DNAPac RP column

Wide-ranging polynucleotide separation capabilities using Reversed Phase particles with variable pore geometry

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Abstract

Purpose: Demonstrate the robustness of a 4µm polymer-based, Reversed Phase particle possessing a distribution of pore sizes that enables the separation of nucleic acids varying in length from 10's to 1,000's of nucleotides in length.

Methods: Ion-Pairing Reversed Phase (IP-RP) for both UV and MS detection are used to evaluate the separation of single stranded and double stranded nucleic acids with lengths ranging from 20-mer up to 1690-mer. Effects of temperature and pH on separation are investigated for analytical columns. Separation range and resolution for a 21.2x150mm semi-prep column at room temperature is also demonstrated.

Results: Polymeric, reversed phase particles with pores sizes from 50Å - 2500Å generally separate polynucleotide samples based on length and associated differences in structural hydrophobicity with mobile phase, temperature and pH capable of tuning resolution of minor variants. Analytical scale separation can be translated to the semi-prep scale.

Introduction

Polynucleotides such as DNA and RNA serve many uses in the areas of basic research, industrial manufacturing, therapeutics to up/down regulate cellular functions, and as therapeutic and vaccine intermediates (i.e., as an instructional template to produce proteins that 1) supplement/replace ineffective proteins or 2) function as antigens). CRISPR technology using guide RNA (gRNA, ~100mer) to directly modify cellular chromosomes was recently leveraged to create a customized treatment within 6-months for an infant with CPS1 deficiency[1]. This approach has provided long-term, life-saving enzyme production negating the need for daily treatments. Associated DNA and RNA such as gRNA and mRNA (1000+mer) require detailed characterization to ensure safety and efficacy (e.g., of sufficient quantity and fidelity in delivery vehicles such as lipid nanoparticles). Here, we present a RP particle with complex pore geometry and the effects of mobile phase and temperature for separating DNA/RNA with therapeutic benefits (siRNA-gRNA-mRNA:~ 20-100-1000+mer). Semi-prep column separations, common for sample recovery for subsequent analyses or sample enrichment for *in vitro* or *in vivo* use, is also shown.

Materials and methods

Sample

Reference text and figures for sample information. All samples were used as-received. Any required dilution of samples was done using 18.2 MΩ deionized water.

Columns

Thermo Scientific™ DNAPac™ RP column, 4 µm Formats (reference figures for specific format used)

Test method(s)

DNAPac RP columns packed with 4 µm DNAPac RP media include 2.1 × 50 mm (P/N 088924), 2.1 × 50 mm (P/N 088924), and 21.2 × 150 mm (P/N 080922-1521232). For instruments, mobile phases, gradient conditions, and other chromatographic operating parameters (flow rate, temperature, injection volume, etc.), please reference the figures and associated text. For all analyses, samples were detected by UV absorbance at 260nm. For 21.2x150mm semi-prep separations, a conventional (U)HPLC chromatography system with a binary pump, autosampler and detector without column compartment (ambient temperature separations) was used for all analyses. Ion Pairing Agents (Cation Anion): TEAA – Triethylamine Acetate, HAA – Hexylamine Acetate, HFIP – Hexafluoroisopropanol.

Data analysis

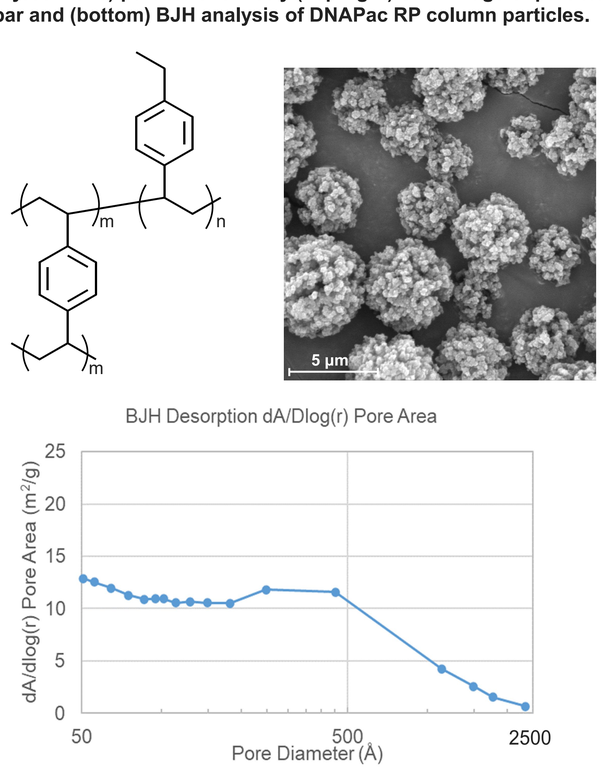
The Thermo Scientific™ Chromeleon™ 7.2.10 or 7.3 Chromatography Data Systems (CDS) software was used for data acquisition and analysis.

