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Application Note

Separation of Short and Long Chain Oligonucleotides Through the Use of Large Pore Diameter, Core-Shell UHPLC Columns

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Introduction

The significance of oligonucleotides in medical research and chromatography is on the rise. siRNA, aptamers, and other oligomers are increasingly utilized in treatments, contributing to the management of genetic disorders such as Duchenne muscular dystrophy, acute porphyria, and familial amyloid neuropathy. To facilitate the advancement of these treatments, successful UHPLC analysis of nucleotides is now paramount. Nevertheless, the diverse chemical structures and molecular weights of oligos pose challenges to achieving optimal separation and resolution during the process.

Separating nucleotides, especially those with multiple negative charges, can be particularly challenging. This difficulty intensifies when trying to distinguish smaller nucleotides like siRNA (7 kDa) from larger ones like sgRNA (32 kDa). However, utilizing a wide-pore, 1000 Å column proves effective in accurately separating longer chain nucleotides, providing high-efficiency peak shapes and enhanced sensitivity.

When dealing with short oligonucleotides, columns with short alkyl chains like C4 do not retain them well. A more suitable approach involves selecting hydrophobic chains such as C18, which improves the retention and resolution of nucleotides. By combining hydrophobic groups with larger pore sizes, the resolution of longer oligomers is further enhanced.

Incorporating large pore size columns into UHPLC assays enables the successful separation of siRNA and sgRNA, overcoming the challenges associated with their different lengths and molecular sizes. This application note demonstrates the use of such a column (BIOshell™ IgG 1000 Å C18) in being able to resolve oligonucleotides of different chain lengths.

Experimental

The sample employed in this study was a 20/100 oligonucleotide ladder. The oligonucleotide ladder samples were acquired from IDT in a dried form. To prevent any loss of sample, the 10 µg sample underwent centrifugation. Following this, the sample was reconstituted in 200 µL of a 10 mM Tris-EDTA solution at pH 8.0. Once reconstitution was complete, the sample was injected for further analysis. All chromatographic assays were performed on a Shimadzu Nexera X2 UHPLC system. The chromatographic conditions used for this work are listed in **Table 1** below.

Table 1: Chromatographic Conditions used for the Analysis of Oligonucleotides

Column:	BIOshell™ IgG 1000 Å C18, 150 x 2.1 mm I.D., 2.7 µm
Mobile Phase:	[A] 100 mM TEAA, pH 7.0; [B] 80:20 Mobile Phase [A] : Acetonitrile
Gradient:	40% B to 62.5% B in 20 min; 62.5% B to 100% B in 2 min; hold at 100% B for 1 min
Flow Rate:	0.2 mL/min
Column temp.:	60 °C
Detector:	UV, 254 nm
Sample:	As indicated in text

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Results

As depicted in **Figure 1**, BIOshell™ IgG 1000 Å C18 column demonstrates excellent retention and resolution of the larger oligonucleotides. The utilization of a 1000 Å pore size contributes to improved peak shapes for these larger oligonucleotides, which elute after the six-minute mark. The separated ladder displays 10 distinct peaks (one being the Tris buffer).

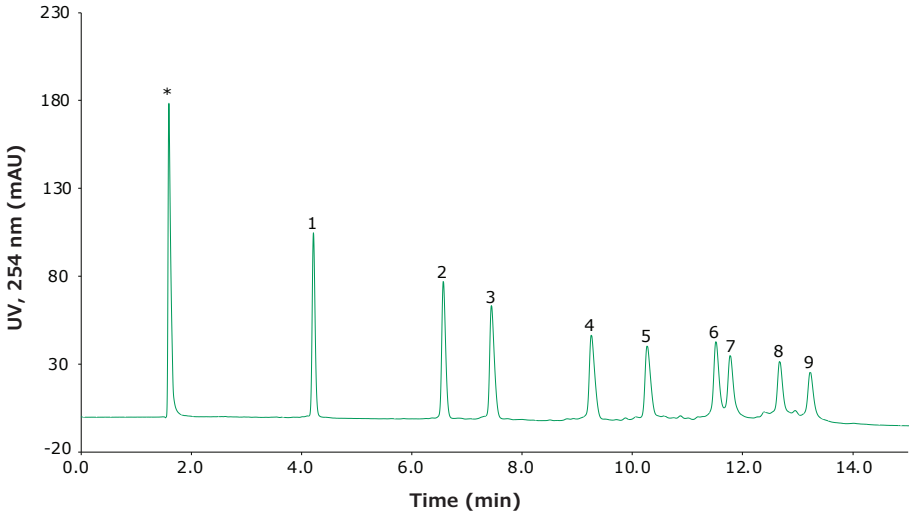

Figure 1: Separation of a 20/100 oligonucleotide ladder using a BIOshell™ IgG 1000 Å C18 column. Elution order 1) 20 Mer; 2) 30 Mer; 3) 40 Mer; 4) 50 Mer; 5) 60 Mer; 6) 70 Mer; 7) 80 Mer; 8) 90 Mer; 9) 100 Mer; * is the Tris buffer

