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The Investigation of DNA and RNA Structural Differences Using Ultra High Performance Liquid Chromatography

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Abstract

DNA and RNA chromatography is extensively used for nucleic acid analysis. To better understand the chromatographic mechanisms by which DNA and RNA oligonucleotides are separated, ion pair reverse-pair ultra-high performance liquid chromatography (IP RP UHPLC) methods were developed. 11mer and 12mer DNA and RNA oligonucleotides of various compositions were used during this study. The first part of this study analyzed 11mer DNA and RNA oligonucleotides to better understand the chromatographic separations of DNA and RNA. The results gathered through the IP RP UHPLC analysis of these oligonucleotides demonstrated the existence of structural features that affect the chromatographic separations of DNA and RNA. This led to the IP RP UHPLC analysis of DNA and RNA oligonucleotides, of equal length and sequence, which either formed a 4 base-pair or 2 base-pair tetraloop secondary structure. The purpose of this investigation is to improve the isolation and purification of nucleic acid mixtures by understanding how DNA and RNA oligonucleotides interact with the stationary support but to also illuminate the role of structural features in nucleic acid separations. The characterization and the separation of the DNA and RNA oligonucleotides were achieved through a variety of methods including temperature melting experiments. The results gathered demonstrated the effectiveness of IP RP UHPLC to analyze the differences between DNA and RNA oligonucleotide separations. The DNA oligonucleotides eluted earlier than the RNA oligonucleotides which demonstrated that RNA has a different chromatographic mechanism than DNA. Differences between nucleic acid separations of fragments with the 2 base-pair tetraloop and 4 base-pair tetraloop structural modifications were also observed. The oligonucleotides with the 4 base-pair tetraloop eluted later than the oligonucleotides with the 2 base-pair tetraloop demonstrating the influence of structural modifications on the separation mechanisms of nucleic acids. The temperature melting experiments performed also confirmed that structural modifications influence the interaction between nucleic acids and stationary support. These results demonstrate the effectiveness of IP RP UHPLC to observe structural differences between DNA and RNA and as an alternative method to traditional methods, such as gel electrophoresis, to analyze oligonucleotides.

Introduction

Ion pair reverse-pair ultra-high performance liquid chromatography (IP RP UHPLC) is used extensively to study nucleic acids such as DNA and RNA [1,2]. Although gel electrophoresis is the most common technique used to analyze nucleic acids, IP RP UHPLC has proved to be a versatile technique for the analysis of nucleic acids [3]. It provides an alternative to gel-based analysis for the separation and purification of nucleic acids based on their sequence composition and also their size [4]. IP RP UHPLC can also be used to analyze nucleic acids under denaturing conditions. Under these conditions, chromatography can be used for the analysis of oligonucleotides, that is, the separation and isolation of single and double-stranded DNA and RNA [2,3,5].

Nucleic acid separation by IP RP UHPLC is maximized by the use of an ion pairing reagent, an amine cation salt that forms a hydrophobic ion pair with the phosphate anion group of the nucleic acid (either DNA or RNA) [1].Triethylammonium acetate (TEAA) and triethylamine hexafluoroisopropanol (TEA-HFIP) are the most common ion pairing reagents used for nucleic acid separations [6]. TEAA contains short alkyl chains which prevents it from entirely covering the stationary phase and in turn preventing the stationary phase from completely retaining its hydrophobic or reverse phase properties. However other pairings are yet to be explored such as hexylammonium acetate (HAA). In this study HAA was the ion-pairing reagent of choice due to its potential for providing higher chromatographic resolution of oligonucleotides compared to TEAA and TEA-HFIP [1,6].

The long alkyl chains of the HAA allows the ion pairing reagent to have a higher affinity for the stationary phase, provide complete coverage of the stationary phase, and allow for size based separation [1,3,6].HAA pairs with the nucleic acid fragments in order to form a

hydrophobic ion-pair and adsorb to the hydrophobic surface of the stationary phase [1,3]. HAA forms this hydrophobic ion-pair between the column stationary phase, composed of C18 groups, and the hydrocarbon chain of the amino cation salt together with an ionic interaction with the negative phosphate group of the nucleic acid. This ion-pairing mechanism allows for efficient size based separations of nucleic acids of different lengths and when the hydrophobicity of the bases plays a minimum role.

However when the goal is to separate nucleic acids of equal length, the hydrophobicity of the bases must be considered. The degree of hydrophobicity is as followed: adenosine (A) > guanosine (G) > cytosine (C) ≈ thymine (T) /uracil (U). Therefore nucleic acids that have a higher percentage of A and G than C and T/U will have a greater degree of hydrophobicity than nucleic acids with a lower percentage of A and G in comparison to C and T/U. Equal length fragments that are more hydrophobic will thereby elute later than fragments that are less hydrophobic due to a stronger interaction with. The separation of the oligonucleotide samples through IP RP UHPLC, using HAA as an ion pairing agent, allows for the characterization of nucleic acid composition.

The current quantification of RNA has been performed by measuring UV absorption at 260 nm, however it only provides information about the impurities and the degradation of RNA in a sample using the 260/280 and 230/260 ratios [11]. IP RP UHPLC is a reliable and accurate method for RNA quantification by isolating the peak of interest from possible contaminants, peak integration, and transcript purification [11]. Traditional RNA isolation procedures have been found to be time-consuming and inefficient. These isolation procedures such as gel electrophoresis, suffers from poor product yields and is unsuitable for high-throughput approaches in contrast to UHPLC techniques [2, 3].

