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Analysis of the Light Responsive Azobenzene Peptide Nucleic Acid Duplexes

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Analysis of the Light Responsive Azobenzene Peptide Nucleic Acid Duplexes

An Honors thesis submitted in partial fulfillment of the requirements for Honors in *the Department of Chemistry and Biochemistry*.

By
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Under the mentorship of *Dr. Nathaniel Shank*

ABSTRACT

Peptide nucleic acids (PNAs) are oligonucleotide analogues in which the sugar-phosphate backbone has been replaced by a pseudopeptide skeleton. Since PNAs use the natural nucleobases (Adenine, Thymine, Cytosine, Uracil, and Guanine) found in either DNA and/or RNA, they are able to hybridize according to Watson-Crick base-pairing to form duplexes. PNA is a promising therapeutic agent because they can function as antigene or antisense chemical agents. To further enhance their utility, we aim to incorporate a photoswitchable moiety using azobenzene. Here, we report the results of the synthesis and purification of a photoswitchable 11 mer PNA along with initial characterization efforts.

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Introduction

Peptide nucleic acid (PNA) are oligonucleotide analogues in which the sugar-phosphate backbone has been replaced by a pseudopeptide skeleton (**Figure 1**). They have become one of the most essential artificial oligonucleotides involved in many aspects of chemical biology, diagnostics, and therapeutics due to their durability of hybridization complexes, metabolic stability, and the ease of synthesis.[1] Antisense PNA oligonucleotides disrupt biological functions in cells and are currently being explored as potential therapeutics.[2] Collectively, PNA have enormous potential across an array of applications that include functioning in gene editing, nucleic acid sensing, genetic probes, gene therapy, and as antisense agents.[1,3] PNA are ideal candidates for this project due to its stability in biological systems and programmability as a molecular handle.

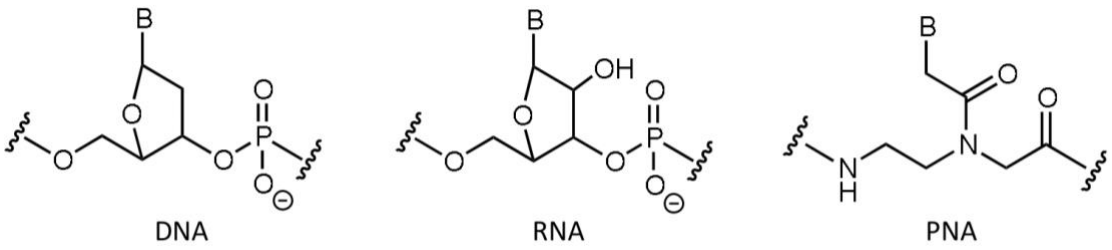


Figure 1. Comparison in Structures of DNA, RNA, and PNA. Left and Middle show the structures of DNA and RNA. Right shows a representative PNA containing a glycine (pseudopeptide) backbone that replaced the typical nucleic acid phosphodiester.

By using the purine and pyrimidine nucleotide bases such as Adenine (A), Thymine (T), Cytosine (C), Uracil (U), and Guanine (G) like DNA and RNA, PNA can participate in Watson-Crick base pairing to form the duplexes (hybridization) (**Figure 2**). In Watson-Crick base pairing, A forms a base pair with T using two hydrogen bonds, and G forms a base pair with C using three hydrogen bonds. Thus, duplexes containing higher GC content are more stable, said to have a higher melting temperature, than duplexes

composed of higher AT pair. High stability of the PNA duplexes allow the short sequences to be used with the high selectivity thus reducing the possibility of off-targeting sequences.[4]

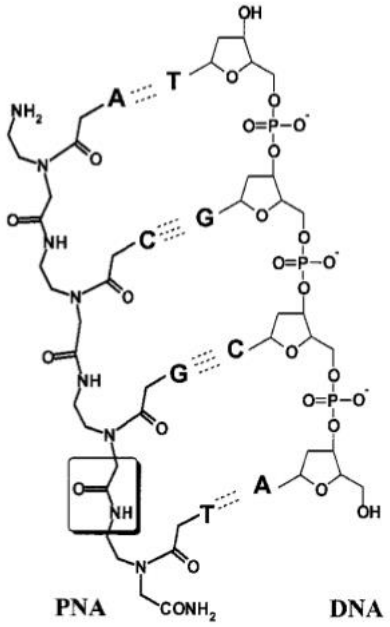
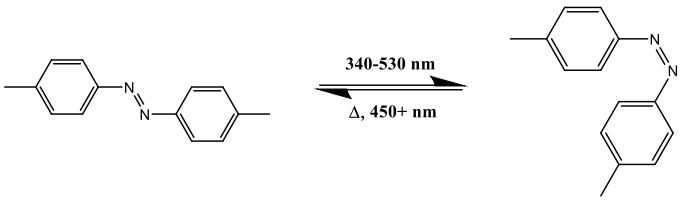


