Chromatography for the Analysis of Oligonucleotides

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With increasing medicinal applications of therapeutic oligonucleotides, and several candidates currently in clinical trials, there is the need to perform analytical characterisation of these drugs, which can be challenging. Two high-resolution chromatographic options for this analysis are the use of either non-porous anion exchange (AEX) or porous ion pair reverse phase (IPRP) UHPLC. Both approaches have their own advantages and also offer differences in selectivities. Here we present chromatographic methodologies for the separation of;

- **single stranded DNA,**
- **failure sequences,**
- **fluorescent dye-labelled DNA,**
- **large double-stranded DNA fragments.**

Introduction

Synthetic oligonucleotide therapeutics are widely used for a variety of applications including DNA amplification and sequencing, in-situ hybridisation, gene silencing and molecular diagnostics. These drugs span a range of oligonucleotide classes, indications, and routes of administration. Synthetic oligonucleotides have motivated drug developers with the promise of rational drug design, lower drug development costs, and the ability to reach targets that conventional small molecule drugs cannot. They also have now emerged as promising therapeutic candidates for diseases including cancer, viral infections, Alzheimer's disease and cardiovascular disorders. Many therapeutic oligonucleotides, including antisense, aptamers and small-interfering RNAs (siRNAs) are being developed. This has created an increased demand for the analysis of these synthetic nucleotide products beyond previous requirements.

The oligonucleotide field is approaching a critical turning point with numerous therapeutics in phase 3 clinical trials. Within the next few years, regulatory approval of multiple oligonucleotide therapeutics seems likely [1]. New start-up companies with novel technologies will likely continue to propel the field forward and fuel the development pipeline with new therapeutic options.

Oligonucleotides for molecular and therapeutic applications demand high resolution purity analysis, as well as identification and quantification of any structural impurities. Therefore, quality control of these synthetic oligonucleotides is critical, requiring stable, high resolution tools to deliver the analytical evaluations required.

siRNA can be used to control translation, and hence the amount of specific target proteins that contribute to disease states. RNA aptamers can have very high binding affinities to specific targets, essentially making them the oligonucleotide analogue of a monoclonal antibody. To be effective in the body these oligonucleotides must be resistant to endogenous nuclease attack that would quickly degrade them. To achieve this, they can be chemically modified by thiolation of the backbone to resist enzymatic breakdown and produce a therapeutically viable biological half-life, which generates further demands on the analytical characterisation of these molecules.

Options for Characterisation

With the increase in medicinal applications, the analytical characterisation required by the regulatory bodies has become more stringent. The higher resolution options for this analysis have proven to be either non-porous anion exchange (AEX) or ion-pair reversed phase (IPRP) UHPLC. Both have their own advantages and differences in selectivities. Because of this, many laboratories employ the use of both techniques to ensure complete characterisation. IPRP has the advantage of direct coupling to mass spectroscopy (MS) [1]. In this methodology overview, we present:

- Fast, High Resolution Separation of Single Stranded DNA
- Separation of Failure Sequences
- Separation of Fluorescent Dye-Labelled DNA
- Separation of Large Double-Stranded DNA Fragments

Fast, High Resolution Analysis of ss- and ds-Nucleic Acids using Reversed-phase UHPLC

Synthetic oligonucleotides are used extensively in laboratories as primers for polymerase chain reactions (PCR) and DNA sequencing, probes to visualise a specific DNA or RNA, tools to study gene function, and biopharmaceutical drugs for treating various diseases [1,2]. Analyses of synthetic oligonucleotides are most commonly performed using ion-pair reversed-phase

chromatography (IP-RP) [4,5] IP-RP utilises ionic interaction between analytes and the ion-pair reagent and the hydrophobic interaction between the stationary phase and the ion-pair reagent. IP-RP provides high-resolution separation of failure sequences and can be directly coupled to mass spectrometry for identification of target oligonucleotides and any related impurities. Here, we look at the HPLC-UV [6] analysis of oligonucleotides using the Thermo Scientific™ DNAPac™ RP column.

