. Cooling
Polyd(A)	-	-	-	-
Polyd(T)	-	-	-	-
Polyd(A)·polyd(T)	-	-	-	-
Polyd(A)·polyd(T)·polyd(T)	-	-	-	-
Polyd(A) - Azamethyl	30	30	30	30
Polyd(T) - Azamethyl	-	-	-	-
Polyd(A)·polyd(T) - Azamethyl	35, 82	28, 69	28, 82	28, 69
Polyd(A)·polyd(T)·polyd(T) - Azamethyl	82	69	82	69
Polyd(A) - Azaethyl	-	-	-	-
Polyd(A)·polyd(T) - Azaethyl	69 very weak	67 very weak	68 very weak	68 very weak
Polyd(A)·polyd(T)·polyd(T) - Azaethyl	61	59	60	59
Polyd(A) - Azapropyl	-	-	-	-
Polyd(A)·polyd(T) - Azapropyl	-	-	-	-
Polyd(A)·polyd(T)·polyd(T) - Azapropyl	54	52	54	52
Polyd(A) - Azaisopropyl	-	-	-	-
Polyd(A)·polyd(T) - Azaisopropyl	-	-	-	-
Polyd(A)·polyd(T)·polyd(T) - Azaisopropyl	-	-	-	-
Polyd(A) - Azabutyl	-	-	-	-
Polyd(A)·polyd(T) - Azabutyl	-	-	-	-
Polyd(A)·polyd(T)·polyd(T) - Azabutyl	53	51	53	51
Polyd(A) - Azaisobutyl	-	-	-	-
Polyd(A)·polyd(T) - Azaisobutyl	-	-	-	-
Polyd(A)·polyd(T)·polyd(T) - Azaisobutyl	33	31	32	29.5

Monitoring of the absorbance change at 260 nm (A_{260}) with varying temperature for polyd(A)·polyd(T) duplex structure led to a single melting transition ($2 \rightarrow 1$) at 68°C in heating and at 67°C in cooling traces, respectively (Figure 3.96, Table 3.6). Under the same conditions, polyd(A)·polyd(T)·polyd(T) by itself had two melting transitions (Figure 3.97, Table 3.6). In heating traces, the first one was at 24°C, corresponding to triplex melting into duplex ($3 \rightarrow 2$), and the second one was at 69°C corresponding to duplex melting to single strands ($2 \rightarrow 1$). In cooling traces, the first one was at 21°C and the second one was around 67°C. It is argued that the changes which are about 2°C between traces are due to the hysteresis in melting (Belozerova and Levicky, 2012; Hoff and Roos, 1972) and the experimental errors.

Except for Azamethyl, the polyd(A)·polyd(T) had almost the same melting point at about 68°C both in heating and cooling traces in the presence of Azacyanines. In other words, none of the Azacyanines had an effect on polyd(A)·polyd(T) except for Azamethyl. Neither the length nor the branching of the alkyl chain length had an influence in polyd(A)·polyd(T)-Azacyanine interactions. In the presence of the Azamethyl however, a single melting transition at around 82°C was observed in the heating traces and a single transition at around 69°C was observed in the cooling traces. At first, this very high melting transition at 82°C was thought to be the stabilization effect of Azamethyl on polyd(A)·polyd(T) duplex. Nevertheless, further investigations of the interactions of Azamethyl with polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) revealed that the transition at 82°C belongs to the polyd(A)·polyd(T)·polyd(T)-Azamethyl complex.

The T_m obtained for polyd(A)·polyd(T)·polyd(T)-Azacyanine samples by monitoring A_{260} reconfirmed that the Azacyanines, except Azamethyl, had no effect on the duplex thermal denaturation. The melting transition of about 68°C was obtained for duplex to single strand transition ($2 \rightarrow 1$) in all the samples except for polyd(A)·polyd(T)·polyd(T)-Azamethyl. On the other hand, it was observed that all the Azacyanines had interactions with polyd(A)·polyd(T)·polyd(T) stabilizing the triplex structure. The stabilization degree notably depended on both the branching and the length of the alkyl chain in the benzimidazole ring. Among the molecules

studied, the Azaisopropyl stabilized the polyd(A)·polyd(T)·polyd(T) triplex with only 3°C increase from 24°C to 27°C melting temperatures. The melting temperature of polyd(A)·polyd(T)·polyd(T) in the presence of isobutyl was 33°C. The melting temperatures for polyd(A)·polyd(T)·polyd(T) in the presence of Azabutyl, Azapropyl and Azaethyl were 53°C, 54°C and 61°C, respectively. The polyd(A)·polyd(T)·polyd(T)-Azamethyl sample had the same single melting transition observed in polyd(A)·polyd(T)-Azamethyl sample at 82°C.

Hence, it was clear that the branching on the benzimidazole chain was negatively affecting the binding of Azacyanines to polyd(A)·polyd(T)·polyd(T). With its shorter length and the decreased flexibility compared to isobutyl, the isopropyl chain stabilized the triplex was less than the others. Since Azacyanines are expected to bind to DNA via intercalation, we believe that the shorter and the less flexible isopropyl chain might prevent the movement of the benzimidazole ring between base pairs resulting in inefficient stacking with the base pairs. In contrast, it was clear that the melting temperature of polyd(A)·polyd(T)·polyd(T) increased with a decrease in the length of the alkyl chain on the benzimidazole ring. Based on the trend obtained with the other Azacyanines, the melting temperature at 82°C turned out to belong to the polyd(A)·polyd(T)·polyd(T)-Azamethyl and not to the polyd(A)·polyd(T)-Azamethyl sample.

The absorbance changes at around 336 nm with varying temperature in polyd(A)·polyd(T)-Azacyanine complexes were also monitored (Table 3.7). No melting transition at that wavelength was observed for polyd(A)·polyd(T) in the presence of Azapropyl, Azaisopropyl, Azabutyl and Azaisobutyl. However, there was a very weak transition at around 68°C in the presence of Azaethyl. In the presence of Azamethyl, two transitions were observed in polyd(A)·polyd(T)'s heating and cooling traces. The transitions were observed at 35°C and 82°C in the first heating trace and at 28°C and 82 °C in the second heating trace, respectively. In both of the cooling traces, the transitions were observed at 28°C and 69°C.

The thermal denaturation profiles were also obtained for polyd(A)·polyd(T)·polyd(T)-Azacyanine samples by monitoring the change in UV-vis absorbances at about 336 nm with varying temperature. No melting transition was observed for polyd(A)·polyd(T)·polyd(T)-Azaispropyl sample at that wavelength. For all the other samples, only one single melting temperature was observed corresponding to the triplex melting (3→2) such that the melting temperatures obtained were exactly the same as the melting temperatures obtained by monitoring the change in the absorbance at 260 nm (Table 3.6 and Table 3.7). In other words, the observed transitions was due to the release of Azacyanines from the triplex structures. These results are in perfect agreement with the obtained results concerning the Azacyanines affinity to bind and stabilize only the triplex but not the duplex structures. The release of Azaisopropyl from the triplex structure was not observable probably due to the weak binding of Azaispropyl to polyd(A)·polyd(T)·polyd(T) triplex.

Overall, all the molecules investigated here were found to increase the stability thus the T_m of polyd(A)·polyd(T)·polyd(T). Yet and more importantly, the increase in the T_m depends on the alkyl chain length and the branching of the benzimidazole ring. The polyd(A)·polyd(T)·polyd(T) thermally denatures itself at 24°C while it's T_m increases in the presence of the molecules in the following order: Azaisopropyl (27°C) < Azaisobutyl (33°C) < Azabutyl (53°C) < Azapropyl (54°C) < Azaethyl (61°C) < Azamethyl (82°C) (Figure 3.97). Eventually, the binding affinity of the Azacyanines to polyd(A)·polyd(T)·polyd(T) was negatively affected with increasing linear alkyl chain length and mainly branching.

Previously, coralyne has been shown to disproportionate polyd(A)·polyd(T) duplex structure to intercalated polyd(A)·polyd(T)·polyd(T) triplex and polyd(A)-self structure. The disproportionation temperature was found to be 35°C. At 35 °C, the duplex disproportionated to polyd(A)·polyd(T)·polyd(T)-coralyne and polyd(A)-coralyne structures that melted at 82°C and 47°C, respectively. The T_m of 35°C and 82°C were the exact transitions obtained by monitoring the A_{336} with varying temperature in the polyd(A)·polyd(T)-Azamethyl sample.

Since our data were indicating the probable disproportionation of polyd(A)·polyd(T) in the presence of Azamethyl, the interactions of polyd(A) with Azacyanines were also investigated. Previously, it has been reported that Azamethyl (named as Aza3 then) binds to poly(A) (RNA) inducing the formation of poly(A) self-structure with a T_m of 55°C (Çetinkol and Hud, 2009). Not surprisingly, here it was observed that Azamethyl was also able to bind to polyd(A) (DNA) and induce the self-structure formation in polyd(A) with a T_m of 30°C. The thermal denaturation temperature indicates that polyd(A)-Azamethyl is not as stable as the polyd(A)-coralyne or poly(A)-Azamethyl complexes. The 300 to 360 nm region of the UV-vis spectra of Azamethyl in the presence and absence of polyd(A) reveal that Azamethyl's binding to polyd(A) can cause hypochromic effect along with about 10 nm redshift in the UV-vis spectrum (Figure 3.14). The red shift and the hypochromic effect were also the signs of coralyne's binding to polyd(A). As expected, similar changes in the presence of polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) were observed (but not in the presence of polyd(T)) supporting the fact that Azamethyl couldn't bind to polyd(T) (Figure 3.18, Figure 3.20 and Figure 3.16). To our surprise, none of the other Azacyanine molecules were neither able to bind nor induce the polyd(A)-self structure under the given conditions.

3.7.3 Determination of the Interactions of Azamethyl Derivatives with polyd(A), polyd(T), polyd(A)·polyd(T) And polyd(A)·polyd(T)·polyd(T) by CD Spectroscopy

The interactions of Azaethyl, Azapropyl, Azaisopropyl, Azabutyl and Azaisobutyl with the polyd(A), polyd(T), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) at 5°C were also investigated via CD spectroscopy and the collected spectra were provided below. For easy comparison of all these molecules, the CD spectra of Azamethyl samples were also given in Figure 2.96. Initially, the CD spectrum at 5°C was collected and compared to the UV-vis results and the spectra of polyd(A), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) in

the absence and presence of Azacyanines were compared. The polyd(T) was only investigated in the absence and presence of Azamethyl and since there was no change or induced band formation in polyd(T) spectrum even in the presence of Azamethyl, its interactions with the other Azacyanines were not further investigated. None of the Azacyanines studied gave rise to intrinsic ellipticity between 230 nm and 400 nm. As a result, the first assessment of the binding was done based on the induced band formation between 300 nm to 360 nm upon binding of Azacyanines to nucleic acids structures. The induced band formation is always considered as the explicit evidence of interaction between the small molecules and nucleic acids (Polak and Hud, 2002; Jain, Polak and Hud, 2003).

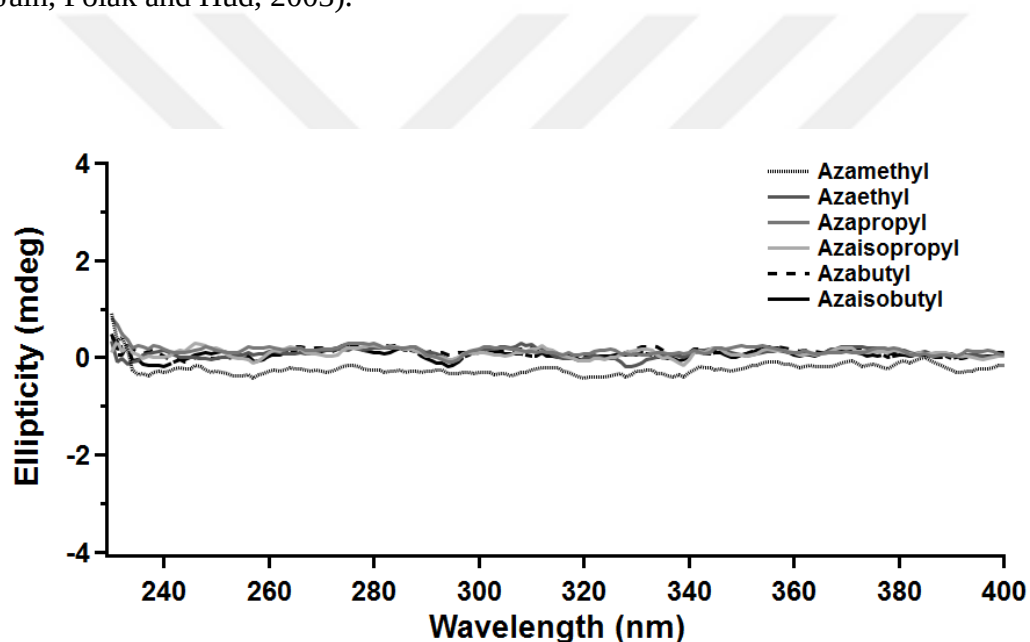


Figure 3.98. CD spectra of all Azacyanines at 5°C.

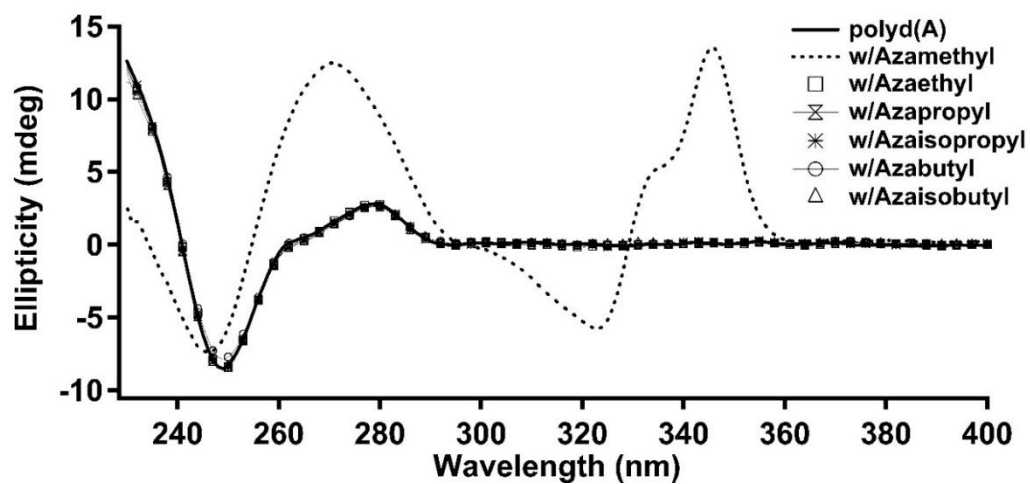


Figure 3.99. CD spectra of polyd(A) in the absence and presence of Azacyanines at 5°C.