Figure 2. Structures of a PNA-DNA Duplex. Amide bonds (box) are characteristic of both peptides and PNAs. The nucleobase complementarily by the adenine (A) – thymine (T) and guanine (G) – cytosine (C) base pairs is indicated by dashed lines designating the total number of hydrogen bonds each contributes to stabilize the duplex.[4]

The photosensitivity regulation of chemical or biological systems targeted by light-induced geometric rearrangement is a classic way to achieve non-invasive control of a process. One particularly useful photosensitive agent is azobenzene, which can undergo cis/trans isomerization when irradiated with light (**Figure 3**). According to the previous studies with peptides, liposomes, nucleic acids, and enzymes, azobenzene was hypothesized to produce comparable results if incorporated into the backbone of a PNA.[5,6]



***trans*-azobezene** ***cis*-azobenzene**

Figure 3. The Conformational Changes of Azobenzene in Response to Light. Trans-azobenzene undergoes a conformational change from the trans- to cis- isomers when irradiated with light in 340 nm to 530 nm spectrum. Cis-azobenzene is reverted to trans-azobenzene when heated or irradiated with light >450 nm.

This project will rely on two major instruments: higher performance liquid chromatography (HPLC) and UV-Vis spectroscopy (UV-Vis). HPLC is an analytical technique used to separate the components of nonvolatile, thermally unstable, and high molecular weight organic mixture. There are two phases in the HPLC: the mobile phase and the stationary phase. The mobile phase is a liquid that carries the injected sample through the separation column and to the detector. The segment of the column that interacts with the target compound is known as the stationary phase. Components are separating the column based on their physicochemical interactions with the mobile and stationary phases, and the elution order is determined by these interactions.

UV-Vis spectrophotometry is an analytical technique that measures the amount of UV or visible light that is transmitted through a sample in comparison to a reference or blank sample. The main components of a UV-Vis are a light source, a sample holder, a dispersive device to separate the different wavelengths of the light, and a suitable detector. According to the Beer-Lambert theory, PNA concentration is determined based on the linear relationship between the concentration and the absorbance of PNA oligomers at 260 nm spectrum by using the equation below:

$$A = \varepsilon \ell c$$

where A is the absorbance (unitless), ε is the molar absorptivity coefficient ($M^{-1}cm^{-1}$), ℓ is the pathlength of the light through the cuvette (cm), and c is the concentration (M).[7]

Materials and Methods

I. Fmoc-Monomer Samples

PNA oligomers can be synthesized using the well-established solid phase peptide synthesis (SPPS) (fluorenylmethyloxycarbonyl)-based (Fmoc) methodologies.[8] The latter allows PNA to interfere with gene expression at the DNA level. Four solutions, N-Methylpyrrolidone (NMP), 0.2 M N, N-Diisopropylethylamine (DIPEA), 0.3 M lutidine, and 0.2 M O-(Benzotriazol-1-yl)-N,N,N',N'- tetramethyluronium hexafluorophosphate (HBTU), were prepared prior to the procedure. The following is a representative coupling cycle for one PNA monomer. In a microcentrifuge tube, the appropriate amount of monomer was weighed out (**Table 1**). NMP, 0.2 M DIPEA, and 0.3 M lutidine (volumes in **Table 1**) were then added into the tube. The solution was mixed until the monomer fully dissolved using a vortex or sonicator. Then 0.2 M HBTU was added, and the monomer was activated for 2.5 minutes before adding the solution to the resin. The sequences were protected with semi-permanent groups orthogonal to the N-terminus to protect the exocyclic amino groups in the A, T, C, and G from interfering with the synthesis (**Figure 4**). At the end of each coupling step the resin was treated with a capping solution (20% piperidone in dimethylformamide (DMF)) to avoid continuing truncated chains.

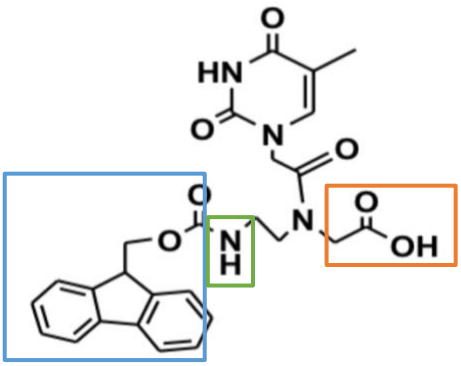


Figure 4. Representative Thymine Monomer Protected with Semi-permanent Groups Orthogonal to the N-terminus. The Fmoc is illustrated by a blue box. Orange box shows the free carboxylic acid. Protected exocyclic amine is shown in a green box.

