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Nouveautés et innovations en chromatographie liquide en 2023

Davy GUILLARME

Quality Assistance
Octobre 2023

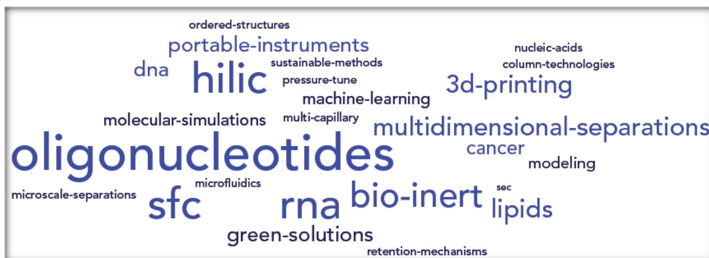
But de la présentation

Selon la demande faite par Arnaud Delobel, le but de cette présentation est de vous donner des informations relatives aux **nouveautés** apparues dans le domaine de la **chromatographie en phase liquide** durant **l'année 2023**.

Ces nouveautés seront dédiées à l'analyse de **petites molécules, d'oligonucléotides, de protéines/mAbs et de nouveaux produits innovants (AAVs, mRNA)**.

Les nouveautés en terme **d'approches innovantes** seront détaillées sur la base des **publications scientifiques** sorties en 2023.

Congrès HPLC 2023



HPLC 2022 San Francisco

HPLC 2023 Dusseldorf

Tendances au congrès HPLC 2023

Biopharmaceuticals and oligonucleotides

- 1 mm I.D. SEC columns
- Column packed with 1.1 µm particles @ 2500 Bar
- Ultra-short RPLC columns of 5 mm length

Green and sustainable analytical chemistry

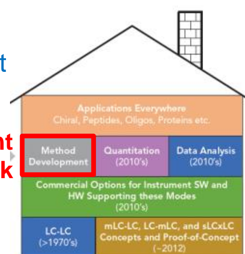
- New greenness metrics
- Alternative solvents (dimethyl carbamate, ethyl lactate))
- Temperature responsive LC with pure water
- SFC, *in silico* modeling

Multidimensional LC and ion mobility

- Need for tools/strategies for method development
- Use of HILIC x RPLC *vs.* RPLC x HILIC

Bioinert

- Various metal passivation strategies



Innovative chromatographic approaches for small molecules analysis

Excellent review on nonpecific adsorption

Analytica Chimica Acta 1250 (2023) 340994

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Managing nonspecific adsorption to liquid chromatography hardware: A review

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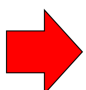
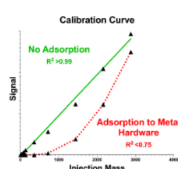
HIGHLIGHTS



- Evaluation of mobile phase additives to reduce nonspecific adsorption.
- The impact of alternative hardware materials on nonspecific adsorption to LC system.
- Strategies to differentiate sources of nonspecific adsorption.

GRAPHICAL ABSTRACT



HILIC sample introduction

Journal of Chromatography A 1700 (2023) 464006

ELSEVIER

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Managing sample introduction problems in hydrophilic interaction liquid chromatography

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ABSTRACT

Sample injection can cause serious problems in hydrophilic interaction liquid chromatography (HILIC) when the injection solvent has higher elution strength than the mobile phase (mp). It can lead to asymmetric peak shapes and poor efficiency. The problem can occur when the mp contains a high proportion of organic e.g. 95% acetonitrile (a weak solvent) whereas the injection solvent contains a higher proportion of water (a strong solvent) that is necessary to dissolve polar samples. We investigated different strategies to overcome this problem. A simple method is pre-column dilution where the injector is programmed to deliver a plug of weak solvent (e.g. pure acetonitrile) along with the sample dissolved in a solvent with higher water content than the mp. Another option is to use alternative organic solvents to acetonitrile in the injection solvent, e.g. isopropanol, acetone or tetrahydrofuran, that may give enhanced sample solubility. The role of the volume of injection solvents was investigated as well as the possible effects of mass overload on the results. The use of small sample volumes is always recommended to reduce mismatch effects.

HILIC sample introduction

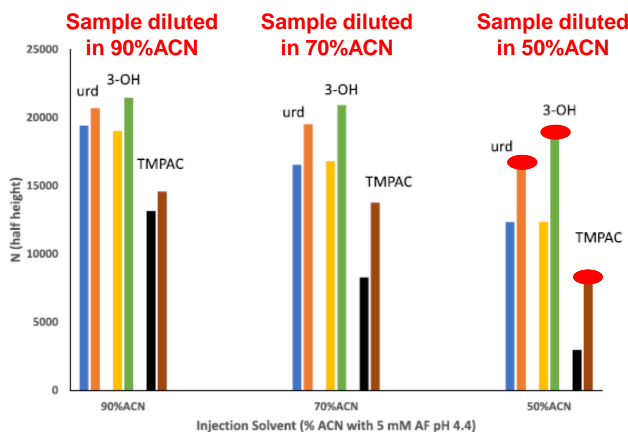

Fig. 1. Pre-column dilution (PCD) using 1 µL of 20 mg/L sample in various concentrations of buffered ACN followed by 4 µL pf pure ACN. Solutes urd = uridine, 3-OH = 3-hydroxybenzoic acid, TMPAC = trimethylphenylammonium chloride. RH bar represents efficiency using PCD; LH bar efficiency using simple 1 µL injection. Column: Premier Amide, mp 5 mM ammonium formate pH 4.4 in 90% ACN at 0.4 mL/min. Detection UV at 210 nm, Temperature 30 °C.

Mobile phase: 90%ACN

Micropillar array columns

analytical chemistry

Pharmafluidics now part of Thermo

pubs.acs.org/ac Article

On-Chip Comparison of the Performance of First- and Second-Generation Micropillar Array Columns

Bert Vankeerberghen, Jeff Op de Beeck, and Gert Desmet*

Cite This: https://doi.org/10.1021/acs.analchem.3c01829 Read Online

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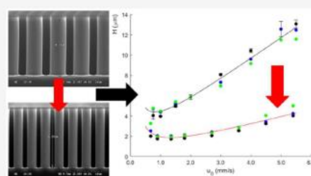
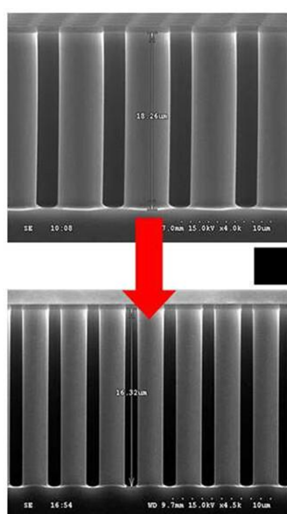
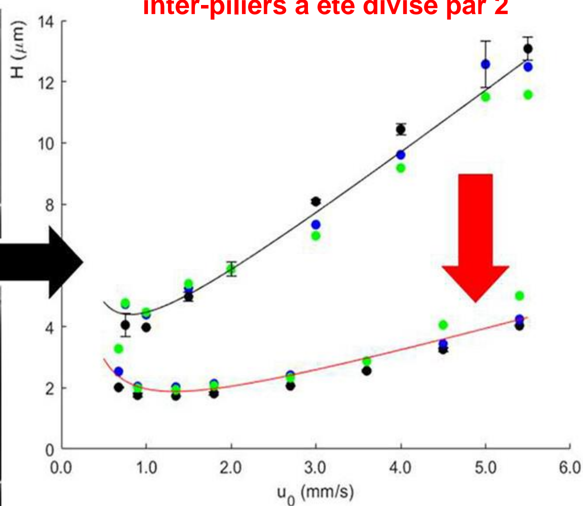


ABSTRACT: Because of its dimensions, the recently introduced micropillar array columns are most suited for high-efficiency liquid chromatography separations in proteomics. Unlike the packed bed columns and capillary-based column formats, the micropillar array concept still has significant room to progress in terms of the reduction of its characteristic size (i.e., pillar diameter and interpillar distance) to open the road to even higher-efficiency separations and their applications. We report here on the on-chip comparison between first-generation (Gen 1) and second-generation (Gen 2) micropillar array columns wherein the pillar and interpillar size have been halved. Because of the on-chip measurements, the observed plate heights H represent the fundamental band broadening, devoid of any extra-column band-broadening effects. The observed reduction of H with a factor of 2 around the u_{opt}-velocity and with a factor of 4 in the C-term dominated regime of the van Deemter-curve is in full agreement with the theoretically expected gain. This shows the pillar and interpillar size reduction could be effectuated without affecting the theoretical separation potential of the micropillar arrays. Compared to Gen 1, Gen 2 offers a 4-fold reduction of the required analysis time around the optimal velocity and about a 16-fold reduction in the C-term-dominated range.

Micropillar array columns

La taille des piliers et la distance inter-piliers a été divisé par 2





Temperature responsive LC columns

analytical chemistry

pubs.acs.org/ac Technical Note

Temperature Responsive × Fast Chiral Comprehensive Liquid Chromatography: a New 2D-LC Platform for Resolving Mixtures of Chiral Isomers

Turaj Rahmani, Adriaan Ampe, and Frédéric Lynen*

Cite This: Anal. Chem. 2023, 95, 8763−8769 Read Online

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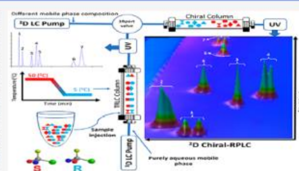
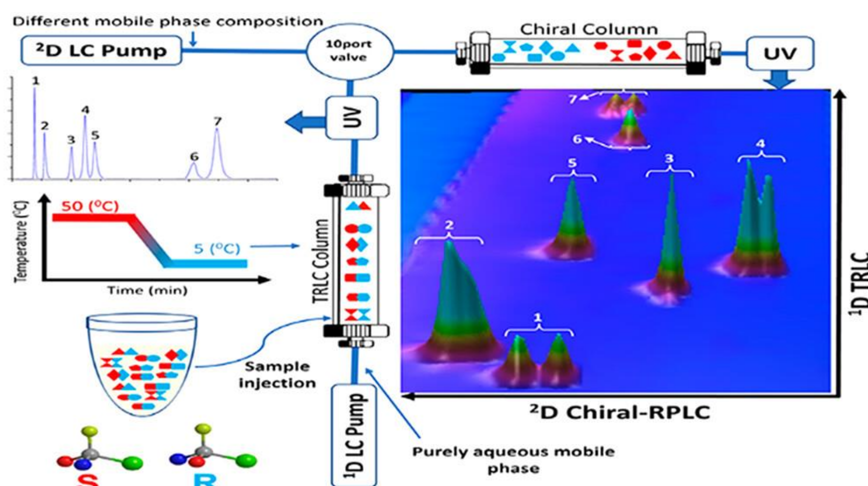


ABSTRACT: Chiral resolution of solutes occurring in mixtures of unrelated species is of relevance in life sciences and in pharmaceutical analysis. While this is conceptually achievable by comprehensive two-dimensional liquid chromatography (LC × LC), few approaches exist whereby the second dimension comprises the chiral separation. The latter is preferable in combination with a conventional reversed phase type of separation in the first dimension as it offers an extension of a conventional achiral analysis. The implementation of such rapid chiral analyses in the second dimension was, thus far, limited by the challenging transfer of the first dimension mobile phase to the second dimension while still achieving chiral separation. In this study, the combination of temperature-responsive and reversed-phase chiral liquid chromatography is assessed in terms of enantioselective separation of a broad range of pharmaceutical compounds. Applying temperature-responsive liquid chromatography (TRLC) in the first dimension allows for analyses to be performed under purely aqueous conditions, which then allows for complete and more generic refocusing of (organic) solutes prior to the second dimension. This offers an enhanced ability to employ fast and broad compositional gradients over the chiral dimension, which broadens the applicability of the technique. In the proposed platform, seven chiral columns (superficially porous and fully porous columns (comprising both polysaccharide and macrocyclic antibiotic phases)) and four mobile phase gradients were screened on a pharmaceutical test mixture. The platform was shown to be able to offer the necessary resolving power for the molecules at hand and offers a new approach for chiral screening of mixtures of unrelated compounds.