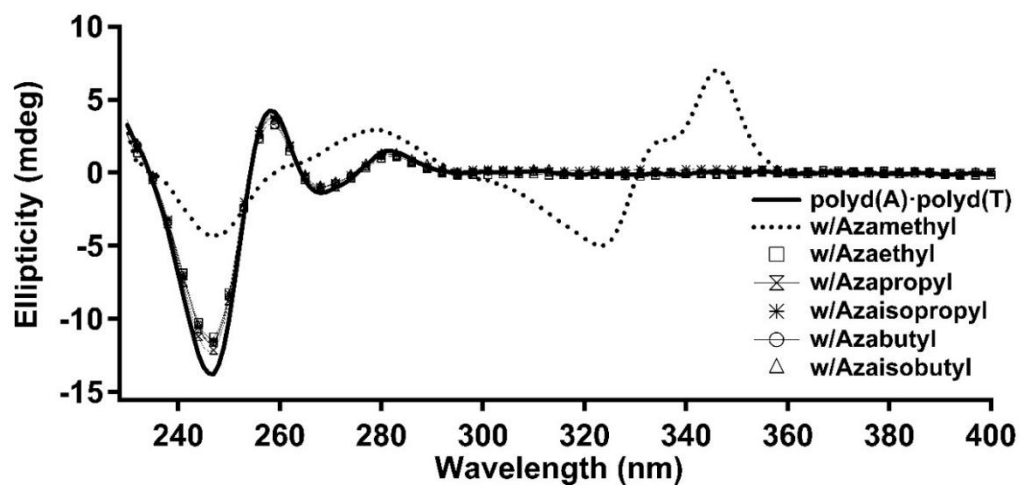


Figure 3.100. CD spectra of polyd(A)·polyd(T) in the absence and presence of Azacyanines at 5°C.

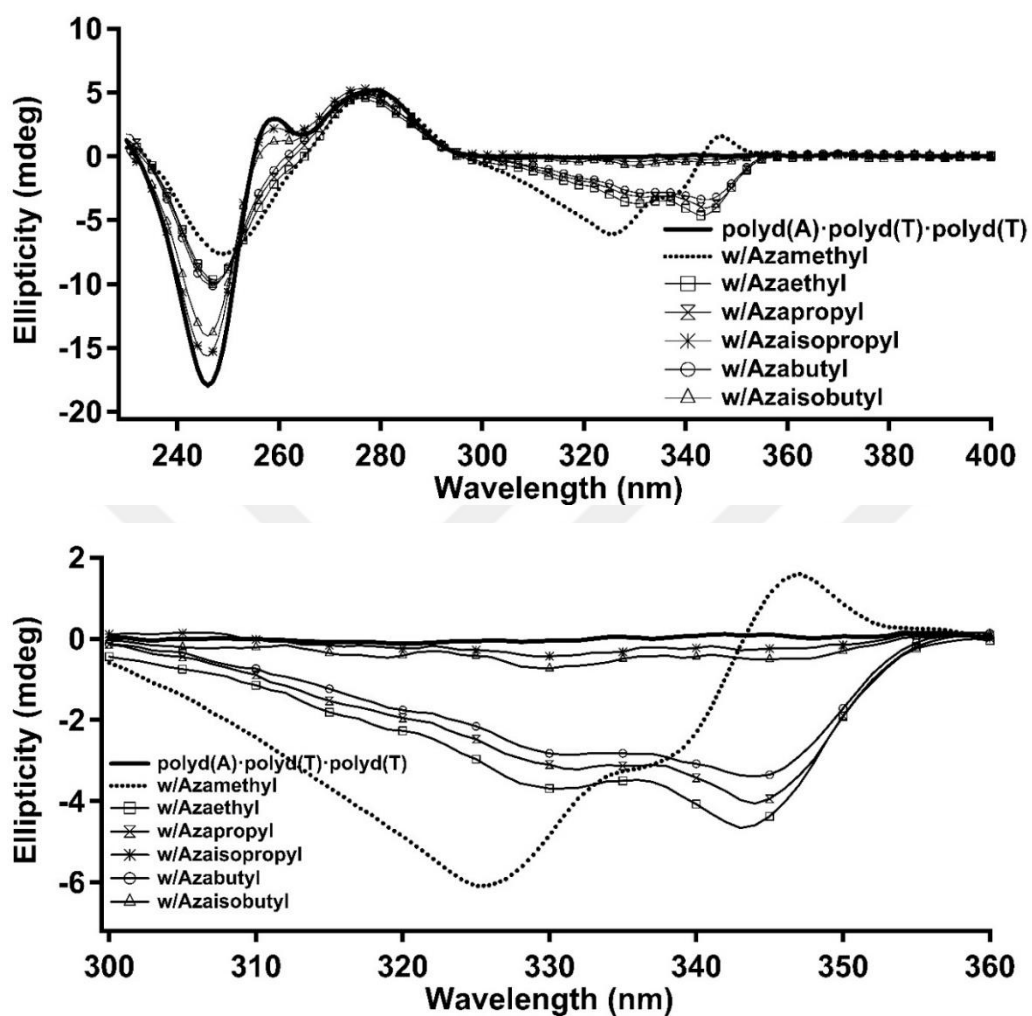


Figure 3.101. CD spectra of polyd(A)·polyd(T)·polyd(T) in the absence and presence of Azacyanines at 5°C.

Top: Full CD spectra of polyd(A)·polyd(T)·polyd(T) at 5°C in the presence and absence of Azacyanines. **Bottom:** Only the 300 nm and 360 nm region of the CD spectra given at the top.

The CD spectra of polyd(A) in the absence and presence of the Azacyanines at 5°C are presented in Figure 3.99. Except for Azamethyl, we did not observe any change in the polyd(A) spectrum in the presence of the other examined Azacyanines which is in good agreement with our UV-vis data suggesting only Azamethyl is able to induce the formation of polyd(A) self-structure meaning only Azamethyl interacts with polyd(A). In the presence of Azamethyl, there was a significant increase in the intensity of the CD band at about 270 nm and there was a slight blue shift in the position of the CD band at about 249 nm. The induced CD band exhibits an intense negative peak between 300 nm and 330 nm followed by another intense positive peak between 330 nm and 360 nm. The area of the induced peak at the positive region almost was twice as high as the peak induced in the negative region. The induced peaks between 300 nm to 360 nm in the presence of Azamethyl is the main signal of the interaction between polyd(A) and Azamethyl. No interactions were observed between the other Azacyanines and polyd(A), such that the CD spectra of polyd(A) in the presence of the other Azacyanines were exactly the same as the CD spectrum of polyd(A) alone (Figure 3.99). There was not any shift in the wavelength of the peaks or change in the intensity.

The CD spectra of polyd(A)·polyd(T) in the absence and presence of Azacyanines at 5°C are given in Figure 3.100. The molecules other than Azamethyl also did not interact with the duplex of polyd(A)·polyd(T) (Figure 3.100). Once again, the induced CD band formation that is a sign of binding was observed in polyd(A)·polyd(T) spectrum only in the presence of the Azamethyl. The induced peak formation was not observed in the presence of other Azacyanines. The observed induced CD was between 300 and 360 nm. The intensity of the negative and the positive induced CD bands were almost the same. The CD spectrum of polyd(A)·polyd(T) in the presence of Azamethyl was distinctly different from the only polyd(A)·polyd(T) spectrum. There was a significant decrease in the intensity of the peaks at around 246 nm and 260 nm despite an increase in the intensity of the peak at about 280 nm. No induced CD band was observed in the polyd(A)·polyd(T) spectrum in the presence of the other Azacyanines. Just a small decrease in the peak

at 249 nm was observed in the presence of Azaethyl, Azapropyl, Azaisopropyl, Azabutyl and Azaisobutyl and there was a slight change in the intensity of the peaks at 246 nm and 260 nm, respectively which might be due to the weak nonspecific interactions between the duplex and these molecules. These changes observed in the CD spectrum of polyd(A)·polyd(T) were also in perfect agreement with our UV-vis thermal denaturation data confirming that only Azamethyl could interact with polyd(A)·polyd(T) inducing a change in its structure.

The interactions of polyd(A)·polyd(T)·polyd(T) with Azacyanines at 5°C were also investigated using CD spectroscopy (Figure 3.101). Upon addition of Azacyanines, there was a significant change in the CD spectrum of polyd(A)·polyd(T)·polyd(T). All the six small molecules were found to be interacting with the triplex of polyd(A)·polyd(T)·polyd(T) (Figure 3.101). The intensity of the CD band at 246 nm and 260 nm decreased upon the addition of Azacyanines. This change is the least in the presence of Azaisopropyl and the highest in the presence of Azamethyl. For instance, the ellipticity at 246 nm was determined for polyd(A)·polyd(T)·polyd(T) as -17.6 mdeg, for polyd(A)·polyd(T)·polyd(T)-Azaisopropyl as -15.5 mdeg, for polyd(A)·polyd(T)·polyd(T)-Azaisobutyl as -14.1 mdeg, for polyd(A)·polyd(T)·polyd(T)-Azabutyl as 10.0 mdeg, for polyd(A)·polyd(T)·polyd(T)-Azapropyl as -9.8 mdeg, for polyd(A)·polyd(T)·polyd(T)-Azaethyl as -9.5 mdeg and for polyd(A)·polyd(T)·polyd(T)-Azamethyl -7.4 mdeg. It was noticed that there were changes in the intensity of the CD spectrum at 246 nm and 260 nm being negatively correlated with their ability to stabilize triplex in the UV-vis thermal denaturation experiments. An induced CD band formation was also recorded between 300 nm to 360 nm in the presence of Azacyanines. Except the Azamethyl, the formed induced band was negative and had two maxima at around 332 nm and 344 nm. The induced bands formed in the presence of Azaisopropyl and Azaisobutyl were extremely weak (less than 1 mdeg almost appeared as null) (Figure 3.101). Still, the intensity of the induced peak for polyd(A)·polyd(T)·polyd(T)-Azaisopropyl as the least triplex stabilizing compound based on our thermal denaturation studies was smaller

than that of the polyd(A)·polyd(T)·polyd(T)-Azaisobutyl. The intensity of the induced CD band for the other Azacyanines increased in the order of polyd(A)·polyd(T)·polyd(T)-Azabutyl, polyd(A)·polyd(T)·polyd(T)-Azapropyl, polyd(A)·polyd(T)·polyd(T)-Azaethyl and polyd(A)·polyd(T)·polyd(T)-Azamethyl. The shape of the induced band in polyd(A)·polyd(T)·polyd(T)-Azamethyl spectrum was distinctly different from the induced CD band in other polyd(A)·polyd(T)·polyd(T)-Azacyanine spectra. In the presence of Azamethyl, a large negative band was formed followed by a small positive band. In addition, the local minimum ellipticity was observed at about 325 nm in the induced negative band, and the maximum ellipticity at about 347 nm in the induced positive band. The change in the shape of the induced band might be an indication of a better arrangement of Azamethyl between the base pairs and its distinct environment. Accordingly, it can be stated that our CD results are in a good agreement with our UV-vis thermal denaturation experiments. It should be noted that the degree of decrease in the intensity of the CD spectrum at 246 nm and 260 nm is positively correlated with the triplex stabilization ability of Azacyanines.

3.8 Investigation of the Interactions of Aza4 and Aza5 with the polyd(A), polyd(T), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) by UV-vis and CD Spectroscopies

The interactions of two benzothiazole derivatives, Aza4 and Aza5, with the polyd(A), polyd(T), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) were also investigated via UV-vis and CD spectroscopy techniques. The interactions of Azamethyl, Aza4 and Aza5 with poly(A) (RNA), and their ability to induce the formation of the self structure previously have been reported in the literature (Persil Çetinkol and Hud 2009). Here, the UV-vis and CD spectra of polyd(A), polyd(T), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) were collected in the absence and presence of these molecules and compared. Since the Aza4 and Aza5 were reported as unstable above 50°C (Persil Çetinkol and Hud 2009), their interactions with polynucleic acids via thermal denaturation experiments were not studied.

3.8.1 Investigation of the Interactions of Aza4 and Aza5 with polyd(A), polyd(T), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) by UV-vis Spectroscopy

The UV-vis spectra of polyd(A), polyd(T), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) at 15°C were collected in the absence and presence of Aza4 and Aza5. The collected spectra were compared to the spectra of only Aza4 and Aza5. The obtained spectra are given in Figure 3.100 - Figure 3.109.