Table 1. Substances in the SPPS based on The Use of 50 mg of Resin.

Substances/Solutions	Amount
Fmoc-Adenine (A)	0.0196 g
Fmoc-Thiamine (T)	0.0137 g
Fmoc-Cytosine (C)	0.0189 g
Fmoc-Guanine (G)	0.0200 g
NMP	135 μL
0.2 M DIPEA	135 μL
0.3 M LUTIDINE	135 μL
0.2 M HBTU	135 μL

II. Kaiser Test

A critical assay used during SPPS is a Kaiser test which is a sensitive colorimetric test for the presence of primary amines. The Kaiser test is performed twice during each coupling round. Before a new monomer is added the Kaiser test is run to ensure that there

is a free amine at the terminus of the growing oligomer. In this case the resin/beads turn blue and indicate a free amine is present and ready to react with the next monomer. After the coupling reaction, the test is performed again. Here, since the new monomer has not been deprotected, there is no primary amine, and the beads will remain yellow in color. Importantly, this indicates that the coupling was success.

The Kaiser test is created by mixing three reagents with a small sample of beads. Three reagents were made to monitor the peptide coupling and capping. Reagent one was made from 0.5 g of ninhydrin dissolved in 10 mL of ethanol. Eight grams of solid phenol was weighed out and added into 2 mL of ethanol for reagent two. Reagent three contained 0.2 mL of 0.01 M aqueous potassium cyanide (KCN) in 9.8 mL pyridine. About 10-15 beads of resin were put in a microcentrifuge tube and two drops of each reagent were added. The microcentrifuge tube was then heated to 90°C for two minutes before observing the color.

III. Cleavage of PNA from Resin

Removing the PNA from the resin, cleavage, was accomplished under acidic conditions. The resin was deprotected with weak base 20% piperidine in DMF to remove the F-moc protecting group on the terminal monomer before cleavage. The cleavage cocktail, 95% Trifluoroacetic Acid (TFA) and 5% *m*-cresol, was added to the reaction vessel and allowed to sit for one hour. The cleavage cocktail was collected and fresh TFA/*m*-cresol was again added to the vessel. The resin/solution was allowed to sit for another hour, and then the second solution was collected. About 15 mL of ether was added to the collected solutions to precipitate the PNA and then the solids were washed

three more times with ether to remove TFA/*m*-cresol. The samples were left under vacuum for an hour and then refrigerated to evaporate the remaining ether.

IV. PNA Purification and Concentration

PNA oligomers were purified by using the Shimadzu LC-2-AD UFLC equipped with a Kinetex column (H17-191743 C18 5 μM 4.6 x 250 mm) and eluted with 0.1% TFA in water (solvent A) and an increasing amount of 9:1 Acetonitrile (ACN):water (solvent B). Typical gradient is 5% solvent B to 40% solvent B over 35 minutes. Samples collected from the HPLC were analyzed by the Liquid Chromatography Mass Spectrometry (LCMS) on a Shimadzu LCMS-9030 to confirm their identity (**Table 2**).

UV-vis spectra were taken on a Agilent Cary 300 UV-Vis spectrophotometer which uses a deuterium light source with a range from 190nm to 900 nm. Samples were heated to 90°C to eliminate secondary structures and then Beer's Law was used to determine sample concentrations at 260nm. The following extinction coefficients: C = 6600 $M^{-1}cm^{-1}$; T = 8600 $M^{-1}cm^{-1}$; A = 13,700 $M^{-1}cm^{-1}$; G = 11,700 $M^{-1}cm^{-1}$.[9]

Table 2. Summary of PNA Oligomers Characteristics

	Sequence	Calculated Mass (μg)	Found Mass (μg)	Concentration (μM)
1	GATCT-**Azo**-ATGCA	3221.33	3219.198	1098
2	TGCAT-**T**-AGATC	3106.27	3105.039	369
3	TGCAT-**A**-AGATC	3130.28	3124.760	857
4	TGCAT-**C**-AGATC	3121.27	3122.111	1024
5	TGCAT-**G**-AGATC	3145.27	3144.399	676
6	GATCT-**Azo**-ATGCA-mPEG-**Rh**	3753.58	3740.456	108.3

V. PNA Synthesis

PNA sequences are listed in **Table 2**.[9] AzoPNA-1 was synthesized prior to the project by previous undergraduate research student, Josh Gerlach. **Figure 5** shows a representative coupling cycle for one PNA monomer.

