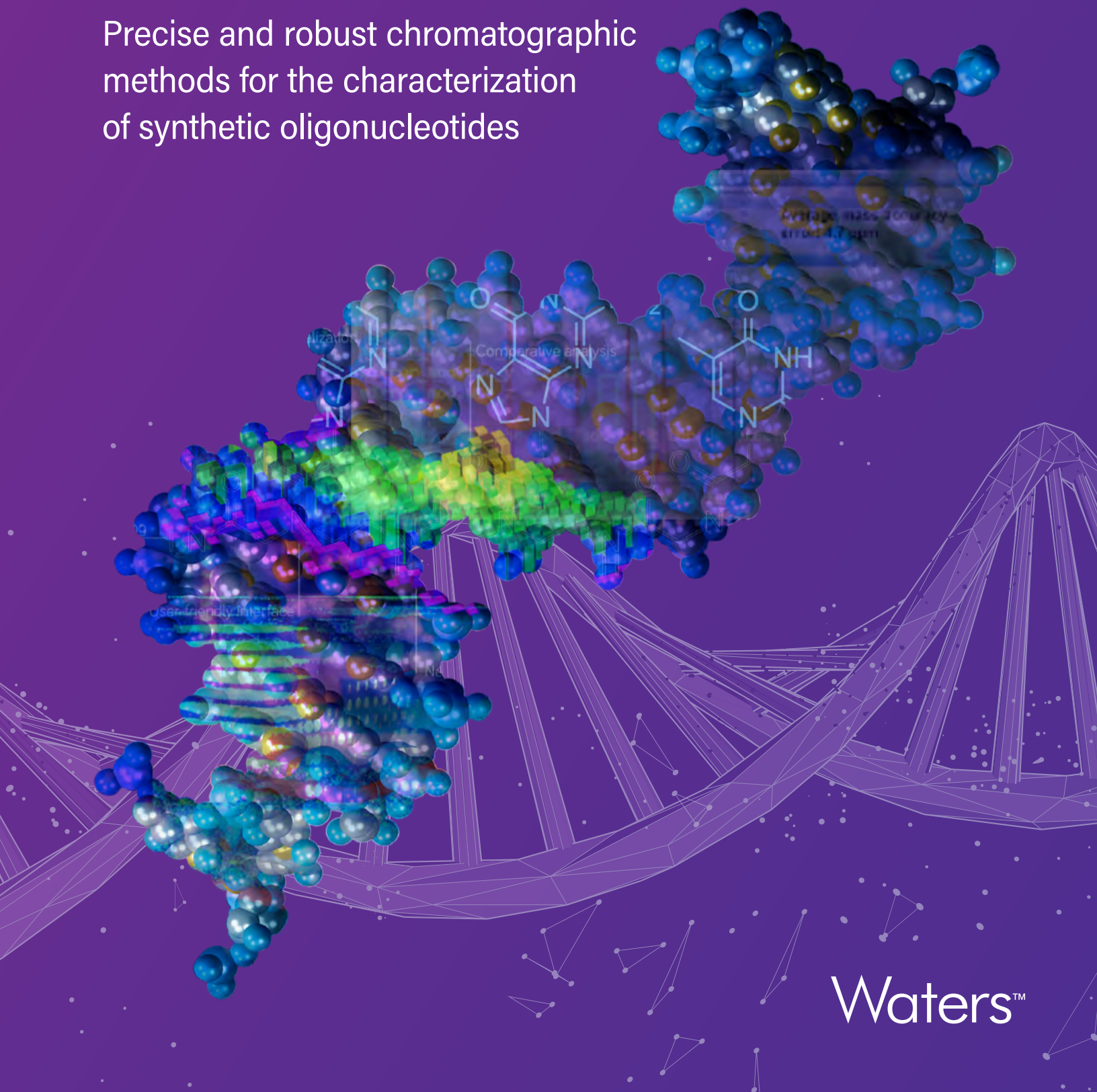
Characterizing Oligonucleotides

Precise and robust chromatographic methods for the characterization of synthetic oligonucleotides



Waters™

A Changing Industry

There has been a resurgence in synthetic oligonucleotides in the past decade, as seen with burgeoning pipelines across the industry. These new drug candidates are providing opportunities to address not only inherited disorders but also historically difficult to treat ailments, such as cardiovascular disease. Synthetic oligos are typically made using solid-phase chemical synthesis, a process that involves the addition of nucleotides one by one to form a growing polymer. The resulting molecules can therefore exhibit n-1 and other types of impurities related to further conjugation and modification. As therapeutic oligos come to be modified with new chemical moieties, the complexity of these impurities also increases. This presents new analytical challenges. Ever improving characterization techniques based on ion pairing reversed phase LC, anion exchange, HILIC and SEC can help.