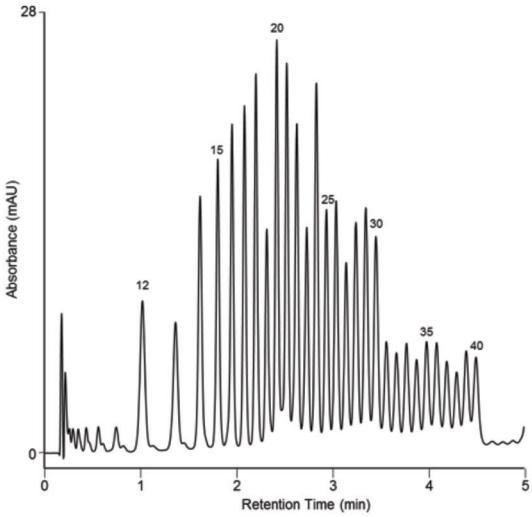
The DNAPac RP column [6] is a reversed-phase column designed for the high resolution separation of oligonucleotides and their failure sequences. These include oligonucleotides with modifications designed to improve stability in biological fluids, such as ribose modification, phosphorothioate (PS) substitution and linkage isomers. This column also supports gel-like separation of dsDNA fragments generated by restriction enzymes for cloning and next generation or high throughput sequencing (NGS, HTS). The column is based on a hydrophobic, polymer resin, so it is stable under high pH and/or high temperature conditions which often provide higher resolution for challenging oligonucleotide samples. In addition, the particles harbour a wide pore size (1000~2000 Å).

The wide pores on the DNAPac RP column have been found to deliver excellent separation of large, double-stranded nucleic acids, up to 10k base pairs, with low carry-over.

The hydrophobic nature of the resin comes from phenyl groups within the Poly(styrene-divinylbenzene) particles. Therefore this column provides alternative selectivity to the conventional silica based C18 columns.

Fast, High Resolution Separation of Single Stranded DNA

It is generally accepted that the ion-pair reagent, such as trimethylamine, interacts with the non-polar stationary phase via hydrophobic interaction which in turn acts as an ion-exchange site for the negatively charged oligonucleotides [1]. At pH values between 6 and 8, standard oligonucleotides have one negative charge for each phosphodiester bond. Therefore, a terminally non-phosphorylated oligonucleotide will harbour a charge equal to one less than the number of bases. Since the number of phosphodiesters, and thus the number of IP reagent molecule interactions is proportional to the length of the oligonucleotide, longer oligonucleotides elute later at neutral pH values. As the oligonucleotides get longer, the percent charge difference between oligonucleotides decreases, so resolution between oligonucleotides decreases with oligonucleotide length. The separation of single stranded poly-deoxythymidine (dT) using IP-RP is illustrated in Figure 1 and Figure 2. Fast Separation of 12-40mer Deoxythymidines (dTs).

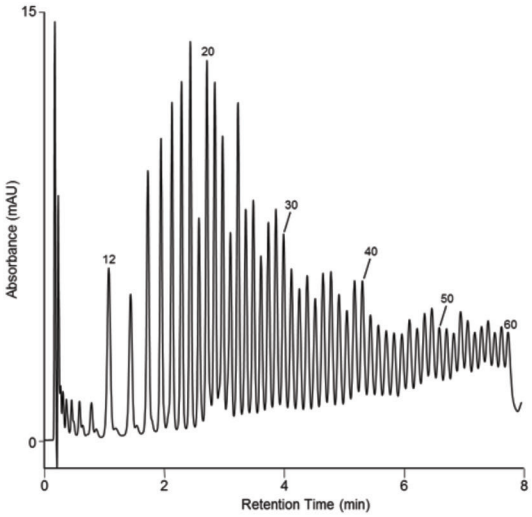


Column:	DNAPac RP, 4 µm
Format:	2.1 × 50 mm
Mobile phase A:	0.1 M TEAA, pH 7.0
Mobile phase B:	0.1 M TEAA in Water / Acetonitrile (75:25 v/v)
Gradient:	

Time (min)	%A	%B
-2.0	76	24
0.0	76	24
4.0	59	41
4.1	10	90
5.0	10	90

Gradient curve:	3
Flow rate:	0.80 mL/min
Inj. volume:	4 µL
Temperature:	80 °C
Detection:	UV (260 nm)
Sample:	poly dT12-40 (1 A/mL)
Peak label:	Length of DNA

Figure 1. Fast Separation of 12-40mer Deoxythymidines (dTs).

