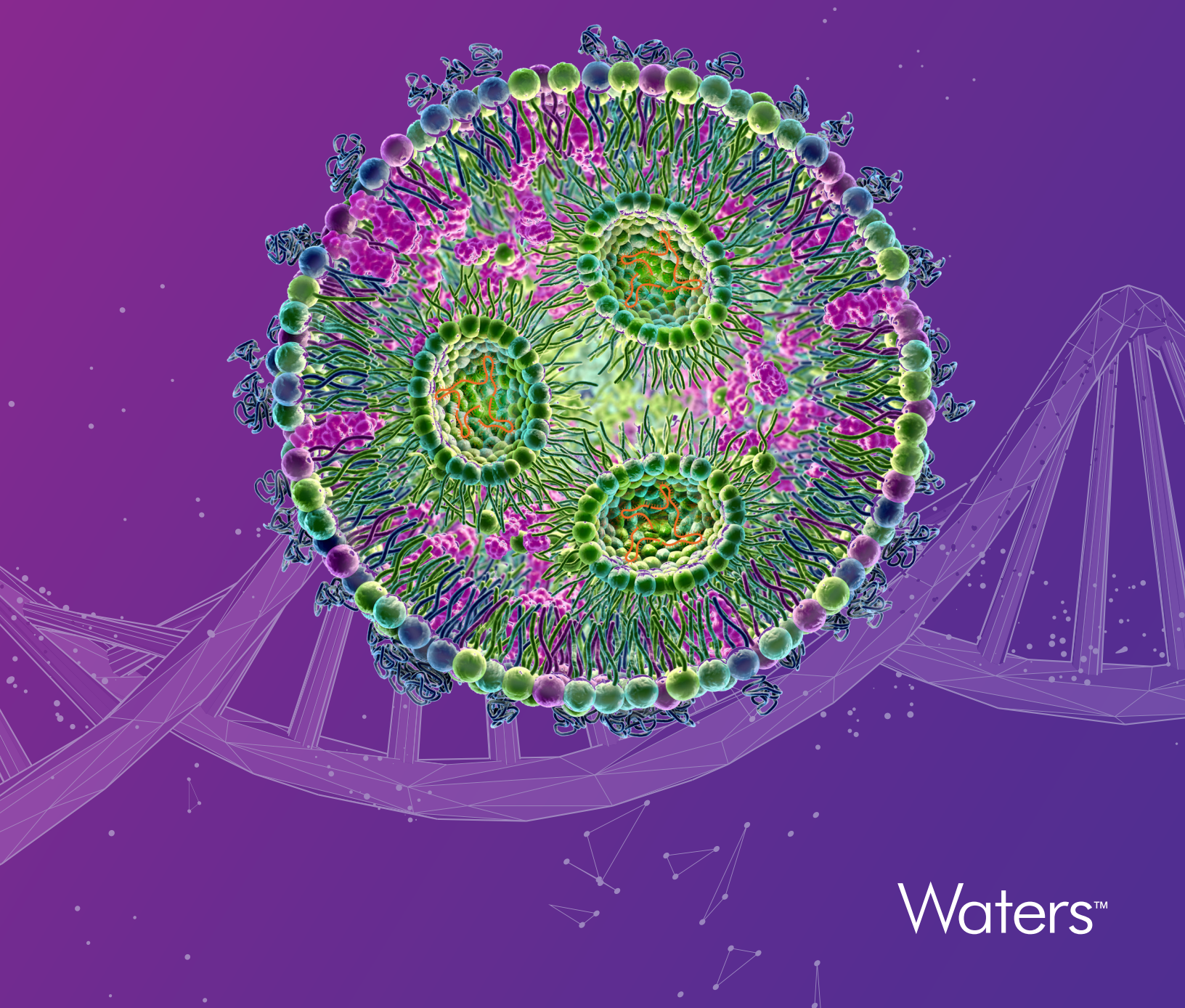
[CONSUMABLES GUIDE BOOK]

Characterizing LNP mRNA

LC tools for purity, identity, integrity, and concentration determining measurements

