Poster Reprint

HPLC 2024
Poster number 274

Emerging Alternative Analytical Techniques for Oligonucleotide Separation and Purification: HILIC & SEC

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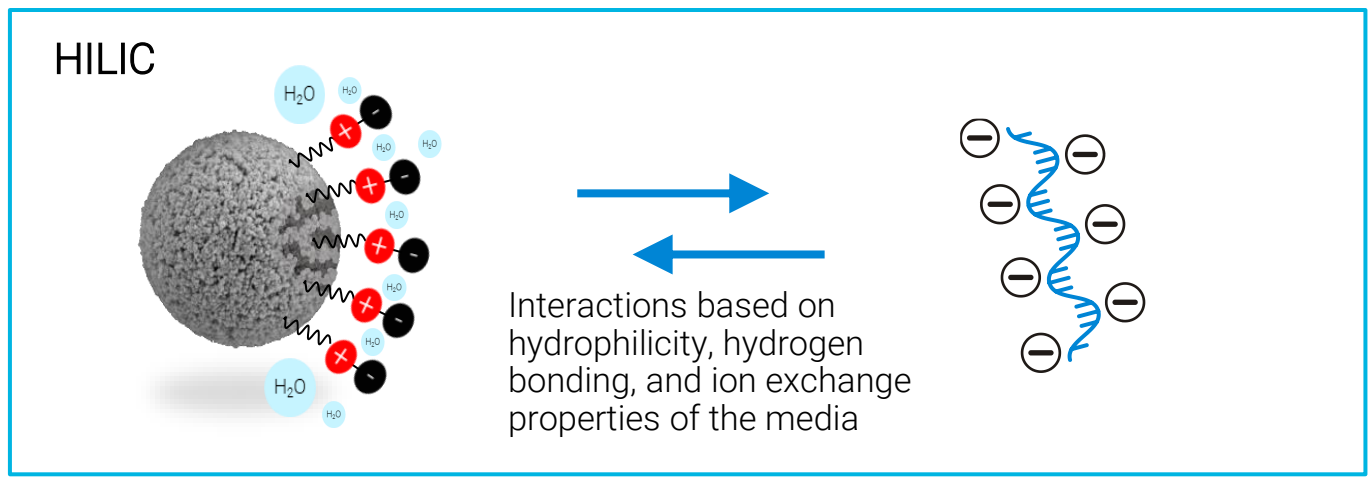
Introduction

Ion-pairing reversed-phase chromatography (IP-RP) and ion-exchange chromatography (IEX) represent the two most common analytical separation techniques for oligonucleotide analysis. However, alterative separation methods are desired as alkylammonium ion-pair reagents used in IP-RP force users to have dedicated instruments, while ion-exchange chromatography (IEX) has its own limitations due to mobile phase incompatibility with MS detection. Hydrophilic interaction chromatography (HILIC) has been proposed as a valuable alternative to IP-RP and IEX as HILIC mobile phases are compatible with MS and provides flexibility in instrument-use especially for MS characterization workflows.

In addition to sequence-based critical quality attributes (CQAs), there is also a need to characterize product and process-related impurities that arise from higher order structures such as aggregation. Size Exclusion Chromatography (SEC) is the gold-standard technique to monitor aggregation. Thus far, SEC has more commonly been used for monoclonal antibody (mAb) and protein aggregate analysis, though it has recently emerged as a viable approach to monitor higher order oligonucleotide structure, especially with longer mRNA oligo therapeutics.

Experimental - HILIC

Understanding Hydrophilic Interaction Liquid Chromatography



Hydrophilic Interaction Liquid Chromatography (HILIC) is an analytical separation technique where analytes are retained due to their polarity as they partition between the organic-rich mobile phase and an aqueous layer created by the hydrophilic stationary phase. The polar nature of oligonucleotides thus lends itself well to this approach.

A wide variety of different HILIC stationary phases exist that leverage different polar functional groups that can also contribute to how the oligo will interact with the stationary phase as it partitions into the water layer near the particle surface.

