Improved LC Separations of Nucleic Acids Using Large Pore Superficially Porous Particles

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Introduction

Problem: Polynucleotides are a rapidly developing therapeutic and diagnostic molecule class with significant separations demands, particularly for longer sequences, and with an expanding repertoire of useful chemical modifications. Resolution of closely related variants could be improved by directed column packing materials design.

Approach: Employ recently developed silica surface hybrid superficially porous particles (SPP) to improve ion pair reversed phase (IP-RP) separations of nucleic acids.

Ion Pairing Reversed Phase Conditions for Polynucleotides

Ion pairing RP for separations of oligonucleotides and other polynucleotides has been in common use for more than 3 decades, but with limited specific design of column packing materials. The range of particle materials and pore sizes has been limited. SPP silica column packing materials stable in the typical mildly alkaline conditions (pH 8-10) and elevated temperatures in common use for polynucleotide separations have recently been developed by us. Previous results from analysis of the benefits of SPP for separations of small molecules as well as larger biological molecules imply that polynucleotide separation efficiency will benefit from the improved mass transfer properties of SPP of appropriate pore size and other particle features. Highly efficient separations using SPP materials allow lower back pressure, and thus longer columns. We show examples wherein very high resolution can be obtained with longer columns.

A broadened range of ion pairing reagents has recently been favored, particularly for DNAs and RNAs longer than about 30 nucleotides (nts). Initially, acetate was used for pH control, but for several reasons, hexafluoroisopropanol (HFIP) is favored as the counterion/additive for IP reagents. We show examples of triethylamine (TEA/acetate and TEA/HFIP), as well as diisobutylamine (DiBA/HFIP) for separations.

Experimental Materials and Conditions

Reagents were obtained from Millipore-Sigma (TEA, 1M TEAA solution, pH 7.0, DiBA) or TCI (TEA, HFIP). LC instruments were Shimadzu Nexera 30 series, using the 20 Series column oven. LC/MS analysis used the Thermo Orbitrap QE/HF hybrid quadrupole. Columns of larger pore fully porous particle (FPP) columns were commercially obtained. SPP columns are produced in house using surface deactivated steel hardware. An Optimize EXP2 Inline filter (0.2 µm) was in front of the columns. Calculations of plates and resolution employed the USP method in Lab Solutions v5.96 SW. Synthetic oligonucleotides (DNA, RNA) were supplied by IDT, or for 30-mer and 90-mer standards, individually purified using 100 mM TEAA (pH 7.0)/acetonitrile with a 1.0 x 10 cm Halo Oligo C18 semi-prep column. Identities were confirmed by online MS analysis, agreeing within 5-10 ppm.

10/60 Standard

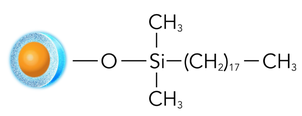
Peak #	Base Length	Sequence
1	10	ATC GCG GAT T
2	15	GCT GCG ACG AGG CTG
3	20	ATC GCG GAT TAG CAC TAC GT
4	25	ATC TCG GAT TAG CAC TAC GCA TCG G
5	30	ATC GCG GAT TAG CAC TAC GCA TCG GTT ACA
6	40	ATC GCG GAT TAG CAC TAC GCA TCG GTT ACA AAC GAG TAC C
7	50	ATC GCG GAT TAG CAC TAC GCA TCG GTT ACA AAC GAG GAC CTG ATG CAC TT
8	60	ATC GCG GAT TAG CAC TAC GCA TCG GTT ACA AAC GAG GAC CTG ATG CAC TTT GAC AGC ATG