Temperature responsive LC columns



TRLC: purely aqueous mobile phase

RPLC x SFC on-line *vs.* off-line

Journal of Chromatography A 1694 (2023) 463907

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Off-line two-dimensional separation involving supercritical fluid chromatography for the characterization of the wastewater from algae hydrothermal liquefaction

Eloïse Teboul[a], Eliise Tammekivi[a], Magali Batteau[a], Christophe Geantet[b], Karine Faure[a,*]

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L'interface est balayée avec du MeOH pur puis ajout de CO_2 après l'interface LCxSFC

Journal of Chromatography A 1697 (2023) 463964

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

On-line reversed-phase liquid chromatography x supercritical fluid chromatography coupled to high-resolution mass spectrometry: A powerful tool for the characterization of advanced biofuels

Jason Devaux[a,b,c], Mélanie Mignot[b,c], Florent Rouvière[a], Isabelle François[d], Carlos Afonso[b,c], Sabine Heinisch[a,*]

Innovative chromatographic approaches for oligonucleotides

Excellent review of ONs in IP-RPLC

Journal of Chromatography Open 3 (2023) 100079

Contents lists available at ScienceDirect

Journal of Chromatography Open

journal homepage: www.elsevier.com/locate/jcoa

Separation of therapeutic oligonucleotides using ion-pair reversed-phase chromatography based on fundamental separation science

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A R T I C L E I N F O

Keywords:
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Preparative chromatography
Separation theory
Ion-pair chromatography
Ion-exchange chromatography

A B S T R A C T

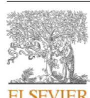
In recent decades, there has been a trend toward using larger biological molecules as new active pharmaceutical ingredients (APIs) instead of the classical small organic API molecules. More recently, this trend has shifted from very large biomolecules toward intermediate-sized APIs, such as oligonucleotide therapeutics. Because of their fundamental role in gene regulation, therapeutic oligonucleotides can be directed against their specific ribonucleic acid (RNA) targets, representing a promising customized approach for the treatment of hitherto incurable diseases. There are several FDA-approved oligonucleotide-based therapeutics and many more are awaiting approval. The complicated synthesis and degradation pathways of oligonucleotides, involving sophisticated new chemical modifications, generate hundreds of impurities, in contrast to classical small APIs, which typically contain only around three to five well-defined impurities (Fig. 1). Therefore, this new class of putative drugs entails challenging separation tasks: for example, a small mass change such as 1 Da must be distinguished in a 10,000 Da parent molecule for purposes of both quantification and purification and at extremely high resolution. All therapeutic oligonucleotides must be chemically modified before entering the body. One such modification is the phosphorothioate (PS) modification, which generates diastereomers: for a 20-nucleotide-long PS oligonucleotide, this exceeds half a million diastereomers.

In this review, we will examine recently published ion-pair liquid chromatographic separation strategies to meet current challenges in oligonucleotide separations. Ion-exchange chromatography will be briefly discussed based on its merits for large-scale purification. The review focuses on studies combining theory and practice and aiming at the analysis and preparative separation necessary for performing reliable quality control as well as purification. All relevant aspects of the separation systems will be discussed, including the stationary phase, pore size, mobile phase, and ion-pairing reagents. We will also discuss how the properties of the oligonucleotide and its impurities can be exploited to increase separation selectivity. A particular focus will be on the adsorption of ion-pairing reagent and the electrostatic surface potential it generates, allowing for interaction with the highly charged oligonucleotides. Furthermore, the effects of various gradient modes to decrease the electrostatic potential and thereby elute oligonucleotides will be covered.

Review exhaustive IP-RPLC of ONs

Characterization of diastereoisomers

Journal of Chromatography A 1708 (2023) 464327



Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Characterization of antisense oligonucleotide and guide ribonucleic acid diastereomers by hydrophilic interaction liquid chromatography coupled to mass spectrometry

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A R T I C L E I N F O

Keywords:
Diastereomers
HILIC
IM-MS
NMR
Oligonucleotides

A B S T R A C T

Oligonucleotides have become an essential modality for a variety of therapeutic approaches, including cell and gene therapies. Rapid progress in the field has attracted significant research in designing novel oligonucleotide chemistries and structures. Beyond their polar nature, the length of large RNAs and presence of numerous diastereomers for phosphorothioate (PS)-modified RNAs pose heightened challenges for their characterization. In this study, the stereochemistry of a fully-modified antisense oligonucleotide (ASO) and partially-modified guide RNAs (gRNAs) was investigated using HILIC and orthogonal techniques. The profiles of three lots of a fully-modified ASO with PS linkages were compared using ion-pairing RPLC (IPRP) and HILIC. Interestingly, three isomer peaks were partially resolved by HILIC for two lots while only one peak was observed on the IPRP profile. Model oligonucleotides having the same sequence of the five nucleotides incorporated to the 3′-end of the gRNA but differing in their number and position of PS linkages were investigated by HILIC, IPRP, ion mobility spectrometry–mass spectrometry (IM-MS) and nuclear magnetic resonance (NMR). An strategy was ultimately designed to aid in the characterization of gRNA stereochemistry. Ribonuclease (RNase) T1 digestion enabled the characterization of gRNA diastereomers by reducing their number from 32 at the gRNA intact level to 4 or 8 at the fragment level. To our knowledge, this is the first time that HILIC has successfully been utilized for the profiling of diastereomers for various oligonucleotide formats and chemical modifications.

Diastereoisomers of ONs

Characterization of diastereoisomers

Different position and number of PS modifications

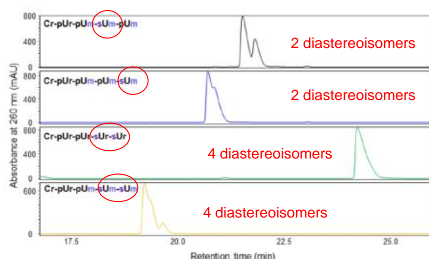

Fig. 2. HILIC-UV profiles obtained for the four model oligonucleotides. The nucleotide three letter code indicates the type of phosphate linkage ("p" is for phosphodiester, while "s" is for phosphorothioate), the nucleobase ("C" is for cytosine, while "U" is for uridine) and the sugar type ("r" is for ribose, while "m" is for 2'-O-methyl chemical modification).

HILIC columns with amide or diol

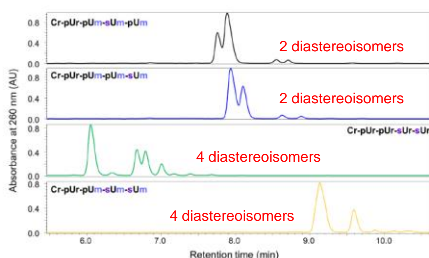

Fig. 3. IPRP-UV profiles obtained for the four model oligonucleotides.

Better selectivity in IP-RPLC

SFC of ONs

Journal of Chromatography A 1708 (2023) 464333

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Tutorial Article

Applicability of supercritical fluid chromatography for oligonucleotide analysis: A proof-of-concept study

Momoka Hayashida [a,b,c], Risa Suzuki [c], Shinnosuke Horie [b,c,d], Junichi Masuda [c], Takao Yamaguchi [a,*], Satoshi Obika [a,*]

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Enhanced fluidity liquid chromatography

ABSTRACT

We evaluated the suitability of supercritical fluid chromatography (SFC) for oligonucleotide analysis using 4-mer oligonucleotides with various phosphorothioate (PS) contents as model compounds. Column screening showed that the diol-modified column was able to separate sequences with different PS contents. Optimization of the column body and additives allowed us to analyze polar oligonucleotides using SFC. Various sequences were also analyzed using the optimized method. A good peak shape was obtained when the guanine plus cytosine content of the analyte was two or less in the 4-mer oligonucleotides. Furthermore, we found that the retention times of the selected sequences were positively correlated with polar surface areas, indicating that oligonucleotides interact with polar stationary phases. In contrast, more hydrophobic full PS sequences were retained more strongly in the diol column than the full phosphodiester (PO) sequences. This suggests that the diol column has unique selectivity for PO and PS linkages. These results indicate that SFC is potentially applicable to oligonucleotide analysis with a separation mechanism that is different from that of ion-pair reversed-phase liquid chromatography.

SFC of ONs

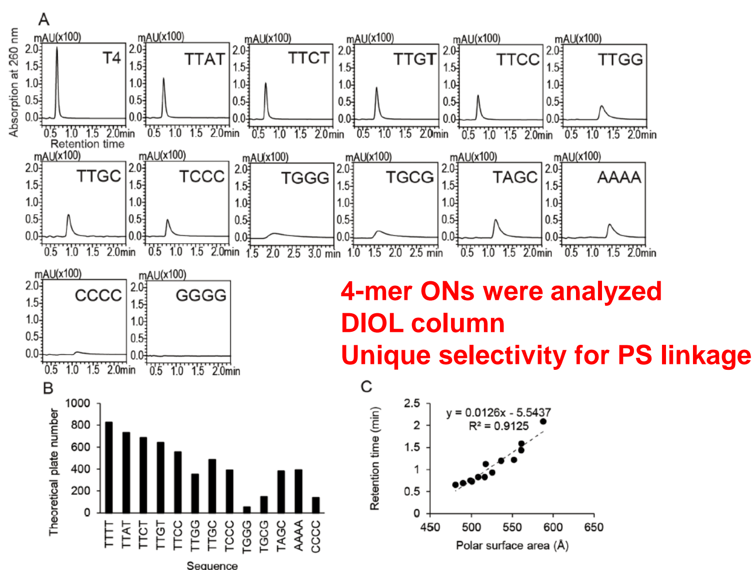

Fig. 6. (A) Chromatograms of the selected sequence. For analytical conditions, see Table S1 (entry 7). (B) Bar chart of theoretical plate number calculated from the chromatograms. (C) Relation between the retention time and topological surface area. Dotted line indicates linear approximation.

ONs analysis in RPLC without ion pair !

Journal of Chromatography A 1694 (2023) 463898

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Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Polybutylene terephthalate-based stationary phase for ion-pair-free reversed-phase liquid chromatography of small interfering RNA. Part 1: Direct coupling with mass spectrometry

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Oligonucleotide
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LC-MS
Patisiran
Impurity profiling

ABSTRACT

Nowadays, ion-pairing reversed-phase liquid chromatography (IP-RPLC) is the dominating generic method for the analysis of nucleic acid related compounds, such as antisense-oligonucleotides (ASO), small-interfering ribonucleic acid (siRNA) or other DNA or RNA type molecules and their conjugates. Despite of its effective performance, the usage of a high concentration of ion-pairing reagent in the eluent in IP-RPLC is unfavorable for the hyphenation with mass spectrometry (MS) which is required for a detailed structural characterization of the analytes and their structurally related impurities. In this work, we tested a polybutylene terephthalate (PBT)-bonded silica-based stationary phase for the separation of generically synthesized Patisiran as siRNA (antisense and sense single strands as well as their annealed double strand) giving some unexpected selectivity without any presence of ion-pairing reagents. Important chromatographic conditions affecting the separation have been investigated and evaluated. Furthermore, MS and tandem MS (MS/MS) characterization was possible without contamination of the MS system with ion-pair agent and related problems.

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Colonne vendue par Chiral Technologies

ONs analysis in RPLC without ion pair !