Five different Agilent HILIC columns were screened with the Tanaka test to characterize the critical interactions of the HILIC separation. These characteristics included hydrophobicity, hydrophilicity, shape/steric selectivity, hydrogen bonding, ion exchange, and acidic-basic nature of the stationary phase. The selectivity values were calculated based on the relative retention of key analyte pairs

Experimental

Agilent HPLC Columns (2.1x 150 mm):

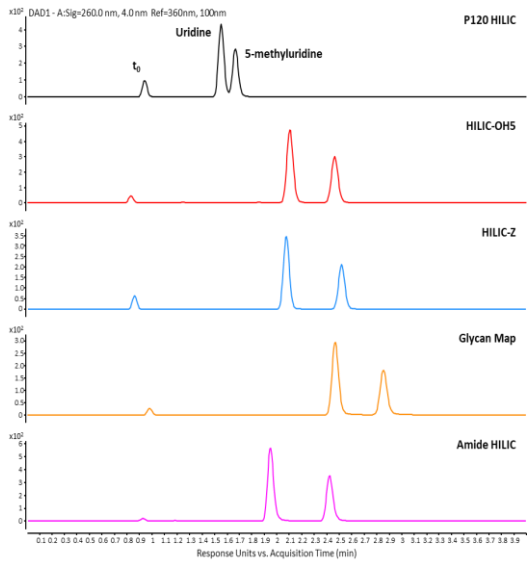
- InfinityLab Poroshell 120 HILIC PN: 693775-901
- InfinityLab Poroshell 120 HILIC-OH5 PN: 683775-601
- InfinityLab Poroshell 120 HILIC-Z PN: 683775-924
- AdvanceBio Glycan Mapping column PN: 683775-913
- AdvanceBio Amide HILIC column PN: 859750-913

Mobile Phase:

- 100 mM ammonium acetate stock solutions were made in waters and adjusted to pH 4.4 or pH 9 with either acetic acid or ammonium hydroxide.
- Mobile phase A = 10% (100 mM stock solution)/ 90% water.
- Mobile phase B = 10% (100 mM stock solution)/ 90% acetonitrile.