In this study we developed IP RP UHPLC methods, using HAA as the ion pairing reagent, additives such as ammonium phosphate, and varying column temperatures, in order to have a better understanding of the chromatographic mechanisms by which DNA and RNA oligonucleotides are separated. 11mer and 12mer DNA and RNA oligonucleotides lengths were used. The 11mer DNA and RNA were complimentary oligonucleotides that lacked secondary structures based on theoretical calculations. These oligonucleotides were analyzed to see if hydrophobicity affected chromatographic separations. The results from the analysis of the 11mer oligonucleotides showed that hydrophobicity is not the only factor in the separation of oligonucleotides.

Based on these results, 12mer DNA and RNA oligonucleotides that formed a secondary structure, a tetraloop, were used in this study. Alterations in the sequence of the 12mer oligonucleotides results in the formation of a 2 base-pair or 4 base-pair tetraloop which allowed for the study of structure stability using chromatography in combination with theoretical calculations. Ultimately our aim is to improve the isolation and purification of nucleic acid mixtures by understanding the mechanisms of interactions with the column but to also illuminate the role of structural features in nucleic acid separations. The results gathered demonstrate the effectiveness of IP RP UHPLC in combination with theoretical models to investigate structural differences between DNA and RNA and its effectiveness as an alternative to traditional methods used to analyze oligonucleotides, such as gel electrophoresis.

Materials and Methods

Materials

HPLC grade acetonitrile (ACN) and ammonium phosphate were purchased from Fisher Scientific. Hexylamine was purchased from ACROS Organics. Glacial acetic acid was purchased

from Sigma-Aldrich. Hexylamine and acetic acid were used to create a 1 liter stock of 1M HAA. The 1M HAA stock was adjusted to pH 7 and filtered. Once filtered the stock of 1M HAA was diluted to 100mM HAA as solvent for, buffer A. Buffer B was composed of 50:50 ACN and 100mM HAA. To test to the effects of ammonium phosphate ions on IP RP UHPLC, 1.0mM ammonium phosphate was added to buffers A and B.

Eight oligonucleotides were purchased from Integrated Diagnostic Technologies (IDT) with standard desalting (Table 1). The D12S and R12S oligonucleotides formed a secondary structure in the form of a 4 base-pair tetraloop while the D12NS and R12NS formed a 2 base-pair tetraloop. Stock solutions of each oligonucleotide were made by adding the appropriate amount of RNAse free water to the oligonucleotide samples. The NanoDrop 2000 UV-Vis Spectrophotometer was used to measure the concentration of each oligonucleotide stock solution by means of absorbance. The extinction coefficient calculated from each sequence using the calculator tool provided by the NanoDrop 2000 UV-Vis Spectrophotometer was then used to obtain the concentration of each oligonucleotide stock solution by applying the Beer-Lambert equation. Stock solutions were diluted to 50 ng/µl working concentrations of the

Table 1: Properties of Oligonucleotides used for IP RP UHPLC

Oligonucleotide	Designation	Sequence	Molecular Weight (g/mol)	Extinction Coefficient (L/(mol x cm))
7mer DNA A	D7A	5'- CGT GCG A -3'	2,121.4	67,200
7mer DNA B	D7B	5'- TCG CAC G -3'	2,081.4	63,100
11mer DNA A	D11A	5'- GAC GTG CGA AG-3'	3406.3	112400
11mer DNA B	D11B	5'- CTT CGC ACG TC-3'	3268.2	93700
11mer RNA A	R11A	5'- GAC GUG CGA AG-3'	3568.2	113000
11mer RNA B	R11B	5'- CUU CGC ACG UC-3'	3402.1	96700
12mer DNA Structure	D12S	5'-CGC GTT TTC GCG-3'	3628.4	102300
12mer DNA Non-Structure	D12NS	5'-CCC GTG TGC GTT-3'	3628.4	103300
12mer RNA Structure	R12S	5'-CGC GUU UUC GCG-3'	3764.3	107500
12mer RNA Non-Structure	R12NS	5'-CCC GUG UGC GUU-3'	3764.3	107300
12mer DNA/RNA mixture	D12/R12	---------	----	--------

oligonucleotides.

UHPLC Analysis

The 11mer and 12mer samples were analyzed by IP RP UHPLC on a Waters Acquity UPLC I-Class System (Waters, Milford, MA, USA). The 11mer and 12mer oligonucleotides were analyzed using Waters Acquity UPLC BEH C18 column with a pore size of 130 Å and incorporated a 1.7 μm bonded phase which consisted of the Ethylene Bridged Hybrid (BEH) particle (Waters, Milford, MA, USA). Samples were detected at 260nm wavelength.

The IP RP UHPLC analysis of the 11mer and 12mer oligonucleotides was performed under the following gradient conditions: buffer A, 100mM HAA, pH 7.0; buffer B, 50:50 ACN: 100mM HAA, pH 7.0. When ammonium phosphate was used, a final concentration of 0.1mM ammonium phosphate was added to buffer A and the HAA portion of buffer B.

Gradient (1) was used to analyze the 11mer and 12mer oligonucleotides at 30°C. Gradient (2) was used to analyze was used to analyze the 12mer oligonucleotides at 30°C, 60°C,

Table 2: Gradients used to analyze the 11mer and 12mer oligonucleotides by IP RP UHPLC

Time (minutes)	Gradient (1) % Buffer A	Gradient (1) % Buffer B	Time (minutes)	Gradient (2) % Buffer A	Gradient (2) % Buffer B
Initial	90	10	0.00	90	10
2.00	65	35	3.00	0	100
15.00	60	40	4.00	0	100
21.00	25	75	4.50	90	10
21.10	0	100	6.00	90	10
23.00	0	100			
23.50	90	10			
25.00	90	10			

and 80°C.