20/100 Standard

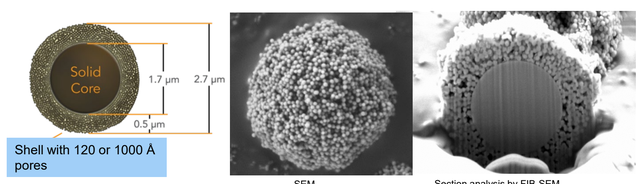
Peak #	Base Length	Sequence
1	20	ATC GCG GAT TAG CAC TAC GT
2	30	ATC GCG GAT TAG CAC TAC GCA TCG GTT ACA
3	40	ATC GCG GAT TAG CAC TAC GCA TCG GTT ACA AAC GAG TAC C
4	50	ATC GCG GAT TAG CAC TAC GCA TCG GTT ACA AAC GAG GAC CTG ATG CAC TT
5	60	ATC GCG GAT TAG CAC TAC GCA TCG GTT ACA AAC GAG GAC CTG ATG CAC TTT GAC AGC ATG
6	70	ATC GCG GAT TAG CAC TAC GCA TCG GTT ACA AAC GAG GAC CTG ATG CAC TTT GAC AGC ATG ATC GGA TGT A
7	80	ATC GCG GAT TAG CAC TAC GCA TCG GTT ACA AAC GAG GAC CTG ATG CAC TTT GAC AGC ATG ATC GGA TGT AAG TTC GAC GC
8	90	ATC GCG GAT TAG CAC TAC GCA TCG GTT ACA AAC GAG GAC CTG ATG CAC TTT GAC AGC ATG ATC GGA TGT AAG TTC GAC GCT AGC CGA TCT
9	100	ATC GCG GAT TAG CAC TAC GCA TCG GTT ACA AAC GAG GAC CTG ATG CAC TTT GAC AGC ATG ATC GGA TGT AAG TTC GAC GCT AGC CGA TCT AGC TTA GCG A

GD 90-mer synthetic:
GAG TTC TTC ATG CCT GGG CTG GTT GAT ACA CAC ATC CAT GCC TCT CAG TAT TCC TTT GCT GGA AGT AGC ATA GAC CTG CCA CTC TTG GAG

HALO® Oligo C18



- Surface modified organo-silane technology for alkaline resistance (Patent Pending).
- Excellent stability under high pH conditions (see below, also obtained for new particles.)
- Commercially available as and end-capped Oligo C18 with 2.7 µm particle size, 0.5 µm thick porous silica shell of 120 Å pore size, on a 1.7 µm core (FusedCore® particle)
- Novel large pore hybrid silica particle similar to previously described Halo 1000Å material (picture below).
- Column hardware is surface-treated to reduce metallic surface interactions and losses.
- Excellent peak shape and increased loading capacity for basic compounds.
- Wide operational use range of pH 2-12 for robust method development.



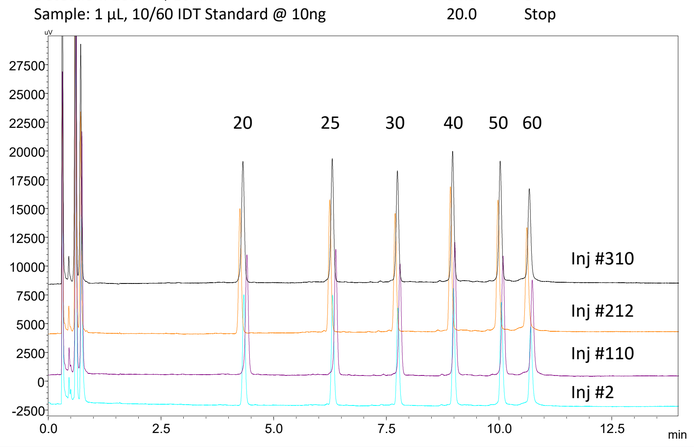
Wagner, Schuster, Boyes, Shields, Miles, Haynes, Kirkland, and Schure. Superficially porous particles with 1000 Å pores for large biomolecule high performance liquid chromatography and polymer size exclusion chromatography J. Chromatogr. A 1485 (2017) 75–85.

Elevated pH and Temperature Testing of Halo Oligo C18

Columns: 2.1 x 50 mm, Oligo C18
Flow Rate: 0.5 mL/min
Temp: 60°C
A- 15 mM TEA/50 mM HFIP, pH 8.9
B- MeOH
Detection: 260 nm, 10 nm
Sample: 1 µL, 10/60 IDT Standard @ 10ng

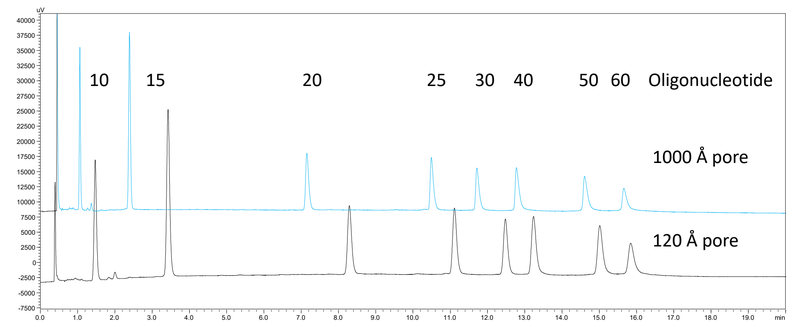
Gradient:

Time	%B
0.0	5.0
14	12
14.1	30
14.4	30
14.5	5
20.0	Stop



Separations of Oligonucleotides Using 120Å and 1000Å Pore Size SPP

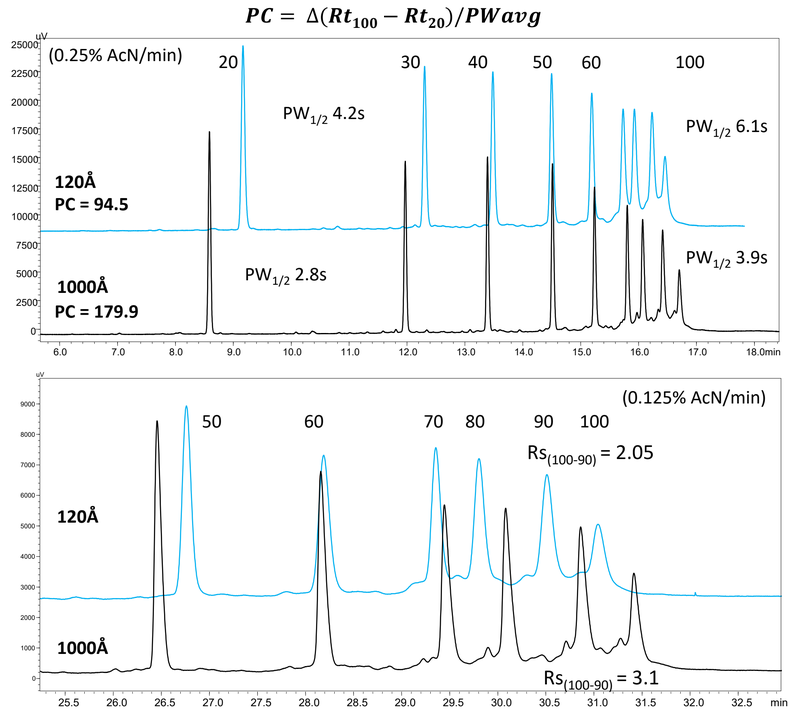
Conditions:
2.1 x 100 mm columns of 2.7 µm particles 60˚C; Mobile Phase: A – 100 mM TEAA; B – Methanol 13.5-20% B in 20 min
Abs 260 nm (10 nm BW); Flow Rate 0.5 mL/min (P=220 bar); 1 µL, 10/60 IDT @ 10ng



- Larger pore SPP exhibits moderate decrease in retention for shorter oligonucleotides, increased gradient range at elution, moderate decrease in peak width

Conditions:
2.1 x 100 mm columns; 60˚C; Mobile Phase: A – 15 mM TEA/50 mM HFIP (pH 8.9); B – AcN, 1.5 – 6.5%B in 20 min (top Panel); 40 min (bottom Panel); Flow Rate 0.5 mL/min (P=220 bar); 1 µL, 20/100 IDT @ 10ng

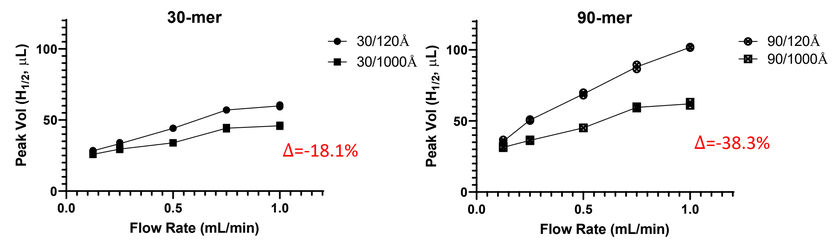
$$PC = \Delta(Rt_{100} - Rt_{20})/PWavg$$



- In TEA/HFIP mobile phase larger pore SPP exhibits a greater decrease in PW, increased gradient range for elution, and overall increase in peak capacity (PC). Resolution of larger oligonucleotides is greater, and increases with lower gradient rate (bottom). At the lower gradient rate resolution is increased by 50% for the 90/100 nts band pair.