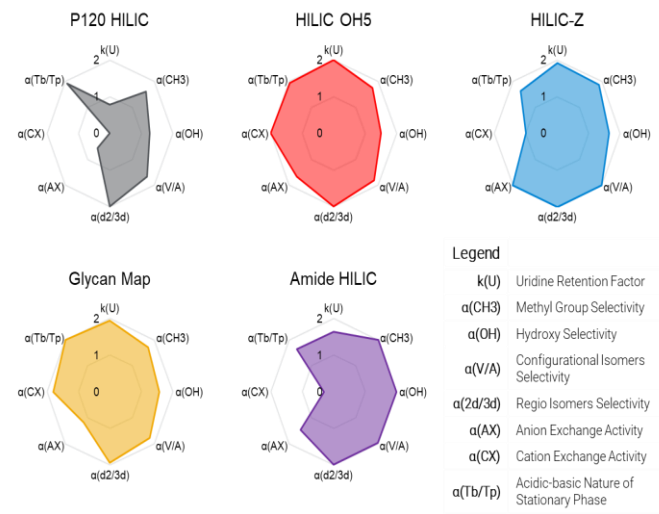
Results and Discussion - HILIC

Chromatographic Separations for the Tanaka Test



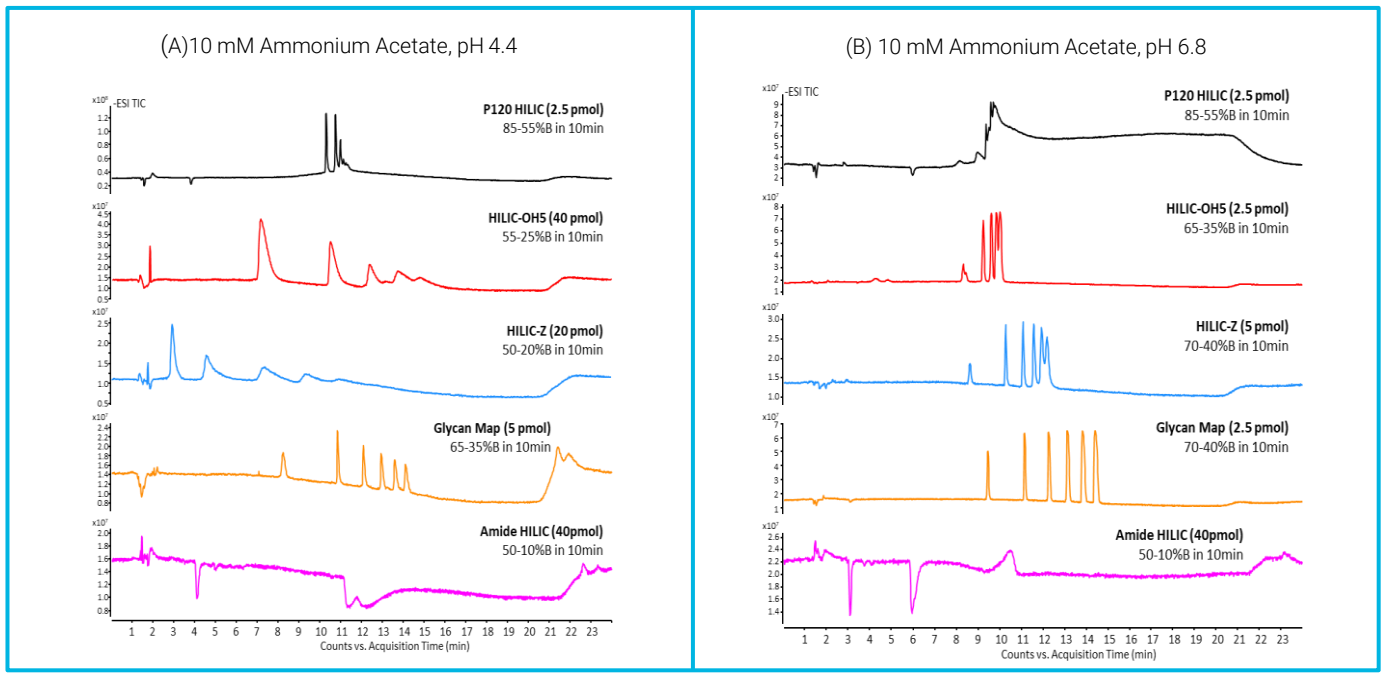
Separation of key analyte pairs to determine attributes that participate in the HILIC retention mechanism.

Characterizing Columns with the Tanaka Test



Radar plots for five stationary phases. Each attribute was normalized to each parameter's maximum value at 2.0, to distinguish each columns' features.