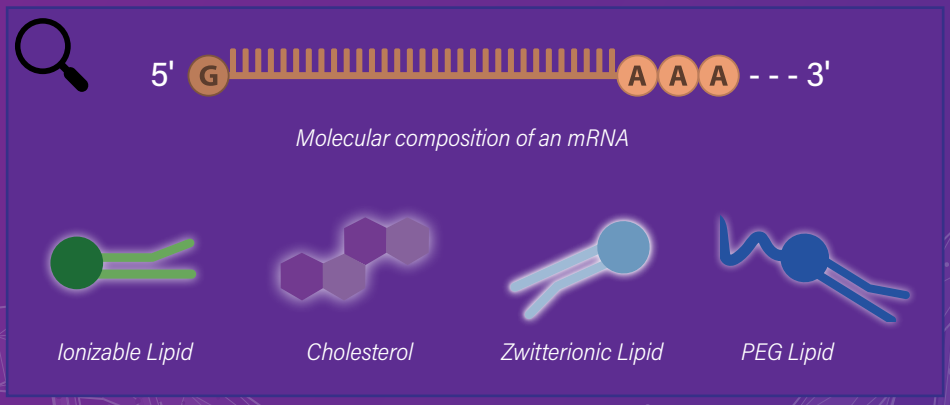


Waters™

A Changing Industry

mRNA is becoming a highly effective drug substance for both *in vivo* and ***ex vivo*** treatment of human infections, diseases and cancers. When encapsulated into a lipid nanoparticle, mRNA can be efficiently delivered to patient cells to be expressed into vaccine antigens, enzyme replacements to bolster a patient's own gene expression, or someday a gene editing apparatus to alter a patient's somatic genome. The FDA approved COVID-19 mRNA vaccines have provided a successful formula for creating potent LNPs. With today's techniques, four types of lipids encase the mRNA. A synthetic ionizable lipid is combined with a zwitterionic phosphatidylcholine lipid, cholesterol and a PEGylated lipid to yield 700–800 Å diameter nanoparticles. mRNA payloads are incorporated that range from 1,000 to up 10,000 nucleotides in length. Given their complexity, these LNP RNA drug products must be comprehensively characterized and tested. Chromatography can expedite the needed assays and thereby confirm physicochemical properties of the intact particle, check their compositions and raw materials for impurities, and confirm the molecular integrity and modifications of the mRNA drug substance.

Structure of lipid Nanoparticle



Chromatography and Method Options

Size Exclusion	**Anion Exchange**	**Poly A Tail LC**
Oligonucleotide Mapping	**5′ Cap Analysis**	**Lipid Testing**

Waters Peer Reviewed Articles

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

SEC to Measure Integrity of mRNA Drug Substance

Size Exclusion Chromatography (SEC) is a widely employed separation technique for isolating species based upon differences in hydrodynamic radius. SEC of larger nucleic acids requires novel method development to test and report on critical quality attributes (CQA) as they relate to the safety and efficacy of the drug. A GTxResolve™ Premier BEH™ SEC 450 Å 2.5 µm Column provides high resolution separations of small to medium sized nucleic acids species in various mobile phase conditions. New SEC separation capabilities with denaturing mobile phases have also come to be established. Formulated LNP samples can be injected onto an SEC column running a buffer comprised of 0.2% SDS + 20% isopropanol to facilitate quick measurements on the nucleic acid payloads within (Figure 2).

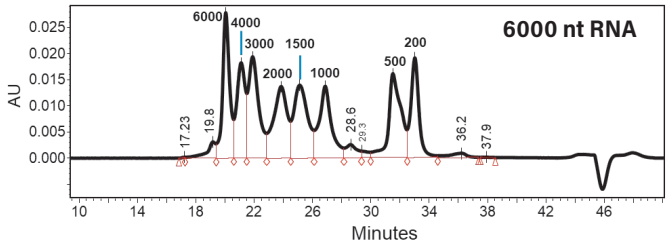


Figure 1. Components of 6000 nt RNA ladder were resolved using a Waters GTxResolve Premier BEH 450 Å SEC Column as reported in Waters Application Note: 720008061.

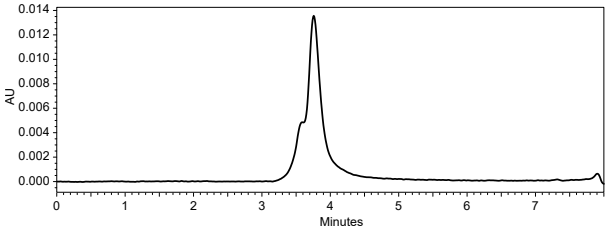


Figure 2. Denaturing SEC performed with a GTxResolve Premier BEH SEC 450 Å 2.5 µm Column and a mobile phase containing 0.2% SDS and 20% isopropanol. Injection of Comirnaty™ COVID-19 mRNA Vaccine 10 µg/dose drug product with simultaneous on-column dissolution of the LNP and detection of the mRNA payload.