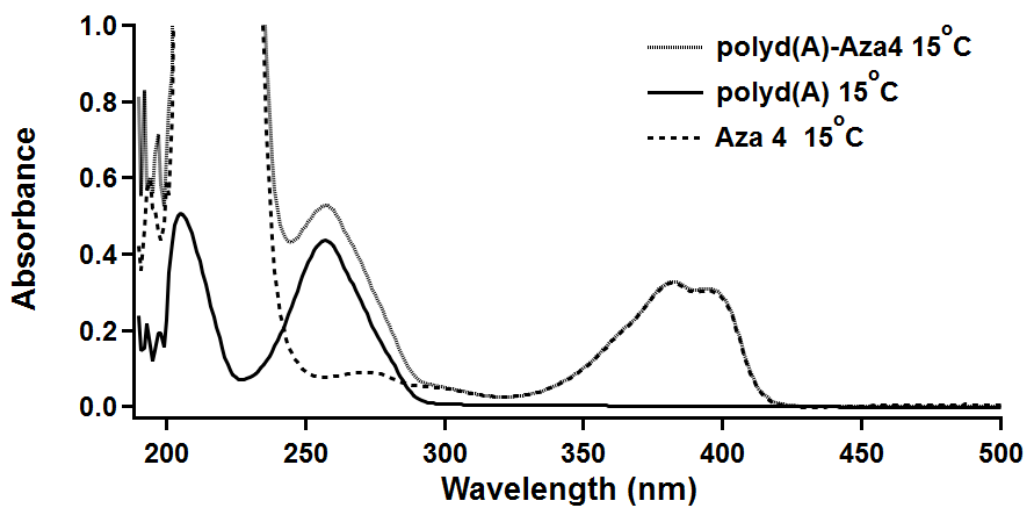


Figure 3.102. UV-vis spectra of polyd(A), polyd(A)-Aza4 and Aza4 at 15°C

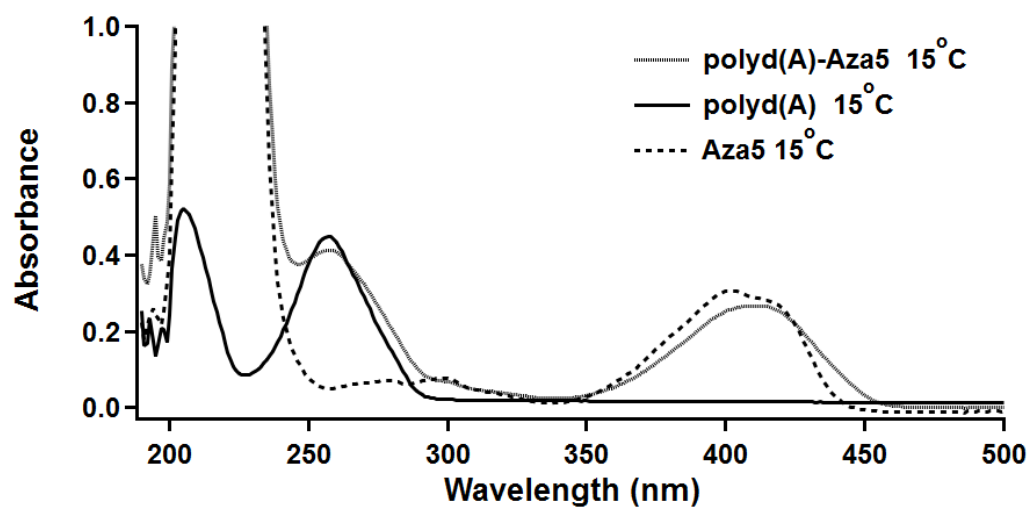


Figure 3.103. UV-vis spectra of polyd(A), polyd(A)-Aza5 and Aza5 at 15°C

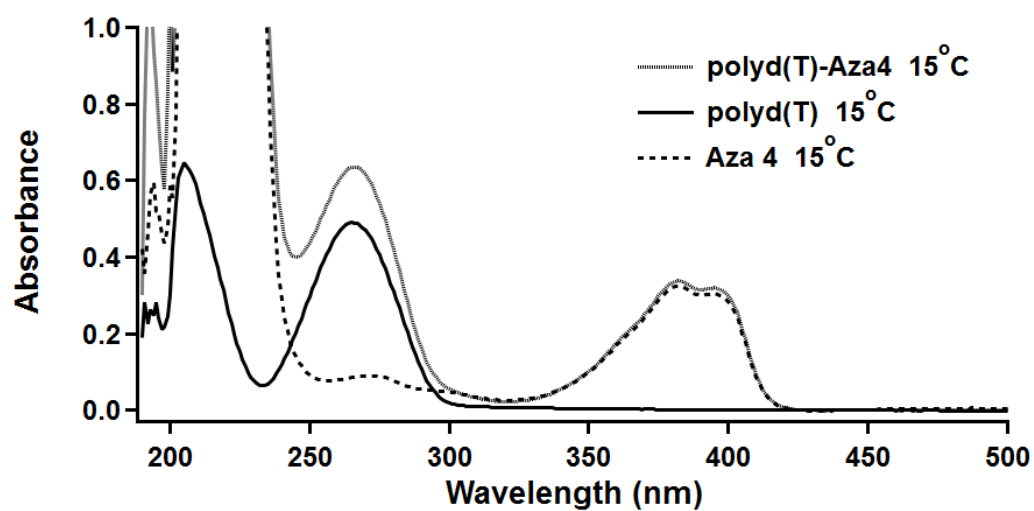


Figure 3.104. UV-vis spectra of polyd(T), polyd(T)-Aza4 and Aza4 at 15°C

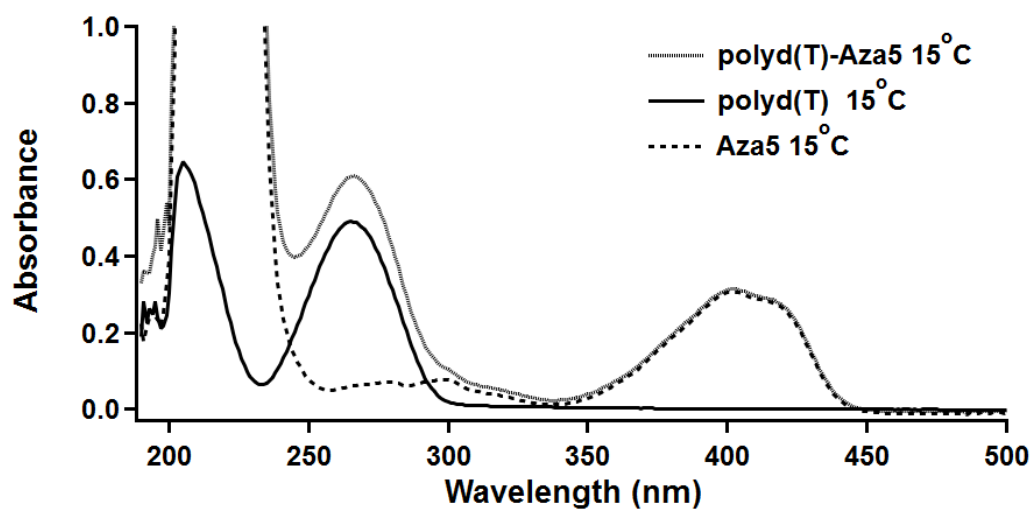


Figure 3.105. UV-vis spectra of polyd(T), polyd(T)-Aza5 and Aza5 at 15°C

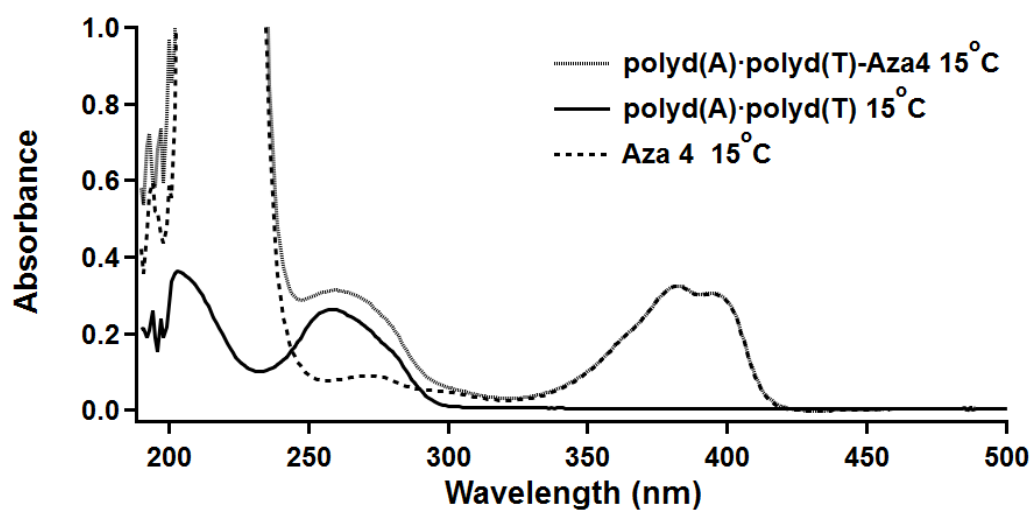


Figure 3.106. UV-vis spectra of polyd(A)·polyd(T), polyd(A)·polyd(T)-Aza4 and Aza4 at 15°C

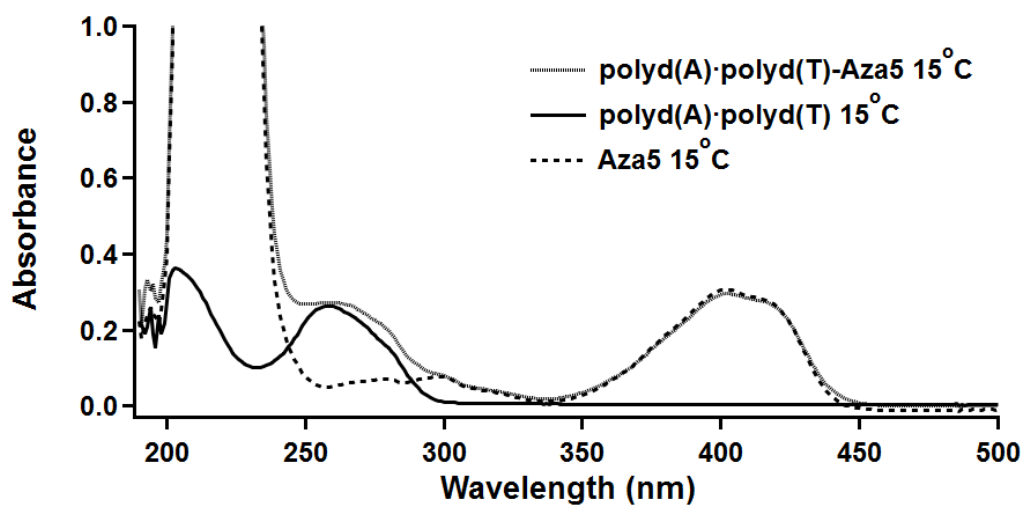


Figure 3.107. UV-vis spectra of polyd(A)·polyd(T), polyd(A)·polyd(T)-Aza5 and Aza5 at 15°C

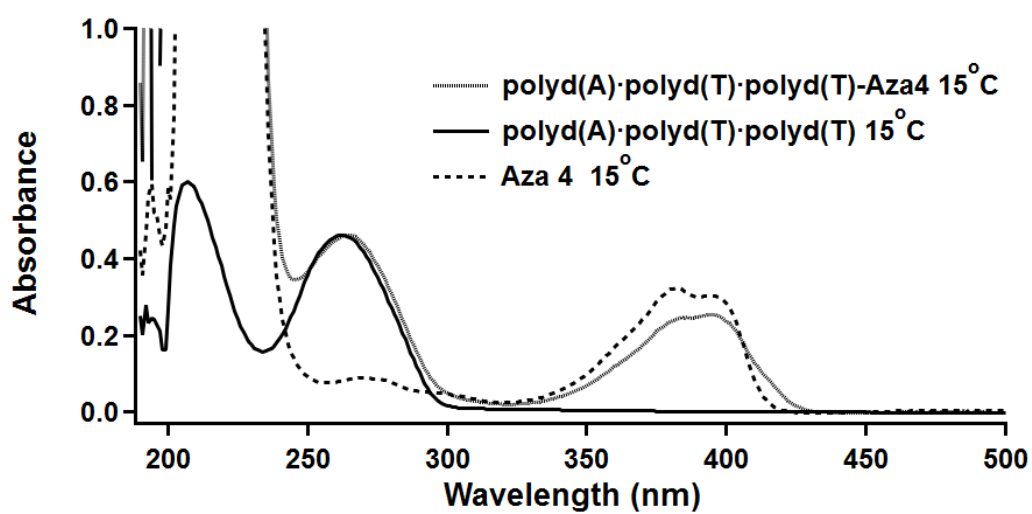


Figure 3.108. UV-vis spectra of polyd(A)·polyd(T)·polyd(T), polyd(A)·polyd(T)·polyd(T)-Aza4 and Aza4 at 15°C

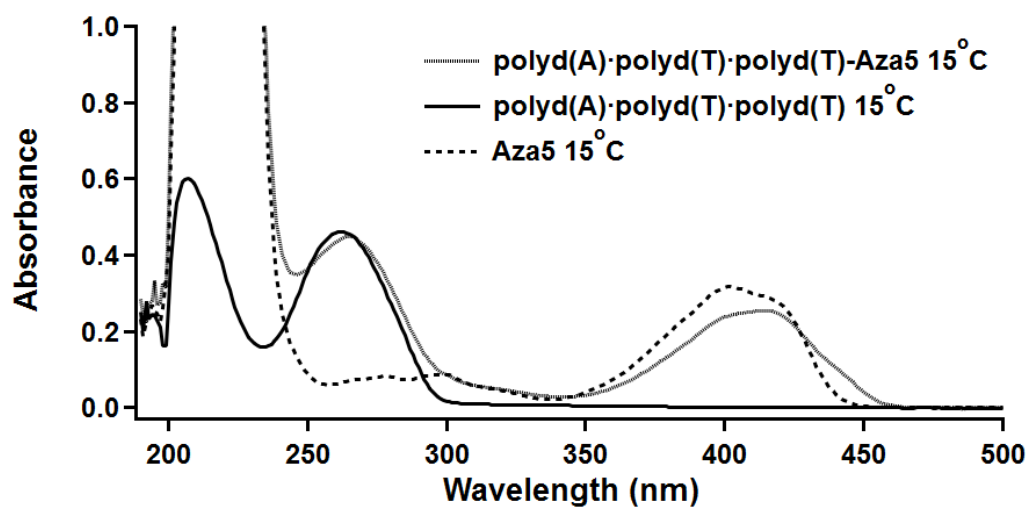


Figure 3.109. UV-vis spectra of polyd(A)·polyd(T)·polyd(T), polyd(A)·polyd(T)·polyd(T)-Aza5 and Aza5 at 15°C

