Separation of Short and Long Chain Oligonucleotides through the use of a Large Pore SPP Column

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HPLC 2022: PT-706

Introduction

Oligonucleotides are becoming increasingly important in medical research and chromatography. The use of siRNA, aptamers, and other oligomers in treatments is widening. These treatments have begun to aid people suffering from genetic diseases like Duchenne muscular dystrophy, acute porphyria, and familial amyloid neuropathy. Successful UHPLC of nucleotides has become critical for these treatments to advance. The chemical structure and molecular weight of oligos varies widely. These variables make oligonucleotides difficult to separate with good resolution.

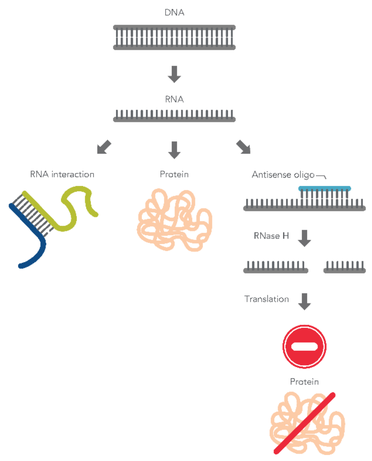


Figure 1: Antisense oligo–mediated cleavage of the target by RNase H. (idtdna.com)

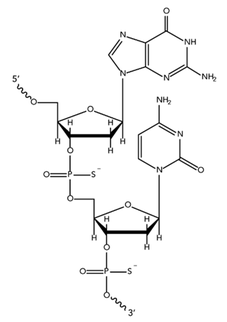


Figure 2: Structure of a 2'-O-modified RNA. (idtdna.com)

Some relatively large nucleotides retain multiple negative charges which makes separation even more difficult. It can be especially difficult to separate both smaller nucleotides like siRNA (7kDa) from larger nucleotides like sgRNA (32kDa). Through the use of a wide pore 1000Å column, longer chain nucleotides can be accurately separated with clean peak shapes and increased sensitivity. Short oligonucleotides are not well retained on columns with short alkyl chains like C4. By selecting more hydrophobic chains such as C18, the retention and resolution of nucleotides improves. Hydrophobic groups and larger pore sizes combine to increase resolution of larger length oligomers. Through the use of large pore size columns, siRNA's and sgRNA's can be separated successfully on UHPLC systems.

Experimental

20/100 Oligonucleotide Ladder

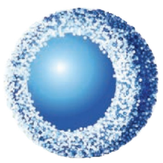
The oligonucleotide ladder samples were purchased through IDT as a dried sample. The 10µg sample was first centrifuged to eliminate loss of sample. Next the sample was reconstituted in 200µL of 10mM Tris-EDTA solution at pH 8.0 (Sigma-Aldrich). The sample was injected after reconstitution.

Instrumentation

All samples were analyzed on a Shimadzu Nexera X2 (Shimadzu Scientific Instruments, USA).

LC Gradient

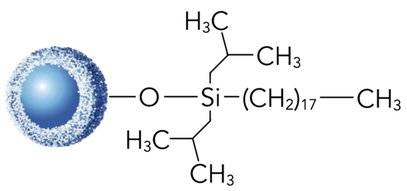
Time	%B
0.00	40
20.00	62.5
22.00	100
23.00	100
23.10	40
28.00	40



1000 Å 2.7 micron particle

Mobile Phase A: 100mM TEAA Buffer pH 7.0
Mobile Phase B: 80% A, 20% ACN

Flow Rate: 0.2 mL/min
Temp: 60°C



Column

A 1000Å, 2.7µm, ES-C18, 2.1x150mm HALO® (Advanced Materials Technology, Inc. Wilmington, DE) column was used for the initial separation.

