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Comparison of MALS-Based Analyses of Large mRNA Separated on Widepore SEC Columns

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Abstract

mRNA is a versatile molecule that is being investigated in multiple clinical contexts for treatment and prevention of diseases. Its high complexity necessitates use of advanced analytical workflows, such as size-exclusion chromatography (SEC) coupled to multi-angle light scattering (MALS) to facilitate their development. The number of in-development mRNA drugs is rapidly increasing. These drug candidates will need to be supported by fit-for-purpose methods capable of high throughput and robust measurements. In this application brief, we compare the set-up and performance of SEC-MALS experiments using two different widepore columns suitable for analysis of large mRNAs: a GTxResolve™ Premier SEC 1000 Å 3 µm Column and a nominally similar manufacturer A 1000 Å 2.7 µm column. Thanks to its broad pore size distribution, the GTxResolve Premier SEC 1000 Å Column allows MALS validation using a common BSA standard, which is problematic in the case of the manufacturer column. Each of these columns were tested using a minimal amount of mRNA sample in a fast measurement under generic SEC conditions. Both columns resolved dimeric species but reliable mass and size characterization required operation at lower noise background, which was only provided by the GTxResolve Premier SEC 1000 Å Column.

Benefits

- GTxResolve Premier SEC 1000 Å Column allows robust MALS analysis
- Lower light scattering noise allows for reliable results even using lower amount of sample

Experimental

A bovine serum albumin (BSA) protein sample was prepared from powder (Sigma-Aldrich, Buchs, Switzerland) at 5 mg/mL in phosphate-buffered saline (PBS) and 50 µg was injected for MALS validation. 1 mg/mL eSpCas9 mRNA (*N*1-Methylpseudouridine/m1Ψ) in 1 mM sodium citrate, pH 6.5 (SC2325, length: 4471 nucleotides, GenScript Biotech Corporation, NJ, USA) was diluted to 0.1 mg/mL in water and 1 µg was injected for MALS measurement.

LC system:	ACQUITY™ Premier System (quaternary) (outfitted with a high pH kit)
MALS detector:	Wyatt DAWN™ Detector (18-Angles Light Scattering)
Concentration detector:	UV absorbance detection at 260 nm
Vials:	QuanRecovery™ with MaxPeak™ HPS 12 x 32 mm Screw Neck Vial, 300 µL, 100/pk, (p/n: 186009186)
Column:	GTxResolve Premier SEC 1000 Å 3 µm 7.8 x 300 mm Column (p/n: 186010738)
Column temperature:	Ambient (22 °C)
Sample temperature:	5 °C
Injection volume:	10.0 µL

Flow rate:	1 mL/min
Mobile phase:	0.2 µm filtered PBS, pH 7.4 (10 mM phosphate, 200 mM KCl, 0.02% NaN3

Column Conditioning Before MALS Measurement

The flow rate was slowly ramped to 1 mL/min during 20 mins and the column was equilibrated at 1 mL/min during 300 mins (20 CV) with PBS. Water was injected as a blank using a standard measurement method (15 mins). For the molar mass calculation, a ε_{260} value of 0.4 mL mg^{-1} cm^{-1} was used for BSA, and 25 mL mg^{-1} cm^{-1} was used for mRNA.

The GTxResolve Premier SEC 1000 Å 3 µm Column and manufacturer A 1000 Å 2.7 µm columns were treated in an identical manner.

These results represent an n=1 comparison. Results may vary. In some extreme cases, it might be necessary to flush an SEC column overnight before achieving the MALS performance demonstrated in this application note. Please refer to the GTxResolve Premier SEC 1000 Å 3 µm Column's Care and Use Manual for additional guidance and considerations.