Gel Electrophoresis

Individual strands of the oligonucleotides and an equimolar mixture were analyzed with a 15% native polyacrylamide gel (PAGE). All gels were buffered using 0.5X TBE. The ladder used was 60 ng/µl microRNA marker (New England BioLabs) which includes a set of a 17mer, 21mer, and 25mer synthetic single stranded residues that have free 5' ends. The 15% native gels were run at 150V and post-stained using a final concentration of 1X SYBR Gold (Life Technologies). All gels were imaged using a Bio-Rad Gel Doc XR+ system.

Results and Discussion

Effects of Ammonium Phosphate on IP RP UHPLC

Various studies have found an effect of phosphate ion addition on nucleic acid separation by IP RP UHPLC. Yamauchi et al. reported that the presence of phosphate was essential for the separation of low molecular weight RNAs (20-500nt) by IP RP UHPLC and proved that in the absence of phosphate ions, RNAs were not able to be detected [8]. They credited this to the fact that trace ions from the column detrimentally adsorb to the phosphate backbone of the RNA and theorized that by adding an excess of phosphate ions to the solutions, trace ions bind to the phosphate ions instead minimizing this effect and allowing for a more sensitive oligonucleotide detection and separation [8]. Chien et al. and David V. McCalley have also reported that the presence of ammonium phosphate ions improve peak shape due to its superior masking effect [9,10].

To evaluate the effect of ammonium phosphate ions on the chromatographic behavior of oligonucleotides by IP RP UHPLC, four 11mer oligonucleotides were studied using Gradient (1), before and after the addition of 0.1mM ammonium phosphate to both buffer A and buffer B. Gradient (1) was an optimized gradient that was used since it provided better resolution.

The addition of ammonium phosphate to both buffer A and buffer B resulted in an overall increase in the intensity of the D11A, D11B, R11A, and R11B oligonucleotides (Figure 1). D11A increased by 36% from 0.22AU to 0.30AU, D11B increased by 20% from 0.20 to 0.24, R11A increased by 150% from 0.05AU to 0.125AU, and R11B increased by 37.5% from 0.08AU to 0.11AU (Table 3). The addition of ammonium phosphate affected DNA and RNA differently—the 11mer RNA experienced a larger increase in absorbance values than the 11mer DNA and the absorbance values of the more hydrophobic oligonucleotides, D11A and R11A, experienced a greater increase in comparison to the less hydrophobic oligonucleotides, D11B and R11B.

The differences observed between DNA and RNA may be due to the trace ions of the C18 column having a greater impact on the backbone of RNA than DNA, therefore when ammonium phosphate ions are added the minimizing effect of these ions are more profound for RNA than DNA. This reasoning can also be applied to the more hydrophobic oligonucleotides, D11A and R11A—these oligonucleotides have a stronger interaction with the C18 column than their less hydrophobic counterparts, D11B and R11B, therefore the minimizing effects of
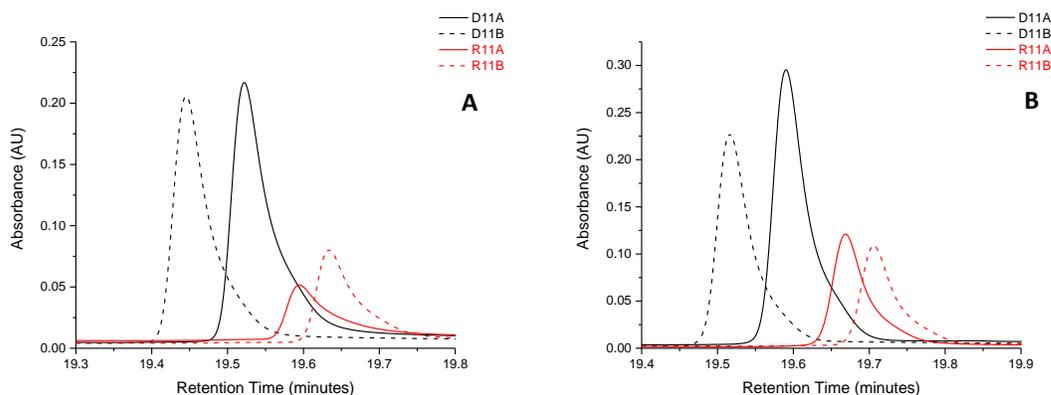

Figure 1: IP RP UHPLC analysis of 11mer DNA and RNA oligonucleotides. The samples were analyzed on a BEH C18 column at 30°C using Gradient 1. (A) Analysis of 11mer oligonucleotides before the addition of ammonium phosphate to buffer A and B. (B) Analysis of 11mer oligonucleotides after the addition of ammonium phosphate to buffer A and B.

Table 3: Oligonucleotide separation obtained at 30°C with and without the addition of ammonium phosphate ions using Gradient 1 at 30°

Oligonucleotide	Before Phosphate Ion Addition		After Phosphate Ion Addition	
	Retention Time (minutes)	Absorbance (AU)	Retention Time (minutes)	Absorbance (AU)
D11A	19.522	0.22	19.590	0.30
D11B	19.445	0.20	19.516	0.24
R11A	19.595	0.05	19.669	0.125
R11B	19.634	0.08	19.706	0.11

ammonium phosphate will be more profound and allow for a more sensitive detection of D11A and R11A. These results indicate the advantages of ammonium phosphate in nucleic acid separations using IP RP UHPLC.