Evaluating HILIC Stationary Phases for Oligo Separation

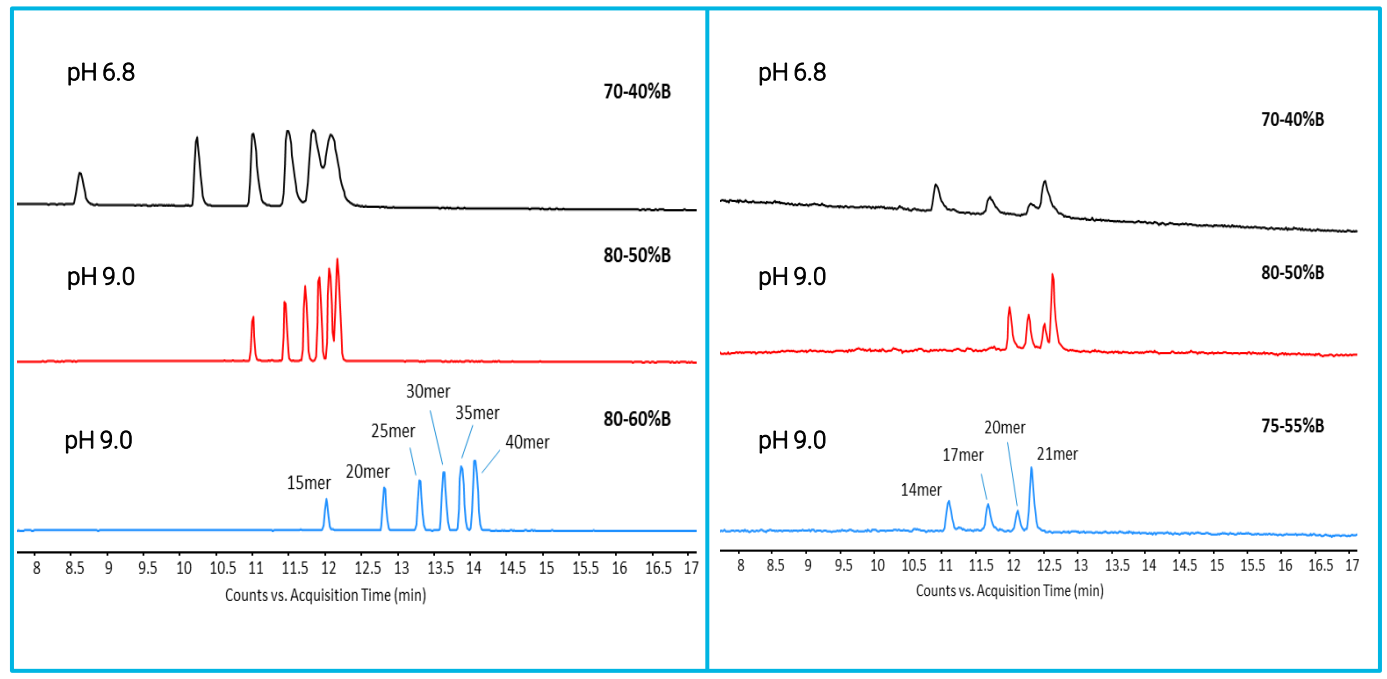


HILIC-LC/MS analysis of 15, 20, 25, 30, 35, and 40 mer DNA standard using HILIC stationary phases with varying chemical properties at (A) pH 4.4 and (B) pH 6.8.

Chromatographic Separation of Oligos at High pH

(A) DNA Oligo Standard

(B) RNA Resolution Standard



Further analysis on the impact of elevated pH was explored with the HILIC-Z column because of its stability at elevated pH. HILIC-LC/MS analysis of (A) DNA and (B) RNA oligo standards at pH 6.8 and pH 9.0 with the HILIC-Z column are shown above. The gradient was modified to improve peak resolution and adjust for retention time shifts when switching the pH of the mobile phase.

Experimental - SEC

Column: AdvanceBio SEC 1000Å, 2.7 µm, 7.8x300 mm, part number PL1180-5302

Mobile Phase: 100 mM Tris acetate, 2.5 mM EDTA or 150 mM phosphate buffer, pH 7.0