Measuring Intact LNP and Large Nucleic Heterogeneity by SEC

Size exclusion chromatography (SEC) is also becoming a powerful technique to assess the heterogeneity and integrity of large nucleic acids and their corresponding LNPs. Contaminating impurities such as truncated and aggregated mRNA have implications on safety and efficacy and must therefore be monitored. In addition, first-of-their kind measurements on the heterogeneity of LNPs are now becoming possible such that new structure-function relationships can be elucidated. GTxResolve Premier SEC 1000 Å 3 µm Columns are built with MaxPeak Premier High Performance Surface Hardware and a novel particle technology to ensure that efficient, high recovery separations can be readily achieved and that new CQAs can be quickly reported.

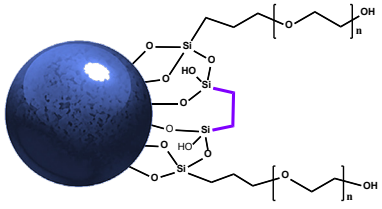


Figure 3. GTxResolve Premier SEC 1000 Å 3 µm Columns contain high efficiency particles modified with a novel ethylene bridged HO-PEO surface chemistry, which produces high recovery analyses of 200 to 1000 Å diameter GTx drugs, such as mRNA, LNPs, and viral vectors. This crosslink protected, hydrophilic surface chemistry also ensures long column lifetimes, low secondary interactions and improved MALS sensitivity.

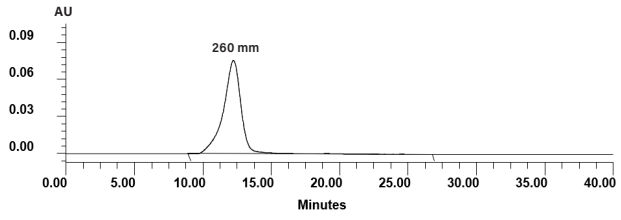


Figure 4. Comirnatry COVID-19 mRNA Vaccine 10 µg/dose drug product and intact LNP heterogeneity measurement using a GTxResolve Premier SEC 1000 Å 3 µm 4.6 x 150 mm Column.

AEX for Purity and Concentration Determining Measurements

Analytical Anion Exchange (AEX) analyses have proven to be a viable solution for assessing the heterogeneity of negatively charged species including nucleic acids. Existing AEX columns for nucleic acid analysis are reported to have challenges in terms of low efficiency and recovery which has limited the development of robust and reliable AEX methods for CQA analysis. Protein-Pak HiRes Q and Gen-Pak™ FAX Columns provide a set of strong and weak anion exchanger columns for the empirical optimization of new anion exchange techniques. Gen-Pak FAX Columns have recently been used to provide higher recovery mRNA analyses as compared to an industry reference monolithic column.

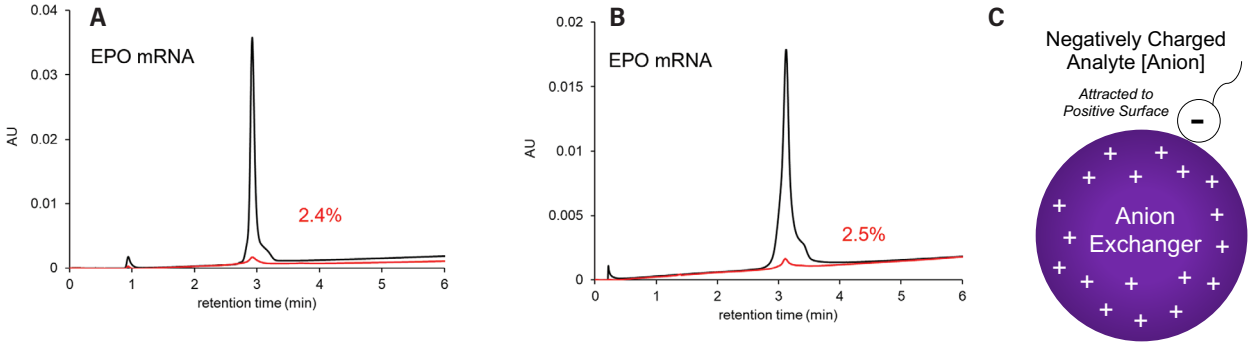


Figure 5. AEX of EPO mRNA using a Gen-Pak FAX (A) versus a monolithic column (B) using a novel approach as reported in Waters Application Note: 720007991. (C) Schematic representation of an anion exchange stationary phase.