IP RP UHPLC Analysis of 11mer Oligonucleotides

The purpose of the IP RP UHPLC analysis of the 11mer oligonucleotides was to optimize the chromatographic separation of complementary oligonucleotides of the same length and to analyze the differences between DNA and RNA chromatographic separation mechanisms. The 11mer DNA and RNA oligonucleotides were analyzed using Gradient (1) at 30°C in order to better understand the effects of base composition on the chromatographic separation of equal length oligonucleotides.

As seen in the chromatogram obtained at 30°C (Figure 2), the 11mer DNA elutes earlier than the 11mer RNA due to a weaker interaction with the C18 column. Regardless of the addition of ammonium phosphate ions, DNA absorbance values were still higher than the equivalent RNA; this may have been
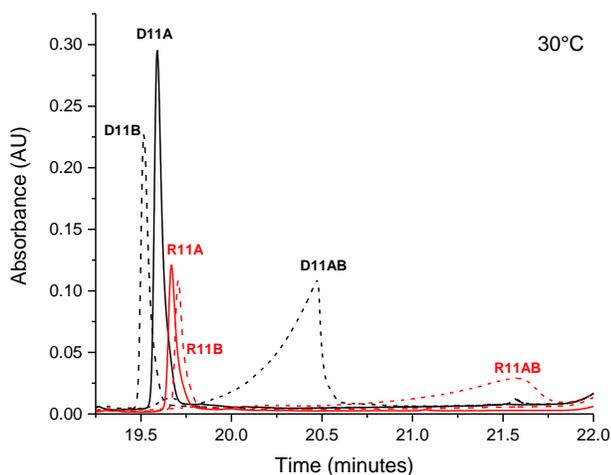

Figure 2: IP RP UHPLC Analysis of the 11mer oligonucleotides. The samples were analyzed on a BEH C18 column at 30°C using Gradient 1.

caused by possible RNA intramolecular folding affecting the transmission of light through the sample and therefore decreasing the

Table 4: Oligonucleotide separation obtained at 30°C using Gradient 1 at 30°

Oligonucleotide	Retention Time (minutes)	Absorbance (AU)
D11A	19.590	0.30
D11B	19.516	0.24
R11A	19.669	0.125
R11B	19.706	0.11

overall absorbance of the UV light (Figure 2, Table 4). D11A eluted later than D11B, falling in line with the degree of hydrophobicity, that is, D11A has a greater number of adenosines and guanosines than the complementary D11B therefore it will have a stronger interaction with the C18 column (Figure 2, Table 4). However in the case of RNA, R11B eluted later than R11A which is the opposite of what would be expected due to its hydrophobic composition. This suggests that structural features may play a role in the separation of RNA oligonucleotides.

The difference in retention time ΔR_T was used to further analyze the differences seen between DNA and RNA in regards to their elution. The ΔR_T for D11A and R11A was 0.073 minutes while the ΔR_T for D11B and R11B was 0.189. The latter increase was due to the R11B having a stronger interaction with the C18 column than D11B and its hydrophobic counterpart, R11A.

The results gathered from the IP RP UHPLC analysis of the 11mer DNA and RNA demonstrated that structural features play a greater role than hydrophobic composition in the separation of equal length oligonucleotides. As theoretical models were unable to predict any secondary structure in any of the 11 mer oligos, 12 mer oligos which structure were previously characterized using molecular dynamic simulations were prepared for subsequent analysis by IP RP UHPLC [12].

PAGE Analysis of 11mer DNA and RNA Oligonucleotides

A 15% native PAGE was performed to compare and asses the results gathered from the IP RP UHPLC analysis of the 11mer oligonucleotides. The 15% native PAGE was performed using 12.5 ng/µl 12mer single oligonucleotide samples and 25.0 ng/µl for the duplex.

When interpreting the results of the electrophoretic analyses we referred to the topology theory of DNA which states that the linking number (Lk) of DNA determines the degree of supercoiling [13]. Lk describes how many times a strand of DNA winds around the helix axis [13]. Lk is determined by the sum of the twist (number of helical turns in circular DNA) and the writhe (shape of the DNA molecule). The more positive the Lk value the more positively supercoiled the DNA will be and vice versa. Supercoiled DNAs are further characterized using the superhelical density (σ) which estimates the number of supercoils per helical turn of DNA. Supercoiling also imposes serious conformational changes to DNA and is therefore energetically unfavorable. Although it is energetically unfavorable, supercoiling allows oligonucleotides to migrate faster in agarose gels. Gel electrophoresis is affected by the difference in shape between supercoiled and relaxed oligonucleotides. During electrophoretic methods circular DNA becomes compact and supercoiling density increases allowing the DNA to migrate faster when compared to the more compact circular counterparts [13].
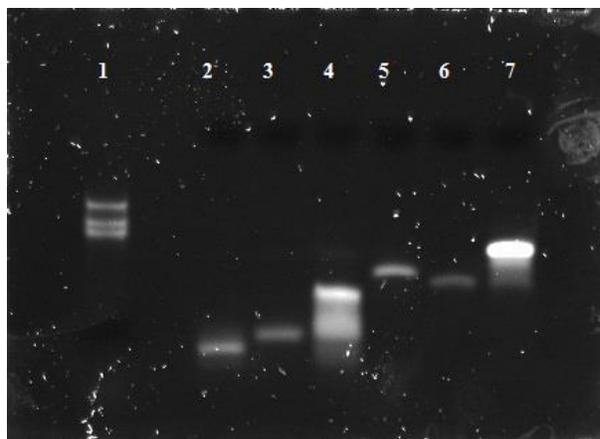