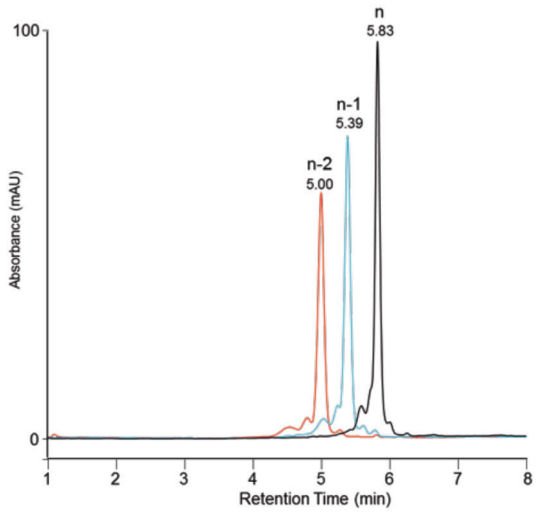


Column:	DNAPac RP, 4 µm
Format:	2.1 × 50 mm
Mobile phase A:	0.1 M TEAA, pH 7.0
Mobile phase B:	0.1 M TEAA in Water / Acetonitrile (75:25 v/v)
Gradient:	

Time (min)	%A	%B
-2.0	76	24
0.0	76	24
7.0	57	43
7.1	10	90
5.0	10	90

Gradient curve:	3
Flow rate:	0.80 mL/min
Inj. volume:	10 µL
Temperature:	80 °C
Detection:	UV (260 nm)
Sample:	poly dT12-60 (0.5 A/mL)
Peak label:	Length of DNA

Figure 2. High Resolution Separation of 12-60mer dTs.



Column:	DNAPac RP, 4 µm
Format:	2.1 × 50 mm
Mobile phase A:	0.1 M TEAA, pH 7.0
Mobile phase B:	0.1 M TEAA in Water / Acetonitrile (75:25 v/v)
Gradient:	

Time (min)	%A	%B
-6.0	80	20
0.0	80	20
8.0	56	44
8.1	10	90
10.0	10	90

Flow rate:	0.25 mL/min
Inj. volume:	10 µL
Temperature:	60 °C
Detection:	UV (260 nm)
Sample:	1) 23mer DNA (n-2) 2) 24mer DNA (n-1) 3) 25mer DNA (n)
Peak label:	Ret. time

Figure 3. Separation of failure sequences.

A poly-dT 12-40mer was separated at high temperature and high flow rate with a 4-minute gradient using triethylammonium acetate (TEAA) as mobile phase. In addition, up to 60mer poly-dT oligonucleotide was separated using a 7-minute gradient. In both cases, a convex gradient was used to maximise the resolution of the longer oligonucleotides in a shorter time.

Separation of Failure Sequences

Among the most common impurities in synthetic oligonucleotides are failure sequences. While each coupling step is highly efficient, the probability of coupling failure increases with each step. Hence, one or two nucleotide 'deletions' can be expected in most synthetic oligonucleotides. Figure 3 shows the separation of a 25mer DNA from its 5' deletion sequences (n-1 and n-2).

Separation of Fluorescent Dye-Labelled DNA

Fluorescent dye-labelled oligonucleotides are used in many applications such as DNA sequencing, PCR, DNA microarrays and in situ hybridisation. In most cases, attachment of a fluorophore increases the overall hydrophobicity of the oligonucleotide sample, resulting in stronger retention on a reversed-phase stationary phase. Figure 4 shows the separation of a fluorescein labelled oligonucleotide and its unmodified form. The unmodified oligonucleotide and other impurities were separated from the main dye-labelled oligonucleotide peak.