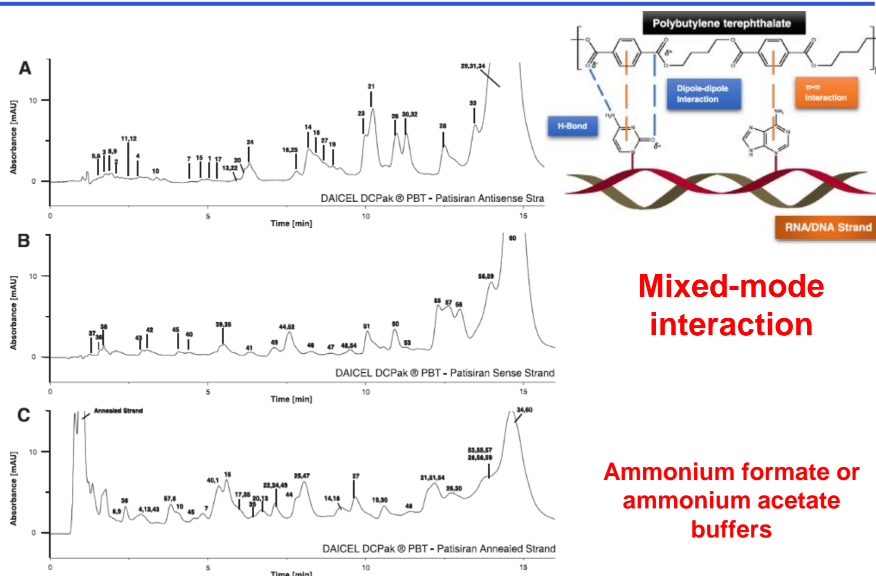

Fig. 4. RPLC-UV chromatograms of Patisiran antisense (guide) strand (A), sense (passenger) strand (B), and annealed siRNA duplex (C) on DCPak® PBT column. The detected peaks have been characterized by ESI-TOF-MS. For compound list see Table 1, 2 and S1. LC-Conditions (A-C): MPA: water, 20 mM AF, ${}^{w}_{w}$pH 6.3; MPB: MeOH/water 9:1 (v/v), 20 mM AF (${}^{w}_{w}$pH 6.3 of the aqueous fraction); Gradient: 10%–45% MPB in 28 min; Temperature: 40 °C; Flow rate: 0.6 ml/min. For MS parameters see chapter 2.4.

Ultra-short columns for ONs

analytical chemistry

pubs.acs.org/ac Article

High-Throughput Chromatographic Separation of Oligonucleotides: A Proof of Concept Using Ultra-Short Columns

Honorine Lardeux, Szabolcs Fekete, Matthew Lauber, Valentina D'Atri, and Davy Guillarme*

Cite This: *Anal. Chem.* 2023, 95, 10448–10456 Read Online

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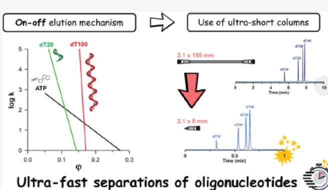
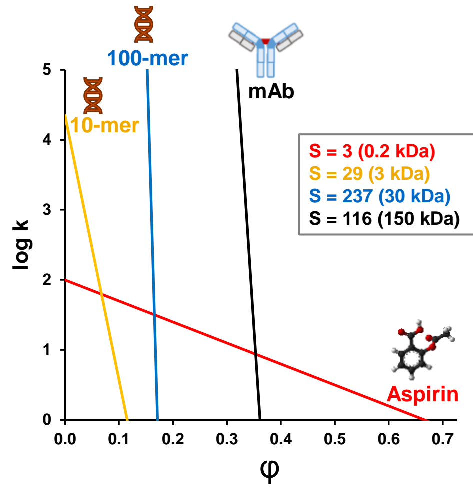
ABSTRACT: Ion-pairing reversed-phase liquid chromatography (IP-RPLC) is the reference separation technique for characterizing oligonucleotides (ONs) and their related impurities. The aim of this study was to better understand the retention mechanism of ONs, evaluate the applicability of the linear solvent strength (LSS) retention model, and explore the potential of ultra-short columns having a length of only 5 mm for the separation of model ONs. First, the validity of the LSS model was evaluated for ONs having sizes comprised between 3 and 30 kDa, and the accuracy of retention time predictions was assessed. It was found that ONs in IP-RPLC conditions follow an "on–off" elution behavior, despite a molecular weight lower than that of proteins. For most linear gradient separation conditions, a column length between 5 and 35 mm was found to be appropriate. Ultra-short columns of only 5 mm were therefore explored to speed up separations by considering the impact of the instrumentation on the efficiency. Interestingly, the impacts of injection volume and post-column connection tubing on peak capacity were found to be negligible. Finally, it was demonstrated that longer columns would not improve selectivity or separation efficiency, but baseline separation of three model ONs mixtures was enabled in as little as 30 s on the 5 mm column. This proof-of-concept work paves the way for future investigations using more complex therapeutic ONs and their related impurities.

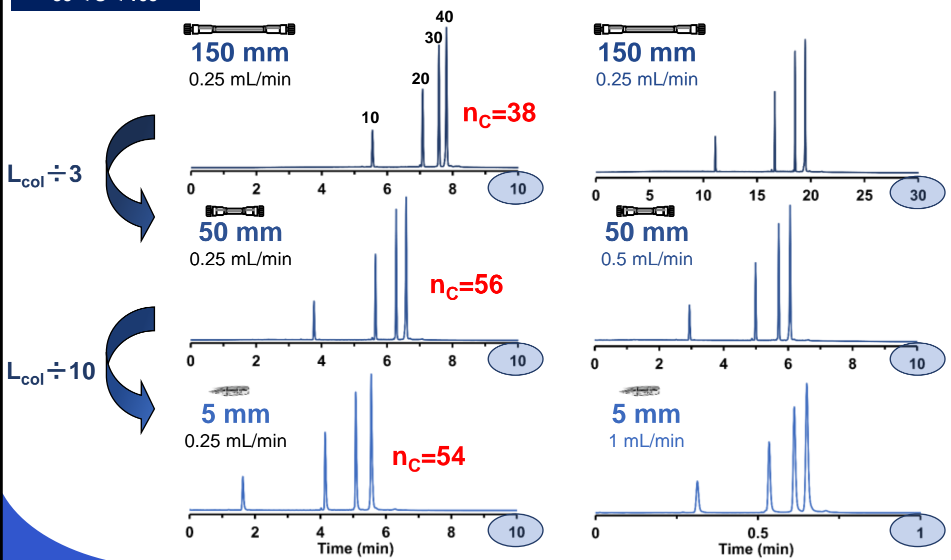


Ultra-short columns for ONs

Test ON samples: poly dT of 10 or 100 nucleotide units



Ultra-short columns for ONs



Pressure enhanced LC of ONs

Pressure–Enhanced Liquid Chromatography as a Suitable Approach to Improve Selectivity for Large Molecule Separations

Waters
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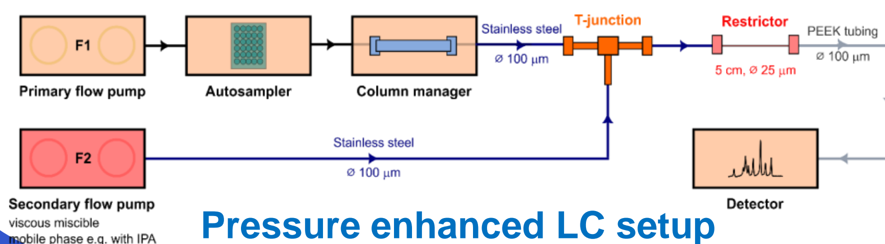
Honorine Lardeux, Davy Guillarme, Mateusz Imiołek, Matthew A. Lauber, and Szabolcs Fekete

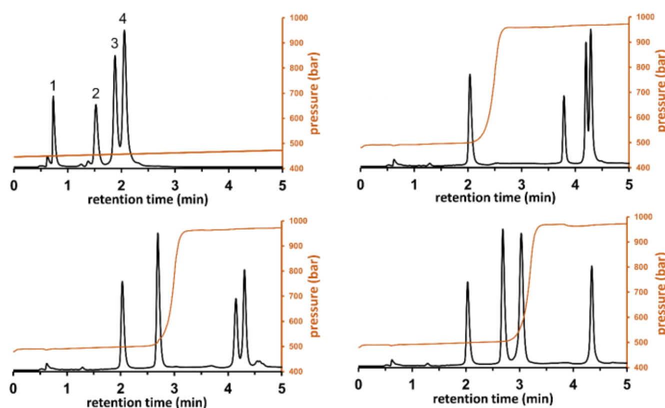
This work describes the application of a pressure–enhanced liquid chromatography (PE-LC) setup to tune the separation of various large molecules comprised of nucleic acids (oligonucleotides, messenger ribonucleic acid [mRNA], and deoxyribonucleic acid [DNA]). When adding pressure as a method development parameter, it indeed becomes possible to modify retention, selectivity, and peak width. As an example, the separation of oligonucleotides having sizes comprised between 40 and 100-mer in ion-pairing reversed-phase liquid chromatography (IP-RPLC) was drastically improved by using a stepwise pressure gradient to selectively shift the retention of a peak or group of peaks. Resolution was increased from 1.5 to 11.8 when setting a rapid high pressure step (twofold increased pressure) during the run. On the other hand, it was also possible to improve the separation of erythropoietin (EPO) mRNA and related impurities under ion-exchange chromatography (IEX). However, with this biomolecule, the best separation was achieved by reducing the pressure in the system. Finally, for another sample (DNA ladder) under IEX conditions, the pressure was found to have a limited impact on the overall selectivity. As highlighted in this work, pressure is an additional parameter that can be successfully used to develop LC methods of large biomolecules.



Pressure enhanced LC setup

Pressure enhanced LC of ONs

- Pressure impacts partial molar volume, conformation and energy of molecular interactions
- **Mobile phase** and **pressure gradient** were combined within the run to improve selectivity in IP-RPLC mode



Sample: dT40, dT60, dT80, dT100 @ 450 bar pressure step

Orthogonality between modes for ONs

Journal of Chromatography A 1705 (2023) 464184

Contents lists available at ScienceDirect

Journal of Chromatography A

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Evaluating orthogonality between ion-pair reversed phase, anion exchange, and hydrophilic interaction liquid chromatography for the separation of synthetic oligonucleotides

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ARTICLE INFO

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ABSTRACT

The orthogonality of separation between ion-pair reversed phase (IP-RP), anion exchange (AEX), and hydrophilic interaction liquid chromatography (HILIC) was evaluated for oligonucleotides. A polythymidine standard ladder was first used to evaluate the three methods and showed zero orthogonality, where retention and selectivity were based on oligonucleotide charge/size under all three conditions. Next, a model 23-mer synthetic oligonucleotide containing 4 phosphorothioate bonds with 2' fluoro and 2'-O-methyl ribose modifications typical of small interfering RNA was used for evaluating orthogonality. The resolution and orthogonality were evaluated between the three modes of chromatography in terms of selectivity differences for nine common impurities, including truncations (n-1, n-2), addition ($n + 1$), oxidation, and de-fluorination. We first evaluated different ion-pairing reagents that provided the best separation of the key impurities while suppressing diastereomer separation due to phosphorothioate linkages. Although different ion-pairing reagents affected resolution, very little orthogonality was observed. We then compared the retention times between IP-RP, HILIC, and AEX for each impurity of the model oligonucleotide and observed various selectivity changes. The results suggest that coupling HILIC with either AEX or IP-RP provide the highest degree of orthogonality due to the differences in retention for hydrophilic nucleobases and modifications under HILIC conditions. IP-RP provided the highest overall resolution for the impurity mixture, whereas more co-elution was observed with HILIC and AEX. The unique selectivity patterns offered by HILIC provides an interesting alternative to IP-RP or AEX, in addition to the potential for coupling with multidimensional separations. Future work should explore orthogonality for oligonucleotides with subtle sequence differences such as nucleobase modifications and base flip isomers, longer strands such as guide RNA and messenger RNA, and other biotherapeutic modalities such as peptides, antibodies, and antibody-drug-conjugates.

