Advanced Superficially Porous Separations Materials for Analyses of Synthetic Oligonucleotides

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ISPPP 2026: P-12
Orlando , Florida

Introduction

Problem: Polynucleotides are a rapidly developing therapeutic and diagnostic molecule class with significant separations demands, particularly for longer sequences. An expanding repertoire of useful chemical modifications have emerged, and the fully synthetic nature of therapeutic oligonucleotides requires analysis of process impurities. Current method development approaches are not well defined, and sample complexity can be high with increasingly complex formulations. Resolution of closely related variants could be improved by directed column packing materials design.

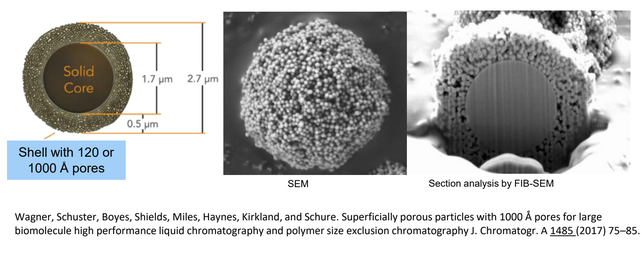
Approach: Employ recently developed silica surface hybrid superficially porous particles (SPP) to improve ion pair reversed phase (IP-RP) separations of nucleic acids.

New Hybrid Silica Materials for Elevated pH Tolerance

Ion pairing RP for separations of oligonucleotides and other polynucleotides has been in common use for more than 4 decades, but with limited specific design of column packing materials. We describe the use of superficially porous particle (SPP) silica column packing materials recently developed at AMT, which are stable in the typical mildly alkaline mobile phases (pH 8-10) and elevated temperatures (>50˚C) in common use for polynucleotide separations. Larger biological molecules exhibit improved separations efficiency resulting from improved mass transfer properties of SPP of appropriate pore sizes and other particle features. Pore sizes are varied using materials shown in Fig. 1. Highly efficient separations using SPP Oligonucleotide C18 materials allow lower back pressure, and thus longer columns. We show examples wherein very high resolution can be obtained with longer columns.

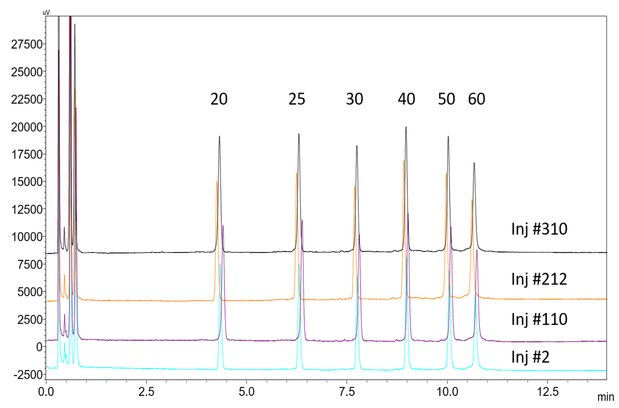
A broadened range of ion pairing reagents has been favored, particularly for DNAs and RNAs longer than about 30 nucleotides (nts). Initially, acetate was used for pH control, but for several reasons, hexafluoroisopropanol (HFIP) is favored as the counterion/additive to improve MS detection. An example of stability of an RP-IP oligonucleotide separation using HALO 120Å OLIGO C18 is shown in Fig 2. Oligo series columns use a new surface-modified organo-silane technology (Elevate) which maintains the morphology and mechanical stability of AMT Fused-Core® particles (Patent Pending).

Fig.1 Superficially Porous Particle Morphology



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.