Conditions:
2.1 x 100 mm 2.7 µm/1000 Å prototype; 60˚C; Mobile Phase: A – 100 mM TEAA (pH 7.0); B – AcN, 6.5-11.5% AcN %B in time and flows shown to yield a 10.0 mL gradient; 1 µL, 20/100 IDT @ 10ng

Fixed Volume Gradient Conditions (10.0 mL); Peak Volume = $PW_{1/2}$ x Flow Rate

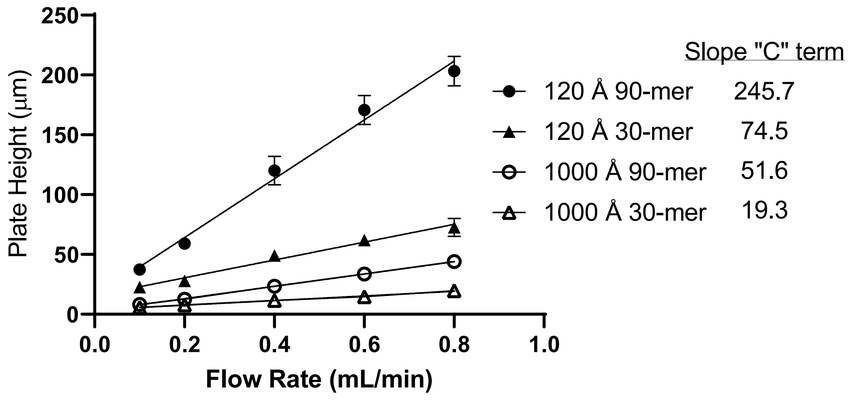


- At constant gradient volume of 10.0 mL, with varied flow rate, band dispersion was compared in TEAA conditions for pore size and chain length in gradient elution with AcN. Band dispersion is lower on the larger pore SPP, relative to smaller, with a greater difference for the large oligonucleotide

Isocratic Flow Rate Measurement: Pore Size and ssDNA Chain Length

- Oligonucleotide IP-RP retention and band width vary significantly with temperature, ion pair concentration and identity, solvent strength and linear velocity (via the system pressure). Interpretation of gradient elution band widths (as shown above) requires caution.
- The effect of pressure on retention complicates the analysis of flow rate (linear velocity) relationship to column efficiency (van Deemter relationship).
- Using an approach similar to that recently described by Stoll et al. (Stoll, Ghimire, Sorensen, and Maloney *J. Chromatogr.* A, 1744 (2025), 465687) column efficiency can be evaluated at constant retention, employing solvent strength (estimated by the linear solvent strength [LSS] relationship) to compensate for flow rate (pressure) effects on retention..
- Very similar column permeabilities are noted for columns packed with these two pore size variant 2.7 µm particles.
- The larger pore material shows much lower loss of efficiency for the 90-mer at high flow, but also shows benefit for the 30-mer band width at lower flow rates, relative to the smaller pore packing material.
- The resistance to mass transfer is ascribed to restricted diffusion for smaller pores, apparent even with the advantageous diffusion path of the SPP particle morphology.
- Similar dependencies for separations efficiency of smaller oligonucleotides on a smaller pore fully porous particle have been noted by Stoll et al. (2025).