Figure 3: 15% PAGE of 11mer DNA and RNA oligonucleotides. Lane 1 contains microRNA marker, Lane 2 contains D11A, Lane 3 contains D11B, Lane 4 contains D11AB, Lane 5 contains R11A, Lane 6 contains R11B, and Lane 7 contains R11AB

DNA resulted in less duplex formation than RNA, suggesting there are stronger

intramolecular interactions within RNA in comparison to DNA (Lane 4 and 7, Figure 3). In addition, we observed that overall, the DNA traveled faster than the RNA which may be due to the thicker double stranded RNA [14,15]. In the case of the duplex and the flexibility of the single strands, RNA tends to fold on itself creating a circular DNA-like structure that slows its motion through the gel. R11B traveled faster than R11A (Lane 5 and 6, Figure 3) suggesting that R11B contains an intramolecular supercoiled DNA-like structure different from the less organized broader structure of R11A thereby causing R11A to travel slower through the gel [14,15]. These results support our hypothesis from the chromatographic analysis where due to possible structural features, R11B elutes later than R11A.

IP RP UHPLC Analysis of 12mer DNA and RNA Oligonucleotides

To investigate the effect of structure on chromatographic behavior and how this effect manifests, whether it is DNA or RNA, we decided to study 12mer DNA and RNA, previously characterized using molecular dynamic simulations [12]. The first set of oligonucleotides, D12S and R12S, contained a sequence forming a well-defined 4 base-pair tetraloop and the second set of oligonucleotides, D12NS and R12NS, contained a the same sequence composition but was altered to form a 2 base-pair tetraloop and therefore more unstable structure

A   B

Figure 4: Structure of 12mer RNA oligonucleotide. A) Structure of the 4 base-pair tetraloop oligonucleotide (R12S) B) Structure of the 2 base-pair tetraloop oligonucleotide (R12NS)

(Figure 4). The analysis of these oligonucleotides was performed at a range of column temperatures from 30°C to 80°C. The gradient was optimized to provide an oligonucleotide separation of interest using a shorter run time than previous gradients (Gradient 2, Table 2)
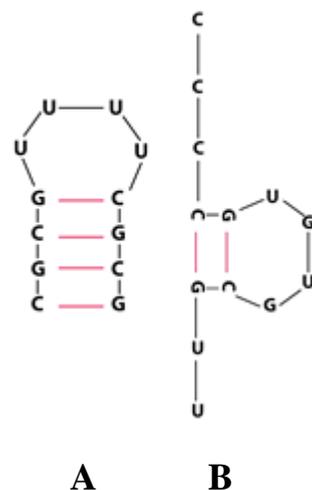
Chromatographic resolution was also used to determine how well the 12mer oligonucleotides were separated during the IP RP UHPLC analysis. A resolution (R_s) of 1.5 or greater between two peaks ensures that oligonucleotides are adequately separated so the height of the peaks can be properly measured. Rs $\geq$ 1.5 can be achieved by changing the mobile phase composition, changing the column temperature or using special chemical effects, such as the addition of ammonium phosphate ions.

As seen in the chromatogram obtained at 30°C, the 12mer DNA eluted earlier than the equivalent 12mer RNA. Furthermore, the 2 base-pair tetraloop, D12NS and R12NS, eluted earlier than 4 base-pair tetraloop, D12S and R12S (Figure 5A, Table 5). This is due to the weaker interaction that the 2 base-pair tetraloop oligonucleotides, D12NS and R12NS, have with the
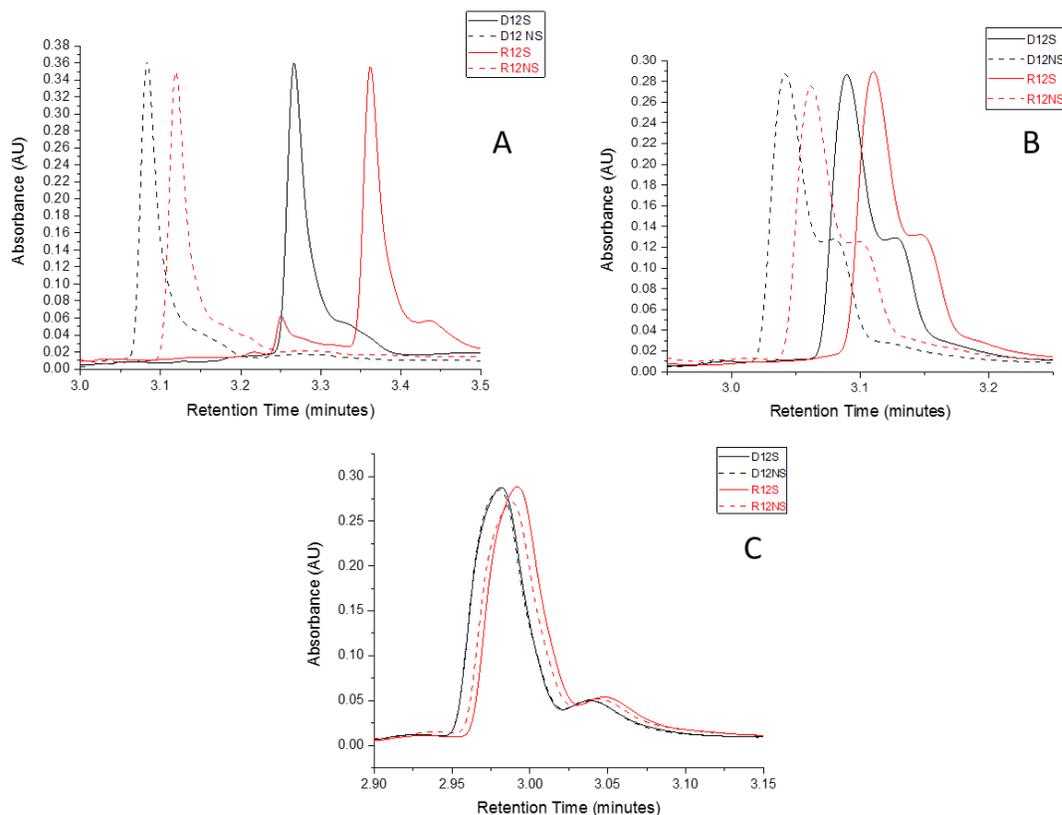