Notes

5′ Cap Analysis and Oligo Mapping Analysis

An mRNA's sequence and its modifications need to be confirmed to ensure the mRNA will reach its target efficacy. The 5′ cap can be characterized after sample prep with DNA probes and RNase H which is specific to DNA/RNA duplexes. Further sequence confirmation can be obtained with mRNA digestion leveraging residue specific endonucleases. Oligo batch tested BEH C_{18} sorbents provide rugged columns for each of the downstream separations. MaxPeak™ High Performance Surfaces ensure quick method starts by eliminating conditioning effects and improving analyte recovery.

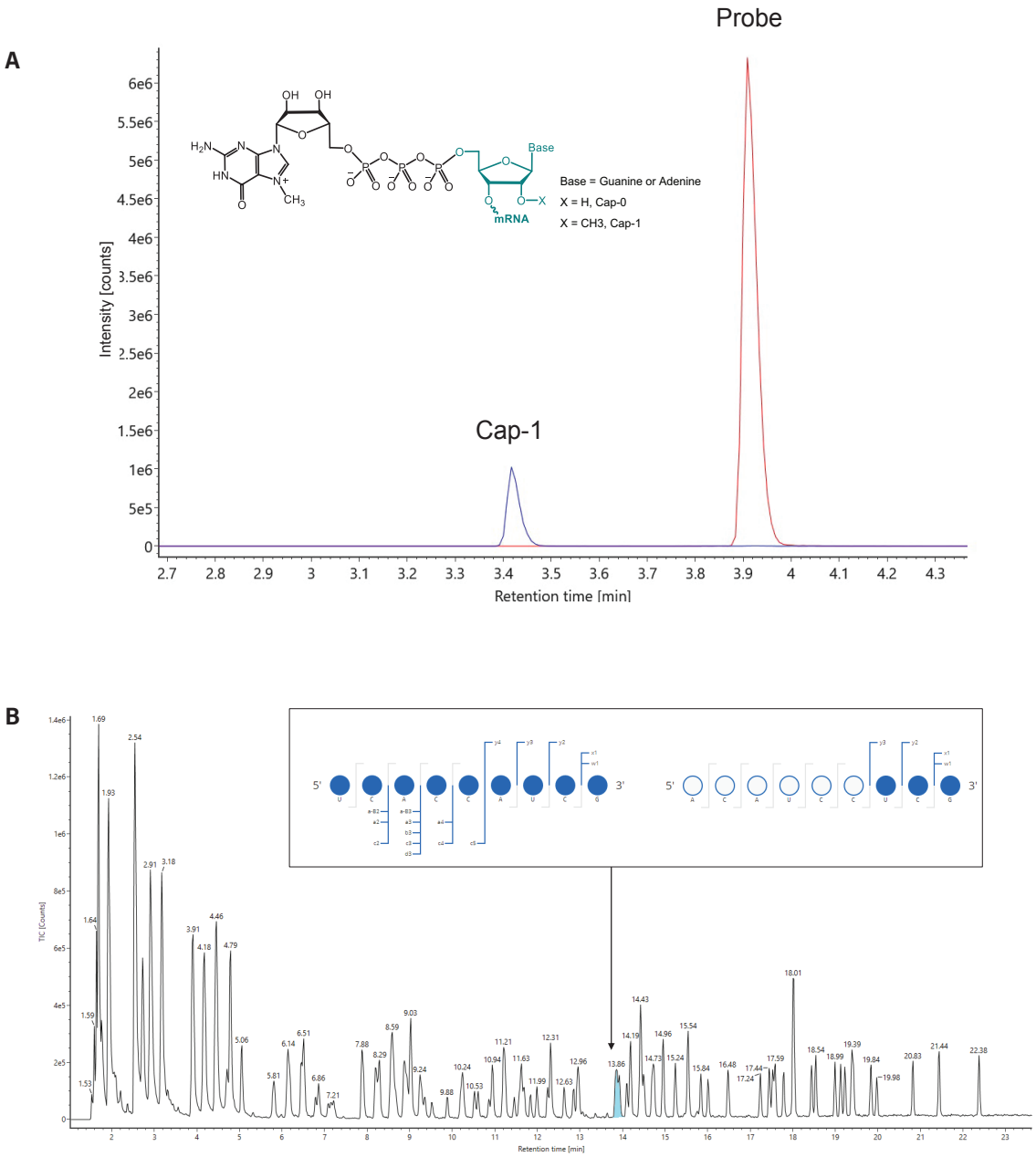


Figure 6. 5′ cap digestion product from RNase H cleavage (A) and and RNase T1 oligonucleotide map of a tail-less luciferase mRNA (B) as published in Waters Application Notes: 720007329 and 720007669.

Poly A Tail Analysis by RPLC

The length and structure of a 3′ poly A tail must be optimized to confer desired half life and ribosome binding affinity properties to an mRNA. RNase cleavage can be applied to digest an mRNA down to its poly A motif. An oligonucleotide batch tested widepore BEH 300 Å C_{18} column provides a high resolution separation of the liberated poly A tail, and the use of strong ion pairing agents makes it possible to achieve single residue resolution.