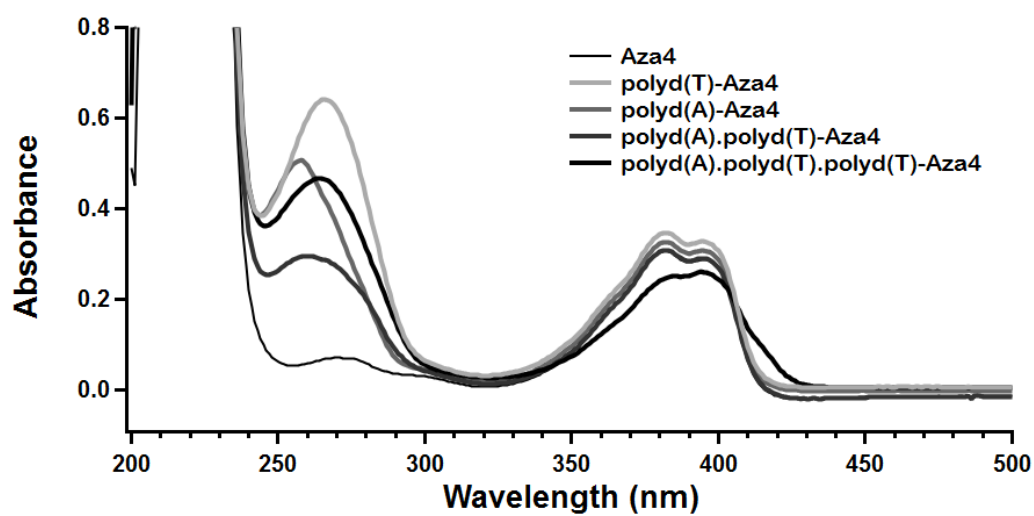


Figure 3.110. UV-vis spectra of all polynucleotides with Aza4 at 15°C

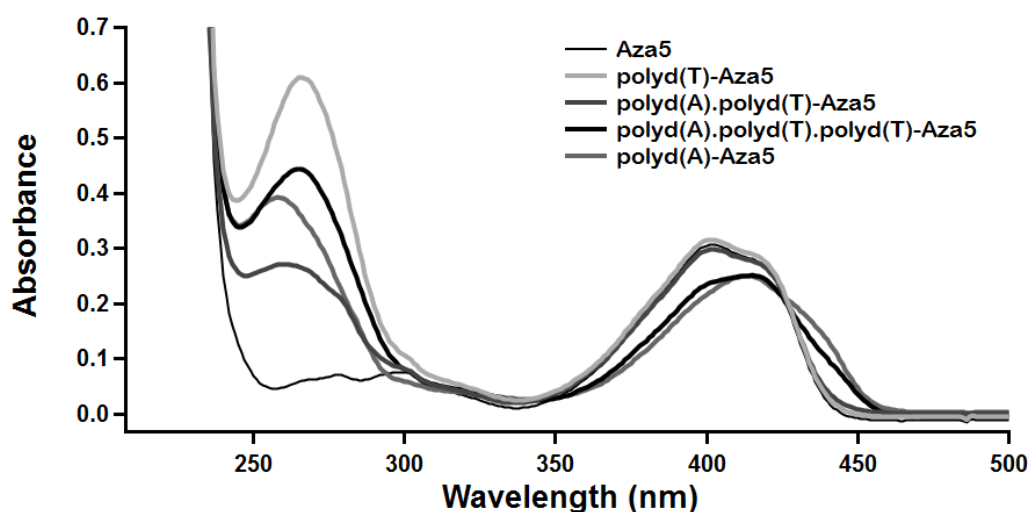


Figure 3.111. UV-vis spectra of all polynucleotides with Aza5 at 15°C

The comparison of the UV-vis spectra revealed no interaction between Aza4 and polyd(A) (Figure 3.102), polyd(T) (Figure 3.104) and polyd(A).polyd(T) (Figure 3.106). On the other hand, Aza4 did not interact with polyd(A).polyd(T).polyd(T) as evident from the red shift and the hypochromic effect observed in the 350 -450 nm region. Aza5 was also had no interaction with the polyd(T) (Figure 3.105) and polyd(A).polyd(T) Figure 3.107). On the other hand, Aza5 was found to be interacting with polyd(A) (Figure 3.103) and polyd(A).polyd(T).polyd(T) (Figure 3.109), once again as evident from the presence of the red shift and hypochromic effect between 350 nm to 450 nm. The changes in that region of the UV-vis spectra are more observable in Figure 3.110 for Aza4 and in Figure 3.111 for Aza5, respectively.

3.8.2 Investigation of Interactions of Aza4 and Aza5 with the polyd(A), polyd(T), polyd(A)·polyd(T) And polyd(A)·polyd(T)·polyd(T) by CD Spectroscopy

The CD spectra of polyd(A).polyd(T). polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) at 5°C were collected in the absence and presence of Aza4 and Aza5 along with the spectra of Aza4 and Aza5. Aza4 and Aza5 are known as achiral molecules and give no rise to any peak formation in CD (Figure 3.112).

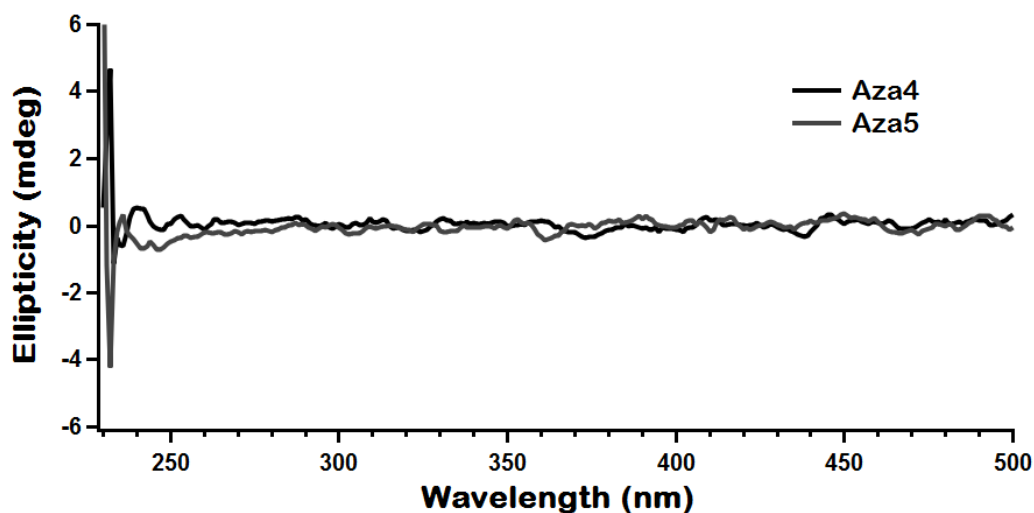


Figure 3.112. CD spectra of Aza4 and Aza5.

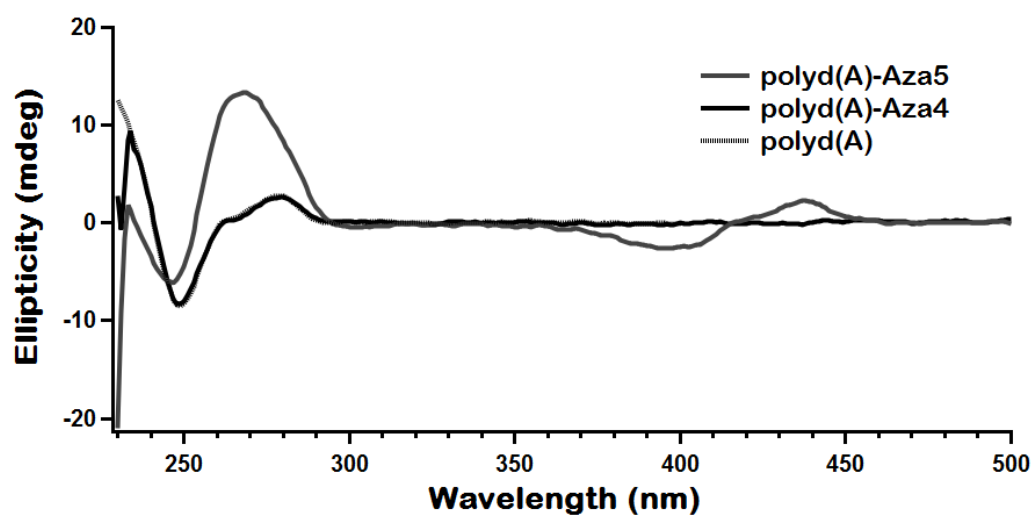


Figure 3.113. CD spectra of polyd(A) in the absence and presence of Aza4 or Aza5.

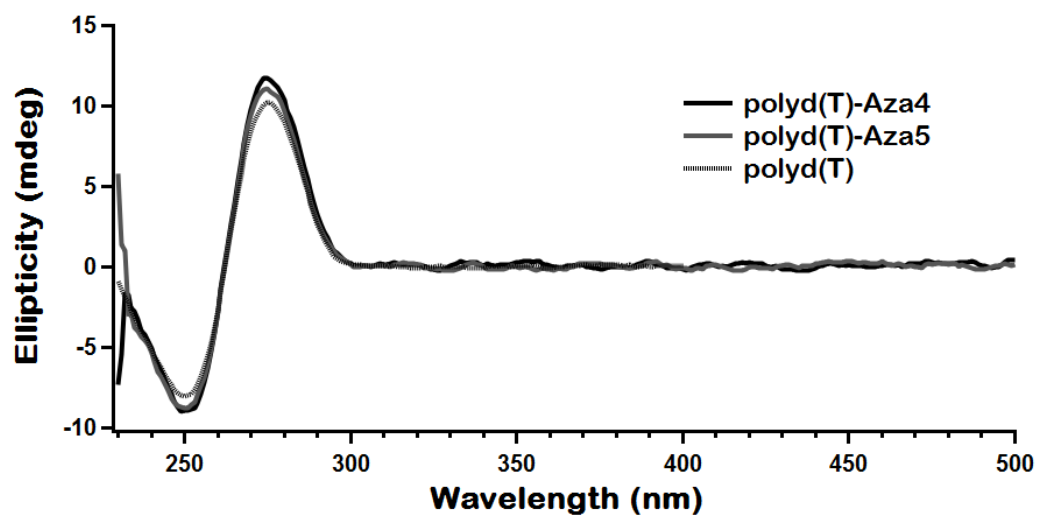


Figure 3.114. CD spectra of polyd(T) in the absence and presence of Aza4 or Aza5.

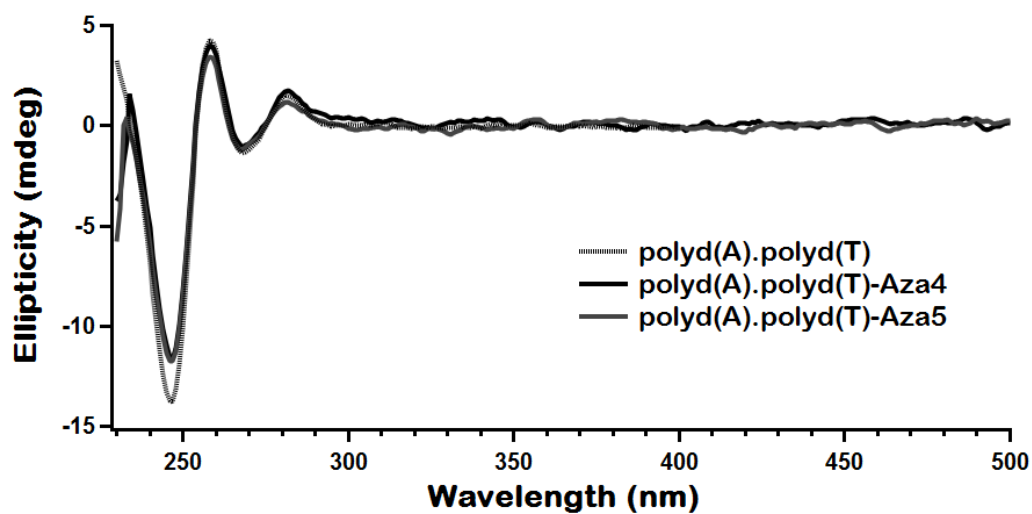


Figure 3.115. CD spectra of polyd(A)·polyd(T) in the absence and presence of Aza4 and Aza5.

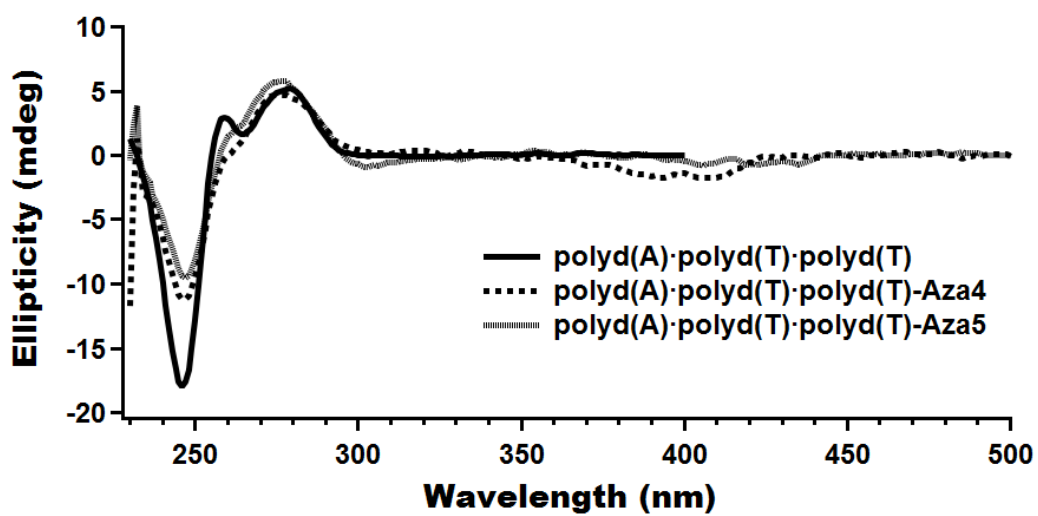


Figure 3.116. CD spectra of polyd(A)·polyd(T)·polyd(T) in the absence and presence of Aza4 and Aza5.

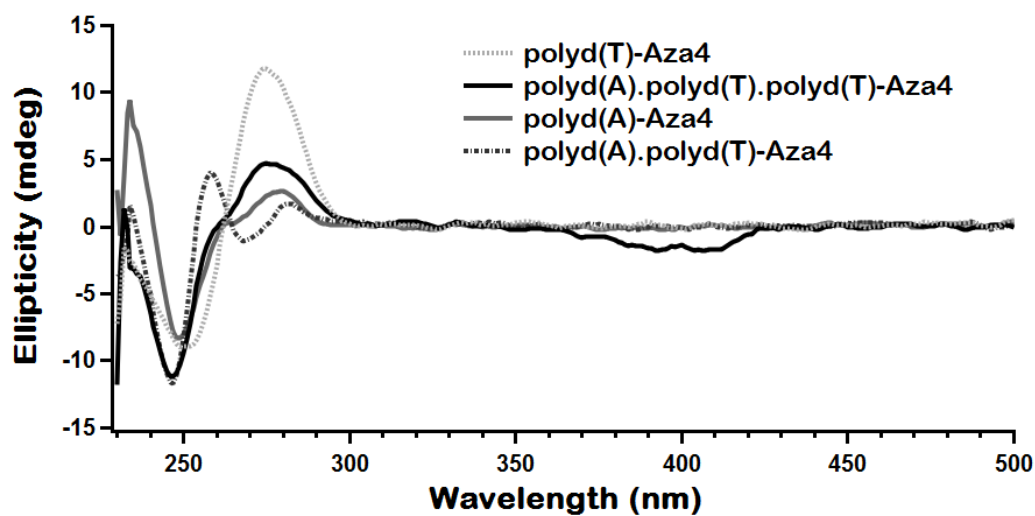


Figure 3.117. CD spectra of all polynucleotides in the presence of Aza4.

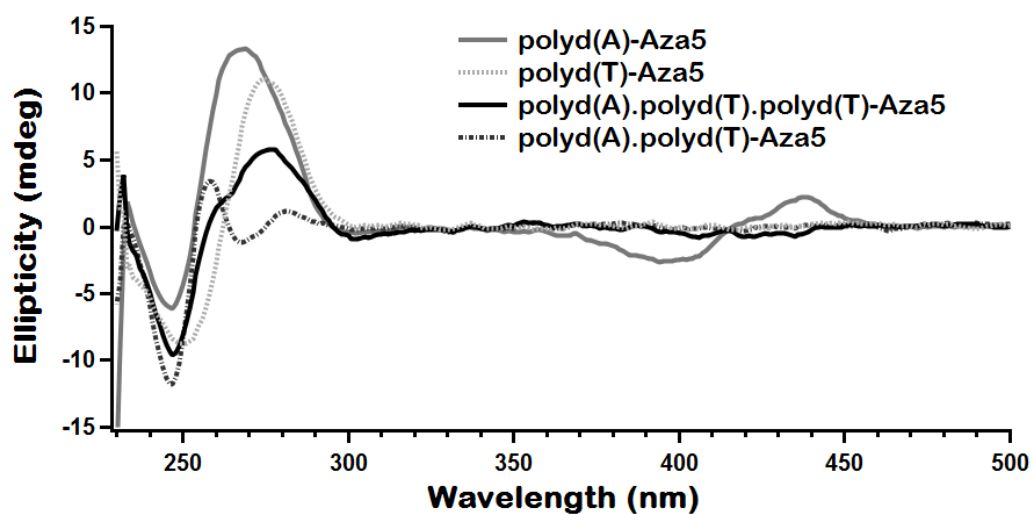


Figure 3.118. CD spectra of all polynucleotides in the presence of Aza5.