To assess the impact of pore size on small and large oligonucleotides, a pore size screening experiment was conducted using the 20/100 oligonucleotide ladder (**Figure 2**). The 160 Å column, which had the same particle size and ligand, exhibited enhanced retention of the larger nucleotides, resulting in broader peak widths. Consequently, this led to the coelution of two nucleotides, likely because the large oligos encountered pores that were too small to traverse adequately.

The increased retention of the smallest nucleotide on the 160 Å column can be attributed to the larger surface area (90 m^2/g) of the 160 Å pore size particle, in contrast to the surface area of the 1000 Å pore size particle (22 m^2/g). This disparity in surface area affects how the smallest nucleotide interacts with the column and influences its elution behavior during the separation.

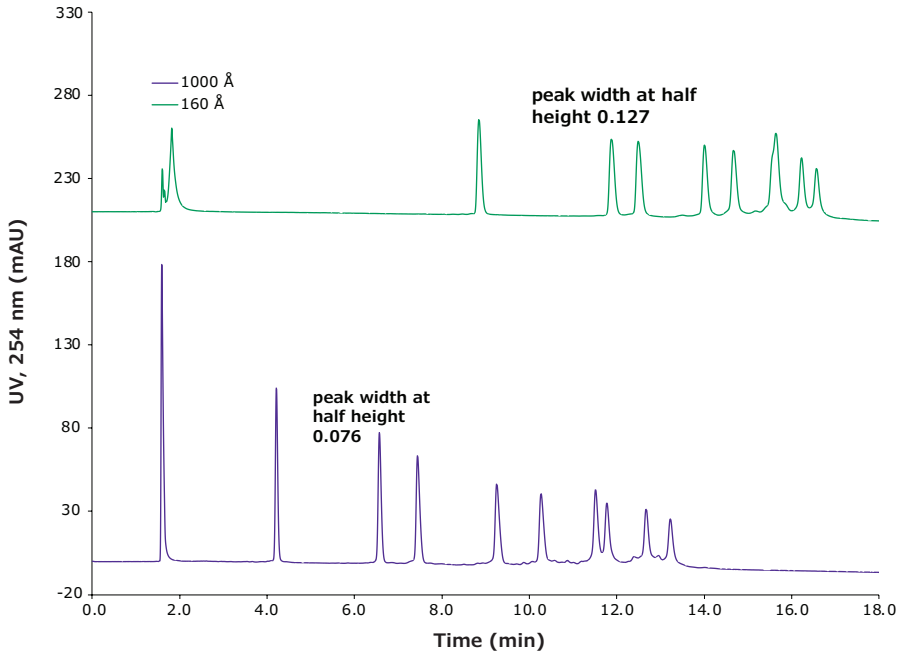

Figure 2: Comparison of columns of similar geometry, particle size, and chemistry, but with different pore diameters, in the resolution of the oligonucleotide ladder. Notice the much narrower peak widths at half height on the 1000 Å column versus the 160 Å column.

To investigate the role column chemistry has on oligonucleotide analysis, an analysis of the 20/100 oligonucleotide ladder was conducted using three different 1000 Å phases in a 150 x 2.1 mm I.D. column geometry (**Figure 3**). Among these phases, the C18 phase (blue trace) exhibited the most favorable separation of the larger oligos. This column maintained high efficiency, allowing the oligos to be retained for up to 14 minutes during a 20-minute gradient.

On the other hand, the Diphenyl phase (purple trace) displayed the highest retention of oligos, but this increased retention compromised peak symmetry for late-eluting analytes. This issue might be remedied by adjusting the mobile phase or gradient conditions.

Meanwhile, the C4 phase (green trace), characterized by shorter alkyl chains, showed reduced retention of longer chain oligonucleotides, resulting in improved peak shapes. However, this decreased retention time of the C4 phase led to co-elution between peaks 6 and 7 which may necessitate further optimization.