Separation of Large Double-Stranded DNA Fragments

Purification and sizing of large double-stranded DNA fragments is an important step in DNA cloning, PCR and for the preparation of DNA libraries for HTS or NGS. Traditionally, these dsDNA fragments are separated by agarose or acrylamide gels, and extracted from the gels for subsequent use. This process requires manual excision of the target DNA from the gel, and extraction of the DNA from the excised gel. This laborious and time consuming step generally produces yields of less than 50%. The DNAPac RP provides reliable separation and higher yields and DNA fragment collection is readily automated. Many reversed-phase columns advertised for such separations harbour small pores which restrict separation of dsDNA fragments over 600-1000 base pairs (bp). The wide-pore DNAPac RP resin produces resolution

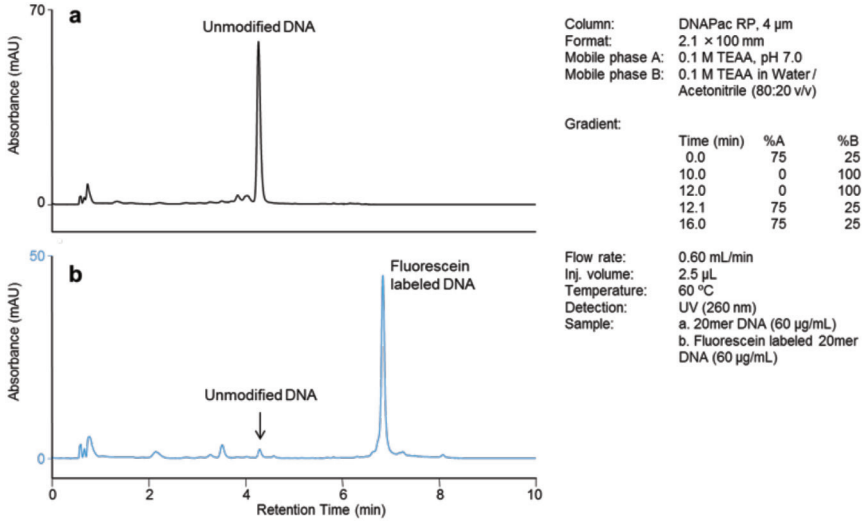


Figure 4. Separation of Fluorescein-Labelled DNA.