The CD analysis revealed that the Aza4 and Aza5 had no interactions with the polyd(T) (Figure 3.114) and polyd(A)·polyd(T) (Figure 3.115) since their addition to the samples did not alter their CD spectra. There were only small

alterations in the intensity of the peaks at 247 nm and 260 nm however, which might be due to some nonspecific interactions.

As for the spectrum of polyd(A) (Figure 3.113), there was no change in the presence of Aza4 while an induced peak formation was observed in the presence of Aza5. Yet, there were notable changes in the wavelength and the intensity of the peaks between 200- to 300 nm indicating the change in the structure of polyd(A).

In the case of the CD spectrum of polyd(A)·polyd(T)·polyd(T) (Figure 3.116), a remarkable decrease was monitored in the intensity of the band at 247 nm upon Aza4 or Aza5 binding in addition to the formation of very weak induced bands between 300 to 500 nm. These results are clearly shown in Figure 3.117 for Aza4 and Figure 3.118 for Aza5. Interestingly, the intensity of the induced CD band for polyd(A)·polyd(T)·polyd(T)-Aza4 complex is higher than that of Aza5. Even though Aza4 and Aza5 are analogous, they exhibit different behavior with regard to the environment they have inside the DNA. The CD results perfectly consistent with the UV-vis results.

3.9 Determination of the Binding Affinities of Azacyanines to polyd(A)·polyd(T)·polyd(T) by Florescence Spectroscopy

The selectivity and affinity (qualitatively) of azacyanines to different nucleic acid structures including polyd(A)·polyd(T)·polyd(T) has been shown previously by competition dialysis experiments (Güloğlu, 2018). In order to get further insights to their affinities to the polyd(A)·polyd(T)·polyd(T), the binding constants of polyd(A)·polyd(T)·polyd(T) to Azamethyl, Azaethyl, Azapropyl, Aza4 or Aza5 were determined using fluorescence titration experiments at room temperature. Since, the binding of Azaisopropyl and Azaisobutyl to polyd(A)·polyd(T)·polyd(T) was very weak, the titrations were not performed with Azaisopropyl and Azaisobutyl. In other words, the complex of polyd(A)·polyd(T)·polyd(T)-Azaisopropyl and polyd(A)·polyd(T)·polyd(T)-Azaisobutyl have the T_m values around 26°C and 33

°C, respectively suggesting that about half of the Azaisopropyl have the ability to bind to polyd(A)·polyd(T)·polyd(T)-Azaisopropyl at room temperature, while the other half will be free in the solution due to chemical equilibrium.

In all the experiments, a solution of small molecules (Azamethyl or its derivative at a concentration of 1.0 μM) was titrated with a concentrated nucleic acid solution (400.0 μM base triplet) containing the same amount of the examined small molecule (1.0 μM). The titration was continued until the emission spectrum became constant and another spectrum was collected after each titration. Then, the integration of the area under the peak were drawn with respect to the nucleic acid concentration using IGOR software and the binding constant (association constant) was calculated with Scatchard analysis (Persil Çetinkol et al., 2008; Chaires et al., 1982).

The fluorescence titration spectra and the binding constant profiles obtained through integration of these spectra are presented in Figure 3.119- Figure 3.128.

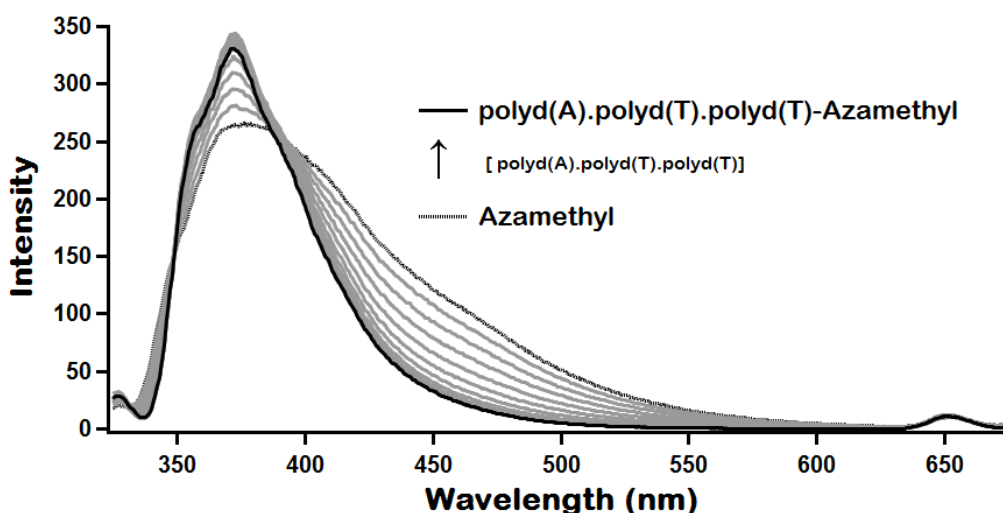


Figure 3.119 Florescence emission spectra of polyd(A)·polyd(T)·polyd(T) – Azamethyl obtained from titration experiments.

The spectra for the first and the last point of titration are demonstrated in dashed black and black lines, respectively.

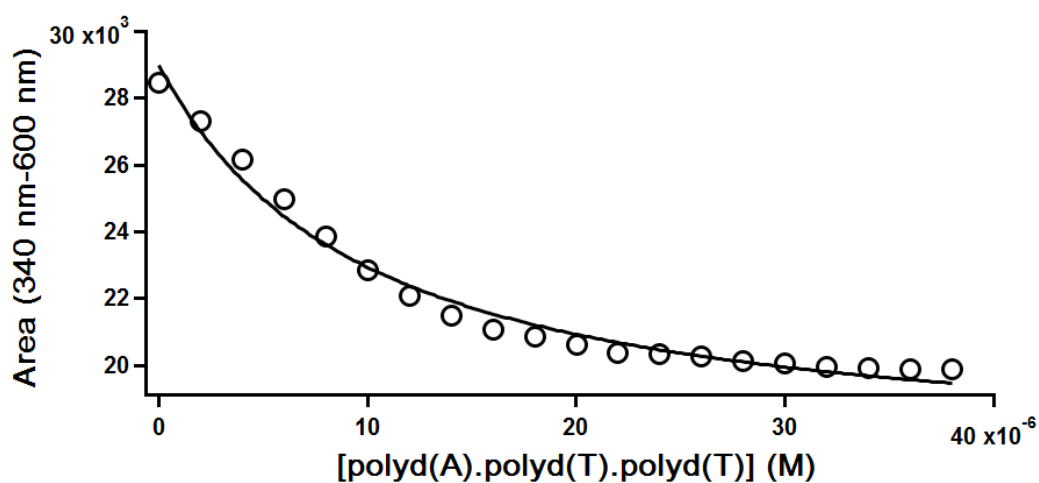


Figure 3.120. Integrated Intensity vs Concentration profile obtained from the titration results in Figure 2.117.

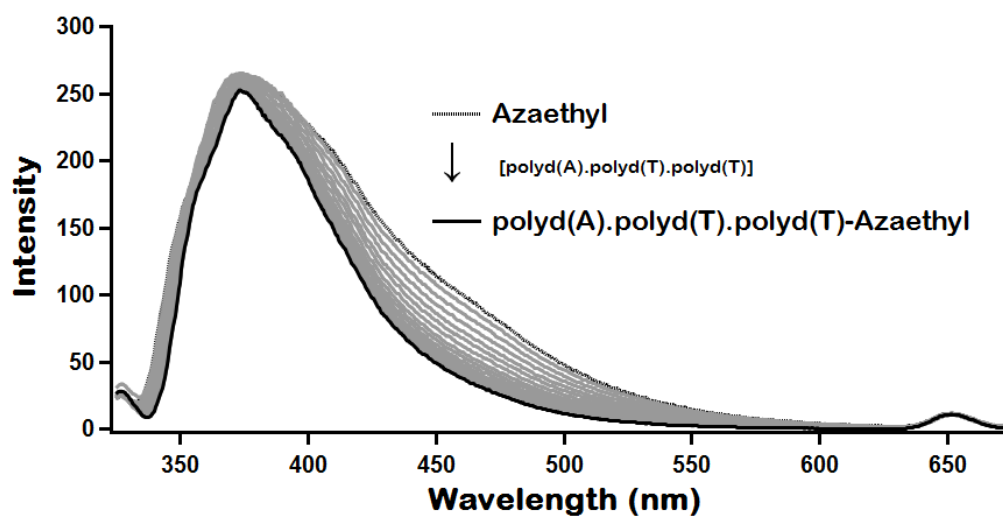


Figure 3.121. Fluorescence emission spectra of polyd(A)·polyd(T)·polyd(T) – Azaethyl

The spectra for the first and the last point of titration are demonstrated in dashed black and black lines, respectively.

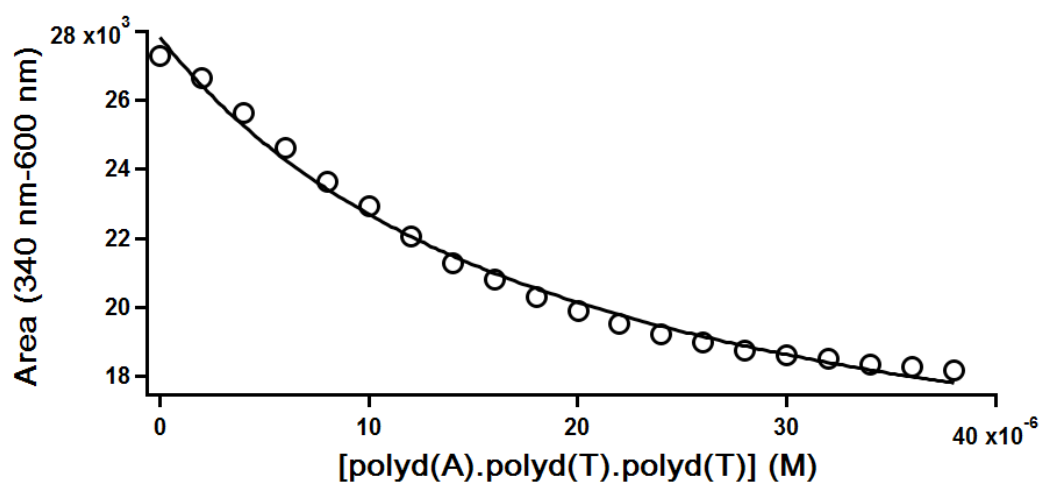


Figure 3.122. Integrated Intensity vs Concentration profile obtained from the titration results in Figure 2.119.

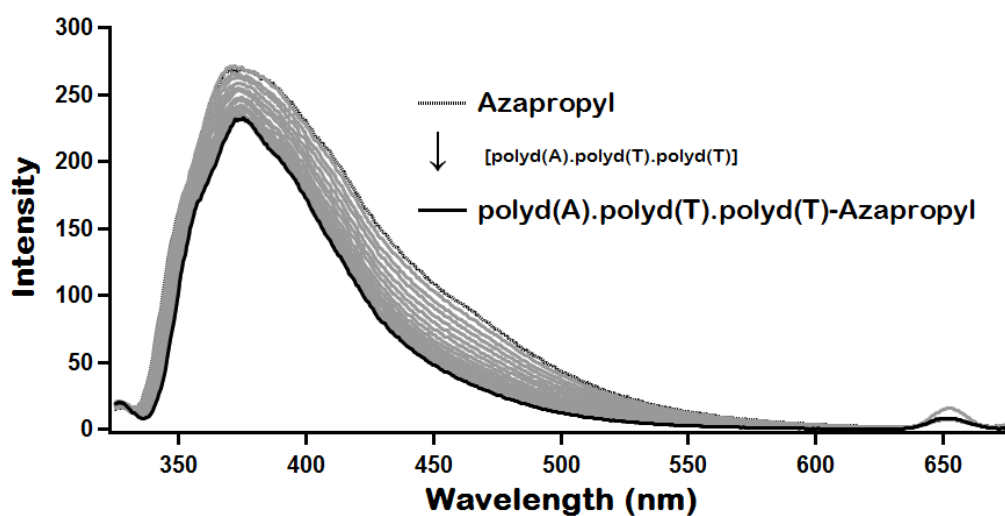


Figure 3.123. Fluorescence emission spectra of polyd(A).polyd(T).polyd(T) – Azapropyl

The spectra for the first and the last point of titration are demonstrated in dashed black and black lines, respectively.