GalNAc Conjugated Small Interfering RNA (siRNA)

Analytical Chromatography Options

Ion Pairing RPLC

HILIC

Anion Exchange

Size Exclusion

Waters Application Notes and Peer Reviewed Articles

Waters scientists and collaborators are publishing on this subject. Make sure to frequent the Resource Tab on our **waters.com/GTx** website to keep up to date on the literature.

Attribute Testing

A synthetic oligonucleotide drug can contain as many as 5,000 atoms arranged in important 5′ to 3′ sequences along with special modifications precisely located at important residue positions. These compositional details must be confirmed along with the purity of the drug substance material. As this field of pharmaceutical science advances so to does the understanding of critical quality attributes.

IDENTITY

The identity of an oligonucleotide drug substance must be confirmed. Chromatographic retention behavior is often confirmatory. Accurate mass analysis confirms elemental composition.

SEQUENCE CONFIRMATION

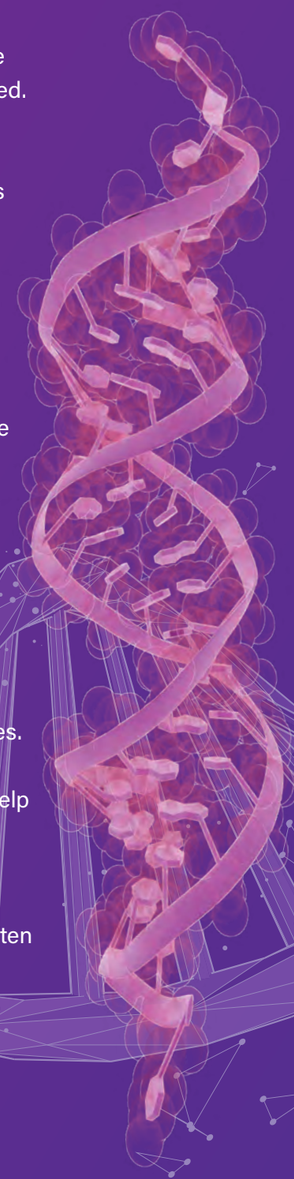
Fragmentation analysis by mass spectrometry can be applied to confirm modifications and their position within an oligonucleotide sequence. Enzymatic digestion and oligo mapping can provide comprehensive characterization data as well.

MODIFICATIONS

Modifications can range from 2' position fluoro and methoxyethyl groups to methylated nucleobases. Precise chromatography and accurate mass information can help pinpoint these molecular details.

PURITY

Synthetic oligonucleotides are often purified by anion exchange and reversed phase chromatography. Nevertheless, they can contain a number of different process and product related impurities.



N-1 SHORTMERS

Early termination and incomplete solid state synthesis of oligonucleotides is common. Chromatography can be applied to measure these so-called n-1 shortmer impurities.

OXIDATION

Many oligonucleotides are modified to contain a phosphorothioate backbone. A PS (phosphorothioate bond) can be oxidized and the sulfur atom can swap back to oxygen.

DEAMINATION

The exocyclic amines of the nucleobases contained within an oligonucleotide can be deaminated into carbonyl residues.

DEPURINATION

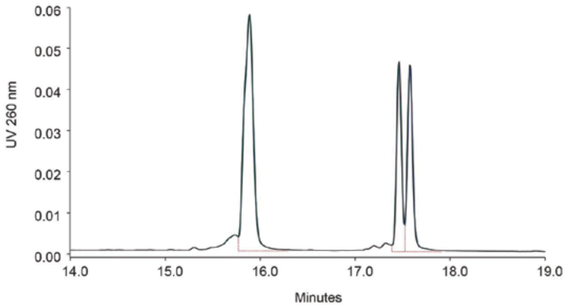
Adenine and guanine can be hydrolyzed from their ribose sugar through a break at the N-glycosidic bond. This is detrimental to the base pairing ability of the oligonucleotide therapy.