Orthogonality between modes for ONs

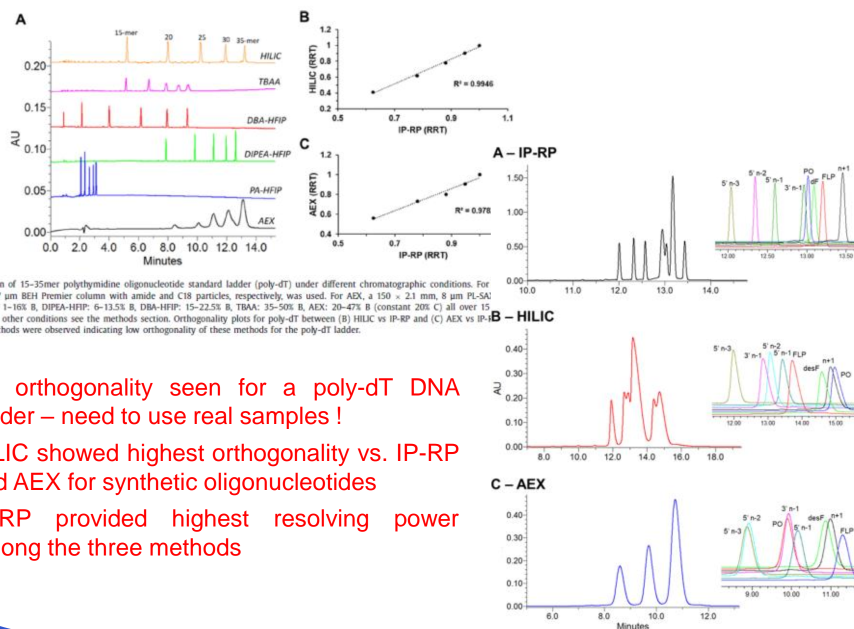

Fig. 2. (A) Separation of 15–35mer polythymidine oligonucleotide standard ladder (poly-dT) under different chromatographic conditions. For a 100 × 2.1 mm, 1.7 µm BEH Premier column with amide and C18 particles, respectively, was used. For AEX, a 150 × 2.1 mm, 8 µm PL-SA conditions: PA-HFIP: 1–16% B, DIPEA-HFIP: 6–13.5% B, DBA-HFIP: 15–22.5% B, TBAA: 35–50% B, AEX: 20–47% B (constant 20% C) all over 15 20 min gradient. For other conditions see the methods section. Orthogonality plots for poly-dT between (B) HILIC vs IP-RP and (C) AEX vs IP- the two selected methods were observed indicating low orthogonality of these methods for the poly-dT ladder.

- ❑ No orthogonality seen for a poly-dT DNA ladder – need to use real samples !
- ❑ HILIC showed highest orthogonality vs. IP-RP and AEX for synthetic oligonucleotides
- ❑ IP-RP provided highest resolving power among the three methods

2D-LC of ONs

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Optimization of orthogonal separations for the analysis of oligonucleotides using 2D-LC

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ARTICLE INFO

Keywords:
Oligonucleotides
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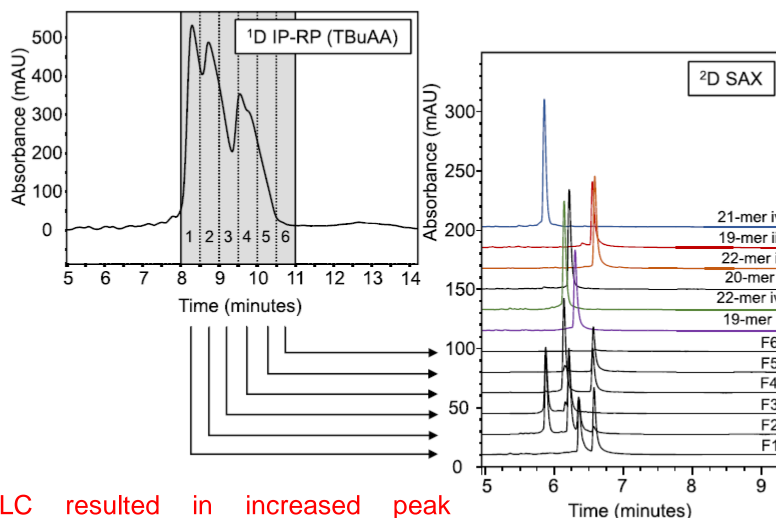
Ion pair reversed phase HPLC
Anion exchange HPLC

ABSTRACT

Oligonucleotides are commonly analysed using one dimensional chromatography (1D-LC) to resolve and characterise manufacturing impurities, structural isomers and (in respect to emerging oligonucleotide therapeutics) drug substance and drug product. Due to low selectivity and co-elution of closely related oligonucleotides using 1D-LC, analyte resolution is challenged. This leads to the requirement for improved analytical methods. Multi-dimensional chromatography has demonstrated utility in a range of applications as it increases peak capacity using orthogonal separations, however there are limited studies demonstrating the 2D-LC analysis of closely related oligonucleotides. In this study we optimised OGN size and sequence based separations using a variety of 1D-LC methods and coupled these orthogonal modes of chromatography within a 2D-LC workflow. Theoretical 2D-LC workflows were evaluated for optimal orthogonality using the minimum convex hull metric. The most orthogonal workflow identified in this study was ion-pair reversed phase using tributylammonium acetate (IP-RP-TBuAA) coupled with strong anion exchange in conjunction with sodium perchlorate (SAX-$NaClO_4$) at high mobile phase pH. We developed a heart-cut (IP-RP-TBuAA)-(SAX-$NaClO_4$) 2D-LC method for analysis of closely related size and sequence variant OGNs and OGN manufacturing impurities. The 2D-LC method resulted in an increased orthogonality and a reduction in co-elution (or close elution). Application of a UV based reference mapping strategy in conjunction with the 2D-LC method demonstrated a reduction in analytical complexity by reducing the reliance on mass based detection methods.

2D-LC of ONs



2D-LC resulted in increased peak capacity, a reduction in co-elution events and improved resolution

Innovative chromatographic approaches for proteins and mAbs

Micro-flow SEC

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Insights on further improving fast size exclusion chromatography separations of biopharmaceuticals using 2.1 millimetre column diameters

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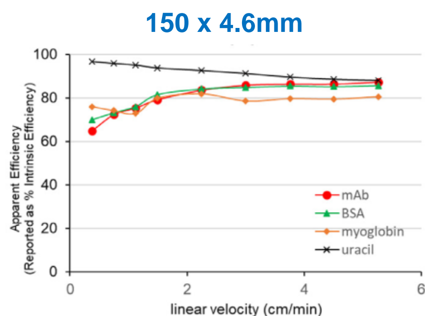
Extra-column dispersion
Therapeutic proteins

ABSTRACT

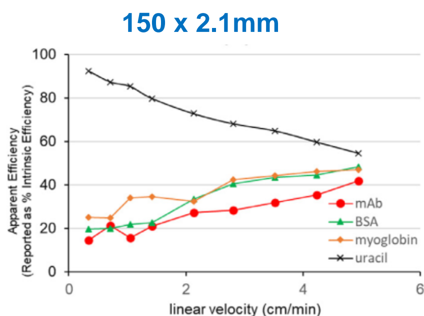
Recent trends in the pharmaceutical and biotechnology industries call for the miniaturization of size exclusion chromatography. The thought of such a future has been tantalizing but there are many practical and theoretical considerations that have impeded progress. Here, the capabilities of a narrow bore 2.1 mm ID SEC column have been studied and compared to reference 150 × 4.6 mm SEC columns when using UV detection. While our study reconfirms the importance of having very low system dispersion for SEC separations, it goes on to show that a 150 × 2.1 mm 1.7 µm particle SEC column can offer a balanced compromise of performance. Despite the fact that the 150 × 2.1 mm ID 1.7 µm column's intrinsic efficiency was not fully utilized, it still performed with an apparent efficiency similar to that of a 150 × 4.6 mm ID 2.5 µm column. Beyond this, our study provides insights on what more will need to be achieved to robustly establish low flow SEC separations. If SEC chromatographers aim to miniaturize sizing separations to 1 mm diameters or below, there is more work to do on chromatographic instruments and flow paths. In order for an instrument to be optimized for 1 mm ID SEC it would need to exhibit a system variance of less than 0.5 µL^2.

Micro-flow SEC

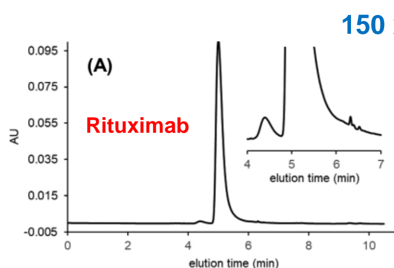
150 x 4.6mm

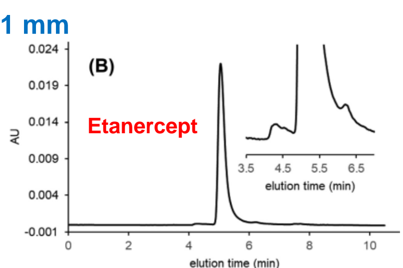


150 x 2.1mm



150 x 2.1 mm





Micro-flow SEC-MS

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Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Micro-flow size-exclusion chromatography for enhanced native mass spectrometry of proteins and protein complexes

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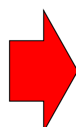
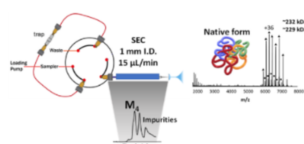
[d] Division of Bioanalytical Chemistry, Amsterdam Institute of Molecular and Life Sciences, Vrije Universiteit Amsterdam, De Boelelaan 1085, 1081, HV Amsterdam, the Netherlands

HIGHLIGHTS

- Micro-flow SEC-native MS offers enhanced sensitivity for the detection of higher-order structures.
- Low flow rates (<15 μL/min) allow milder ionization conditions ensuring native MS of proteins.
- Micro-flow SEC allows the use of higher concentration of volatile salts, preventing protein adsorption more efficiently.
- Trapping the protein before SEC-native MS eliminates the adverse chromatographic effects of large volume injections.



GRAPHICAL ABSTRACT



1mm SEC column
D < 15 μL/min

Micro-flow SEC-MS

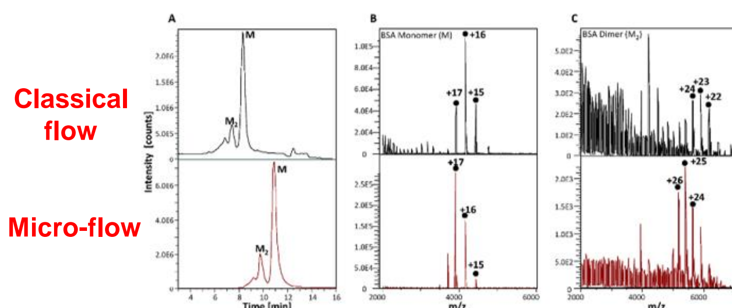


Fig. 1. A) Extracted-ion chromatograms (EICs) of BSA analysed with conventional-flow (top) and micro-flow (bottom) SEC-MS with a mobile phase of 50-mM ammonium-acetate; B) corresponding mass spectra of BSA monomer (M) and C) dimer (M2). Conditions: Conventional-flow SEC-MS: flowrate, 200 µL/min; injected BSA concentration, 60 µM; Micro-flow SEC-MS: flowrate 15 µL/min, injected BSA concentration, 15 µM.