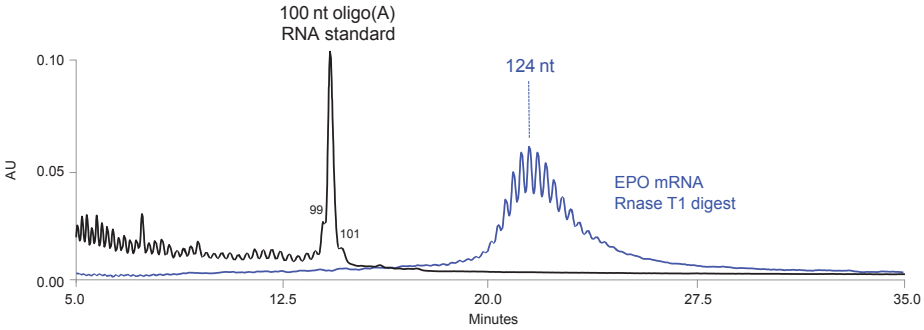


Figure 7. IP-RPLC chromatogram of a T1 digested mRNA sample and its constituent poly A tail as obtained with an ACQUITY Premier Oligonculeotide BEH 300Å 1.7 µm column (Waters Application Note: 720007873; Anal. Chem. 2023, 95, 38, 14308–14316).

Ultrafast RPLC to Increase Throughout

Your assay could achieve a new level of throughput and productivity with adoption of short bed length columns. Waters has developed low adsorption ACQUITY™ Premier 2.1 x 20 mm Columns packed with 1.7 µm oligonucleotide batch tested and selected stationary phases. These columns have been carefully optimized for both efficiency and mechanical durability, offering improved throughput for purity, identity and critical quality attribute testing. A key insight from recently published work shows that throughput can be significantly improved by selecting the appropriate column size. The larger the oligonucleotide or mRNA digestion component the more likely they are to exhibit a bind and elute chromatography mechanism.

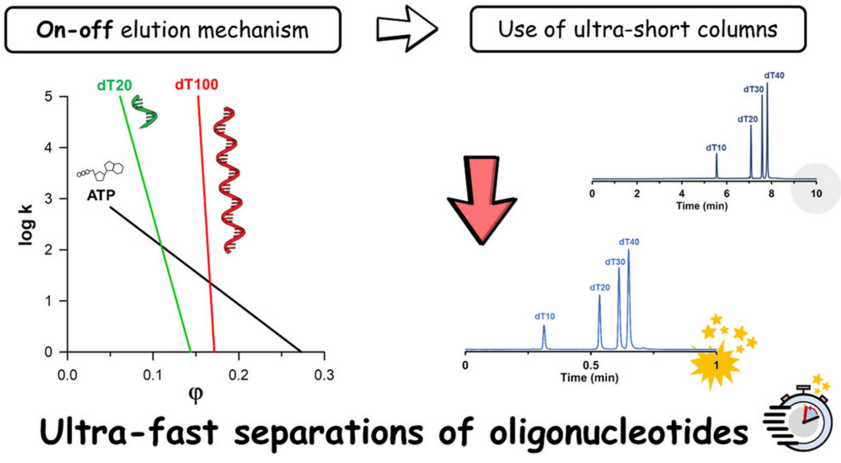


Figure 8. Isotherms of a 20 and 100-mer oligonucleotide that demonstrate the quick change of the analyte from an adsorbed to desorbed state upon an increase in eluent strength. A example 1-minute IP-RPLC analysis. (Anal. Chem. 2023, 95, 27, 10448-10456).