Separation of Oligonucleotide Ladder on 1000Å ES-C18

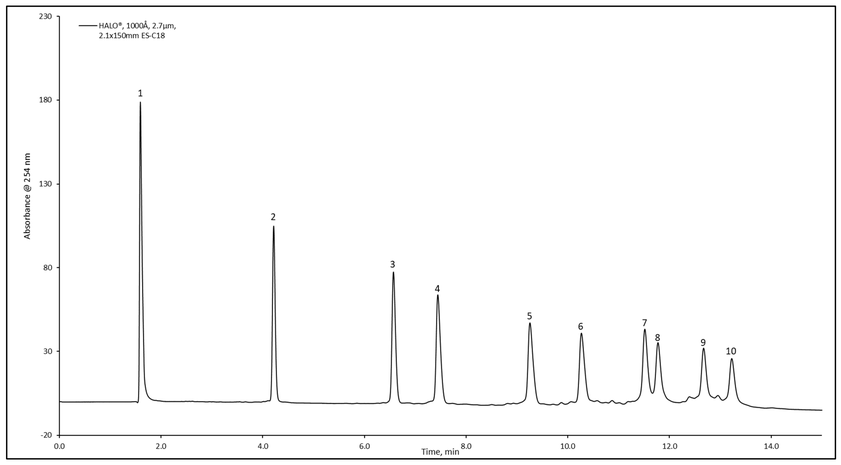
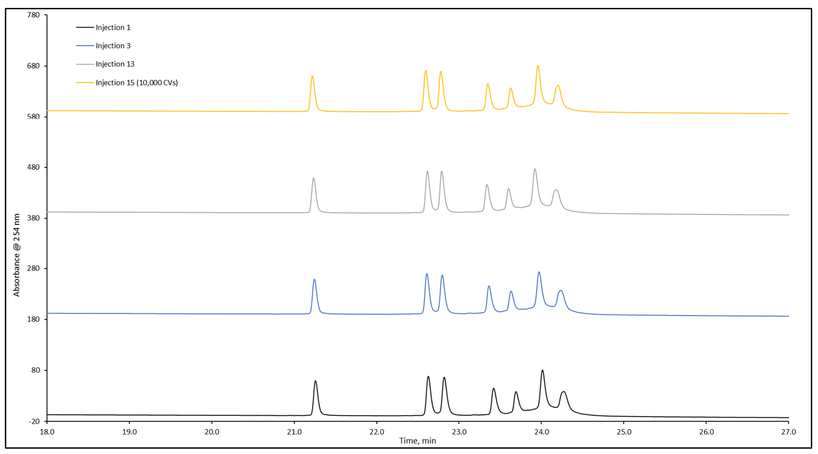


Figure 3: A separation of a 20/100 oligonucleotide ladder on a HALO 1000Å, 2.7µm, ES-C18 2.1x150mm column

HALO 1000Å ES-C18 shows great retention and resolution of the larger oligonucleotides. The 1000Å pore size allows for better peak shapes of the larger oligonucleotides which elute after the 6 minute mark. The ladder being separated shows 10 peaks with the extra peak potentially being an impurity or intermediate oligonucleotide.

*We are currently in the process of determining the peak identities of the ladder as well as any impurities or potential extra oligonucleotides within the sample.

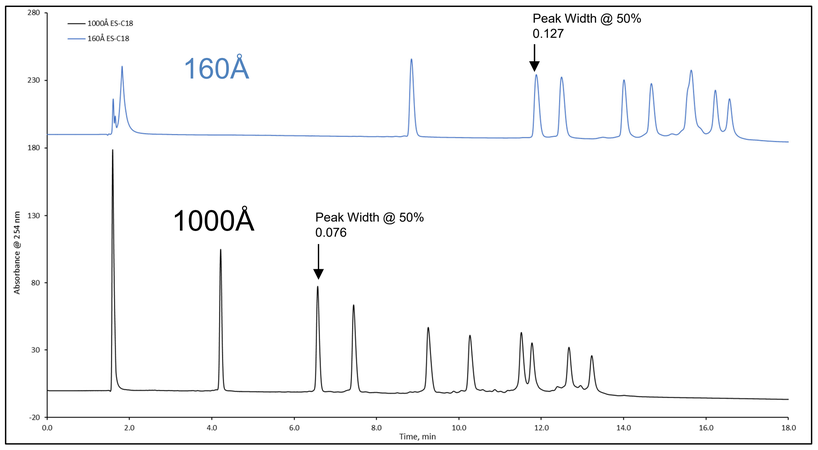
1000Å ES-C18 Stability



A HALO 1000Å ES-C18 column shows excellent stability using an oligonucleotide ladder ranging from 20 to 100 bases in length. No change in back pressure was observed and retention time and peak shape were constant over the course of 10,000 column volumes.