Figure 5: IP RP UHPLC analysis of 12mer DNA and RNA oligonucleotides. The samples were analyzed on a BEH C18 column at (A) 30°C, (B) 60°C, and (C) 80°C using Gradient 2.

C18 column in comparison to the 4 base-pair tetraloop oligonucleotides, D12S and R12S. The chromatographic resolution between the DNA and RNA were calculated to further illustrate these differences: for the 12mer DNA D12S/NS R_s= 2.83 and for the 12mer RNA S/NS R_s= 4.25. The larger Rs observed on the RNA samples, indicated a stronger interaction of the RNA 4 base-pair tetraloop, D12S, compared to the DNA equivalent.

Similar trend were observed when the column temperature was raised to 60°C (Figure 5B, Table 5). The 12mer DNA eluted earlier than the equivalent 12mer RNA and the 2 base-pair tetraloop oligonucleotides eluted earlier than the 4 base-pair tetraloop oligonucleotides. However at 60°C, the difference in retention times between the 4 base-pair tetraloop and 2 base-pair tetraloop 12mer oligonucleotides decreased in comparison to those obtained at 30°C (Table 5). The chromatographic resolution for the 12mer DNA NS/S R_s= 0.9895 and the RNA NS/S R_s= 1.59. Based on these values we can see the effect of temperature by observing how some of the structural features that were separating the 2 base-pairs to the 4 base-pairs in time were minimized in both DNA and RNA bringing the resolution between the pairs close together but still seeing differences between DNA and RNA.

Lastly, the 12mer DNA and RNA oligonucleotides were analyzed at 80°C to confirm if the results gathered at 30°C and 60°C were due to the effects of structural features, as at such high temperatures the effects of structure should be completely eliminated. The chromatographic resolution for the 12mer DNA NS/S R_s= 0.0133 and RNA NS/S R_s= 0.9766 indicating how the differences in retention time between the 4 base-pair tetraloop and 2 base-pair tetraloop oligonucleotides were indistinguishable due to the reduction of the effects of structure but also the differences between DNA and RNA (Figure 5C).

Table 5: Oligonucleotide separation obtained using Gradient 2 at 30°C, 60°C, and 80°C.

Oligonucleotide	30°C		60°C		80°C	
	Retention Time (minutes)	Absorbance (AU)	Retention Time (minutes)	Absorbance (AU)	Retention Time (minutes)	Absorbance (AU)
D12S	3.267	0.36	3.089	0.29	2.981	0.29
D12NS	3.083	0.36	3.042	0.29	2.980	0.29
R12S	3.374	0.36	3.197	0.29	3.091	0.29
R12NS	3.119	0.36	3.062	0.28	2.998	0.27

Separation of 12mer DNA/RNA Mixture

The chromatographic separation of each individual oligo solution was evaluated to study if the same separation was consistent to that when all the 12mer oligonucleotides were mixed in a single solution. The 12mer DNA/RNA mixture was analyzed by IP RP UHPLC using Gradient 1 at 30°C (Figure 6). The retention times observed for the individual 12mer DNA and RNA strands were fairly consistent with those observed for the
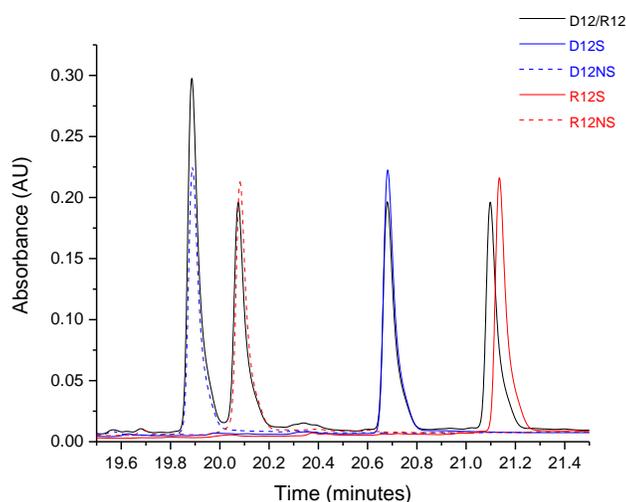

Figure 6: IP RP UHPLC analysis of the 12mer DNA/RNA mixture, 12mer DNA, and 12mer RNA using Gradient 1 at 30°C. The chromatogram is an overlay of the 12mer DNA/RNA mixture with the D12 and R12 oligonucleotides.