Fig.2 Highly stable ssDNA separations (20-60 bases) using Elevated pH and Temperature IP-RP Conditions



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

Materials and Methods

Reagents were obtained from Millipore-Sigma (TEA, 1M TEAA solution, pH 7.0, DiBA, DIPEA) 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 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. Therapeutic siRNAs were obtained from MedChem Express. 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

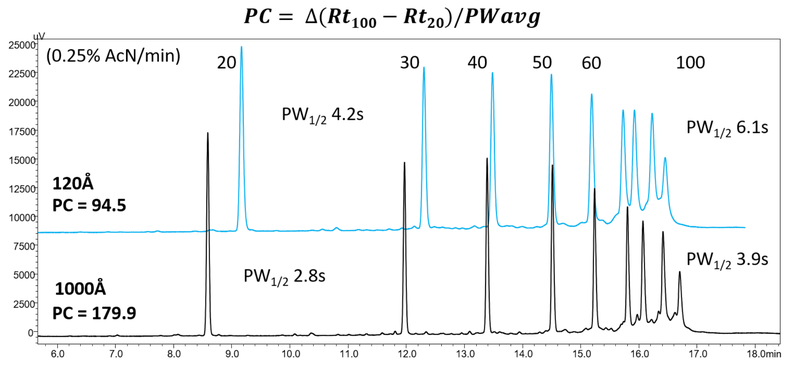
20/100 Standard

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

Pore Size Effects on Oligonucleotide Separations

Separation performance was evaluated for 120 and 1000Å pore size Oligo packing materials using the 20/100 mixture of oligonucleotides in gradient elution with acetonitrile as the strong solvent. The chromatograms below show narrowed peaks (PW1/2 height), and corresponding increased peak capacity. Longer sequences show a greater effect.

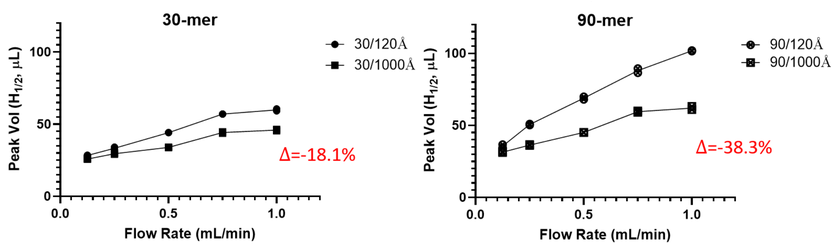
$$PC = \Delta(Rt_{100} - Rt_{20})/PWavg$$



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

- 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.

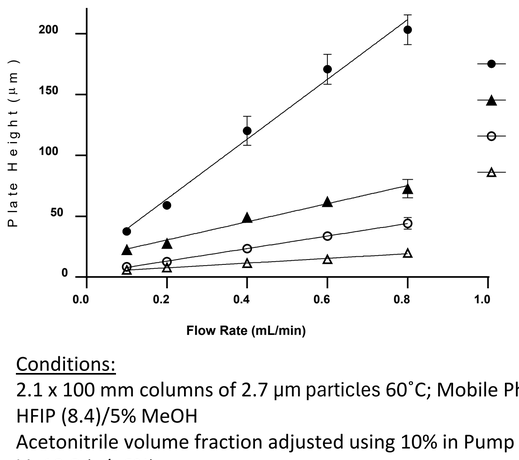
Fixed Volume Gradient Conditions (10.0 mL); Peak Volume = $PW_{1/2}$ x Flow Rate



Conditions: 2.1 x 100 mm 2.7 µm/HALO 1000 Å OLIGO C18; 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

- 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

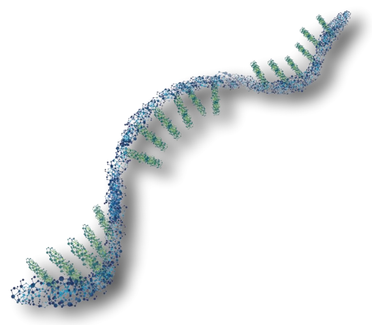
Flow Rate Effect on Column Efficiency



	Slope "C" term
120 Å 90-mer	245.7
120 Å 30-mer	74.5
1000 Å 90-mer	51.6
1000 Å 30-mer	19.3