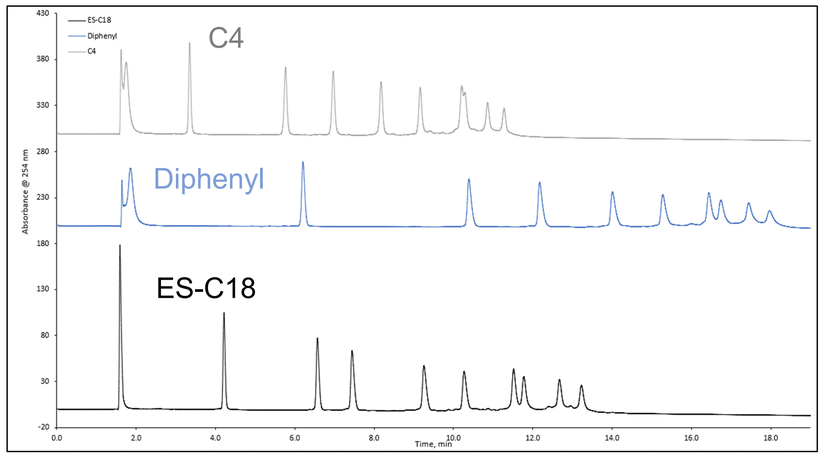
Stability Test Conditions:
Column: HALO 1000Å ES-C18, 2.7µm, 2.1x30mm
Part Number: 92712-302
Mobile Phase A: 100mM TEAA Buffer pH 7.0
Mobile Phase B: Methanol
Flow Rate: 0.25 mL/min (Sample Injection)
0.80 mL/min (Stability Gradient)
Temperature: 50°C
Detection: UV, 254nm
Initial Back Pressure: 49 bar
Final Back Pressure: 49 bar
Injection Volume: 1.0µL
Sample: 20/100 Ladder (IDT)

HALO® ES-C18 Pore Size Comparison



A pore size screening was performed using the 20/100 oligonucleotide ladder to evaluate the effect of pore size on small and large oligonucleotides. The 160Å column has increased retention of the larger nucleotides which increases the peak widths. The increase in peak widths causes 2 nucleotides to coelute, possibly due to the large oligos navigating through pores that are too small. The increased retention of the smallest nucleotide on the 160Å is due to the greater surface area (90 m^2/g) of the 160Å pore size particle compared to the surface area of the 1000Å pore size particle (22 m^2/g).

1000Å Phase Comparison of Oligonucleotide Ladder



A 20/100 ladder was run on three different 1000Å phases in 2.1x150mm column dimension. The ES-C18 phase (black trace) has the best separation of the larger oligos. The phase maintains peak shape while retaining the oligos up to 14 minutes over a 20 minute gradient. The Diphenyl phase (blue trace) has the most retention on the oligos, but with increased retention the peak shape is less symmetrical for the later eluters. The peak shapes observed with the Diphenyl could potentially be fixed with a change in mobile phase or gradient conditions. Finally the C4 phase (gray trace) with shorter alkyl chains has reduced retention of the longer chain oligonucleotides which improves peak shape. The decreased retention time of the C4 phase comes at the cost of coelution between peaks 7 and 8.

Conclusions

Over the past few years, with the emergence of Covid-19, oligonucleotide research and characterization has become a topic of increasing interest. The current knowledge surrounding oligonucleotides is developing and successful synthesis of these nucleotides requires a strong understanding. In order for scientists and corporations to confirm the synthesis for lab and drug ready oligonucleotides, robust chromatography methods are necessary. Chromatographers can take advantage of multiple large pore size column chemistries in order to obtain improved chromatographic results when running oligonucleotides. As we learn more, we may be able to increase the successful synthesis of larger oligonucleotides, expanding the possibilities of these drugs potentially saving many lives in the process. In order to confirm the synthesis of the larger oligonucleotides, large pore size column chemistries can be crucial for the successful separations of the oligonucleotides.