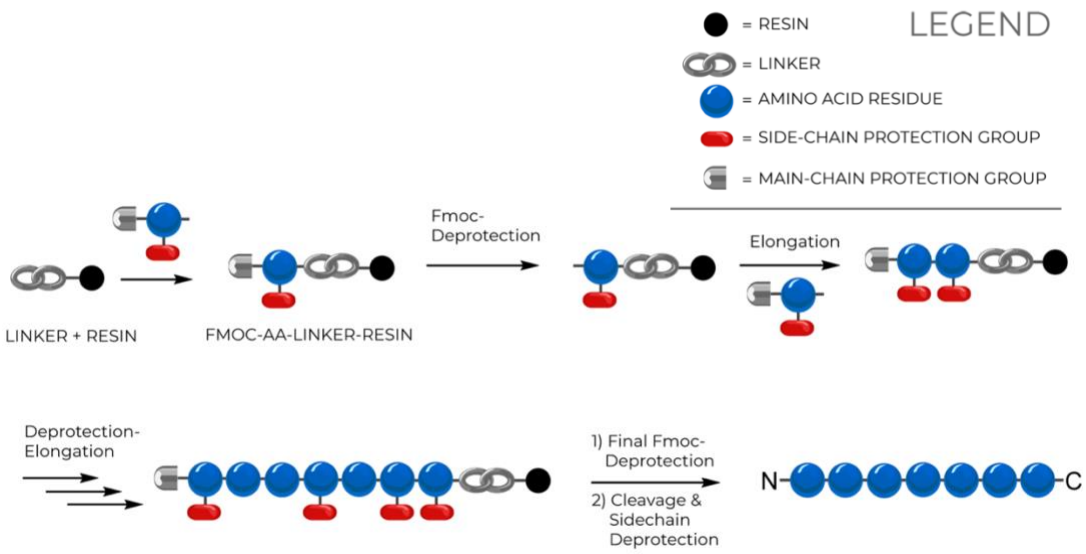


Figure 5. The Symbolic Scheme of the Solid-Phase Peptide Synthesis (SPPS). The process begins with the immobilization protected Fmoc amino acid to the Fmoc-PAL-mPEG resin. The linker is the rink amide that attaches to the resin. Fmoc-deprotection is performed using 20% piperidone in DMF for seven minutes, yielding a free amine. The resin was then washed with DMF (x3) and DCM (x4) before performing the Kaiser test. The elongation of PNA is accomplished by adding the prepared Fmoc-monomers (A, T, C, G) accordingly to the sequences (Table 2) followed by an hour of coupling. A spacer mPEG and fluorescent dye were coupled in a similar manner. Following elongation of all PNA monomers, the PNA is cleaved from the resin, purified by reverse-phase HPLC, and lyophilized to form a fluffy white powder.[10]

VI. Thermal Denaturation Studies

The PNA duplexes were formed by incubating the PNA (**Table 2 entries 2-5**) with the complementary AzoPNA (**Table 2 entries 1 or 6**). The samples were heated to 90°C, held there for five minutes, and then cooled to 15°C at 1°C/min. The data points were collected at the wavelength of 260 nm every 0.5°C. All the strand concentrations were analyzed at 1 μM in 1 x PBS (pH 7.4).

VII. Visualizing PNA Duplexes by Gel Electrophoresis

Agarose gel electrophoresis of PNA was carried out to visualize the PNA duplexes formation. Agarose gel electrophoresis is normally used to separate the nucleic acid

fragments based on their size. Negatively charged nucleic acid migrates through the pores of an agarose gel towards the positively charged end of the gel when an electrical current is applied. Agarose is a linear polymer that is comprised of alternating D- and L-galactose joined by α(1-3) and β(1-4) bonds with an anhydro bridge between the 3 and 6 positions.[11] During gelation, agarose polymers associate noncovalently and form a network of bundles whose pore sizes to determine a gel's molecular sieving properties.[11] A 1% agarose gel was run for 45 minutes in the 1X TAE buffer. PNA samples, as single strands, or annealed duplexes, were loaded in the agarose gel to resolve PNA duplexes based on their molecular weight. The gel will be gelatinized by heating with buffer and casted in a tray with combs in. The gel was stained with Coomassie brilliant blue dye overnight to visualize and analyze by using the UVP Illuminator at 365 nm spectrum.