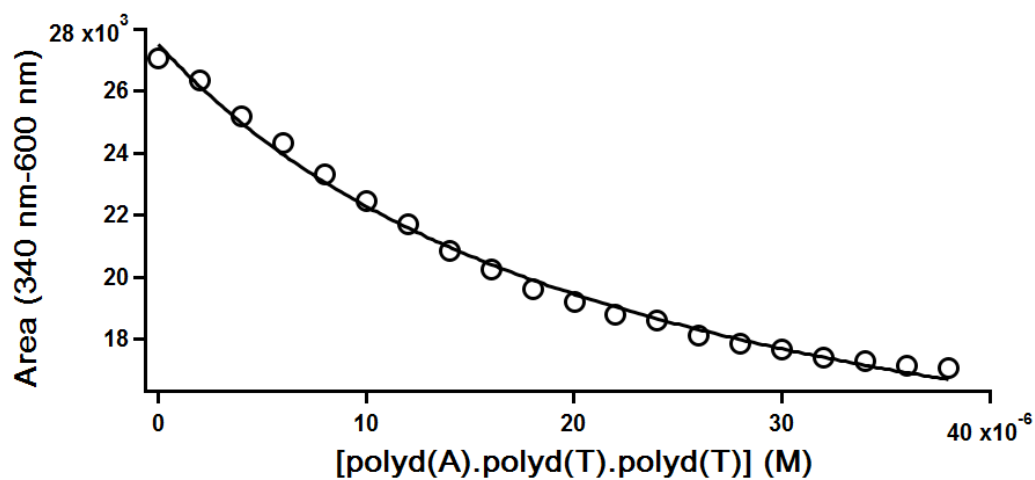


Figure 3.124. Integrated Intensity vs Concentration profile obtained from the titration results in Figure 2.121

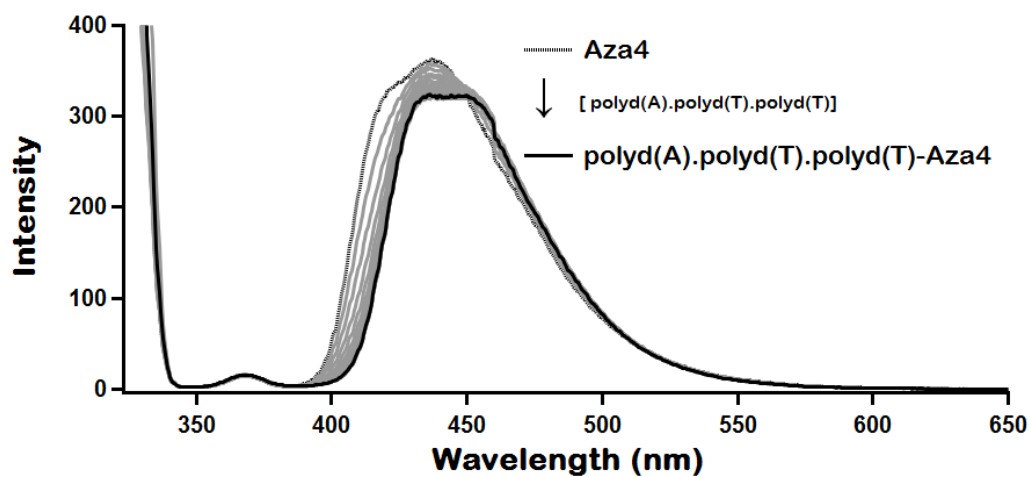


Figure 3.125. Fluorescence emission spectra of polyd(A).polyd(T).polyd(T) – Aza4. The spectra for the first and the last point of titration are demonstrated in dashed black and black lines, respectively.

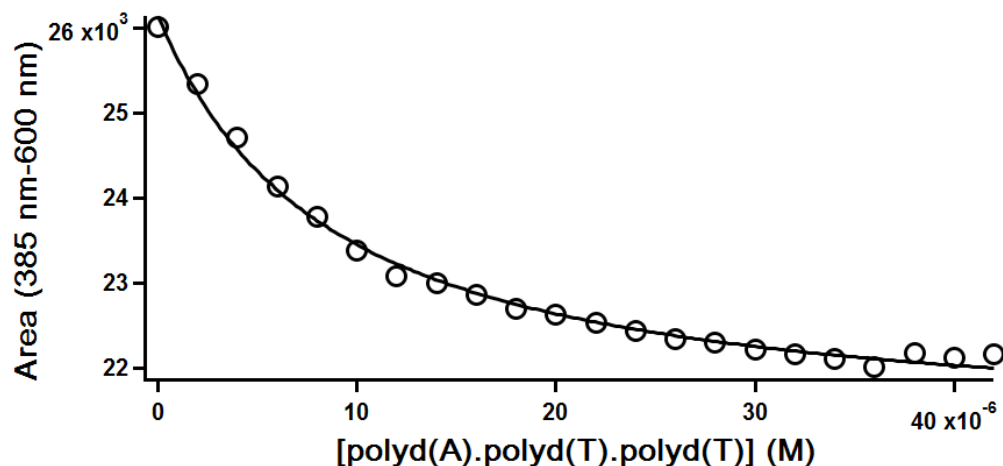


Figure 3.126. Integrated Intensity vs Concentration profile obtained from the titration results in Figure 2.123.

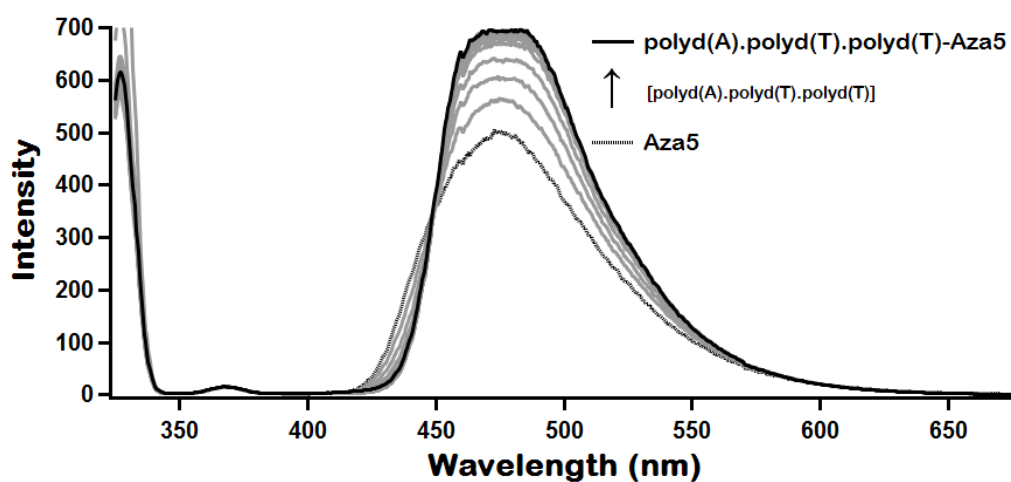


Figure 3.127. Fluorescence emission spectra of polyd(A)·polyd(T)·polyd(T) – Aza5. The spectra for the first and the last point of titration are demonstrated in dashed black and black lines, respectively.

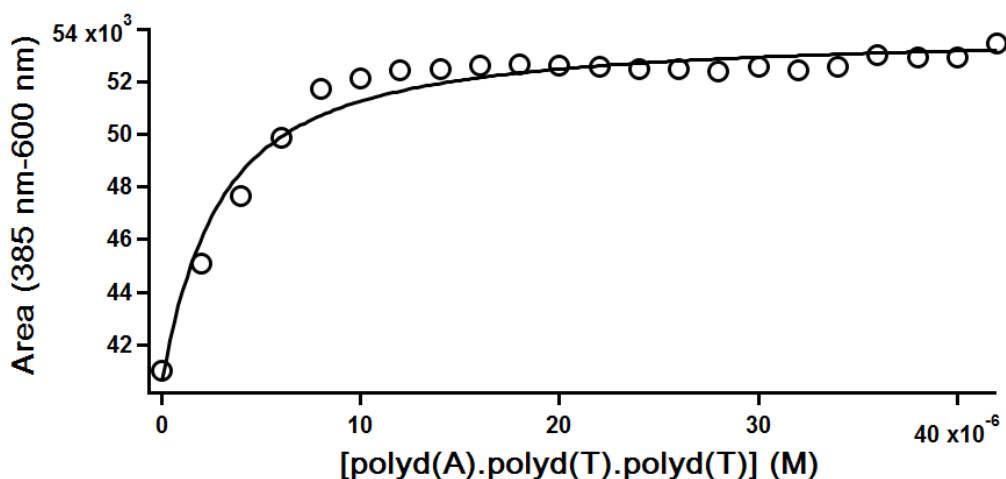


Figure 3.128. Integrated Intensity vs Concentration profile obtained from the titration results in Figure 2.125

Azamethyl (Figure 3.119) represents an increase followed by a decrease in the intensity, whereas Azaethyl (Figure 3.121) and Azapropyl (Figure 3.123) represent a decrease in the intensity. As can be seen from Figure 3.125, Aza4 showed a red shift accompanied by a decrease in intensity, whereas Aza5 (Figure 3.127) revealed an increase in intensity similar to Azamethyl. Their graphics are given in Figure 3.120, Figure 3.122, Figure 3.124, Figure 3.126 and Figure 3.128, respectively.

The binding constants obtained were represented collectively in Table 3.8.

Table 3.8 Association constants of polyd(A)·polyd(T)·polyd(T)-Azacyanine Complexes

Azacyanine	Association Constant (M^{-1})
Azamethyl	$1.10 \times 10^5 \pm (9.29 \times 10^3)$
Azaethyl	$5.83 \times 10^4 \pm (6.44 \times 10^3)$
Azapropyl	$4.47 \times 10^4 \pm (4.73 \times 10^3)$
Aza4	$1.26 \times 10^5 \pm (9.29 \times 10^3)$
Aza5	$4.40 \times 10^5 \pm (7.08 \times 10^4)$

Among the examined small molecules, Aza5 was found to have the highest affinity to polyd(A)·polyd(T)·polyd(T) with a K_a of $4.40 \times 10^5 \pm (7.08 \times 10^4)$.

Among the benzimidazole based Azacyanines, Azamethyl had the highest affinity ($1.10 \times 10^5 M^{-1}$) followed by Azaethyl ($5.83 \times 10^4 M^{-1}$) and Azapropyl (4.47×10^4).

The obtained association constant values were in a good agreement with the UV-vis thermal denaturation experiments, in which polyd(A)·polyd(T)·polyd(T)-Azamethyl was found to denature at 82°C, polyd(A)·polyd(T)·polyd(T)-Azaethyl at 61°C and polyd(A)·polyd(T)·polyd(T)-Azapropyl at 54°C.

3.10 Determination of the Binding Mode via Viscosity Measurements

Viscosity experiments were carried out to specify the binding mode where each Azacyanine was titrated gradually onto polyd(A)·polyd(T)·polyd(T) solution, and the viscosity was measured after each titration (Figure 2.127). In all of the samples, there is an increase in the viscosity of polyd(A)·polyd(T)·polyd(T) when the Azacyanine molecule is added. It should be taken into consideration that the increase in the amount of viscosity of Azacyanines differs fairly from each other. The increase in the viscosity implies the intercalative binding mode (Bloomfield, Crothers and Tinoco Jr., 1999), while it is not negligible that different binding modes can be seen due to the observed differences.

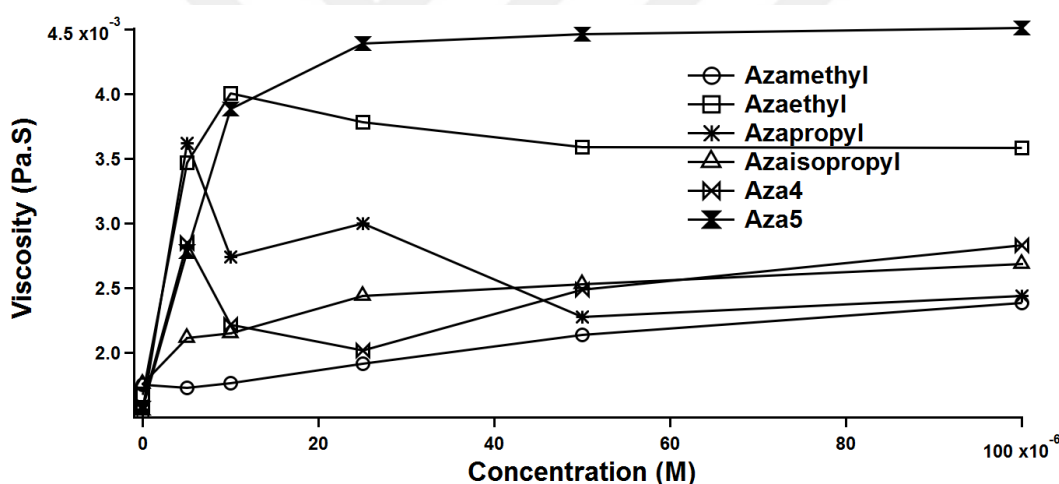


Figure 3.129. Viscosity values of polyd(A)·polyd(T)·polyd(T) with respect to Azacyanine concentration.

There is an increase in the viscosity of polyd(A)·polyd(T)·polyd(T) upon binding of all Azacyanines mostly in Aza5.

CHAPTER 4

FINDINGS

4.1 Main Purpose

In this thesis, we have investigated the interactions of five new Azacyanine molecules (Azaethyl, Azapropyl, Azaisopropyl, Azabutyl, and Azaisobutyl) and the Aza4 and Aza5 with polyd(A), polyd(T), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) structures. We have also investigated the interactions of the previously-studied Azamethyl with the same sequences under the same conditions for the first time.

The significance of triplex structures that was unheeded until 1990s increased conspicuously by the anti-gene strategies proposed by Hélène (Hélène, 1991). Hélène had declared that a transcription of a gene could be inhibited or a chromosome could be severed on a certain point by the transformation of triplex forming oligonucleotides (TFO) to the cell nuclei. Also, Hélène proposed that such anti-gene strategies could be used in triggering apoptosis (normal cell death) or in smothering certain genes in cancer-cells. Hélène demonstrated the possible importance of triplex forming oligonucleotides in therapy (Hélène, 1991; Jain et al., 2008). However, there is a need of existence of such molecules that bind to triple helices. The main purpose of this study has been to discover new molecules with specific binding affinity to the triple helices. One of the main purposes of this study has been to shed light on the hypothesis proposed by Prof. Dr. Lobachev and his research group. They suggest that the Azacyanines cause chromosome loss and halt the cell division in (GAA)_n yeast cells. They hypothesized that this might be due to the binding of Azacyanines to the triple helix structures (Kim, 2009). So, in order to shed light onto the studies of Kim, that suggested the possible binding of Azacyanines to triple helical DNA structures in vivo, we investigated the interactions

of Azacyanines in vitro under in vivo-like conditions. Our studies revealed that Azacyanines have particularly high affinities to the polyd(A)·polyd(T)·polyd(T) triplex structure. Furthermore, when the benzimidazole-based Azacyanine derivatives are compared, it is exerted that the affinity to the polyd(A)·polyd(T)·polyd(T) is affected negatively by the chain length and branching of the benzimidazole ring. This reveals that the structure of the nucleic acid (DNA or RNA)-oriented molecules are very important in recognition of the nucleic acids and their discrimination. This knowledge might provide new insights in the drug/molecule design e.g. in the design of DNA-helix-oriented molecules.

4.2 The Interactions of Azamethyl with Nucleic Acids

By evaluating all the obtained data through UV-vis and CD experiments, it is concluded that the Azamethyl binds to the d(A)₃₂, d(A)₃₂·d(T)₃₂·d(T)₃₂, polyd(A) and polyd(A)·polyd(T)·polyd(T). Therefore, similar to coralyne, Azamethyl has an affinity to the polyd(A)·polyd(T)·polyd(T) triplex structure more than the polyd(A)·polyd(T) duplex structure, and like coralyne, Azamethyl also denatures the polyd(A)·polyd(T) duplex forming the triplex (Polak and Hud, 2002; Jain, Polak and Hud, 2003). The binding of Azamethyl was not highly affected by the pH between pH=3-7. There is evidently no decrease in the CD intensity of polyd(A)·polyd(T)·polyd(T)-Azamethyl, while there is an obvious decrease in the intensity at pH=2. On the other hand, the increase in the salt concentration ([NaCl]) gradually decreases the affinity of the Azamethyl to polyd(A)·polyd(T)·polyd(T) triplex. This is an expected result since NaCl neutralizes the negative phosphate backbone of the DNA. As the concentration of NaCl increases, the affinity of the positively-charged Azamethyl to the negative phosphate backbone decreases. The Job plot analysis shows that one Azamethyl binds to one of the two base triplets conforming to the nearest neighbor exclusion principle observed in the intercalative binding mode. The viscosity experiments demonstrate that Azacyanines increase the viscosity of polyd(A)·polyd(T)·polyd(T) supporting the intercalative binding mode (Bloomfield, Crothers and Tinoco Jr., 1999).