12mer DNA/RNA mixture. The slight differences in the retention times between the D12/R12 mix and the 12mer single stranded oligonucleotides were due to human error when making

Table 6: 12mer DNA/RNA and 12mer single stranded oligonucleotide separation obtained using Gradient 1 at 30°C

D12/R12 Mix	Retention Time (minutes)	Absorbance (AU)	Oligonucleotide	Retention Time (minutes)	Absorbance (AU)
D12NS	19.886	0.30	D12NS	19.889	0.22
R12NS	20.075	0.19	R12NS	20.082	0.19
D12S	20.759	0.19	D12S	20.682	0.22
R12S	21.098	0.19	R12S	21.135	0.19

solutions such as a new buffer B solvent.

PAGE Analysis of 12mer DNA and RNA Oligonucleotides

A 15% native PAGE was performed as an alternative method to analyze the D12/R12 mixture, D12S, D12NS, R12S, and R12NS samples. The concentrations used were 12.5 ng/µl for the 12mer single oligonucleotide samples and 25.0 ng/µl for the 12mer DNA/RNA mixture.
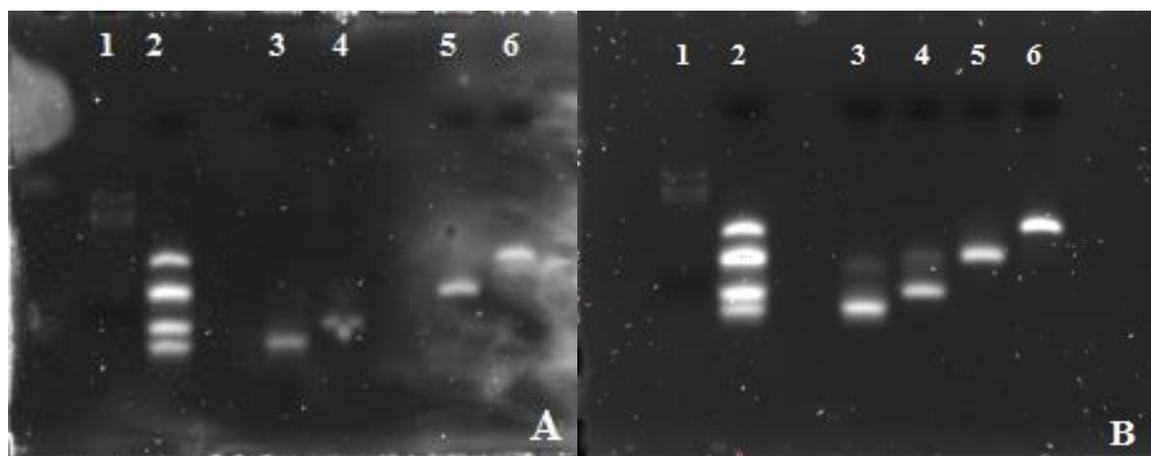

Figure 7: PAGE of 12mer DNA and RNA oligonucleotides. Lane 1 contains microRNA marker, Lane 2 contains D12/R12 mixture, Lane 3 contains D12S, Lane 4 contains D12NS, Lane 5 contains R12S, and Lane 6 contains R12NS (A) 15% native PAGE analysis of 25.0 ng/µ D12/R12 mixture and 12.5 ng/µl 12mer DNA and 12mer RNA. (B) 15% native PAGE analysis of 12mer 25.0 ng/µl D12/R12 mixture, 12mer DNA, and 12mer RNA.

The 12mer DNA oligonucleotides traveled faster than the 12mer RNA oligonucleotides (Lane 3,4 and 5,6, Figure 7), as observed previously with the 11mer, probably due to RNA having wider helical structures when compared with DNA making it travel slower during electrophoresis. However, for both DNA and RNA oligonucleotides, those with the 4 base-pair tetraloop (Lane 3 and 5, Figure 7) traveled faster than those with the 2 base-pair tetraloop (Lane 4 and 6, Figure 7). The 4 base-pair tetraloop has a longer stem region forming more coils than the equivalent 2 base-pair therefore simulating a supercoiled DNA-like structure that allows it to travel faster. The unpaired bases of the 2 base-pair tetraloop probably causes the DNA and RNA to be restricted as they travel through the gel pores, therefore slowing it down in comparison to

the fully paired stem of the 4 base-pair tetraloop. A second 15% native PAGE was performed

using the 25.0 ng/µl 12mer oligonucleotide samples to test the reliability of the results found in

the previous experiments (Figure 7B). The trends observed were reproducible therefore

confirming the reliability of the results.