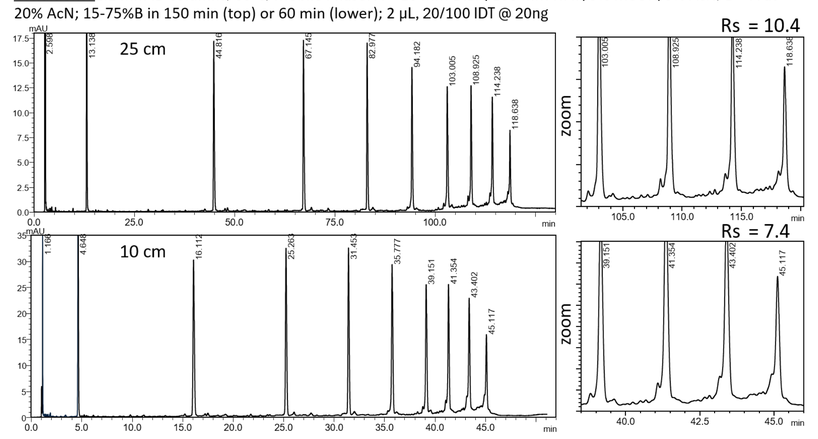
Conditions:
2.1 x 100 mm columns of 2.7 µm particles 60˚C; Mobile Phases are 10 mM DiBA/100 mM HFIP (8.4)/5% MeOH
Acetonitrile volume fraction adjusted using 10% in Pump A and 20% in Pump B to yield k'=10.0 (+/- 6%)

- Oligonucleotide IP-RP retention and band widths vary with temperature, ion pair identity and concentration, solvent strength and linear velocity (via the system pressure).
- Pressure effect on retention complicates the analysis of flow rate (linear velocity) relationship to column efficiency (van Deemter analysis).
- Isocratic column efficiency can be measured by varying solvent strength to maintain constant retention (Stoll, Ghimire, Sorensen, and Maloney J. Chromatogr. A, 1744 (2025), 465687).
- The larger pore material shows much less loss of efficiency for the 90-mer at higher flow, but also shows benefit for the 30-mer ssDNA at lower flow rates, relative to the smaller pore packing material.
- The resistance to mass transfer (C-term) describes restricted diffusion for smaller pores, apparent even for these SPP particle materials.
- Significant resistance to mass transport of smaller oligonucleotides using a fully porous particle have been recently noted by Stoll et al. (2025).



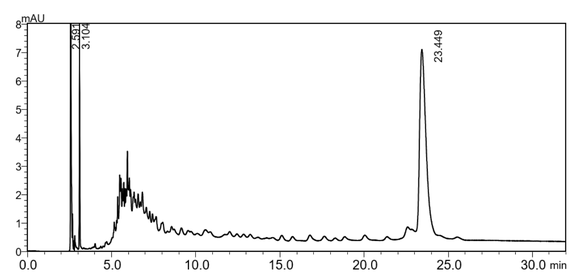
Utility for Resolution of ssDNA and RNA Samples

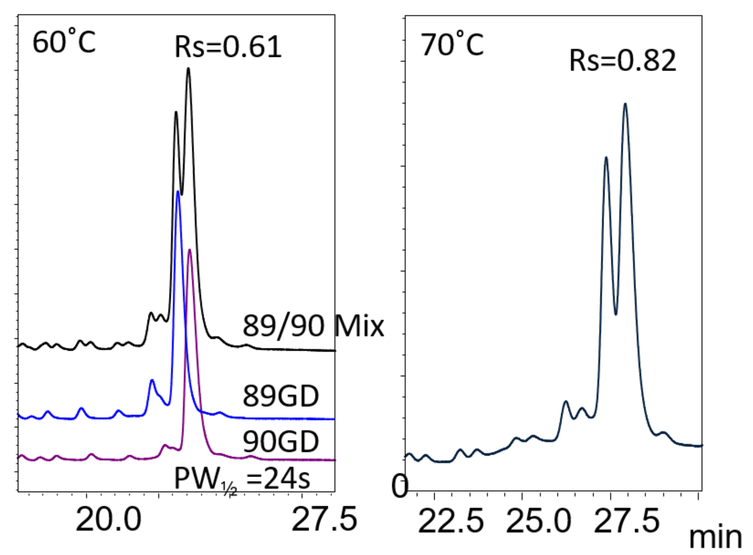
Conditions: 2.1 mm ID columns; 60˚C; Mobile Phase: A – 10 mM DiBA/100 mM HFIP/5% MeOH/5% AcN; B – A at 20% AcN; 15-75%B in 150 min (top) or 60 min (lower); 2 µL, 20/100 IDT @ 20ng