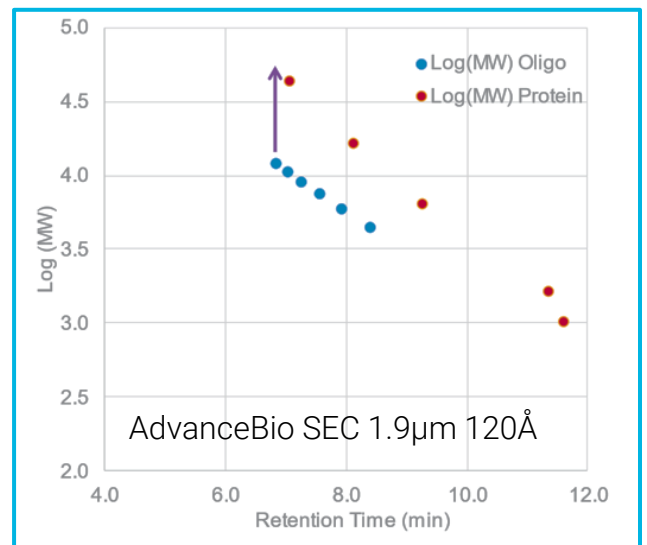
Flow Rate: 0.6 mL/min

Column Temperature: 40 °C

Results and Discussion - SEC

Larger Pore Sizes for Oligos

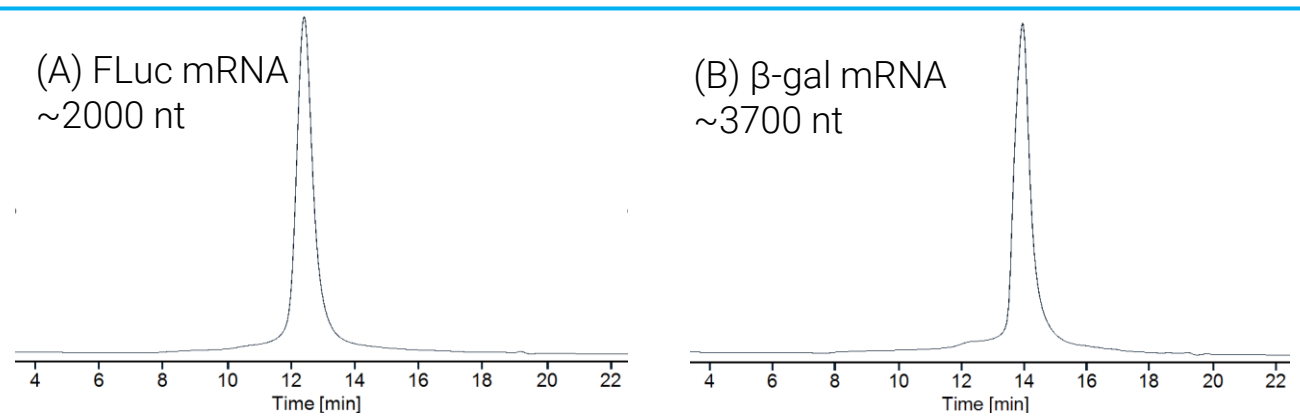
Because SEC measures size of an analyte in solution, and oligonucleotides are often much more elongated than globular proteins, necessitating larger pores for the same molecular weight. A 12.1 kDa poly dT standard elutes at an elution time corresponding to a 50-60 kDa globular protein. Pore size guidance is emerging, but optimum pore size may still need to be empirically determined, shown for the mRNA examples below.



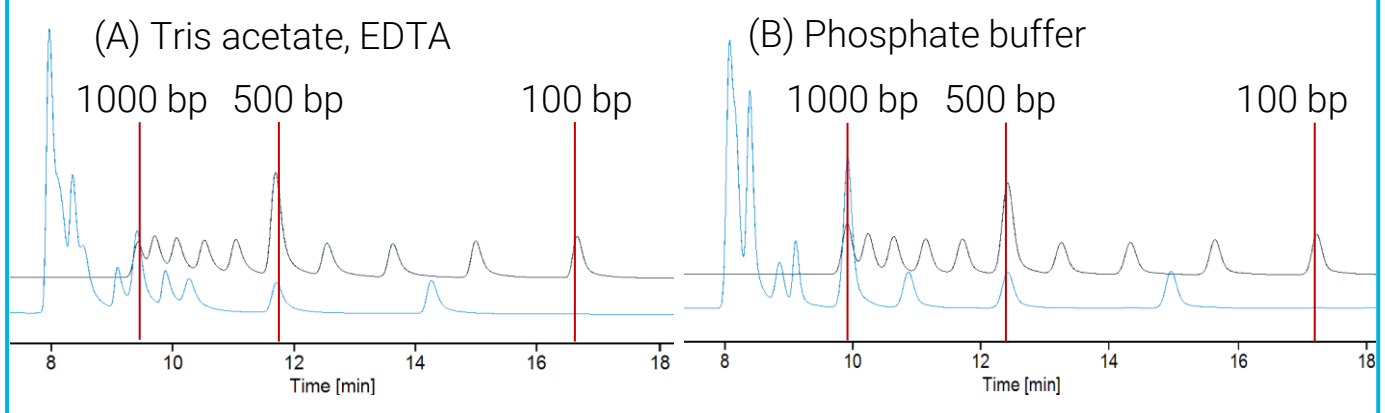
Elution times of a poly dT oligo ladder (dT_{15}-dT_{40}) compared to a mix of globular proteins (1-44 kDa)

Separations of Large Nucleic Acids

For large oligonucleotides, the large pore volume and high exclusion limit of the new AdvanceBio SEC 1000 Å columns enable high resolution separations of mRNA and large DNA. Single strand RNA can have a complex 3D structure due to localized complementary binding, resulting in hydrodynamic diameter that does not uniformly correlate with sequence length, and is also significantly smaller in solution than a double helix DNA of the same sequence length.



Despite its shorter sequence, Fluc mRNA (A) elutes earlier than β-gal mRNA (B), indicating it is more elongated in solution



Phosphate buffer shows lower tailing than tris acetate mobile phase, improving resolution at 100 bp DNA ladder (black), 1 kbp DNA ladder (blue)

Conclusions

As the therapeutic oligonucleotide market continues to mature, the need for alternative analytical approaches emerge to meet those demands. As demonstrated in this work, HILIC and SEC both represent effective emerging techniques for oligonucleotide workflows.

DE84038528

Published in USA, July 25, 2024

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