Results and Discussions

Using the previously published protocols, six PNA oligomers were synthesized based on the solid phase support synthesis as shown in **Figure 6** and **Figure 7**.[12]

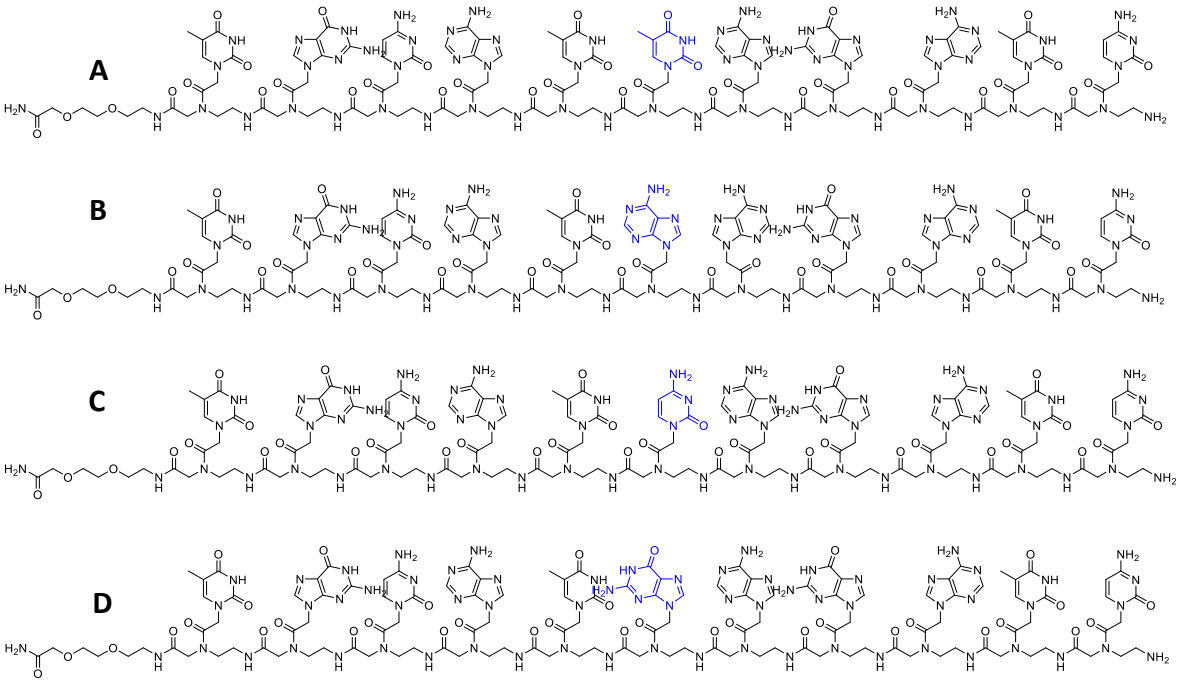


Figure 6. The Chemical Structures of PNA Oligomers with the attached of F-moc monomers. Differences are indicated with blue highlighted heterocyclic nitrogenous base in **A)** Thymine. **B)** Adenine. **C)** Cytosine. **D)** Guanine attached to PNA backbone.

Azobenzene is an organic photoswitchable moiety that contains a diazene (H_2N_2) derivate with two hydrogens replaced by phenyl group that was attached to the PNA backbone of AzoPNA-1 and AzoPNA-6 (**Figure 7**). AzoPNA-6 was created later in the project with an addition of mPEG spacer, and a rhodamine substituent on the AzoPNA-6 to serve as a fluorescence tag allowing the visualization of PNA duplexes formation on the agarose gel electrophoresis.

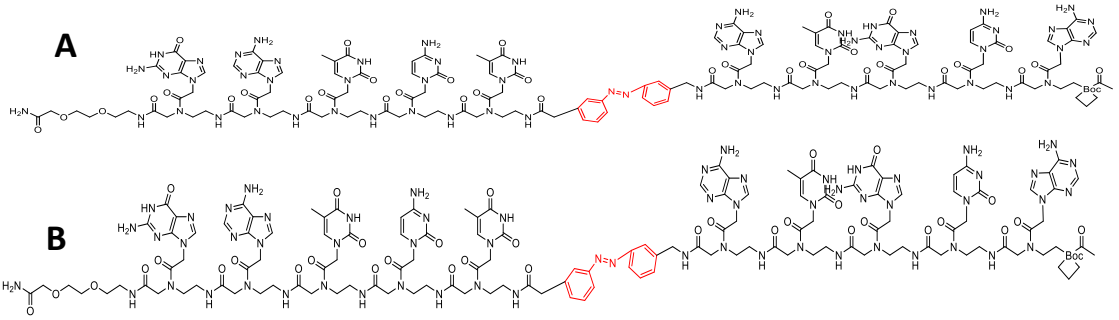


Figure 7. Chemical Structure of PNA oligomers on ChemDraw 19.1. as **A.** AzoPNA-1. **B.** AzoPNA-6. Single azobenzene (red) is attached to the backbone of both PNA oligomers. AzoPNA-6 was synthesized with a mPEG spacer along with a rhodamine substituent (magenta).