Results

DNAPac RP particle characterization – chemistry and pore structure

Figure 1 summarizes the chemical and physical properties of the resin, which is based on polymeric divinylbenzene (top left). Unlike conventional silica particles, the polymeric resin is not susceptible to hydrolytic degradation at neither extreme pH (1-14) nor high temperature (90+°C). The aromatic groups also provide π- π bonding with aromatic nucleotides giving different selectivity from conventional alkyl phases incapable of such interactions. The SEM image visually shows the highly variated porous structure of the particles. Barrett-Joyner-Halenda (BJH) N_2 adsorption analysis details the surface area contributions of the different pore sizes. From this analysis it is clear that a continuum of pores from 50Å to 2500Å (suitable for small molecules up to macromolecule adsorption, respectively) contributes to the overall surface area of the particle. Based on the complex geometry and resulting pore structure, we commonly refer to the media as a *supermacroporous* (SMP) particle.

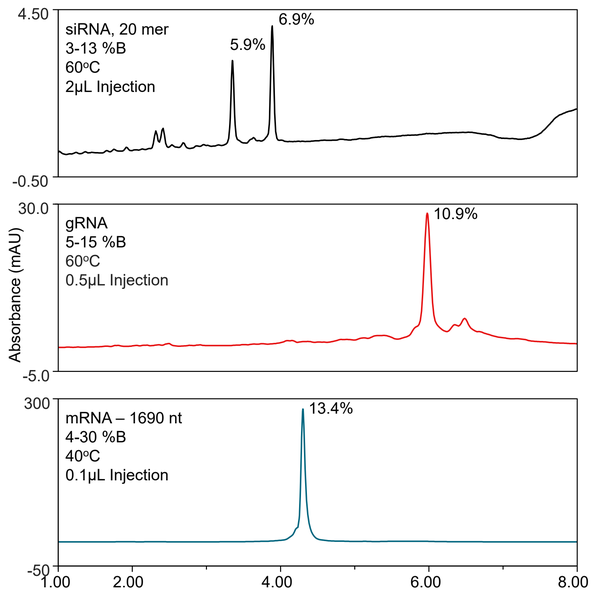
Figure 1 Particle properties overview showing (Top left) poly(divinylbenzene-co-ethylvinylbenzene) particle chemistry (Top right) SEM image of particles with 5µm scale bar and (bottom) BJH analysis of DNAPac RP column particles.



Variable pore size enables separation of short and long polynucleotides

In general, the elution order of polynucleotides is based on length with retention time being directly proportional to the length of the sample for HILIC, RP, and anion exchange chromatography[2]. Figure 2 shows the analysis of siRNA (top, 20 mer separated into sense and anti-sense oligos), gRNA (middle, ~100mer) and mRNA (bottom, 1690 nt) to highlight this principle with the peak labels indicating % acetonitrile (ACN) of sample elution. In contrast to what is achieved with the porous particle, silica is typically limited to smaller pore sizes (e.g., 300Å) limiting their ability to resolve sample lengths as they increase beyond approximately 100mer [2,3].

Figure 2 Separation of an (top) siRNA, (middle) gRNA, and (bottom) mRNA sample on a 2.1x50mm DNAPac RP column. Peak label indicates % mobile phase B/% acetonitrile at which the sample elutes.