Lipid Raw Material and LNP Composition Analysis

Today's vaccines are prepared with a special, 4-component lipid formulation. It is important to examine these LNP components and confirm their relative abundances. In addition, it is important to check the raw materials used to manufacture these LNPs for impurities and degradants. A Charge Surface Hybrid (CSH™) Phenyl Hexyl Particle provides a unique repulsive effect against the ionizable cationic lipid found in these formulations and is the recommended starting point for new chromatography methods.

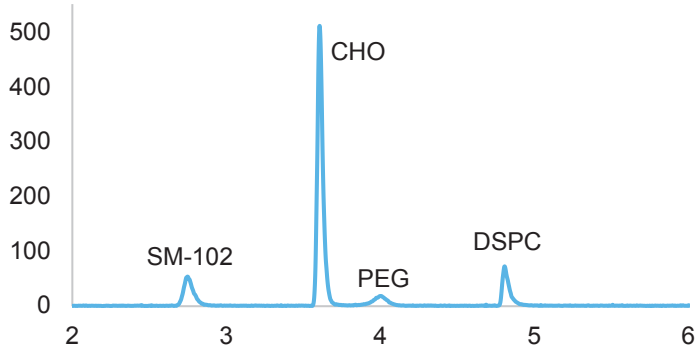


Figure 9. Evaporative light scattering chromatogram of lipids separated with an ACQUITY Premier CSH Phenyl Hexyl Column (Waters Application Note: 720007740; Journal of Chromatography B Volume 1234, 15 February 2024, 124005).

Notes

Ordering Information

The columns, standards, and reagents that can help you characterize LNP mRNA are provided below.

Quality and Design Matters:
Use consumables built and quality control tested for the highly ionic analytes comprised within cell and gene therapy drug substances and products.

MaxPeak High Performance Surfaces are based on a vapor deposited organosilica material that is incorporated into Waters column hardware to yield higher analyte recoveries and improved peak shapes.

SEC

GTxResolve Premier BEH SEC 450 Å 2.5 µm Columns

	Dimension	150 mm	300 mm	Guard
Standard Column	4.6 mm	186010584	186010585	186010583
	7.8 mm	186010586	186010587	186010583

GTxResolve Premier SEC 1000 Å 3 µm Columns

	Dimension	150 mm	300 mm	Guard
Standard Column	4.6 mm	186010735	186010736	186010733
	7.8 mm	186010737	186010738	186010733

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

STANDARDS AND REAGENTS

Standards and Reagents

	P/N
dsDNA 50 to 1350 Ladder	186010778
ssDNA 10 to 60 Ladder	186009449
ssDNA 20 to 100 Ladder	186009448
ssDNA 20-mer LC-MS Standard	186009451

OLIGO RPLC

ACQUITY Premier Oligonucleotide BEH C_{18} 1.7 µm Columns

	Diameter	50 mm	100 mm 130 Å	150 mm	50 mm	100 mm 300 Å	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

	Diameter	50 mm	100 mm 130 Å	150 mm	50 mm	100 mm 300 Å	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

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

	Dimension	130 Å	300 Å
Standard Column	2.1 x 20 mm	186011115	186011021

LIPID RPLC

ACQUITY Premier CSH Phenyl Hexyl 1.7 µm Columns

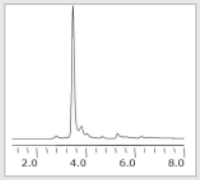
	Diameter	50 mm	100 mm 130 Å	150 mm
Standard Column	2.1 mm	186009474	186009475	186009476
VanGuard FIT Column	2.1 mm	186009477	186009478	186009479

LIPID RPLC

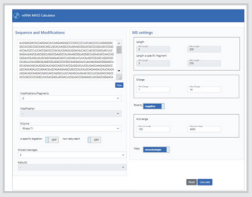
XSelect™ Premier CSH Phenyl Hexyl 2.5 µm Columns

	Diameter	50 mm	100 mm 130 Å	150 mm
Standard Column	2.1 mm	186009879	186009880	186009881
	4.6 mm	186009886	186009887	186009888
VanGuard FIT Column	2.1 mm	186009882	186009883	186009884
	4.6 mm	186009889	186009890	186009891