- Micro-flow SEC-native MS offers **enhanced sensitivity** for the detection of higher-order structures
- Low flow rates **(≤ 15 µL/min)** allow milder ionization conditions ensuring native MS of proteins
- Micro-flow SEC **allows the use of higher concentration of volatile salts**, preventing protein adsorption more efficiently
- **Trapping the protein** before SEC-native MS eliminates the adverse chromatographic effects of large volume injections

Recycling SEC

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Resolution limits of size exclusion chromatography columns identified from flow reversal and overcome by recycling liquid chromatography to improve the characterization of manufactured monoclonal antibodies

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Flow reversal
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Long-range velocity biases
Characterization of monoclonal antibodies
Twin-column recycling chromatography

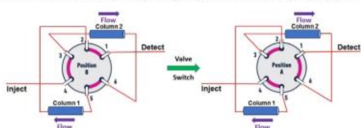
ABSTRACT

The flow reversal (FR) technique consists of reversing the flow direction along a chromatographic column. It is used to reveal the origin (such as poor column packing, active sites, or slow absorption/escape kinetics) for the resolution limit of 4.6 mm × 150 mm long columns packed with 1.7 µm 200 Å Bridge-Ethylene-Hybrid (BEH^{TM}) Particles. These columns are used to separate manufactured monoclonal antibodies (mAb, ~ 150 kDa) from their close impurities (or IdeS fragments, ~ 100 kDa) by size exclusion chromatography (SEC). FR unambiguously demonstrates that the resolution limit of these SEC columns is primarily due to long-range flow velocity biases covering distances of at least 500 µm across the column diameter. This confirms the existence of center-to-wall flow heterogeneities which cause undesirable tailing for the mAb peak. Because the transverse dispersion coefficient ($D_t = 1.1 \times 10^{-6}$ cm^2/s) of mAbs across the column diameter is intrinsically low, the bandspreading of the mAb in a single flow direction is in part reversible upon reversing the flow direction. For the very same residence time in the column, the column efficiency is found to increase by +85% relative to that observed under conventional elution mode. The observed peak tailing of the mAb and its sub-units is not caused by active surface sites or by slow absorption/escape from the BEH Particles. Therefore, the most critical mAb impurities (hydrolytic degradation $F_{ab/c}$ and IdeS $F_{(ab')_2}$ fragments) can only be successfully separated and quantified with acceptable accuracy by adopting alternate pumping recycling liquid chromatography (APRLC). APRLC enables the full baseline separation of the mAb and 100 kDa mAb fragments and partial separation of $F_{ab/c}$ and $F_{(ab')_2}$ fragments after increasing the number of cycles to ten. It was made possible to accurately measure the relative abundances of the mAb (99.0 ± 0.1%), $F_{(ab')_2}$ fragment (0.88 ± 0.03%), and $F_{ab/c}$ immunogenic fragment (0.13 ± 0.02%) in less than 45 min for a total mAb sample load of only 5 µg. Still, further improvements are needed to increase the sensitivity of the APRLC method and to reduce the solvent consumption by adopting narrow-bore 2.1 mm i.d. SEC columns.

Recycling SEC

Two twin SEC columns of 150 x 4.6 mm

Alternate Pumping Recycling Liquid Chromatography (APRLC)



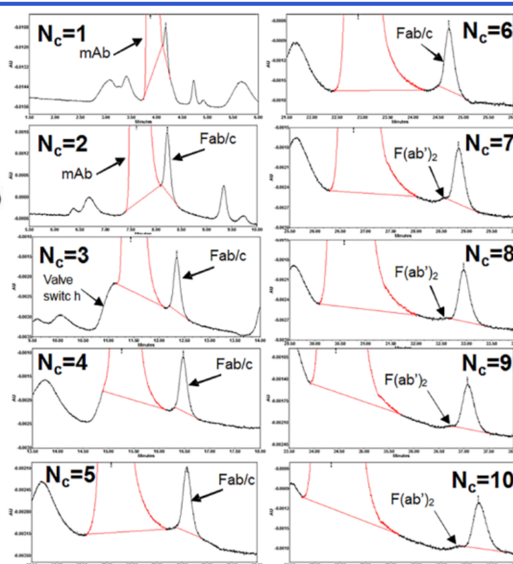


Fig. 5. APRLC experiments using two twin 4.6 × 150 mm 1.7 μm 200 Å BEH Columns. The number of cycles was increased from 1 to 10 and the 10 different experimental chromatograms are shown. Same experimental conditions as those in Fig. 2. For better visualization of the gain in peak resolution as the number of cycles is increasing, the time x-axis covers the same width of 4.5 min and is centered at a time equal to half the sum of the retention times of the mAb and that of the 100 kDa fragments ($F_{ab/c}$ and $F_{(ab')_2}$). Note the clear separation and accurate quantification of the two low-molecular-weight fragments $F_{ab/c}$ and $F_{(ab')_2}$ for a number of cycles larger than 2 and 7, respectively.

APRLC simulates the use of very long columns by transferring a targeted separation zone from one the second column

SEC-CDMS

Charge-detection mass spectrometry (CDMS) determines the mass of ions directly (not *m/z*) and enables direct measurement of the charge of an ion alongside its mass-to-charge ratio

analytical chemistry

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Exploring Charge-Detection Mass Spectrometry on Chromatographic Time Scales

Lisa Strasser,[#] Florian Füssl,[#] Tomos E. Morgan, Sara Carillo, and Jonathan Bones*

Cite This: https://doi.org/10.1021/acs.analchem.3c03325 Read Online

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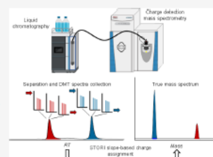
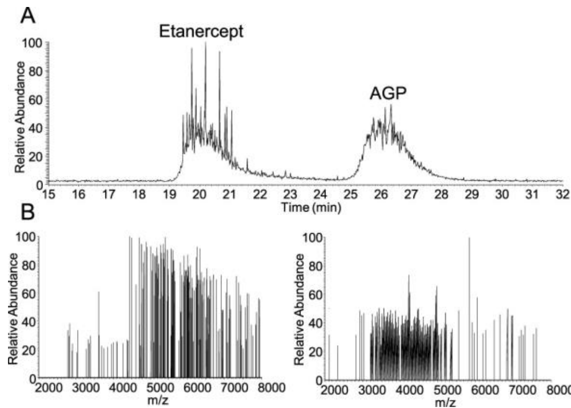
ABSTRACT: Charge-detection mass spectrometry (CDMS) enables direct measurement of the charge of an ion alongside its mass-to-charge ratio. CDMS offers unique capabilities for the analysis of samples where isotopic resolution or the separation of charge states cannot be achieved, i.e., heterogeneous macromolecules or highly complex mixtures. CDMS is usually performed using static nano-electrospray ionization-based direct infusion with acquisition times in the range of several tens of minutes to hours. Whether CDMS analysis is also attainable on shorter time scales, e.g., comparable to chromatographic peak widths, has not yet been extensively investigated. In this contribution, we probed the compatibility of CDMS with online liquid chromatography interfacing. Size exclusion chromatography was coupled to CDMS for separation and mass determination of a mixture of transferrin and β-galactosidase. Molecular masses obtained were compared to results from mass spectrometry based on ion ensembles. A relationship between the number of CDMS spectra acquired and the achievable mass accuracy was established. Both proteins were found to be confidently identified using CDMS spectra obtained from a single chromatographic run when peak widths in the range of 1.4−2.5 min, translating to 140−180 spectra per protein were achieved. After demonstration of the proof of concept, the approach was tested for the characterization of the highly complex glycoprotein α-1-acid glycoprotein and the Fc-fusion protein etanercept. With chromatographic peak widths of approximately 3 min, translating to ~200 spectra, both proteins were successfully identified, demonstrating applicability for samples of high inherent molecular complexity.



SEC-CDMS

- SEC-CDMS method (with MS friendly mobile phase) was developed to separate β-galactosidase (466 kDa) and transferrin (80 kDa)
- The method was then applied to AGP and etanercept (highly glycosilated species)



Homodimers separation of bsAbs

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Journal of Chromatography B

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Effectively removing the homodimer in bispecific antibodies by weak partitioning mode of anion exchange chromatography

Xiaoying Liang, Qingquan He, Guohong Qin, Guozhu Li, Qian Li, Huanghong Tan, Zichen Wang, Mengni Fan, Dan Xu *

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ARTICLE INFO

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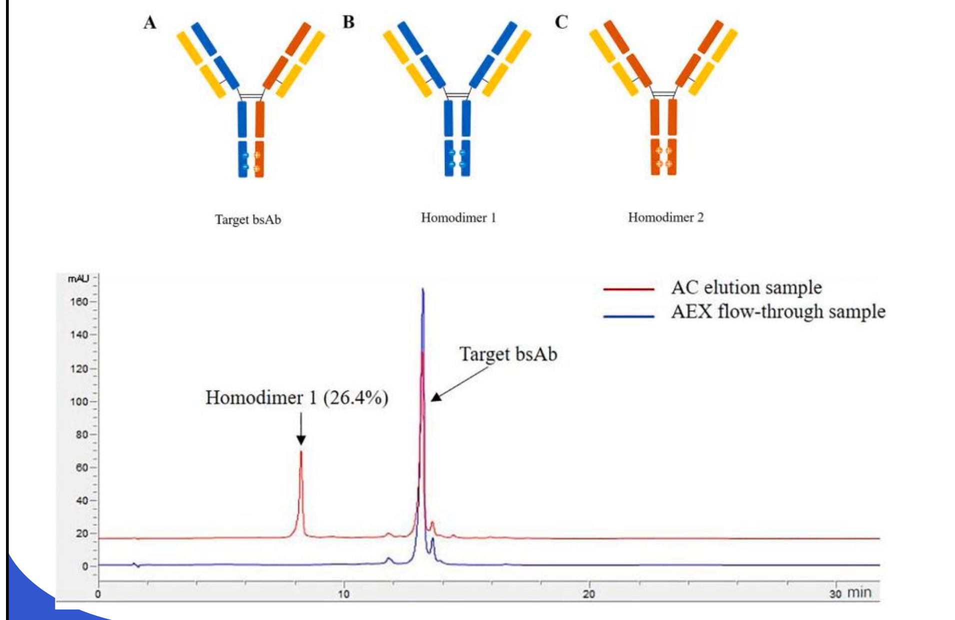
Bispecific antibody (bsAb)
Homodimer
Anion exchange (AEX) chromatography
Weak partitioning mode
Design of experiments (DoE)
High-throughput process development (HTPD)

ABSTRACT

Small amounts of by-products are nevertheless created during the recombinant production of IgG-like bispecific antibodies due to imbalanced chain expression and improper chain pairing, despite the employment of molecular strategy techniques to promote accurate pairing. Among them, homodimers represent the species that are more difficult to remove due to their physical and chemical properties being similar to the target antibody. Homodimer by-products are always produced even though various technologies can significantly increase the expression of heterodimers, so a robust purification process to recover high-purity heterodimers is required. Most of the chromatography methods commonly adopt the bind-and-elute mode or two-step to separate homodimers, which has numerous drawbacks such as prolonged process times and limited dynamic binding capacity. Flow-through mode of anion exchange is a frequently-used polishing step for antibodies, but it is typically regarded as being more effective for host-cell protein or host-cell DNA removal rather than other product-related impurities such as homodimers and aggregates. This paper demonstrated that single-step anion exchange chromatography allows high capacity and effective clearance of the homodimer byproduct to be simultaneously achieved, suggesting that weak partitioning was a better polishing strategy for achieving a high level of heterodimer purity. And robust operation range of anion exchange chromatography steps for homodimer removal was also developed by leveraging the design of experiments.