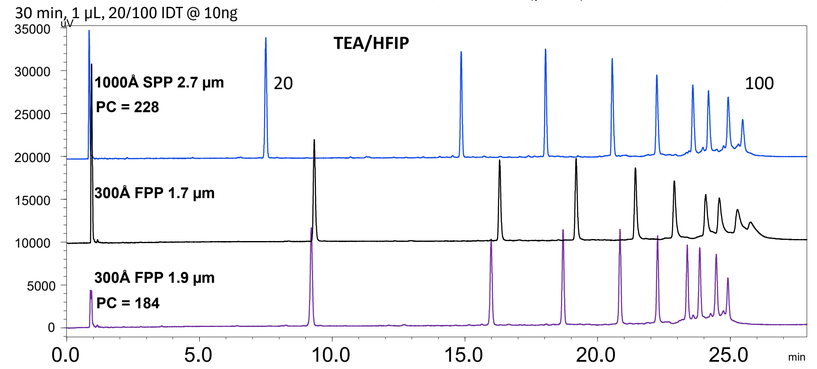
Flow Rate Effect on Column Efficiency



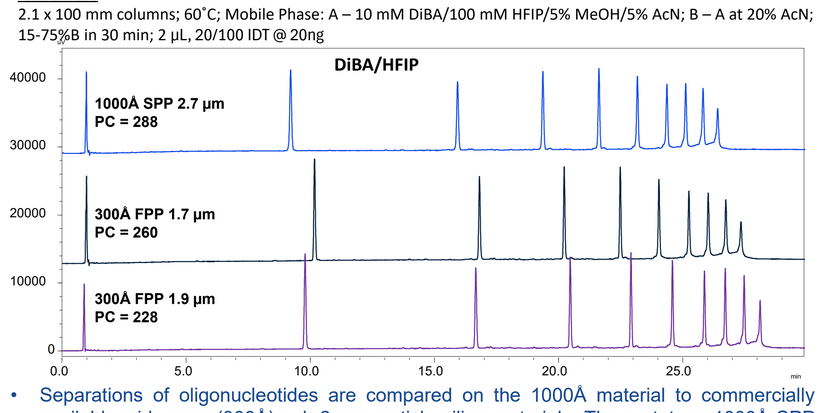
Conditions:
2.1 x 100 mm columns of 2.7 µm particles 60˚C; Mobile Phases are 10 mM DiBA/100 mM HFIP (8.4)/5% MeOH
Acetonitrile volume fraction adjusted using 10% in Pump A and 20% in Pump B to yield k'=10.0 (+/- 6%)

SPP 1000Å versus FPP 300Å

Conditions:
2.1 x 100 mm columns; 60˚C; Mobile Phase: A – 15 mM TEA/50 mM HFIP (pH 8.9); B – 50%AcN/Water, 5 – 12%B in 30 min, 1 µL, 20/100 IDT @ 10ng



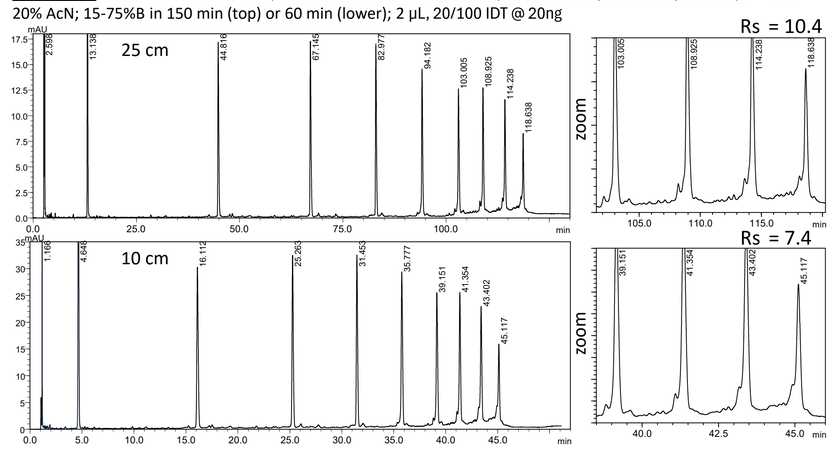
Conditions:
2.1 x 100 mm columns; 60˚C; Mobile Phase: A – 10 mM DiBA/100 mM HFIP/5% MeOH/5% AcN; B – A at 20% AcN; 15-75%B in 30 min; 2 µL, 20/100 IDT @ 20ng



- Separations of oligonucleotides are compared on the 1000Å material to commercially available wide pore (300Å) sub-2 µm particle silica materials. The prototype 1000Å SPP exhibited modest improvements in peak widths in TEA/HFIP, and greater gradient range difference between the 20 and 100 base oligonucleotide, contributing to the higher PC.
- Peak shapes improved for larger oligonucleotides using all of the columns, (particularly for the 1.7 µm material), by use of the more hydrophobic DiBA/HFIP mobile phase condition.
- Peak capacities were larger on the 1000Å SPP material, due to both lower PW1/2 and larger gradient span of the separation.