Molecular Dynamic Simulations of R12S

Molecular dynamic (MD) simulations were applied to further understand the

chromatographic and electrophoretic results and how they can be related to structure. First the

12mer RNA with the 4 base-pair tetraloop was placed to be surrounded by HAA to see which

parts of the structure interact more with the ion pairing agent. A nanosecond timescale

simulation revealed that HAA is more likely to bind to the loop region than the stem region of

R12S (Figure 8A, B). We also determined that on average ~7 molecules of HAA are bound to
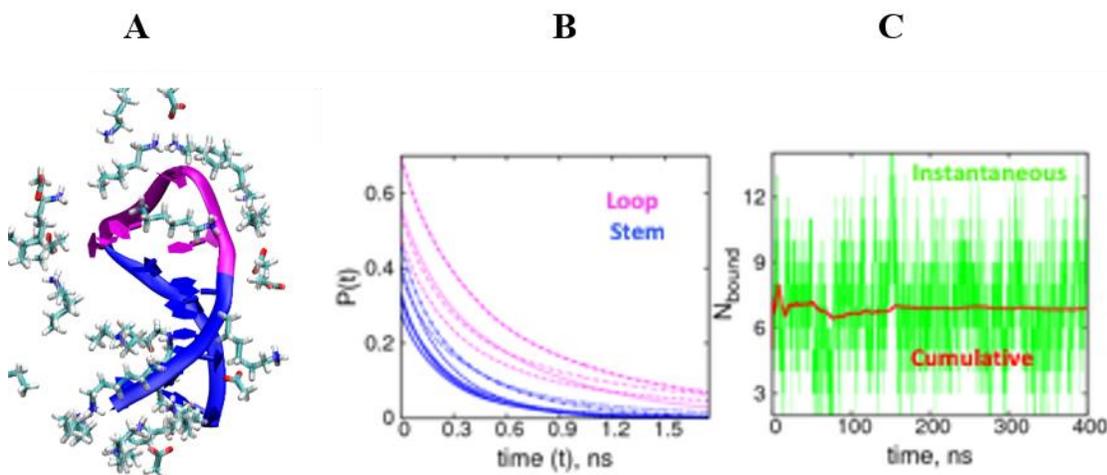
the loop region of R12S (Figure 8C).

Figure 8: MD Simulation of R12S oligonucleotide. A) Simulation of R12S oligonucleotide B) Nanoscale timescale
stimulation of the probability of HAA binding to R12S stem and loop regions C) Plot of instantaneous and
cumulative amount of HAA bound to the loop region of R12S

Being that the 4 base-pair tetraloop has a more robust and stable structure than the 2 base-

pair tetraloop, we could assume, based on these theoretical results, that HAA interacts to a higher

extent with the 4 base-pair tetraloop producing a stronger interaction with the C18 column and a later elution than its 2 base-pair counterpart. Further studies will include simulations of the 2 base-pair tetraloop and its interaction with HAA and modifications of the C18 column with HAA and how the oligonucleotides interact with the treated surface.

Conclusions

The results gathered have demonstrated the effectiveness of IP RP UHPLC to analyze the difference between DNA and RNA chromatographic separation mechanisms. Furthermore this study demonstrated the difference between nucleic acid separations of fragments with well-defined 4 base-pair tetraloop and those with a less-defined 2 base-pair tetraloop. These results showed the effective use of IP RP UHPLC as an alternative and complementary method to other more conventional methods that have been used to analyze oligonucleotides.

Complimentary 11mer oligonucleotides were first studied to better understand the effect of hydrophobicity on the nucleic acid separation of equal length oligonucleotides. The results of this study yield unexpected results. The 11mer RNA with the lower degree of hydrophobicity, R11B, eluted later than the 11mer RNA with a greater degree of hydrophobicity, R11A. A 15% native PAGE was performed as alternative method of analysis to corroborate the results gathered by IP RP UHPLC. The topology theory of DNA was used to analyze the results from the native PAGE. It was observed that the R11B traveled faster than R11A suggesting that R11B contains an intramolecular supercoiled DNA-like structure while the R11A contained a more circular DNA-like structure causing it travel slower through the gel. These results support our hypothesis from the results gathered through the chromatographic analysis where R11B eluted later than R11A due to the presence of structural features thereby having an effect on chromatographic mechanisms alongside base composition.

12mer oligonucleotides with a define structure based on theoretical models, 4 base-pair, D12S and R12S, and 2 base-pair tetraloop, D12NS and R12NS, were analyzed by IP RP UHPLC at a range of column temperatures. The results demonstrated that there are differences in the chromatographic mechanisms by which DNA and RNA are separated and that structural features will influence nucleic acid separations. In general we observed how the 12mer DNA oligonucleotides eluted earlier than the 12mer RNA oligonucleotides as observed with the 11mer experiment. The D12S and R12S eluted later than the D12NS and R12NS, which demonstrated the influence of structural features on the separation mechanisms of nucleic acids. These trends were progressively reduced as the temperature of the column was increased from 30°C to 60°C and 80°C. As the temperature as closer to denaturing conditions, therefore exceeding the melting temperature of the oligonucleotides of interest, those differences were eliminated. These results suggest that RNA interacts with the C18 column in a different manner than DNA and that structural features do affect the separation mechanisms of oligonucleotides.

Two 15% native gels were performed to confirm the results gathered from the 12mer oligonucleotide IP RP UHPLC analysis. The 4 base-pair tetraloop oligonucleotides travelled faster than the 2 base-pair tetraloop oligonucleotides showing that the 4 base-pair oligonucleotides have a higher helicity than the 2 base-pair tetraloop DNA and RNA. These results complemented the chromatographic results and demonstrated how the effect of structure influences chromatographic separations.

Preliminary MD simulation of the R12S oligonucleotide gathered how HAA was more likely to be bound to the loop than the stem region suggesting that the interaction between HAA and the loop region of R12S may affect its chromatographic separation compared to that of the R12NS. Further MD simulations will be investigating the interaction between HAA, the

oligonucleotides, and the C18 column to better understand the chromatographic separation mechanisms of RNA and how structural features affects these mechanisms.

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