Homodimers separation of bsAbs



4D-LC of mAbs

analytical chemistry

pubs.acs.org/ac Article

Novel Multidimensional Liquid Chromatography Workflow with In-Loop Enzymatic Digests of Multiple Heart-Cuts for Fast and Flexible Characterization of Biotherapeutic Protein Variants

Kilian Mayr, Thomas Weindl, Achim Gärtner, Julien Camperi, Thomas Maetzke, Markus Förster, Thomas Nachtigall, Frank Steiner, Annette Vogt, Fabian Hosp,* and Michael Mølhøj

Cite This: https://doi.org/10.1021/acs.analchem.2c04467 Read Online

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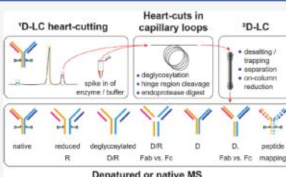
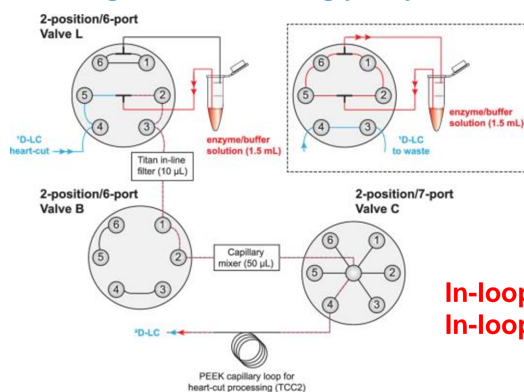


ABSTRACT: Multidimensional liquid chromatography (mD-LC) is becoming a powerful tool for complete characterization of individual peaks and protein variants through separation methods such as nondenaturing ion exchange (IEC) or size-exclusion chromatography coupled to reversed-phase (RP) chromatography. The flexibility of commercially available and customized mD-LC systems is still limited in terms of enzymatic peak processing between chromatographic dimensions. In this regard, only a few column-immobilized proteases are available for detailed peak characterization by mD-LC coupled to mass spectrometry (mD-LC−MS). Here, we present a purpose-built and automated multiple heart-cutting mD-LC design with a novel analytical workflow involving in-loop enzymatic heart-cut digestion between the first-dimensional column and transfer to the second dimension before MS or MS/MS analyses. The setup facilitates the spike-in of any enzyme to multiple heart-cuts for multilevel analysis, for example, for peptide mapping, fragment generation, or deglycosylation, to reduce heterogeneity and provide maximum flexibility in terms of incubation time for optimal peak characterization. We demonstrate the application of IEC coupled to RP-LC−MS and automated in-loop deglycosylation and on-column reduction of an IgG antibody combined with upper hinge region cleavage for Fab generation. We further employ mD-LC−MS and mD-LC−MS/MS to assess post-translational modifications of a bispecific antibody and to support molecule selection by evaluating the best downstream purification strategy. The novel design and automated workflow of the mD-LC system described here offers enhanced flexibility for in-solution processing and real-time monitoring of multiple heart-cuts enabling streamlined characterization of unknown biotherapeutic charge and size variants.

4D-LC of mAbs

The setup facilitates the spike-in of any enzyme to multiple heart-cuts for multilevel analysis, for example, for peptide mapping, fragment generation, or deglycosylation



In-loop deglycosylation
In-loop trypsine digestion

Schematic illustration of the automated loop transfer of heart-cuts with simultaneous spike-in of enzyme and buffer.
The enzyme-buffer solution pumped in circulation by two mixing tees is combined with the 1D-LC heart-cut using the 2-position/6-port valve L.
All in-line digests were performed using 600 µL/min (heart-cut peak) and 300 µL/min (enzyme-buffer blend) flows.
The blend is mixed horizontally using a 10 µL Titan in-line filter integrated between the valves L and B
The mixture is then transferred to a variable size PEEK-capillary loop in the column compartment for temperature-controlled digestion.

Analytical / functional testing of mAbs

analytical chemistry

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In-Depth Characterization of mAb Charge Variants by On-Line Multidimensional Liquid Chromatography-Mass Spectrometry

Zhuoyu Liu, Yanjing Cao, Lei Zhang,* Ya Xu, and Zhongli Zhang*

Cite This: https://doi.org/10.1021/acs.analchem.3c00791 Read Online

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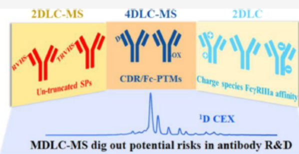
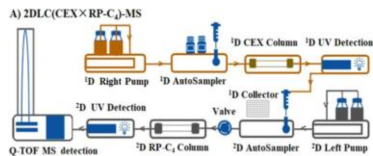
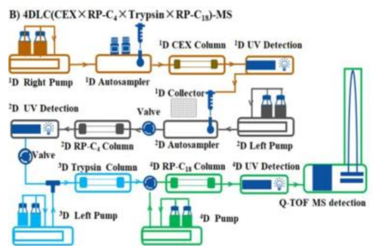
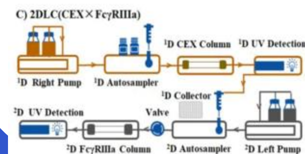


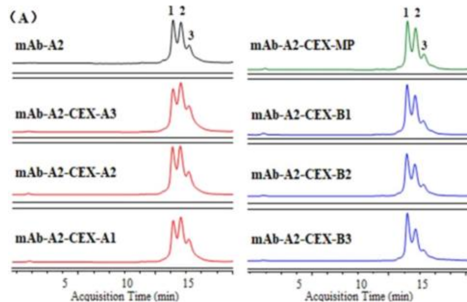
ABSTRACT: In-depth characterization of charge heterogeneity is a pivotal step desired in the therapeutics antibody development. To this end, a novel on-line multidimensional liquid chromatography-mass spectrometry (MDLC-MS) method for charge variant characterization was developed to dig out potential risks on safety and efficacy. This method implemented 96-well plate fractionation and on-column preconcentration by multi-injection, thereby facilitating detection of charged species at low abundance. Eleven charge variants of mAb-A were preliminarily characterized by 2DLC(CEX × RP-C_4)-MS. TRVHS and RVHS signal peptide variants of mAb-A were found in basic peaks of the CEX profile. The results supported process development in a timely manner, and the signal peptide-containing variants with potential immunogenicity were successfully removed by an optimized purification process. The retained seven charge variants of mAb-A were further characterized by 4DLC(CEX × RP-C_4 × Trypsin×RP-C_{18})-MS. Post-translational modifications including deamidation, cyclization of N-terminal glutamine, C-terminal lysine truncation as well as proline amidation, and methionine oxidation were identified, and their potential risks were evaluated. Biological activity of the seven charge variants was evaluated by 2DLC (CEX × FcγRIIIa). Increased FcγRIIIa receptor binding affinity was observed in the acidic variants. The MDLC-MS detection can be completed in 72 h with 1.25 mg of mAb, demonstrating to be sample-economic, time-effective, and labor-saving. It provided a powerful and timely tool for charge variant characterization and met the aggressive timeline desired for antibody development.

Analytical / functional testing of mAbs









FcγRIIIa affinity chromatograms of charge variants by 2D-LC (CEX × FcγRIIIa) analysis

Evaluation of ADCC effector function (affinity to the Fc receptor)

Affinity chromatography – functional testing

Development of C1q Affinity Chromatography for the Study of C1q–IgG Interactions

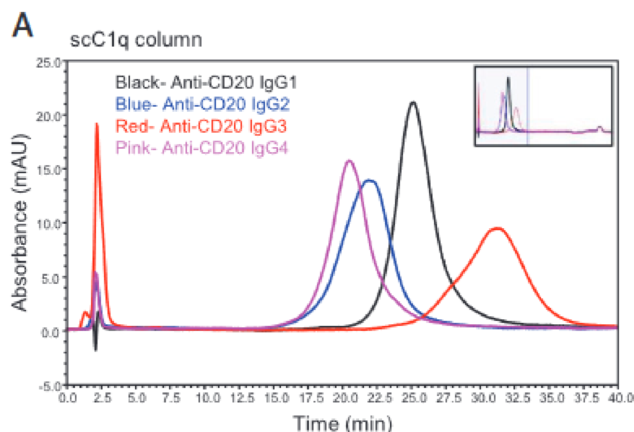
Michael J. E. Marshall,* Alexander Knaupp,[†] Christian Spick,[†] Ilker Koese,[‡,§] Maria Maier,[¶] Mark S. Cragg,* Florian Cymer,[§] and Tilman Schlothauer[†]

The classical complement system represents a central effector mechanism of Abs initiated by the binding of C1q to target bound IgG. Human C1q contains six heterotrimeric globular head groups that mediate IgG interaction, resulting in an avidity-driven binding event involving multiple IgG molecules binding a single C1q. Accordingly, surface bound IgG molecules are thought to assemble into noncovalent hexameric rings for optimal binding to the six-headed C1q. To study the C1q–Fc interaction of various Abs and screen for altered C1q binding mutants, we developed, to our knowledge, a novel HPLC-based method. Employing a single-chain form of C1q representing one C1q head group, our HPLC methodology was able to detect the interaction between the single-chain monomeric form of C1q and various ligands. We show that, despite a narrow window of specific binding owing to the low affinity of the monomeric C1q–IgG interaction, this approach clearly distinguished between IgG subclasses with established C1q binding properties. IgG3 displayed the strongest binding, followed by IgG1, with IgG2 and IgG4 showing the weakest binding. Fc mutants known to have increased C1q binding through oligomerization or enhanced C1q interaction showed greatly increased column retention, and IgG glycovariants displayed a consistent trend of increasing retention upon increasing galactosylation and sialylation. Furthermore, the column retention of IgG isotypes and glycovariants matches both the cell surface recruitment of C1q and complement-mediated cytotoxicity induced by each variant on an anti-CD20 Ab backbone. This methodology therefore provides a valuable tool for testing IgG Ab (glyco)variants for C1q binding, with clear relevance for therapeutic Ab development. *The Journal of Immunology*, 2023, 210: 1837–1848.

Direct evaluation of complement dependent cytotoxicity (CDC) without the need for cell-based assays

Affinity chromatography – functional testing

Comparison of the IgG binding properties of the scC1q column to cell-based assays of complement activation



FcγIIIa, FcRn and C1Q affinity columns are now available from Roche (expensive !)

A4F-MS

analytical chemistry

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Article

Characterizing Non-covalent Protein Complexes Using Asymmetrical Flow Field-Flow Fractionation On-Line Coupled to Native Mass Spectrometry

Iro Konstantina Ventouri,* Wayne Chang, Florian Meier, Roland Drexel, Govert W. Somsen, Peter J. Schoenmakers, Bart de Spiegeleer, Rob Haselberg,* and Alina Astefanei*