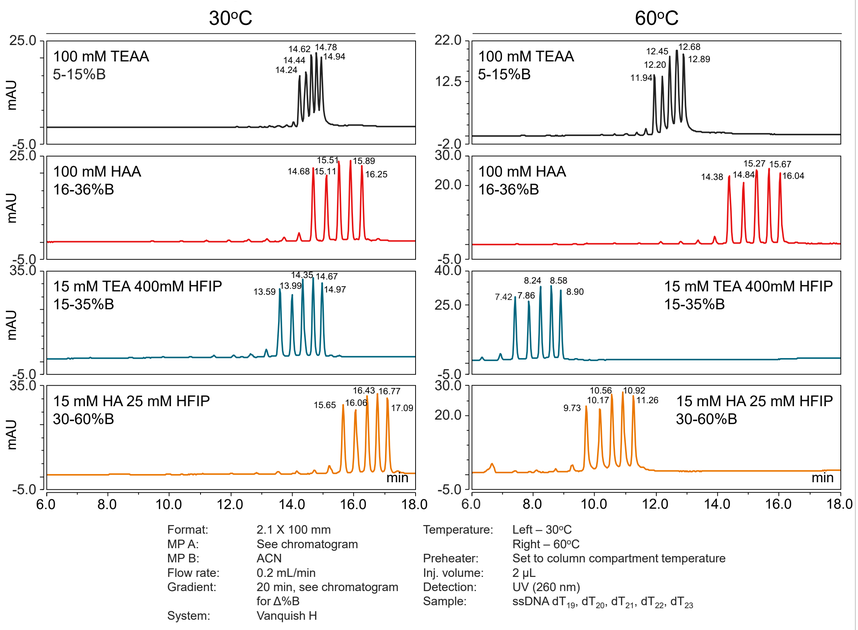


Format:	2.1 X 50 mm
Column:	Top: DNAPac RP, 4 µm
MP A:	0.1M TEAA
MP B:	ACN
Gradient:	See chromatogram
System:	Vanquish H
Temperature:	See chromatogram
Preheater:	Set to temperature
Flow rate:	0.4 mL/min
Inj. volume:	5 µL
Detection:	UV (260 nm)
Sample:	Top – siRNA, 20 µg/mL Middle – gRNA, 10 µM Bottom – mRNA 1690 nt 3.5 mg/mL

Mobile phase and temperature effects on separation

Choice of ion-pairing agent (IP) and temperature can significantly affect sample resolution as shown in Figure 3. The weak IP TEAA provides poor resolution with very shallow gradients relative to HAA with a steeper gradient for n+1 and other variants. Higher quantities of HFIP (a toxic chemical used for MS) are required with TEA to give similar resolution to HA IP systems. Temperature also influences separation with HA generally being less affected than TEA IP agents.

Figure 3 Separation of dT_{19-23} ssDNA sample mix on a 2.1x100mm column using different mobile phases common for UV and MS detection. Peak label indicates retention time of sample elution.

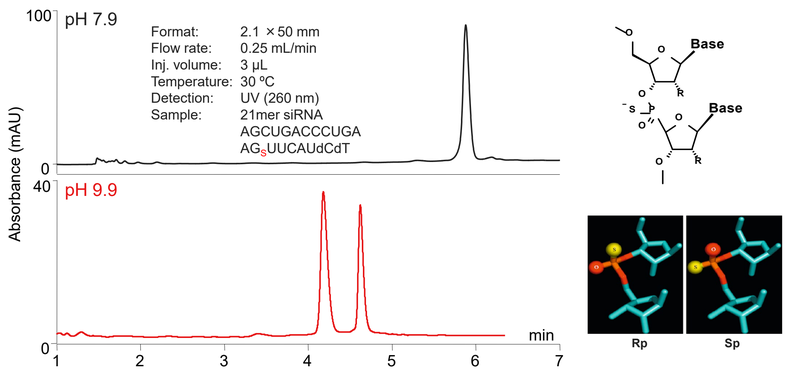


Format:	2.1 X 100 mm	Temperature:	Left – 30°C Right – 60°C
MP A:	See chromatogram		
MP B:	ACN	Preheater:	Set to column compartment temperature
Flow rate:	0.2 mL/min	Inj. volume:	2 µL
Gradient:	20 min, see chromatogram for Δ%B	Detection:	UV (260 nm)
System:	Vanquish H	Sample:	ssDNA dT_{19}, dT_{20}, dT_{21}, dT_{22}, dT_{23}

Mobile phase pH – tuning separation of stereoisomers

Mobile phase pH can be used to tune the separation to resolve stereoisomers. Figure 4 highlights this example showing pH 7.9 separation of an siRNA molecule possessing a single phosphorothioate bond with a mixture or Rp and Sp stereoisomers to yield a single peak. Increasing the pH to 9.9 resolves the two stereoisomers (MS data not shown confirms exactly equivalent masses).