Six PNA oligomers were purified by the reverse-phase HPLC after the cleavage of the resin. **An** example of HPLC purification chromatogram of 11 mers is shown in **Figure 8**. Based on the HPLC chromatogram, full-length PNA was clearly separated from all the truncated sequences at 12 to 14 minutes. However, the shorter purification times could be established by using steeper gradients, so the product elutes faster. Purified PNA was analyzed on the LC-MS to determine the found mass.

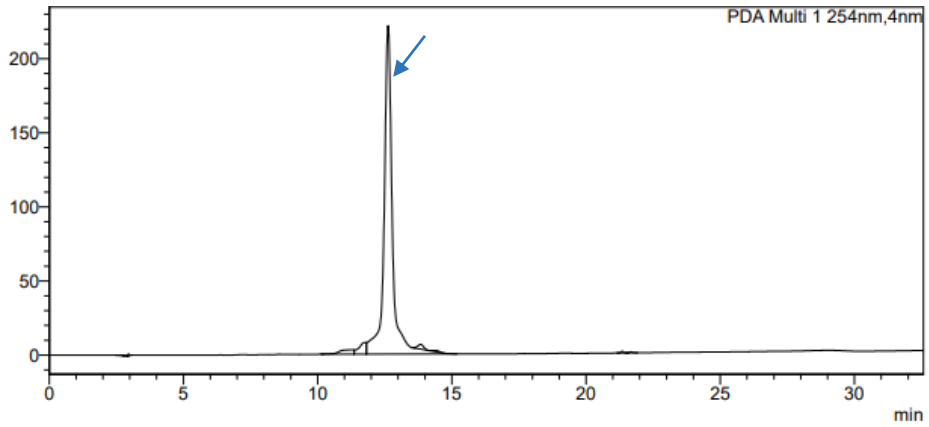


Figure 8. Representative Chromatogram of Reverse-phase HPLC of Purified PNA Oligomers. PNA oligomers were purified by using the Shimadzu LC-2-AD UFLC equipped with a Kinetex column (H17-191743 C18 5 μM 4.6 x 250 mm) and eluted with 0.1% TFA in water (solvent A) and an increasing amount of 9:1 Acetonitrile (ACN):water (solvent B). Typical gradient is 5% solvent B to 40% solvent B over 35 minutes. The peak of interest was indicated by blue arrow.

There was a correlation between the found mass and the calculated mass from the skeletal structure indicating the purification of the isolated PNA (**Table 2**). Based on **Table 2**,

PNA concentrations were determined to be in the low mmol range which is the typical yield of PNA from 50 mg of starting resin. Specifically, precipitation of azoPNA-6 indicated that the concentration exceeds the solubility of the PNA which was resulted in the low concentration (**Table 2 entry 6**). The stability of each complementary PNA were evaluated using thermal denaturation on the UV-Vis. By measuring the PNA duplexes absorbance at 260 nm and pH 7.5 as a function of temperature, typical sigmoidal UV melting curves were obtained as shown in **Figure 9**. According to the spectra, the melting point (T_m) of all PNA duplexes was observed in the range from 40°C to 45°C. Due to the number of hydrogen bonds formed, the higher the length of PNA duplexes, the higher melting temperature; therefore, the T_m of 11mers PNA duplexes is reasonably low.