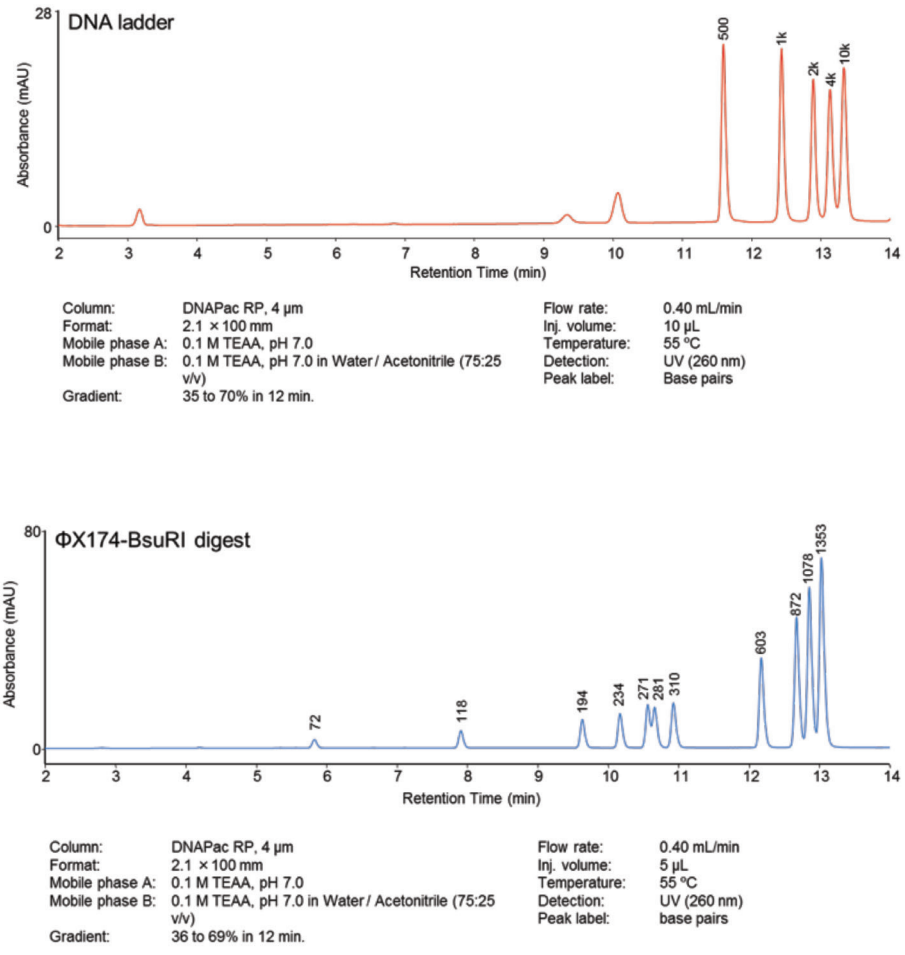


Figure 5. Separation of dsDNA fragments , showing a restriction enzyme digest and a DNA ladder. [2].

comparable to many DNA gels up to 10K bp, and in similar times. Figure 5 shows the separation of dsDNA fragments generated from a restriction enzyme (bottom panel) and a DNA ladder (top panel). Fragments ranging from 72 bp to 10,000 bp were separated on the DNAPac RP within 15 minutes [6].

Summary

The therapeutic use of oligonucleotides will likely arrive at the markets within the next few years if all goes well with current phase 3 trials, offering hope to those whom these therapies seek to help. With the complexity and analytical challenges that

these compounds pose, it is perhaps a relief to those who must perform characterisation and quality monitoring that there are robust column technologies available that afford both rapid and high resolution separations of a vast range of oligonucleotides, including single and double stranded DNA, those labelled with fluorescent dye, and failure sequences.

Here we have demonstrated modern and innovative chromatographic strategies for the analysis of this class of compounds for development and quality control purposes.

References

1. Offord, C., Oligonucleotide Therapeutics Near Approval, The Scientist, December 2016 http://www.the-scientist.com/?articles.view/articleNo/47499/title/Oligonucleotide-Therapeutics-Near-Approval/

2. Baek, J., Thayer, J., Wang, H., Birznieks, I., Liu, X., High Resolution LC/MS Analysis of Therapeutic Oligonucleotides on a New Porous Polymer-Based Reversed Phase Column, Poster: https://tools.thermofisher.com/content/sfs/posters/PN-21515-LC-MS-Therapeutic-Oligonucleotides-WCBP2016-PN21515-EN.pdf

3. Dias, N.; Stein, C.A. Antisense Oligonucleotides: Basic Concepts and Mechanisms, Molecular Cancer Therapeutics, 2002, 1, 347-355.

4. Resnier, P.; Montier, T.; Mathieu, V.; Benoit, J.P.; Passirani, C. A review of the current status of siRNA nanomedicines in the treatment of cancer, Biomaterials, 2013, 34, 6429-6443

5. Jose V. Bonilla, G. Susan Srivatsa, Handbook of analysis of oligonucleotides and related products, p10, 2011 CRC Press

6. Baek, J., Thayer, J., Lin, S., Liu, X., Separation of Large Double-Stranded DNA (dsDNA) Fragments Using a Wide-Pore Reversed-Phase Chromatography Column, Application note: https://tools.thermofisher.com/content/sfs/brochures/AN-21533-LC-