Figure 4 Separation of 21-mer siRNA possessing a racemic mixture (Rp and Sp) at the phosphorothioate bond using a 2.1x50mm column with either pH 7.9 (top) or pH 9.9 (bottom) mobile phases common MS detection.



Top Chromatogram

Mobile phase A:	15 mM TEA, 400 mM HFIP, **pH 7.9**
Mobile phase B:	15 mM TEA, 400 mM HFIP in Water / Methanol (75:25 v/v)

Gradient:

Time (min)	%A	%B
0.0	65	35
5.0	42	58
5.1	10	90
7.0	10	90
7.1	65	35
13.0	65	35

Bottom Chromatogram

Mobile phase A:	35 mM TEA, 40 mM HFIP, **pH 9.9**
Mobile phase B:	35 mM TEA, 40 mM HFIP in Water / Methanol (75:25 v/v)

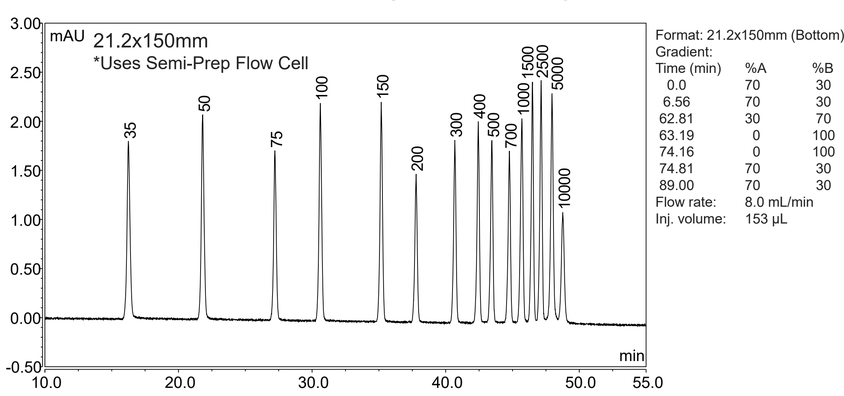
Gradient:

Time (min)	%A	%B
0.0	93	7
5.0	52	48
5.1	10	90
7.0	10	90
7.1	93	7
13.0	93	7

Separation power of SMP particle maintained for 21.2 X 150mm format

Figure 5 demonstrates the separation of a dsDNA sample mix containing analytes ranging from 35 base pair (BP) up to 10,000 base pair. As demonstrated for the analytical columns, the SMP particle separates peaks in excess of 100 BP in length. Here, the column is run at a reduced scaled linear flow rate relative to the analytical format. Despite the reduction in flow rate, peaks remain well resolved indicating efficient mass transfer to and from the surface of the complex porous topology of the SMP particles. A benefit of the reduce sample flow rate is increased sample concentration when collected post-column.

Figure 5. Evaluation of dsDNA separation ranging from 35 – 10,000 BP on 21.2x150mm columns. Sample: 0.03 mg/mL for each analyte.



Format: 21.2x150mm (Bottom)
Gradient:

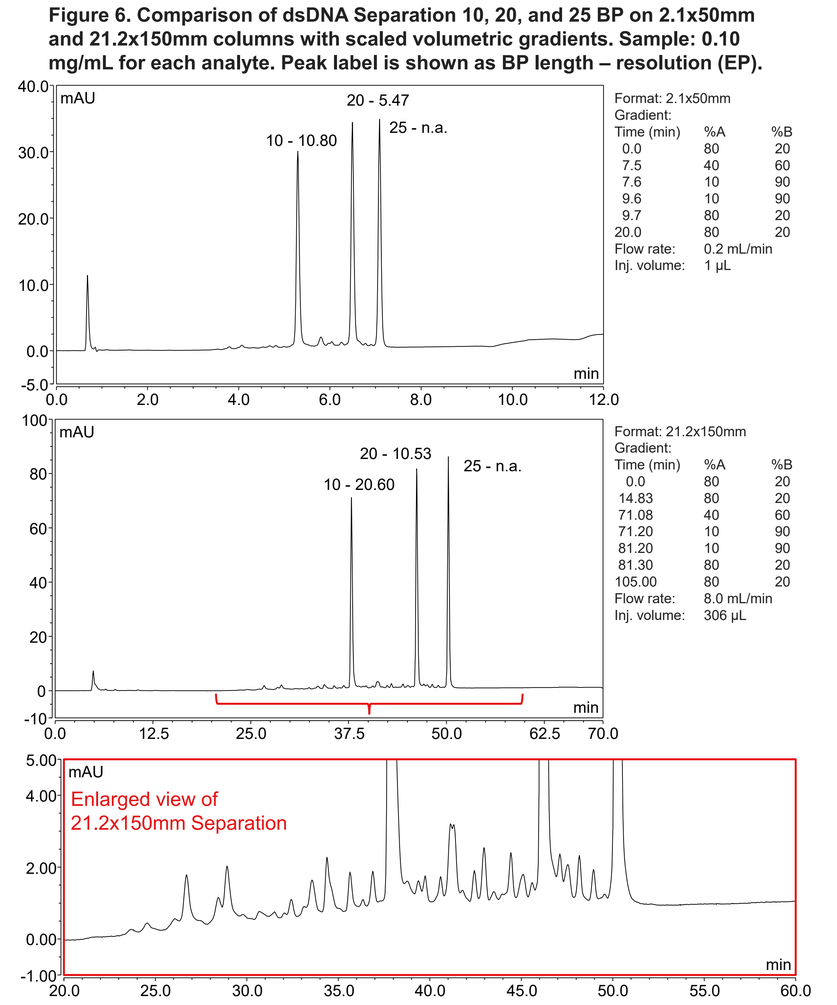
Time (min)	%A	%B
0.0	70	30
6.56	70	30
62.81	30	70
63.19	0	100
74.16	0	100
74.81	70	30
89.00	70	30

Flow rate: 8.0 mL/min
Inj. volume: 153 µL

Analysis of short (10, 20, 25 BP) dsDNA

Purification and sample recovery are essential applications for semi-prep and prep columns. Samples typically have a multitude of variants/impurities due to errors in the manufacturing process or latent modification of the sample intended or unintended. Evaluation of these variants and the fidelity of the target molecule is critical to understanding the manufacturing process and necessitates high resolution separations to collect these low abundance species. Figure 6 shows the scaling of an analytical method from a 2.1x50mm column to a 21.2x150mm semi-prep column with preservation of the volumetric gradient based on column bed volume. Despite the reduced linear flow rate, the 21.2x150mm column achieves equivalent if not superior resolution (partially attributable to column length) as highlighted in the red-boxed enlarged view for the semi-prep column. This data highlights the separation power of the SMP media for short oligos as well as long oligos shown above.

Figure 6. Comparison of dsDNA Separation 10, 20, and 25 BP on 2.1x50mm and 21.2x150mm columns with scaled volumetric gradients. Sample: 0.10 mg/mL for each analyte. Peak label is shown as BP length – resolution (EP).



Format: 2.1x50mm
Gradient:

Time (min)	%A	%B
0.0	80	20
7.5	40	60
7.6	10	90
9.6	10	90
9.7	80	20
20.0	80	20

Flow rate: 0.2 mL/min
Inj. volume: 1 µL

Format: 21.2x150mm
Gradient:

Time (min)	%A	%B
0.0	80	20
14.83	80	20
71.08	40	60
71.20	10	90
81.20	10	90
81.30	80	20
105.00	80	20

Flow rate: 8.0 mL/min
Inj. volume: 306 µL

Conclusions

- High resolution separation of nucleic acids varying in length from 10 to 10,000 nucleotides is achieved using an SMP particle with a variable pore structure.
- Polymeric SMP particles are compatible with different mobile phase IP compositions, high pH and high separation temperature, which are critical parameters for optimizing the separation of polynucleotides
- SMP particles provide high resolution separations in both analytical and semi-prep formats

References

1. K. Musunuru, M.D., et al., Patient -Specific In Vivo Gene Editing to Treat a Rare Genetic Disease. *New England Journal of Medicine*, 2025.
2. J. Maurer; Malburet, C.; Francois-Heude, M.; Guillarme, D.; Evaluation of ion pairing reversed-phase liquid chromatography for the separation of large RNA molecules. *Journal of Chromatography A* 1740 (**2025)** 465574
3. Ma, K. et al., (June 2023). *A versatile reversed phase platform for short, intermediate, and long nucleic acid analysis*, HPLC 2023, Duesseldorf, Germany.

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