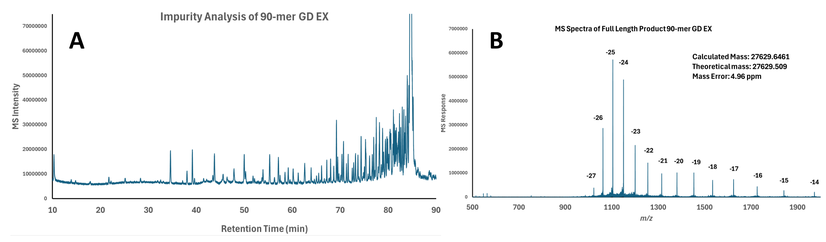
Utility for Resolution of Longer Chain Nucleic Acids

Conditions: 2.1 mm ID columns; 60˚C; Mobile Phase: A – 10 mM DiBA/100 mM HFIP/5% MeOH/5% AcN; B – A at 20% AcN; 15-75%B in 150 min (top) or 60 min (lower); 2 µL, 20/100 IDT @ 20ng



- Resolution of the oligonucleotide mixture on a longer column (25 cm) using a shallow gradient, resolving out to 100 nts. Resolution scales close to the √L for column length using the gradient rate scaled to L (150 min to 60 min) for comparing the 25 and 10 cm columns (across the range increase by 1.34; expected 1.58).

Conditions: 2.1 x 150 mm, 1000Å prototype; 0.2 mL/min; 60°C; Mobile Phases:
A- 10 mM DiBA/100 mM HFIP/5% MeOH/5% AcN; B – A at 20% AcN; 0-32%B in 120 min.



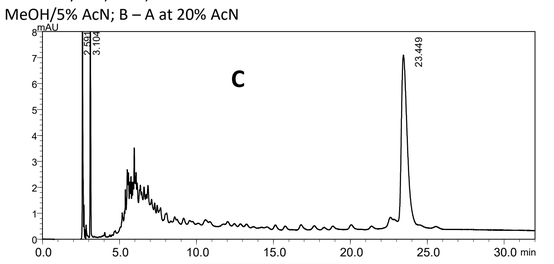
- An impurity profile a 90-mer (GD EX) on a 15 cm column by LC/MS, identifying truncates and other impurities (Panel A). The MS spectrum for the full length 90-mer (Panel B). Various truncated species are resolved, including the (n-1) 89 nt 3'-end failure.

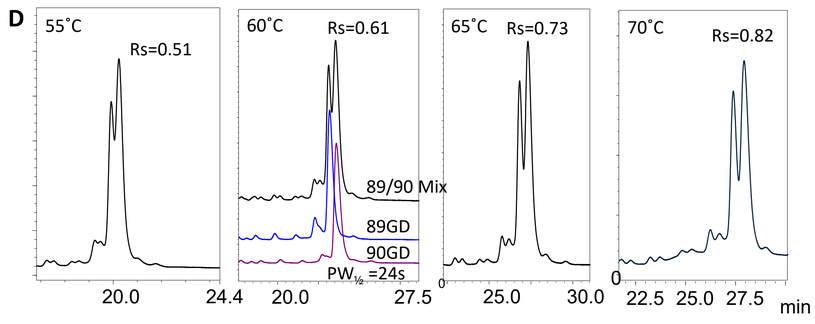
- Resolution the GD EX 90-mer and synthesis impurities on a 25 cm column of the 1000Å SPP material (Panel C), on a 25 cm column, with a shallow gradient (0.015% AcN/min). A synthetic 89-mer (-G at 3'-end of GD EX) was obtained. Separation of the 89/90-mer pair is difficult, even with a shallow gradient (0.015% AcN/min). Resolution and retention is surprisingly dependent on temperature, as shown below in Panel D.

Conditions: 2.1 x 250 mm,1000Å; A - 0.2 mL/min; 60°C; Mobile Phase:
A- 10 mM DiBA/100 mM HFIP/5% MeOH/5% AcN; B – A at 20% AcN

Gradient:

Time	%B
0.0	40.0
1.0	40
2.0	64
30.0	67
31.0	100
33.0	100
34.0	40.0
40.0	STOP





Summary

- HALO® Oligo C18 is an elevated pH stable column for HPLC, UHPLC, and LC-MS separations. Conditions favorable for oligonucleotide separations are well-tolerated with this hybrid SPP particle for both small and larger pore size column packing materials.
- A novel hybrid silica wide pore SPP particle is described, exhibiting the efficiency and speed benefits in oligonucleotide separations previously shown by us and other large biomolecule separations using SPP particles.
- Both gradient and isocratic analysis of column efficiencies demonstrate benefits for separations using wider pore SPP packed columns.
- Large synthetic ssDNA analyses exhibit very high resolution.