Cite This: https://doi.org/10.1021/acs.analchem.2c05049

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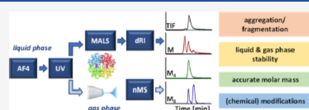
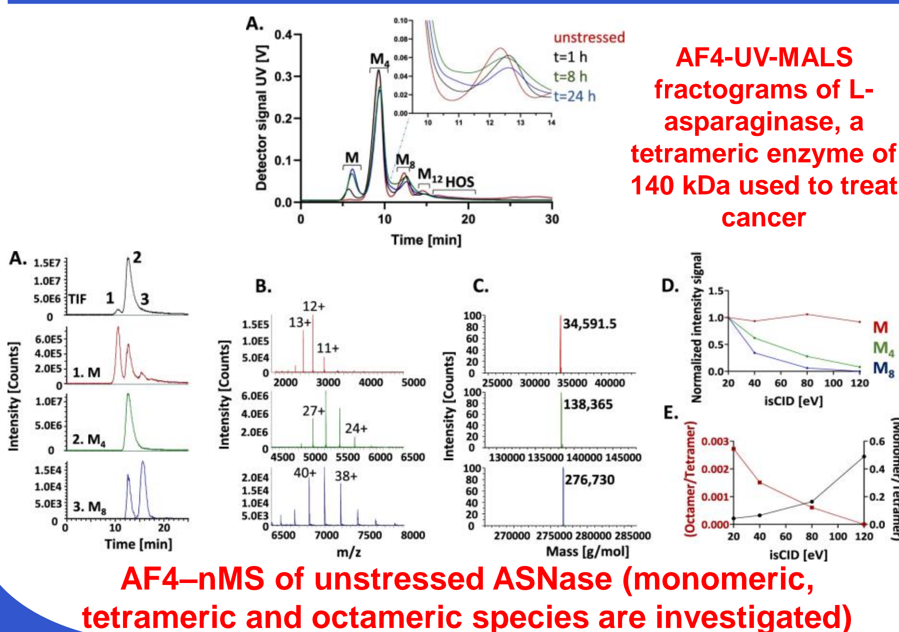
ABSTRACT: We report an online analytical platform based on the coupling of asymmetrical flow field-flow fractionation (AF4) and native mass spectrometry (nMS) in parallel with UV-absorbance, multi-angle light scattering (MALS), and differential-refractive-index (UV–MALS–dRI) detectors to elucidate labile higher-order structures (HOS) of protein biotherapeutics. The technical aspects of coupling AF4 with nMS and the UV–MALS–dRI multi-detection system are discussed. The "slot-outlet" technique was used to reduce sample dilution and split the AF4 effluent between the MS and UV–MALS–dRI detectors. The stability, HOS, and dissociation pathways of the tetrameric biotherapeutic enzyme (anticancer agent) L-asparaginase (ASNase) were studied. ASNase is a 140 kDa homo-tetramer, but the presence of intact octamers and degradation products with lower molecular weights was indicated by AF4–MALS/nMS. Exposing ASNase to 10 mM NaOH disturbed the equilibrium between the different non-covalent species and led to HOS dissociation. Correlation of the information obtained by AF4–MALS (liquid phase) and AF4–nMS (gas phase) revealed the formation of monomeric, tetrameric, and pentameric species. High-resolution MS revealed deamidation of the main intact tetramer upon exposure of ASNase to high pH (NaOH and ammonium bicarbonate). The particular information retrieved from ASNase with the developed platform in a single run demonstrates that the newly developed platform can be highly useful for aggregation and stability studies of protein biopharmaceuticals.



A4F-MS



AF4-UV-MALS fractograms of L-asparaginase, a tetrameric enzyme of 140 kDa used to treat cancer

AF4–nMS of unstressed ASNase (monomeric, tetrameric and octameric species are investigated)

Lab of the future: automation

ACS Partner Journal

Journal of the American Society for Mass Spectrometry

pubs.acs.org/jasms

Research Article

"Lab of the Future"—Today: Fully Automated System for High-Throughput Mass Spectrometry Analysis of Biotherapeutics

Published as part of the Journal of the American Society for Mass Spectrometry virtual special issue "High-Throughput in Mass Spectrometry".

Hans E. Waldenmaier,* Elsa Gorre, Michael L. Poltash, Harsha P. Gunawardena, Xianglin Alex Zhai, Jing Li, Bo Zhai, Eric J. Beil, Joseph C. Terzo, Rose Lawler, A. Michelle English, Marshall Bern, Andrew D. Mahan, Eric Carlson, and Hirsh Nanda

Cite This: https://doi.org/10.1021/jasms.3c00036 | Read Online

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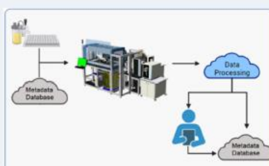
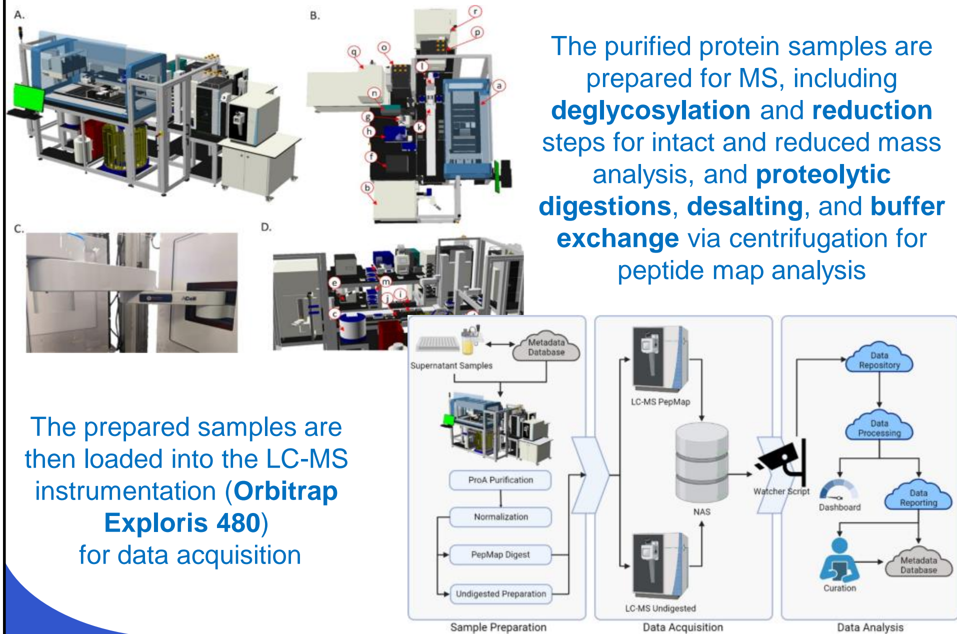


ABSTRACT: Here we describe a state-of-the-art, integrated, multi-instrument automated system designed to execute methods involved in mass spectrometry characterization of biotherapeutics. The system includes liquid and microplate handling robotics and utilities, integrated LC-MS, along with data analysis software, to perform sample purification, preparation, and analysis as a seamless integrated unit. The automated process begins with tip-based purification of target proteins from expression cell-line supernatants, which is initiated once the samples are loaded onto the automated system and the metadata are retrieved from our corporate data aggregation system. Subsequently, the purified protein samples are prepared for MS, including deglycosylation and reduction steps for intact and reduced mass analysis, and proteolytic digestions, desalting, and buffer exchange via centrifugation for peptide map analysis. The prepared samples are then loaded into the LC-MS instrumentation for data acquisition. The acquired raw data are initially stored on a local area network storage system that is monitored by watcher scripts that then upload the raw MS data to a network of cloud-based servers. The raw MS data are processed with the appropriately configured analysis workflows such as database search for peptide mapping or charge deconvolution for undigested proteins. The results are verified and formatted for expert curation directly in the cloud. Finally, the curated results are appended to sample metadata in the corporate data aggregation system to accompany the biotherapeutic cell lines in subsequent processes.

Lab of the future: automation



Innovative chromatographic approaches for AAVs and mRNA

Review on LC of AAVs

Trends in Analytical Chemistry 164 (2023) 117088

Contents lists available at ScienceDirect

Trends in Analytical Chemistry

journal homepage: www.elsevier.com/locate/trac

Chromatographic strategies for the analytical characterization of adeno-associated virus vector-based gene therapy products

Szabolcs Fekete [a], Megane K. Aebischer [b, c], Mateusz Imiołek [a], Tobias Graf [d], Raphael Ruppert [d], Matthew Lauber [e], Valentina D'Atri [b, c], Davy Guillarme [b, c, *]

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[b] Institute of Pharmaceutical Sciences of Western Switzerland, University of Geneva, CMU - Rue Michel Servet 1, 1211, Geneva 4, Switzerland
[c] School of Pharmaceutical Sciences, University of Geneva, CMU - Rue Michel Servet 1, 1211, Geneva 4, Switzerland
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Review on AAVs

A R T I C L E I N F O

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Empty capsid
Viral proteins
Ion exchange chromatography

A B S T R A C T

In recent years, the biopharmaceutical industry's interest in gene therapy modalities has increased dramatically. To warrant their quality during manufacturing and upstream/downstream process, fit-for-purpose analytical methods play a crucial role in the overall control strategy. However, characterization of gene therapy products remains challenging due to their large size, structural complexity, heterogeneity, potential instability, and limited sample availability. In addressing some of these challenges with innovative approaches, liquid chromatography (LC) based methods have become an integral part of the currently used analytical toolbox. This review focuses on both established methods and emerging trends in the LC analysis of adeno-associated virus (AVV) vector-based gene therapy products. Each method is discussed to highlight their advantages, drawbacks, and unique capabilities in the analysis of AAV gene transfer vehicles and their corresponding impurities. Taken together, this review provides guidance on the selection of LC-based methods for routine testing and extended characterization of gene therapy products.

Micro-flow LC for AAVs

Applied Biochemistry and Biotechnology
https://doi.org/10.1007/s12010-023-04656-x

ORIGINAL ARTICLE

Characterization of Adeno-Associated Virus Capsid Proteins by Microflow Liquid Chromatography Coupled with Mass Spectrometry

Xi Qin[1] · Xiang Li[1] · Lingsheng Chen[2] · Tie Gao[2] · Ji Luo[2] · Lihai Guo[2] · Sahana Mollah[3] · Zoe Zhang[3] · Yong Zhou[1] · Hong-Xu Chen[2]

Accepted: 4 July 2023
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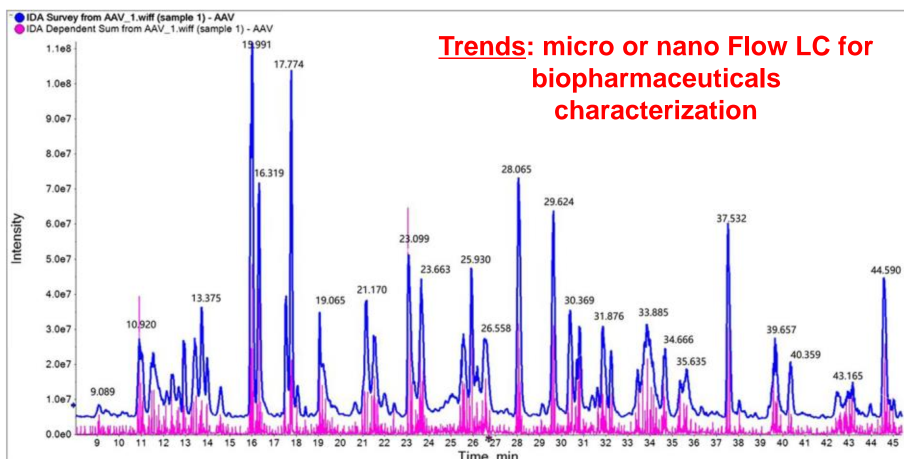
Abstract

Adeno-associated virus (AAV) has been widely used to treat various human diseases as an important delivery vector for gene therapy due to its low immunogenicity and safety. AAV capsids proteins are comprised of three capsid viral proteins (VP; VP1, VP2, VP3). The capsid proteins play a key role in viral vector infectivity and transduction efficiency. To ensure the safety and efficacy of AAV gene therapy products, the quality of AAV vector capsid proteins during development and production should be carefully monitored and controlled. Microflow liquid chromatography coupled with mass spectrometry provides superior sensitivity and fast analysis capability. It showed significant advantages in the analysis of low- concentration and large numbers of AAV samples. The intact mass of capsid protein can be accurately determined using high-resolution mass spectrometry (MS). And MS also provides highly confident confirmation of sequence coverage and post-translational modifications site identification and quantitation. In this study, we used microflow liquid chromatography-tandem mass spectrometry (LC–MS/MS) for the characterization of AAV2 capsid protein. we obtained nearly 100% sequence coverage of low-concentration AAV2 capsid protein (8×10^{11} GC/mL). More than 30 post-translational modifications (PTMs) sites were identified, the PTMs types included deamidation, oxidation and acetylation. From this study, the proposed microflow LC–MS/MS method provides a sensitive and high throughput approach in the characterization of AAVs and other biological products with low abundance.

Micro-flow LC-MS/MS for AAV2 capsid protein

Column of 0.3 x 150 mm, 2.6 μm @ 5 μL/min



Trends: micro or nano Flow LC for biopharmaceuticals characterization

Low sample consumption, high sensitivity
100% sequence coverage, more than 30 PTMs identified !