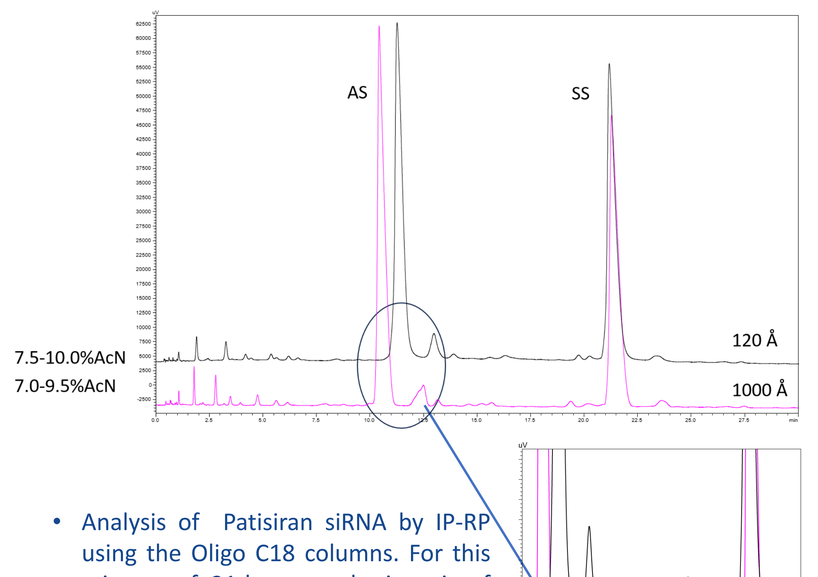
- Resolution of the 20/100 oligonucleotide mixture on a longer column (25 cm) using a shallow gradient, resolving out to 100 nts.
- Resolution of 90/100 nt pair close to expected for √L dependence. The gradient rate is scaled to L (150 min to 60 min) for comparing the 25 and 10 cm columns (across the range increase by 1.34; expected 1.58).

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

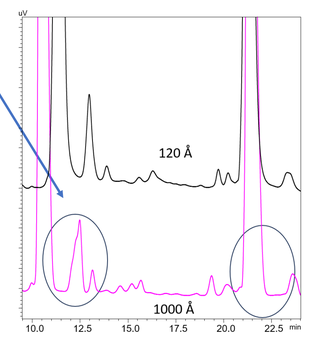




- Resolution of GD EX 90-mer using the 1000Å SPP material. A synthetic 89-mer (-G at 3'-end of GD EX) was obtained. Separation of the 89/90-mer pair requires a shallow gradient (0.015% AcN/min).



- Analysis of Patisiran siRNA by IP-RP using the Oligo C18 columns. For this mixture of 21-base synthetic pair of sense (SS) and anti-sense (AS) RNAs, modest decrease in the PW1/2 using the 1000Å pore, but useful shift in resolution of truncated SS RNAs relative to the target strands are noted.



Conditions: 2.1 x 50 mm columns; 70˚C; Flow Rate 0.25 mL/min.; Patisiran, 0.5 µL, 1mg/mL; Mobile Phase: A – 5 mM DIPEA/50 mM HFIP; B – AcN, %B in 30 min gradient

Conclusions

- A novel hybrid silica wide pore SPP particle is described, exhibiting the efficiency and speed benefits in oligonucleotide separations previously shown by us for large protein and conjugate separations using SPP particles.
- HALO® Oligo C18 are elevated pH stable columns for HPLC, UHPLC, and LC-MS separations. Conditions favorable for high resolution oligonucleotide separations are well-tolerated with this hybrid SPP particle for both small and larger pore size column packing materials.
- Both gradient and isocratic analysis of column efficiencies demonstrate benefits for separations using wider pore SPP packed columns. At lower flow rates (0.1-0.2 mL/min) efficiency nears the theoretic limit.
- Large synthetic ssDNA analyses exhibit very high resolution, permitting unit resolution of >90 bases.

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