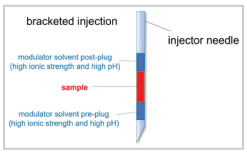
Application Notes



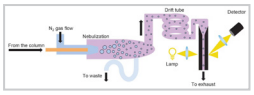
[Properties of the Gen-Pak™ FAX Column and Its Utility for Anion Exchange Analysis of Large Molecule Biologics]



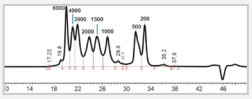
[Synthetic mRNA Oligo-Mapping Using Ion-Pairing Liquid Chromatography and Mass Spectrometry]



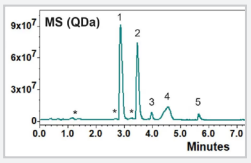
[Salt Plug Injection Methods for Improved Anion Exchange Analyses of Large Nucleic Acids]



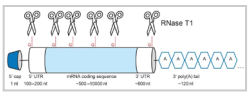
[Optimized ELSD Workflow for Improved Detection of Lipid Nanoparticle Components]



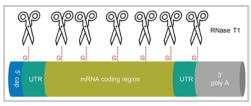
[Suitability of XBridge™ Premier GTx BEH™ SEC 450 Å 2.5 µm Column for Size-based Separations of Nucleic Acids]



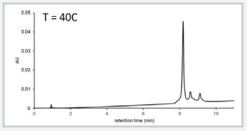
[Lipid Nanoparticle Analysis: Leveraging MS to Reduce Risk]



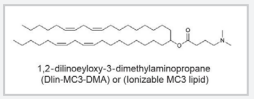
[Ion-Pair Reversed-Phase Liquid Chromatography Method for Analysis of mRNA Poly(A) Tail Heterogeneity]



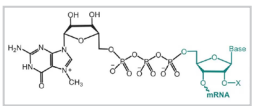
[Size-Exclusion Chromatography Method for Poly(A) Tail Analysis of mRNA]



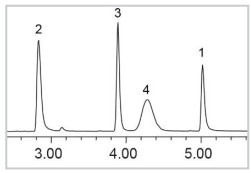
[Methods for the Anion Exchange Chromatographic Analysis of mRNAs]



[Rapid Analysis of Lipid Nanoparticle Components Using BioAccord LC-MS System]

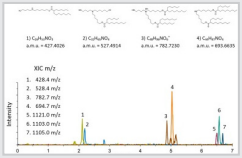


[Rapid Analysis of Synthetic mRNA Cap Structure Using Ion-Pairing RPLC with the BioAccord LC-MS System]

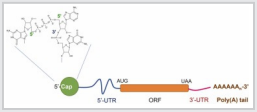


[Lipid Nanoparticle Compositional Analysis Using Charged Surface Hybrid Phenyl-Hexyl Separation With Evaporative Light Scattering Detection]

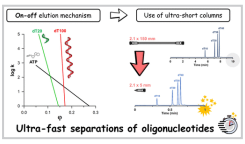
Waters Journal Articles



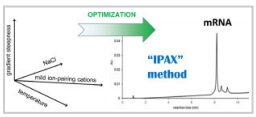
"Monitoring stability indicating impurities and aldehyde content in lipid nanoparticle raw material and formulated drugs"
J Chrom B. 2024, 1234, 124005.



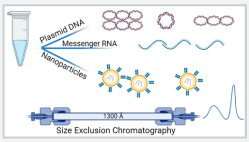
"Challenges and emerging trends in liquid chromatography-based analyses of mRNA pharmaceuticals"
J Pharm Biomed Anal. 2023, 224, 115174.



"High-Throughput Chromatographic Separation of Oligonucleotides: A Proof of Concept Using Ultra-Short Columns"
Anal. Chem. 2023, 95, 27, 10448–10456.

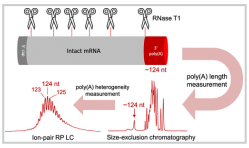


"Salt gradient and ion-pair mediated anion exchange of intact messenger ribonucleic acids" J Chrom Open, 2022, 2, 100031.