2D-LC of AAVs

analytical chemistry

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Development of a Two-Dimensional Liquid Chromatography-Mass Spectrometry Platform for Simultaneous Multi-Attribute Characterization of Adeno-Associated Viruses

Zhijie Wu, Hongxia Wang,* Andrew Tustian, Haibo Qiu,* and Ning Li

2D-LC for AAVs

Cite This: *Anal. Chem.* 2022, 94, 3219−3226 Read Online

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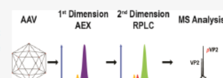
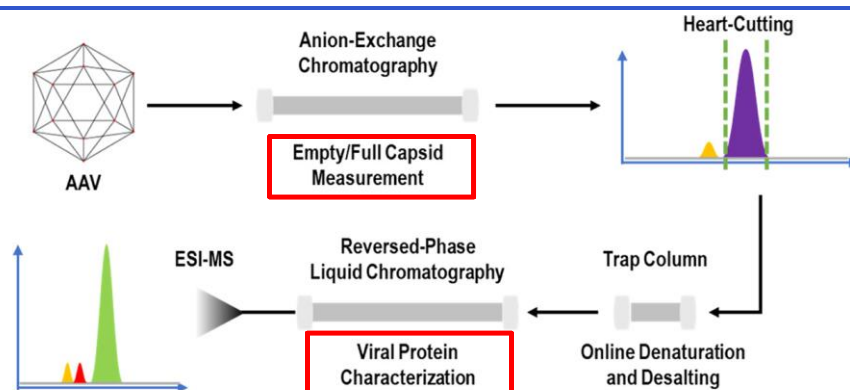


ABSTRACT: Adeno-associated viruses (AAVs) are non-enveloped, single-stranded DNA viruses that have recently emerged as an attractive vector for delivering genetic materials to hosts for gene therapy applications. Due to their ability to transduce a wide range of species and tissues *in vivo*, low risk of immunotoxicity, and mild innate and adaptive immune responses, AAVs are currently used in research and clinical studies as a monotherapy or with other biomolecules to perform gene editing, replacement, addition, and silencing. As AAVs are a new and complex therapeutic modality with molecular weights into the megadalton range, new analytical techniques are therefore needed to support process development, product characterization, and release. In this study, an online two-dimensional liquid chromatography-mass spectrometry (2DLC-MS) method was developed for AAV characterization. Our method uses high-resolution anion-exchange chromatography (AEX) in the first dimension to separate and measure empty and full capsids in AAV samples, followed by reversed-phase liquid chromatography coupled with mass spectrometry (RPLC-MS) to separate and characterize viral proteins. In this technique, online denaturation and removal of MS-incompatible salt were performed following AEX. The viral proteins present in the peak of interest after first-dimensional AEX are subjected to intact protein separation on the second-dimensional RPLC column and then characterized by MS. The 2DLC-MS method demonstrated in this study allows for high-throughput and multi-attribute AAV characterization in a single run, with minimal sample handling required for different AAV serotypes.

2D-LC of AAVs



Schematic of the 2D LC-MS platform used for **multi-attribute characterization** of adeno-associated viruses (AAVs).

In this platform, AAV samples first undergo AEX separation for **empty and full capsid measurements**. Using the heart-cutting technique in 2D-LC, peaks of interest are stored in different trapping loops, and fractions are transferred to second-dimension LC, where the fractions are subjected to **online denaturation and desalting in a trap column**, followed by separation using RPLC-MS (**viral proteins characterization**).

Review on LC of mRNA

Journal of Pharmaceutical and Biomedical Analysis 224 (2023) 115174

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Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.journals.elsevier.com/journal-of-pharmaceutical-and-biomedical-analysis

Review

Challenges and emerging trends in liquid chromatography-based analyses of mRNA pharmaceuticals

Szabolcs Fekete [a], Catalin Doneanu [b], Balasubrahmayam Addepalli [b], Maissa Gaye [b], Jennifer Nguyen [b], Bonnie Alden [b], Robert Birdsall [b], Duanduan Han [b], Giorgis Isaac [b], Matthew Lauber [b,*]

[a] Waters Corporation, Geneva, Switzerland
[b] Waters Corporation, Milford, MA, USA

Review on mRNA

A R T I C L E I N F O

Keywords:
Lipid nanoparticles
mRNA
Anion exchange
Ion-pairing reversed phase
Oligo mapping
Lipid compositional analysis

A B S T R A C T

Lipid encapsulated messenger RNA (LNP mRNA) has garnered a significant amount of interest from the pharmaceutical industry and general public alike. This attention has been catalyzed by the clinical success of LNP mRNA for SARS-CoV-2 vaccination as well as future promises that might be fulfilled by the biotechnology pipeline, such as the in vivo delivery of a CRISPR/Cas9 complex that can edit patient cells to reduce levels of low-density lipoprotein. LNP mRNAs are comprised of various chemically diverse molecules brought together in a sophisticated intermolecular complex. This can make it challenging to achieve thorough analytical characterization. Nevertheless, liquid chromatography is becoming an increasingly relied upon technique for LNP mRNA analyses. Although there have been significant advances in all types of LNP mRNA analyses, this review focuses on recent developments and the possibilities of applying anion exchange (AEX) and ion pairing reversed phase (IP-RP) liquid chromatography for intact mRNAs as well as techniques for oligo mapping analysis, 5' endcap testing and lipid compositional assays.

mRNA poly(A) tail length and heterogeneity

analytical chemistry

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Article

Liquid Chromatography Methods for Analysis of mRNA Poly(A) Tail Length and Heterogeneity

Martin Gilar,* Catalin Doneanu, and Maissa M. Gaye

Cite This: https://doi.org/10.1021/acs.analchem.3c02552

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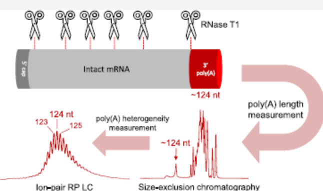
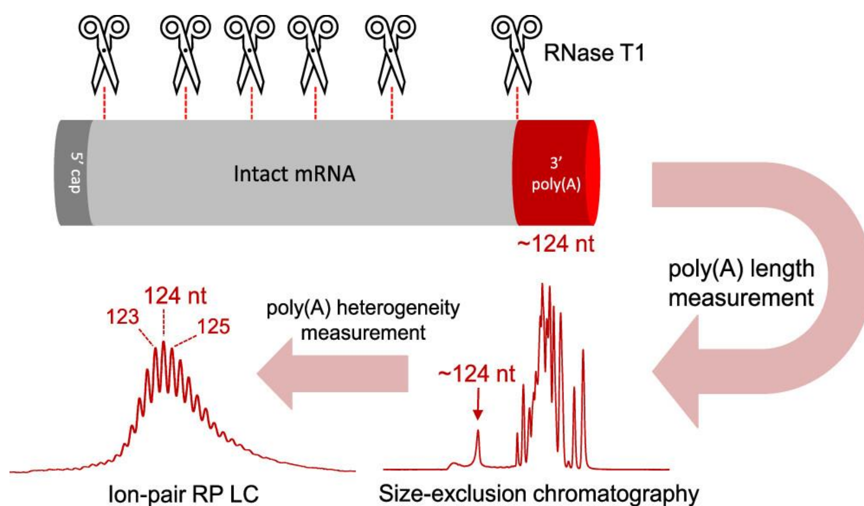
ABSTRACT: Messenger RNA (mRNA) is a new class of therapeutic compounds. The current advances in mRNA technology require the development of efficient analytical methods. In this work, we describe the development of several methods for measurement of mRNA poly(A) tail length and heterogeneity. Poly(A) tail was first cleaved from mRNA with the RNase T1 enzyme. The average length of a liberated poly(A) tail was analyzed with the size exclusion chromatography method. Size heterogeneity of the poly(A) tail was estimated with high-resolution ion-pair reversed phase liquid chromatography (IP RP LC). The IP RP LC method provides resolution of poly(A) tail oligonucleotide variants up to 150 nucleotide long. Both methods use a robust ultraviolet detection suitable for mRNA analysis in quality control laboratories. The results were confirmed by the LC-mass spectrometry (LC MS) analysis of the same mRNA sample. The poly(A) tail length and heterogeneity results were in good agreement.



mRNA poly(A) tail length and heterogeneity



- Average length measured with SEC
- Heterogeneity was measured with IP-RPLC (resolution up to 150 bp)
- SEC and IP-RPLC results were in good agreement

Ultra-wide pore SEC columns for mRNA

analytical chemistry

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Article

Separation of Plasmid DNA Topological Forms, Messenger RNA, and Lipid Nanoparticle Aggregates Using an Ultrawide Pore Size Exclusion Chromatography Column

Alexandre Goyon,* Shijia Tang, Szabolcs Fekete, Daniel Nguyen, Kate Hofmann, Shirley Wang, Whitney Shatz-Binder, Kiel Izabelle Fernandez, Elizabeth S. Hecht, Matthew Lauber, and Kelly Zhang

Cite This: https://doi.org/10.1021/acs.analchem.3c02944 Read Online

SEC for very large species

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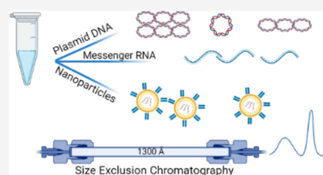
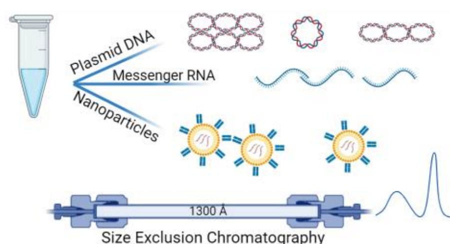


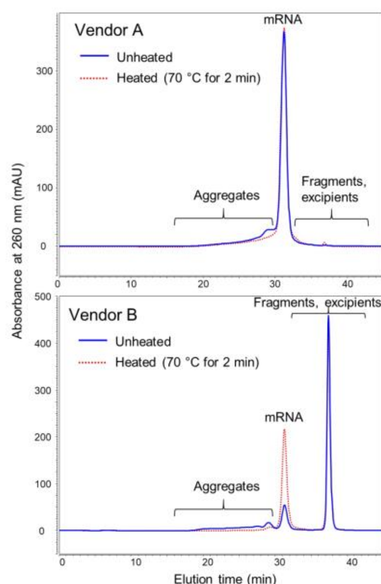
ABSTRACT: Health authorities have highlighted the need to determine oligonucleotide aggregates. However, existing technologies have limitations that have prevented the reliable analysis of size variants for large nucleic acids and lipid nanoparticles (LNPs). In this work, nucleic acid and LNP aggregation was examined using prototype, low adsorption ultrawide pore size exclusion chromatography (SEC) columns. A preliminary study was conducted to determine the column's physicochemical properties. A large difference in aggregate content (17.8 vs 59.7 %) was found for a model messenger RNA (mRNA) produced by different manufacturers. We further investigated the nature of the aggregates via a heat treatment. Interestingly, thermal stress irreversibly decreased the amount of aggregates from 59.7 to 4.1% and increased the main peak area 3.3-fold. To the best of our knowledge, for the first time, plasmid DNA topological forms and multimers were separated by analytical SEC. The degradation trends were compared to the data obtained with an anion exchange chromatography method. Finally, unconjugated and fragment antigen-binding (Fab)-guided LNPs were analyzed and their elution times were plotted against their sizes as measured by DLS. Multi-angle light scattering (MALS) was coupled to SEC in order to gain further insights on large species eluting before the LNPs, which were later identified as self-associating LNPs. This study demonstrated the utility of ultrawide pore SEC columns in characterizing the size variants of large nucleic acid therapeutics and LNPs.

Ultra-wide pore SEC columns for mRNA

2.5 µm, 1300 Å



Low adsorption column hardware



Comparison of 2 mRNA samples

Merci de votre attention !