DUPLEX VS. SINGLE STRANDS

Small interfering RNA must be formulated to be intact duplex molecules in order to be functionally active and loaded into a RISC (RNA-induced silencing complex) subunit.

IP-RPLC to Confirm Identity and Purity

Ion pairing (IP) reversed phase (RP) liquid chromatography is the *de facto* approach for confirming the identity and purity of an oligo sample. Standard pore size stationary phases are used to separate up to 30-mer oligos, while widepore (300Å) stationary phases are available for larger species; both are available as oligo QC-tested and batch selected materials. IP-RP methods have historically leveraged triethylamine, though hexylamine and diisopropylethylamine have more recently become preferred options. With these methods, MaxPeak™ High Performance Surfaces have provided a step change in separation performance and robustness.



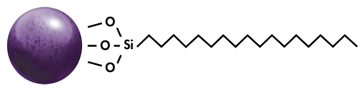
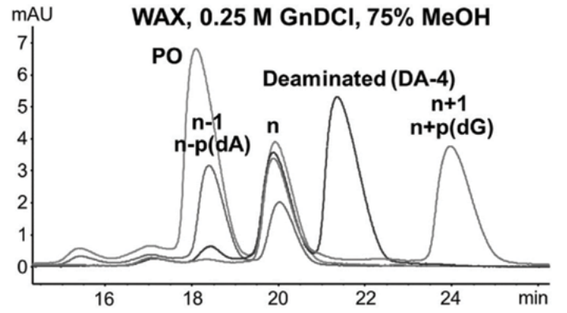


Figure 2. Denaturing IP-RPLC separation of a phosphorothioated siRNA as obtained with an ACQUITY™ Premier Oligonucleotide BEH C_{18} 300Å Column as reported in Waters Application Note 720007362.

AEX for Charge Based LC and New Selectivity

Anion exchange is an inherently good fit for the analysis of oligos and is compatible with a myriad of elution mechanisms. Research has uncovered both non-denaturing uses as well as new types of denaturing methods. Weak anion exchangers show promise for unique selectivity through pH adjustments and gradient tuning. Moreover, ion pairing agents can be added to the mobile phase to attenuate adsorption and partitioning. Ultimately, options for retentivity matter when it comes to developing new AEX methods. The Gen-Pak FAX Column is a weak anion exchanger that has a uniquely low retentivity, while the Protein-Pak HiRes Q Column and its SAX sorbent has comparatively strong retentivity. Both are useful tools for your analytical toolbox.



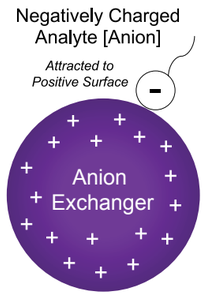
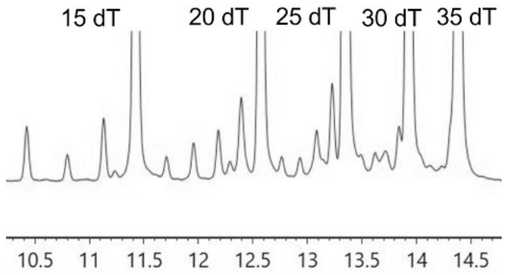


Figure 3. UV Chromatograms obtained for model oligonucleotide mixtures as obtained with a Gen-Pak FAX column. Used with permission from Anal. Biochem. 659 (2022) 114956.

HILIC as an Alternative

Hydrophilic interaction chromatography (HILIC) has garnered significant attention in recent years, due to the strong adsorptive interactions that oligonucleotides have with the associated stationary phases. LC-MS methods with ammonium acetate mobile phases have come to be preferred for ion-pairing free techniques with performance improvements coming from MaxPeak HPS hardware. An amide bonded BEH™ particle provides high efficiency separations that are based on direct H-bonding effects and the partitioning of an oligonucleotide into and out of an adsorbed water layer.



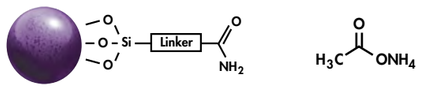


Figure 4. Separation of oligo dT species with an ACQUITY™ Premier BEH Amide 130Å Column as reported in Waters Application Note 720007395.