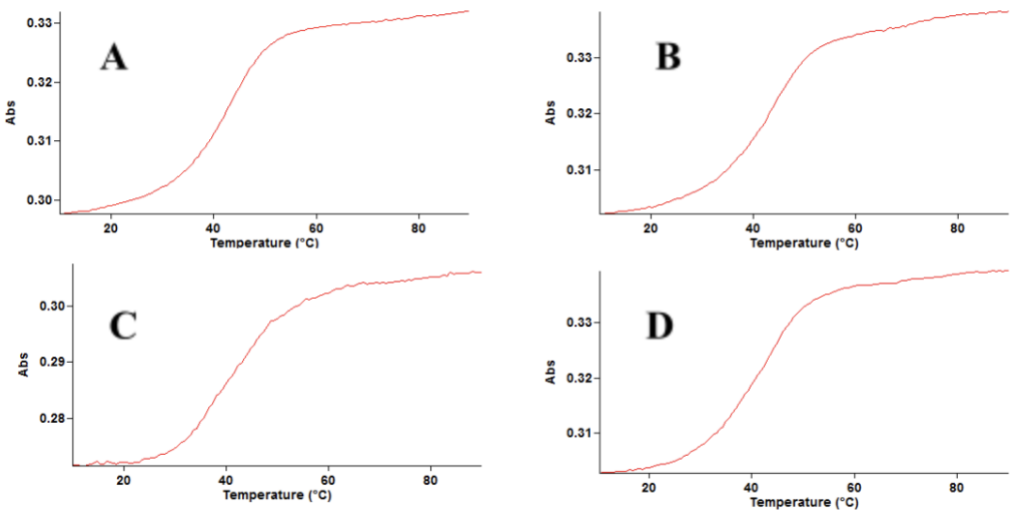


Figure 9. Thermal Denaturation Spectra of PNA Duplexes. Red curves indicate the formation of PNA duplexes as **A.** AzoPNA-1+PNA-2 **B.** AzoPNA-1+PNA-3 **C.** AzoPNA-1+PNA-5 **D**. AzoPNA-1+PNA-4.

To assess if the azobenzene is capable of disrupting the duplex we looked at performing gel assays. **Figure 10** and **11** illustrated the results of initial runs of the azoPNA duplexes. In **Figure 10**, there was only one broad band of AzoPNA-1 (10 μg) duplex

near the wells in lane one demonstrating a weak signal of the presence of azoPNA-1 oligomer at 10 μg. Additionally, there was no detection on the presence of azoPNA duplexes at 20 μg in lanes two and three. In other hand, several bands were shown on the second gel (**Figure 11**); however, there was not enough evidence to conclude the formation of PNA duplexes on gel electrophoresis. In **Figure 11**, lanes one and two contained multiple bands of PNA-2 (100 μg and 10 μg), which shifted to the left lanes of the gel. Lane three did not show any band indicating the concentration of PNA-2 sample (1 μg) was too low to detect the presence of PNA. One band was present from lanes four to six corresponding to azoPNA-1 (100 μg, 10 μg, and 1 μg) samples. Subsequently, azoPNA-6 was created with the rhodamine dye added to the backbone, allowing the PNA duplexed to fluoresce itself in electrophoretic analysis. AzoPNA-6 duplexes were hypothesized that would be detected on the 1% agarose gel. Nonetheless, there was no presence of bands on the agarose gel to support the hypothesis that the rhodamine dye had successfully served as a fluorescent component in this project.