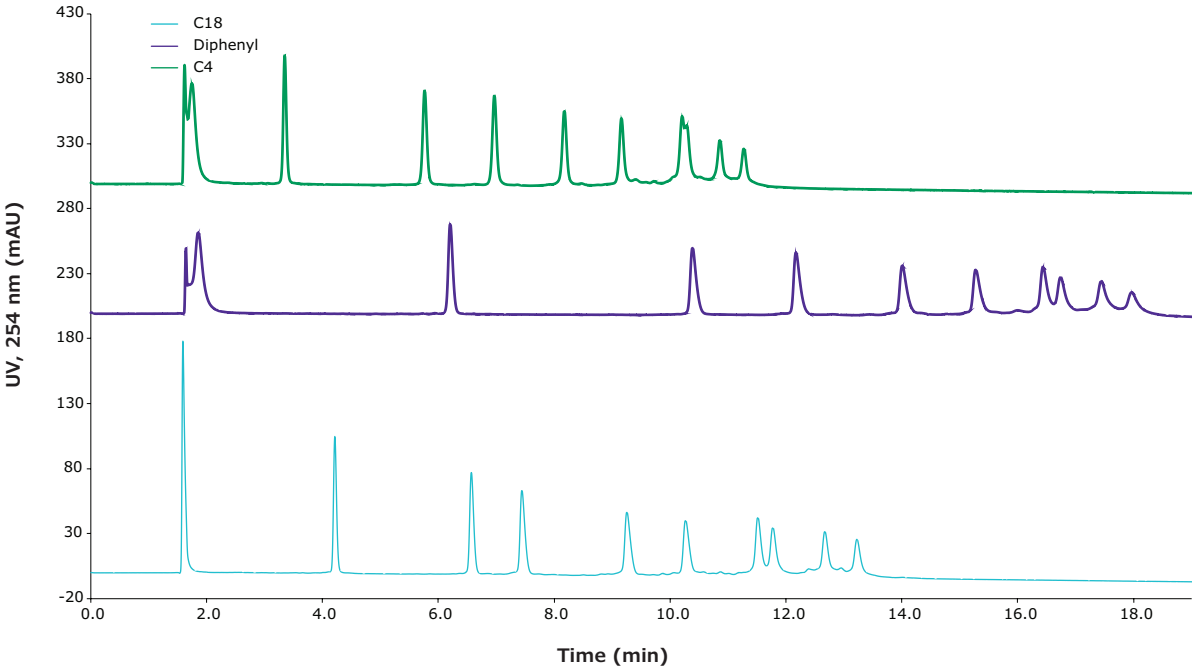

Figure 3: Analysis of oligonucleotide ladder on three different BIOshell™ IgG columns.

Conclusion

In recent years, the emergence of COVID-19 has sparked a growing interest in oligonucleotide research and characterization. The understanding of oligonucleotides is continuously evolving, and their successful synthesis demands a profound comprehension of the subject. To validate the synthesis of lab and drug-ready oligonucleotides, robust chromatography methods are essential for both scientists and corporations.

Chromatographers can capitalize on the utilization of multiple large pore size column chemistries to achieve enhanced chromatographic outcomes when working with oligonucleotides. As our knowledge expands, we have the potential to improve the successful synthesis of larger oligonucleotides, opening new possibilities for drug development and potentially saving countless lives in the process.

Large pore size column chemistries play a crucial role in confirming the synthesis of larger oligonucleotides, enabling successful separations, and facilitating advancements in oligonucleotide-based therapeutics.

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Description	Cat. No.
BIOshell™ IgG 1000 Å C18, 150 x 2.1 mm I.D., 2.7 µm	**582703-U**
BIOshell™ IgG 1000 Å C4; 150 x 2.1 mm I.D., 2.7 µm	**63289-U**
BIOshell™ IgG 1000 Å Diphenyl; 150 x 2.1 mm I.D., 2.7 µm	**577421-U**
BIOshell™ A160 Peptide C18; 150 x 2.1 mm I.D., 2.7 µm	**66905-U**
Water, for chromatography (LC-MS grade), LiChrosolv®	**1.15333**
Acetonitrile, hypergrade, for LC-MS LiChrosolv®	**1.00029**
Triethylammonium acetate buffer, suitable for HPLC, 0.98 - 1.02 M	**69372**
Tris-EDTA buffer solution, BioUltra, for molecular biology, pH 8.0	**93283**

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