SEC for Native LC-UV-MS

Size exclusion chromatography (SEC) has not yet been put to regular use for the analysis of synthetic oligonucleotides. However, there is potential for it to become a powerful tool. It can be applied to achieve fast desalting native SEC-MS and be used to assay duplex siRNA for product-related, single stranded impurities. Columns constructed with 100 to 300Å pore diameter packing materials have a fractionation range suitable for separating 5 to 150-mer oligonucleotides.

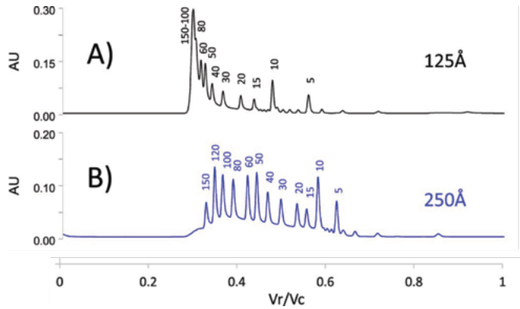


Figure 5. SEC separation of dA 5 to 150-mer species as published in Anal. Chem. 2023, 95, 38, 14308–14316.

Ordering Information

Information on the tools you need to comprehensively characterize an oligonucleotide can be found below.

RPLC

ACQUITY™ Premier Oligonucleotide BEH™ C_{18} 1.7 µm Columns

		130 Å			300 Å		
	Diameter	50 mm	100 mm	150 mm	50 mm	100 mm	150 mm
Standard Column	2.1 mm	186009484	186009485	186009486	186010539	186010540	186010541
VanGuard™ FIT Column	2.1 mm	186010685	186010686	186010687	186010754	186010755	186010756

XBridge™ Premier Oligonucleotide BEH C_{18} 2.5 µm Columns

		130 Å			300 Å		
	Diameter	50 mm	100 mm	150 mm	50 mm	100 mm	150 mm
Standard Column	2.1 mm	186009836	186009837	186009838	186010542	186010543	186010544
	4.6 mm	186009901	186009902	186009903	186010545	186010546	186010547
VanGuard FIT Column	2.1 mm	186010688	186010689	186010690	186010757	186010758	186010759
	4.6 mm	186010691	186010692	186010693	186010760	186010761	186010762

AEX

Anion Exchange Columns

	Dimension	P/N
Protein-Pak HiRes Q Column	4.6 x 100 mm	186004931
Gen-Pak FAX Column	4.6 x 100 mm	WAT015490

OLIGONUCLEOTIDE STANDARDS

	P/N
MassPREP™ Oligonucleotide Standard	186004135
ssDNA 10 to 60 Ladder	186009449
ssDNA 20 to 100 Ladder	186009448
ssDNA 20-mer LC-MS Standard	186009451
Lipid Conjugated ASO LC-MS Standard	186010747

HILIC

ACQUITY Premier BEH Amide 130 Å 1.7 µm Columns

	Diameter	50 mm	100 mm	150 mm
Standard Colum	2.1 mm	186009504	186009505	186009506
VanGuard FIT Column	2.1 mm	186009507	186009508	186009509

XBridge Premier BEH Amide 130 Å 2.5 µm Columns

	Diameter	50 mm	100 mm	150 mm
Standard Column	2.1 mm	186009928	186009929	186009930
	4.6 mm	186009935	186009936	186009937
VanGuard FIT Column	2.1 mm	186009931	186009932	186009933
	4.6 mm	186009938	186009939	186009940

SEC

ACQUITY Premier Protein SEC 250 Å 1.7 µm Columns

	Diameter	150 mm	300 mm	Guard
Standard Column	4.6 mm	186009963	186009964	186009969

ACQUITY UPLC Protein SEC 125 Å 1.7 µm Columns

	Diameter	150 mm	300 mm	Guard
Standard Column	4.6 mm	186006505	186006506	186006504

XBridge Premier Protein SEC 250 Å 2.5 µm Columns

	Diameter	150 mm	300 mm	Guard
Standard Column	7.8 mm	186009961	186009962	186009969

XBridge Protein SEC 125 Å 2.5 µm Columns

	Diameter	150 mm	300 mm	Guard
Standard Column	7.8 mm	186009159	186009160	186009158

waters.com/GTx

Waters™

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
waters.com