Like coralyne again, Azamethyl drives polyd(A) to form a homoadenine self-structure. Furthermore, Azamethyl also binds to the parallel triplex forming DTR1, and stabilizes it in addition to the anti-parallel polyd(A)·polyd(T)·polyd(T), which alludes to the idea that Azamethyl can be used as a potential molecule for the identification of triplex forming structures. More importantly, Azamethyl can be used in the anti-gene strategies (i.e. to suppress particular genes in cancer cells which give rise to the apoptosis) as it binds to the triplex structures with a higher affinity and stabilizes them more.

Conversely, Azamethyl did not bind to the d(T)₃₂, polyd(T), d(A)₃₂·d(T)₃₂ and polyd(A)·polyd(T) structures which is important for designing the molecules with binding capability only to the triplex nucleic acid structures.

4.3 The Interactions of Azamethyl Derivatives with Nucleic Acids

Our studies revealed that Azacyanines did not really exhibit any affinity towards polyd(T) and polyd(A)·polyd(T). We did not observe any change in ellipticity or in thermal denaturation due to the lack of interactions between Azacyanines and polyd(A)·polyd(T) structure. Among the Azacyanines, only Azamethyl showed affinity towards polyd(A). Azamethyl was able to induce polyd(A) self-structure with a thermal denaturation of about 30°C. None of the other Azacyanines were able to stabilize/induce polyd(A) self-structure.

When the interactions of Azaethyl, Azapropyl, Azaisopropyl, Azabutyl and Azaisobutyl with the polyd(A), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) were considered, all of the five molecules bound only to polyd(A)·polyd(T)·polyd(T) triplex, and stabilized it in different ratios. They had an affinity towards polyd(A)·polyd(T)·polyd(T) and stabilized the triplex structure. The polyd(A)·polyd(T)·polyd(T)-Azamethyl had the highest temperature of thermal denaturation and the increase in the chain length in the benzimidazole ring led to a decrease in the T_m. The lowest T_m for the polyd(A)·polyd(T)·polyd(T)-Azaisopropyl revealed that branching affected the binding negatively. Among them, the affinity of

the Azamethyl, Azaethyl, Azapropyl and Azabutyl were also investigated by fluorescence titration experiments. The results were in agreement with the values obtained from thermal denaturation, that the chain length affected the binding and T_m negatively. Since the complex of polyd(A)·polyd(T)·polyd(T)-Azaisopropyl and polyd(A)·polyd(T)·polyd(T)-Azaisobutyl had a thermal denaturation point near room temperature, the association constant of this complex was not consistent with the titration experiments.

4.4 The Interactions of Aza4 and Aza5 with Nucleic Acids

The interactions of the Aza4 and Aza5 molecules with the polyd(A), polyd(T), polyd(A)·polyd(T) and polyd(A)·polyd(T)·polyd(T) were investigated. The very high association constant of them to the polyd(A)·polyd(T)·polyd(T) demonstrated that Aza4 and Aza5 also had an affinity to the triplex structures. According to the titration experiments, Aza5 had the highest affinity which agrees with the result of a previous research (Kim, 2009). Furthermore, the CD experiments displayed that Aza4 and Aza5 did not bind to the polyd(T) and polyd(A)·polyd(T), while only Aza5 bound to the polyd(A).

4.5 Final Remarks and Recommendations

To sum up, the current study mainly focuses on four areas as to study the binding of the Azacyanines to the singlet, duplex and triplex nucleic acid structures using CD, UV-vis and Fluorescence spectroscopy techniques, support the *in vivo* studies, investigate the relationships between the binding of the examined molecules to triplex structures and their effects in yeast cells and finally, find out the relationship between the structure of a given molecule and its binding affinity as potential anti-tumor agents.

Azacyanines binding to the triple helix structures and the molecular structure have a direct effect on their binding affinity. As they do not bind to the duplex structures, they can be used in the studies intended for anti-gene therapies and the

triple helix-oriented drug design. One of the basic concepts in the sensitive drug design is to decrease the side-effects. Therefore, Azacyanines are candidate molecules and have potentials to be used as discriminative agents for triple nucleic acids in vivo. Since their affinities to bind to polyd(A)·polyd(T)·polyd(T) triplex structures (evaluated by fluorescence spectroscopy) are less than the most common and routine anti-cancer molecules, it can be pointed out that their genotoxicities will be less as they do not bind to duplex structures. According to a previous study on yeast cells (Kim, 2009) their genotoxicities were shown to be negligible. Therefore, investigations on their effect on the cancer cells are envisioned as our next perspective.

In the light of all these results, it becomes apparent that aromatic ring structure should always be taken into account on the new drug design as very little changes in the molecule structure could leave a great importance on the identification of the nucleic acid structures. In similar structures with the Azacyanine (with regard to plural aromatic rings), the intercalation/binding have been negatively affected due to the presence of isopropyl and isobutyl groups. Conversely, these groups can be preferred for blocking the DNA grooves to prevent the binding.

The binding of the Azamethyl to triplex structures, especially to the structures containing GAA·TTC repeats exhibited the same effects which have been observed in vivo experiments (Kim, 2009). It was concluded that the triplex structures consisting of $(\text{GAA})_n \cdot (\text{TTC})_n$ repeats have a role in the genetically neurodegenerative diseases such as Friedreich's Ataxia which is an orphan disease and one of the strategies to develop a drug to indicate the Friedreich's Ataxia ability to increase the frataxin synthesis through preventing the formation of GAA·TTC triplex sequences in vivo. At this point, Azacyanines have an opposite effect and cannot be utilized as a drug candidate within this scope. On the other hand, Azacyanines especially Azamethyl and Aza5 which have high affinities to the triplex structures and desired fluorescence properties that can be utilized for the determination of the triplex structures in vivo.



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APPENDICES

A. CD-Melting Profiles of d(A)₃₂, d(A)₃₂·d(T)₃₂ and d(A)₃₂·d(T)₃₂·d(T)₃₂ with Azamethyl

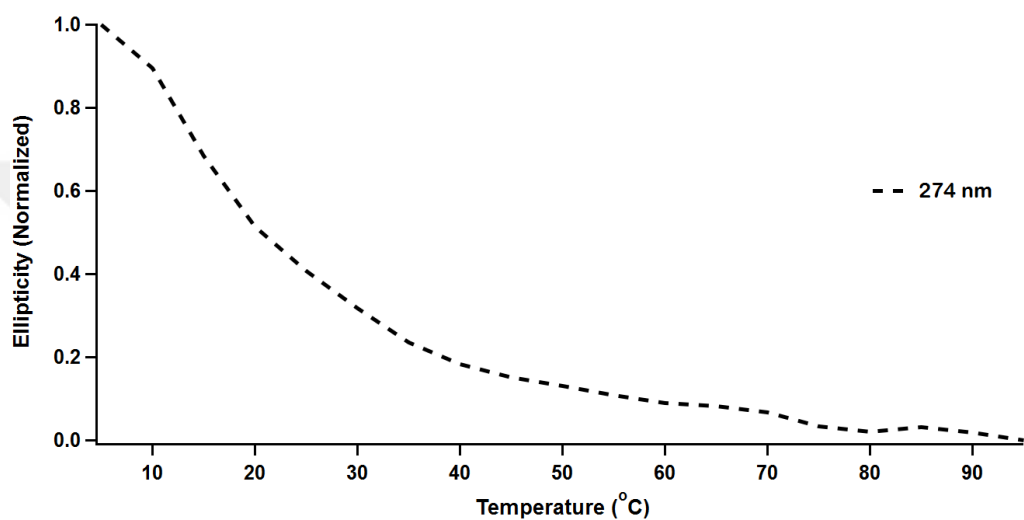


Figure A1. Melting profile of d(A)₃₂-Azamethyl between 5°C and 95°C at 274 nm obtained via CD

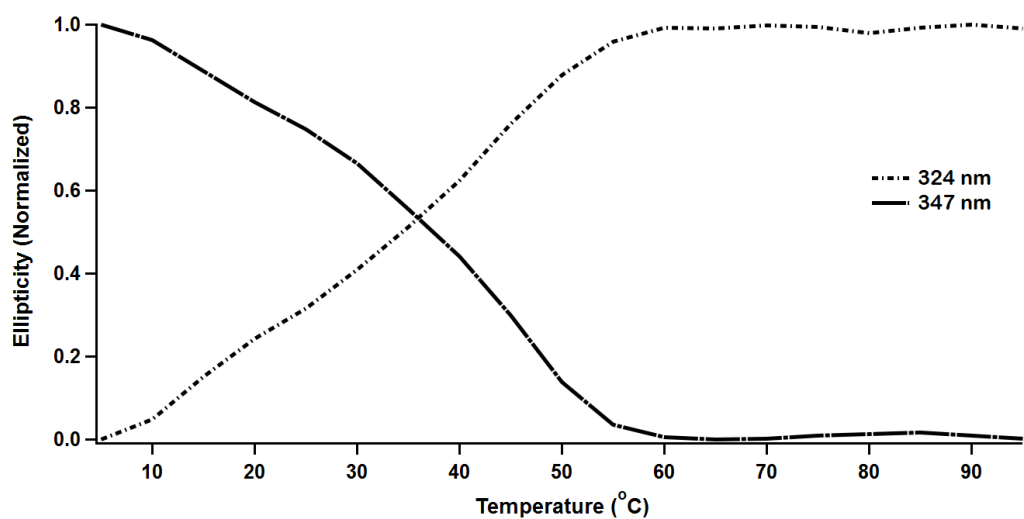


Figure A2. Melting profiles of d(A)₃₂-Azamethyl between 5°C and 95°C at 324 and 347 nm obtained via CD

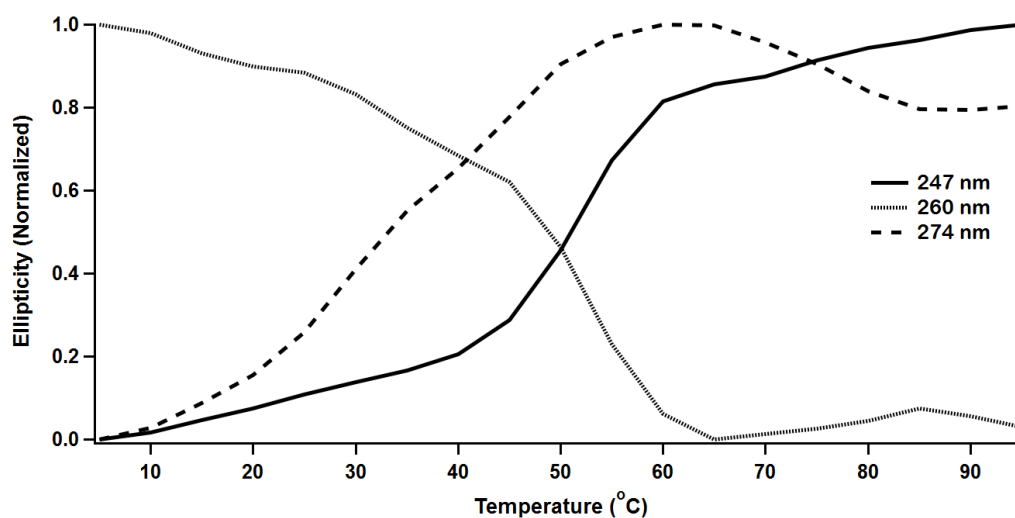


Figure A3. Melting profile of $d(A)_{32} \cdot d(T)_{32}$ between 5°C and 95°C at 247, 260 and 274 nm obtained via CD

The ellipticity at 260 nm decreases with the rising temperature while increases at 247 and 274 nm.

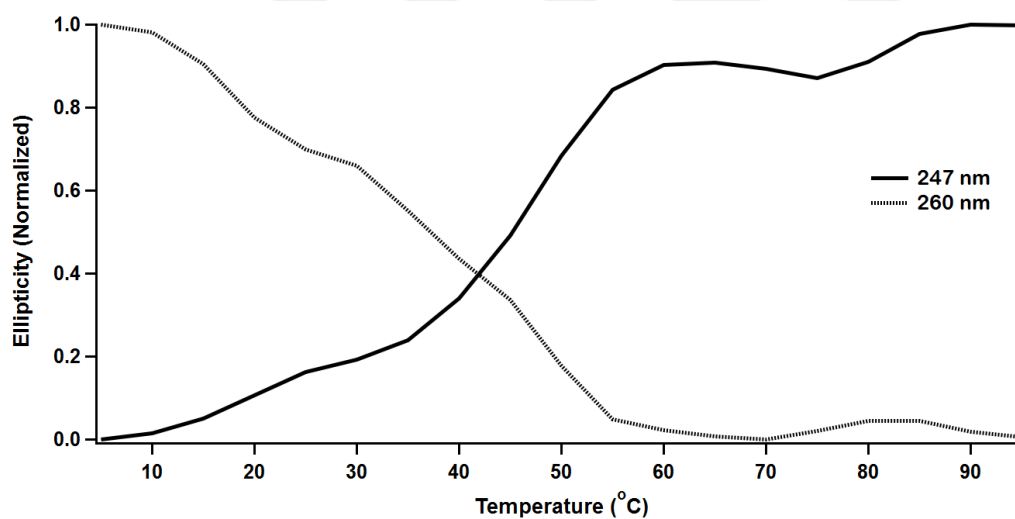


Figure A4. Melting profiles of $d(A)_{32} \cdot d(T)_{32}$ -Azamethyl between 5°C and 95°C at 260 and 274 nm obtained via CD