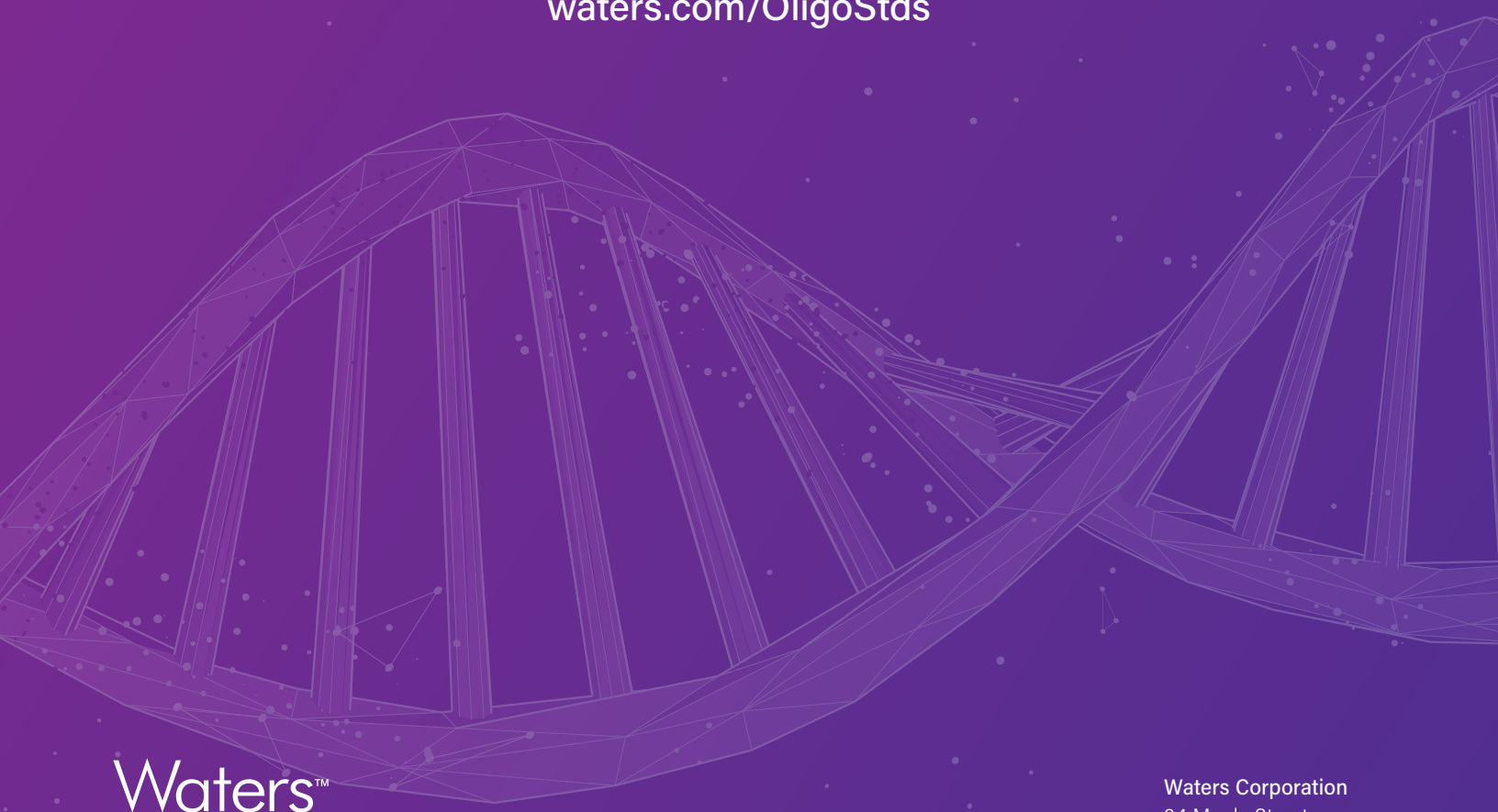
"Separation of Plasmid DNA Topology Forms, Messenger RNA, and LNP Aggregates Using an Ultrawide Pore Size Exclusion Chromatography Column"
Anal. Chem. 2023.

"Analyzing Encapsulated mRNA with LC, MS, and Calorimetry"
Genetic Engineering News, 2021.



"Liquid Chromatography Methods for Analysis of mRNA Poly(A) Tail Length and Heterogeneity"
Anal. Chem, 2023, 95, 38, 14308–14316.

waters.com/GTx
waters.com/GTxResolveSEC
waters.com/OligoRPLC
waters.com/OligoStds



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