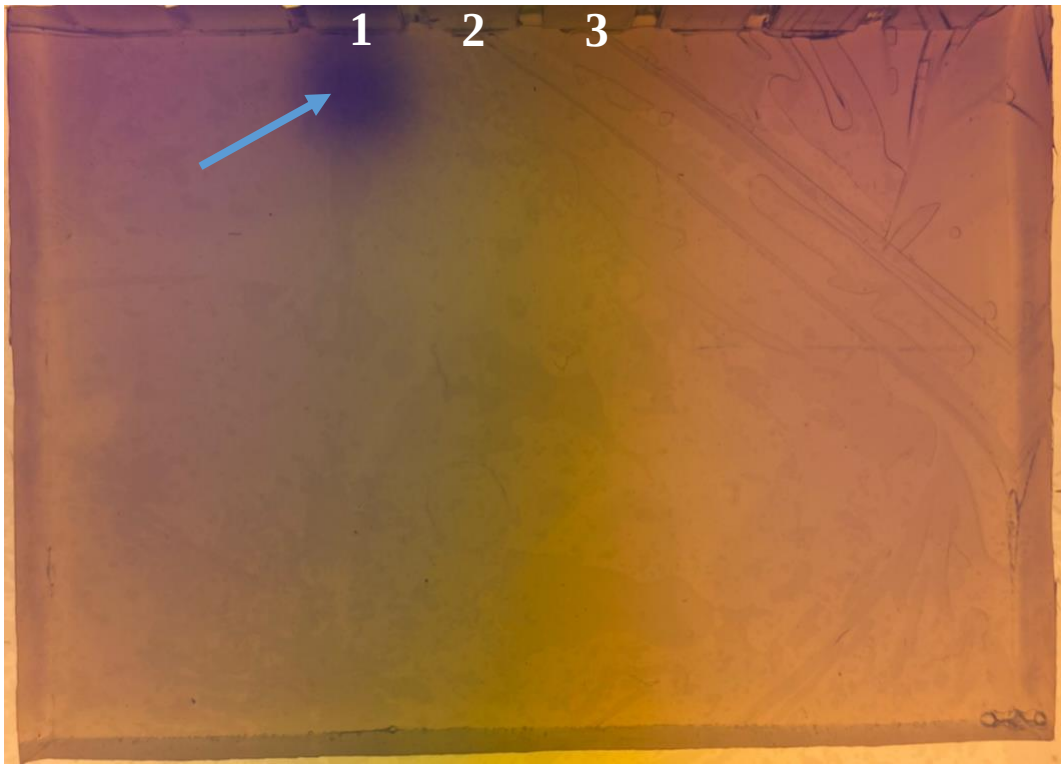


Figure 10. Result of 45 Minute Run of 3% Agarose Gel under the UVP Illuminator. Three samples were loaded in lane one, two and three. In lane one, the presence of the broad band indicating the detection of the AzoPNA-1 (10 µg) (blue arrow). In lanes two and three, there was no band displayed the detection of the azoPNA-1+PNA-2 (20 µg) and azoPNA-1+PNA-3 (20 µg) duplexes.

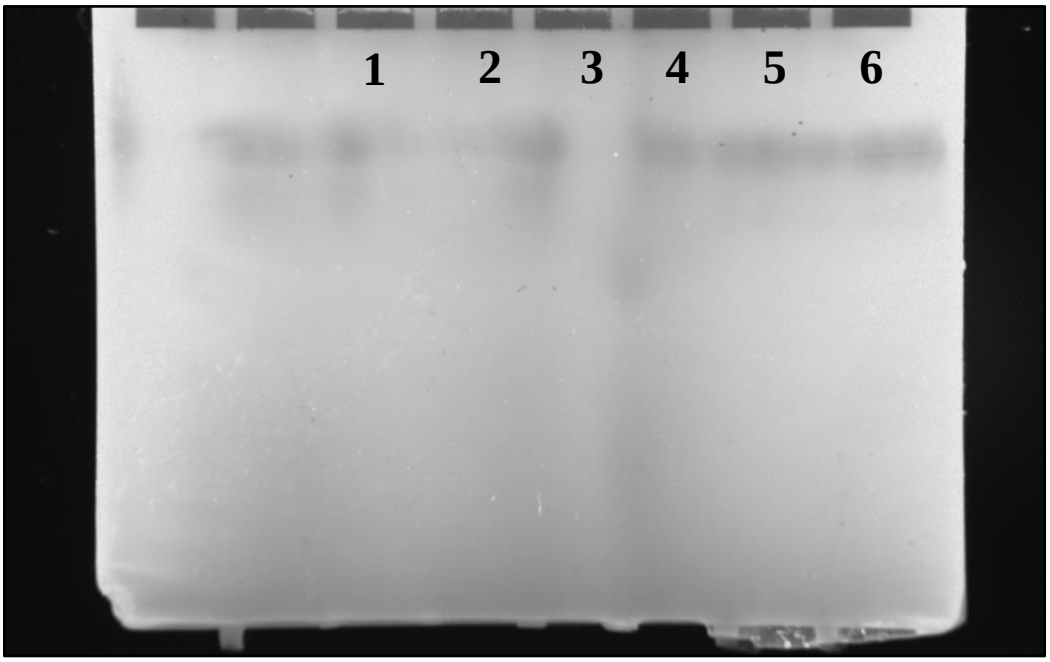


Figure 11. Result of 45 Minute Run of 3% Agarose Gel under the Protein Gel Electrophoresis Illuminator. Six samples were loaded in from lanes one to six. In lanes one and two, PNA-2 (100 μg and 10 μg) were loaded. Lane three contained PNA-2 sample (1 μg). From lanes four six, azoPNA-1 (100 μg, 10 μg, and 1 μg) samples were loaded into the wells.

Overall, the purpose of this project was to focus on the synthesis and characterization of an 11mer PNA that undergoes conformational change upon irradiation due to the presence of a photoswitchable moiety. Six PNA oligomers were successfully synthesized and purified by using HPCL and LCMS. The concentration of PNA oligomers were determined to verify the yield of PNA from the starting resin. PNA duplexes formation was detected by the sigmoidal curve on the UV-Vis spectra. In spite of this, there was no strong evidence of visualization on the gel electrophoresis to confirm the presence of PNA duplex formation. However, the outcomes of this research project play an important aspect in the future studies.

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