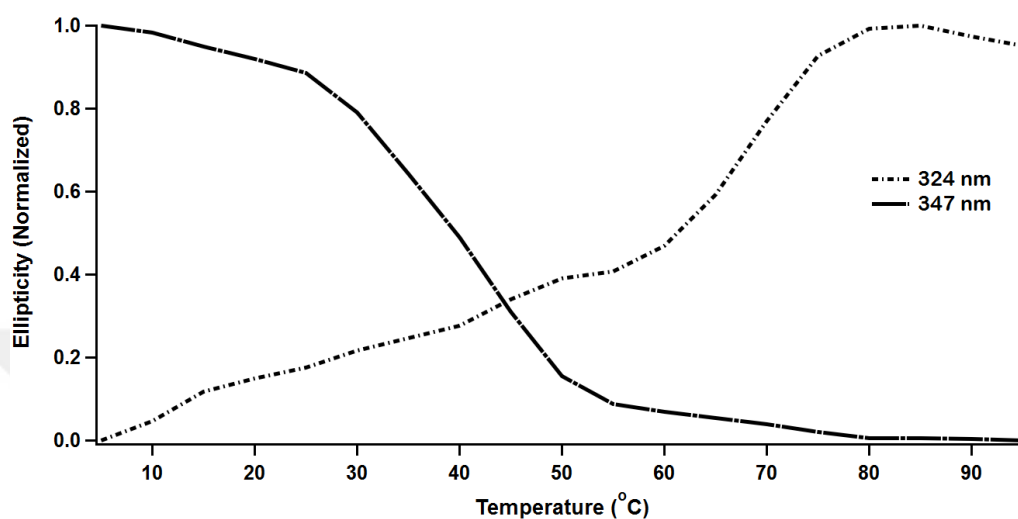


Figure A5. Melting profiles of $d(A)_{32} \cdot d(T)_{32}$ -Azamethyl between 5°C and 95°C at 324 and 347 nm obtained via CD

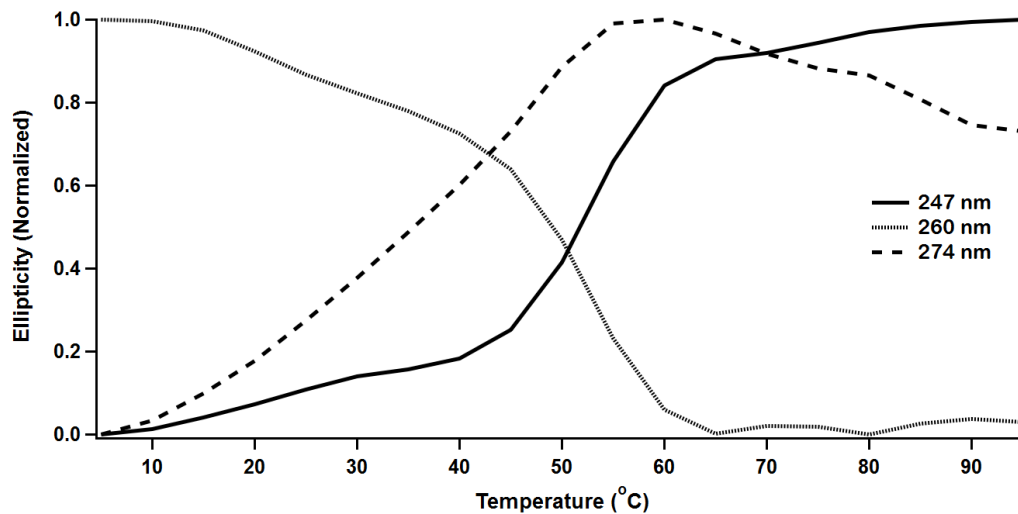


Figure A6. Melting profile of $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ between 5°C and 95°C at 247, 260 and 274 nm obtained via CD

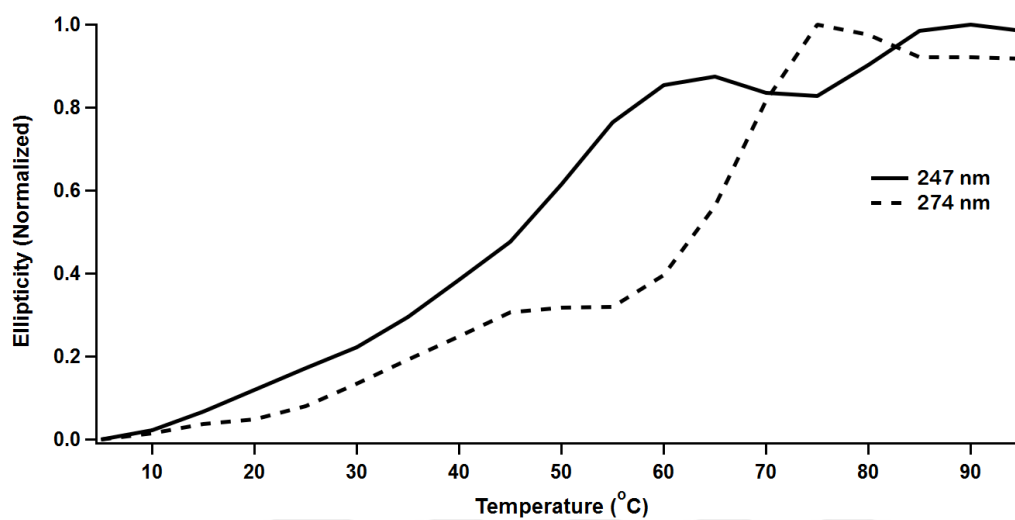


Figure A7. Melting profile of $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ -Azamethyl between 5°C and 95°C at 247 and 274 nm obtained via CD.

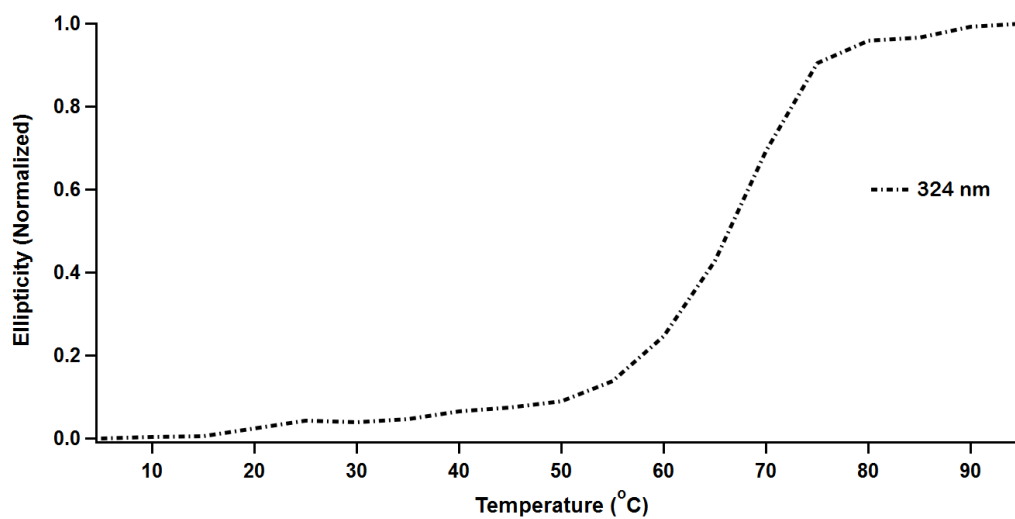


Figure A8. Melting profile of $d(A)_{32} \cdot d(T)_{32} \cdot d(T)_{32}$ -Azamethyl between 5°C and 95°C at 324 nm obtained via CD

B. CD Spectra of SS1, SS2, SS3, DD1 and DTR1 with Azamethyl

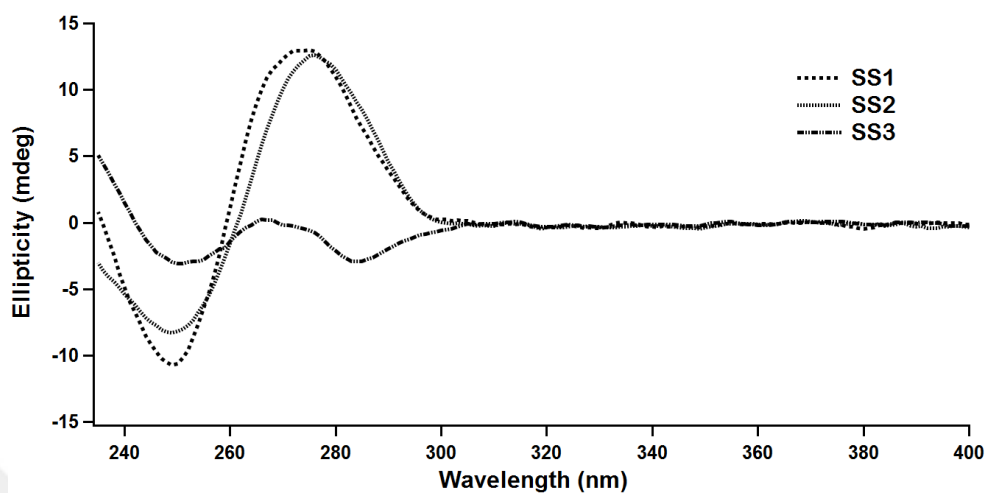


Figure B1. CD spectra of SS1, SS2 and SS3 at 5°C.

There exists a negative and a positive peak at 250 and 275 nm, respectively for SS1 and SS2 while for SS3 two negative peaks can be seen at 250 and 285 nm.

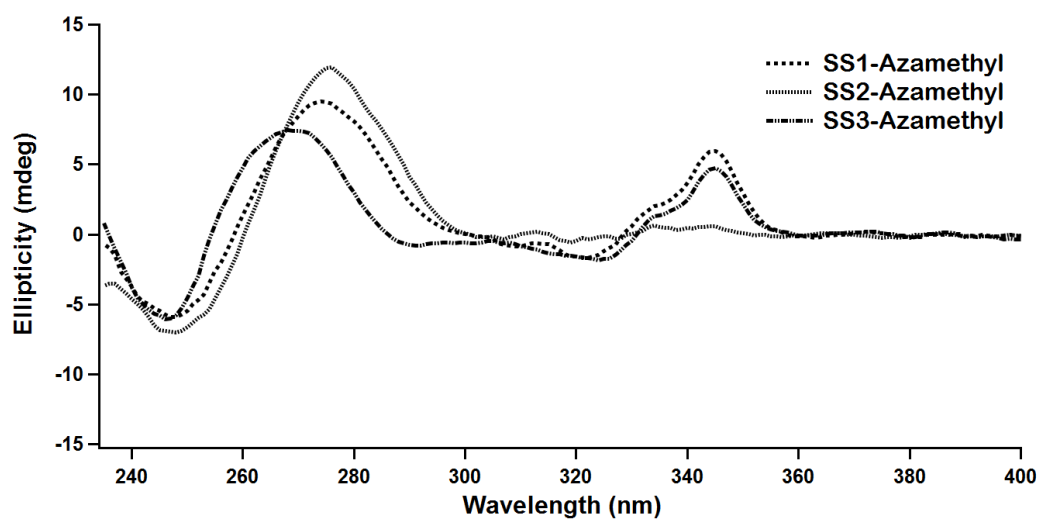


Figure B2. CD spectra of SS1-Azamethyl, SS2-Azamethyl and SS3-Azamethyl at 5°C.

After Azacyanine binding, there exist a negative peak at 250 nm and a positive peak between 260 to 280 nm for SS1, SS2 and SS3 while an induced negative and positive peak are visible at 325 and 345 nm, respectively for just SS1 and SS3.

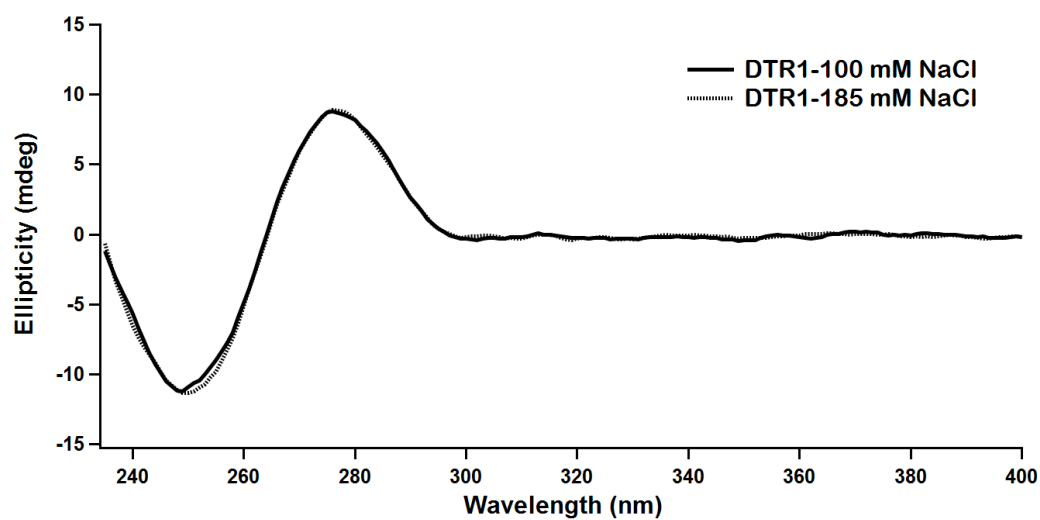


Figure B3. CD spectra of DTR1 at 5°C in 100 mM and 185 mM NaCl. There exists almost no change between the spectra of DTR1 in 100 mM NaCl and in 185 mM NaCl individually.

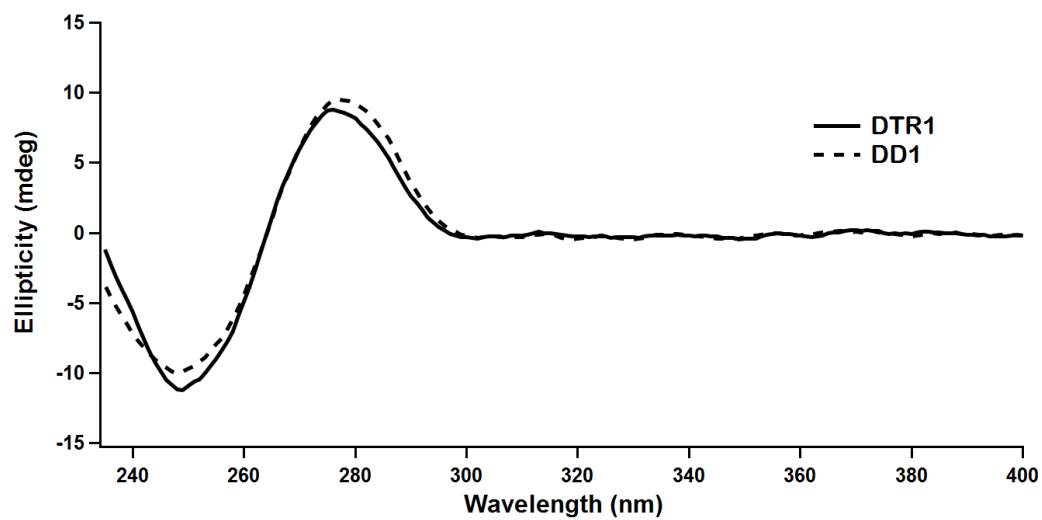


Figure B4. CD spectra of DTR1 and DD1 together at 5°C.