Results and Discussion

Column Equilibration and Noise Evaluation

Size-exclusion chromatography resolves analytes according to their hydrodynamic volume. MALS is a powerful characterization technique enabling absolute determination of molar mass and size of macromolecules in solution. Coupled together, SEC-MALS affords a versatile platform for the analysis of new modality drugs, such as mRNA therapeutics.[1] However, its effective use requires a couple of considerations regarding appropriate operation conditions, such as achieving minimal MALS noise and validating the system suitability.

Light scattering (LS) is sensitive to background particulates, therefore certain chromatographic columns may need to be extensively flushed to establish stable baseline performance and low background signal.

Typically, flushes between 12–24 hours or a minimum of 20–40 column volumes (CV) are required to properly equilibrate the column. 20 CVs of freshly prepared, 0.2 µm filtered phosphate buffer saline mobile phase was passed through the columns and 90° LS signal was checked for noise levels. A comparison of results from the GTxResolve Premier SEC 1000 Å Column and manufacturer A column obtained under identical conditions is provided in Figure 1. This test revealed that the Waters column displayed almost 4 times lower MALS noise after the equilibration procedure, making it suitable for robust MALS measurement. This manufacturer A column would require lengthier flushing to improve its MALS readiness.

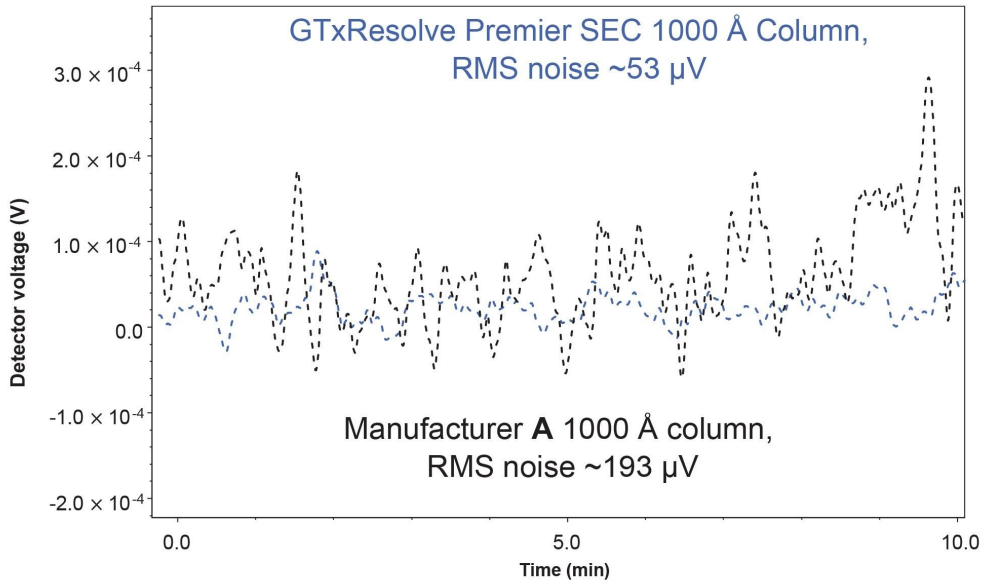

Figure 1. Background noise evaluation after 20 CV flush for GTxResolve Premier SEC 1000 Å Column (blue) and manufacturer A 1000 Å (black) column with measured RMS noise values indicated.

Establishing SEC-MALS Instrument Constants and System Suitability

Like all analytical techniques, SEC-MALS requires that system suitability is established using a standard sample prior to sample analysis. Specifically, this process consists of instrument constant determination (normalization, alignment, band broadening) and system suitability verification. Normalization relates the various detectors' signals to the 90° detector signal and the instrument calibration constant, , alignment accounts for interdetector delay volumes, and band broadening corrects for inter-detector mismatches from peak broadening.

Conveniently, all steps may be performed in a single experiment in the same mobile phase as the subsequent sample analysis by using a standard with known molar mass and size < 10 nm radius that yields a well-resolved monodisperse peak on all detectors used for the analysis, such as bovine serum albumin (BSA).[2]

Injecting BSA onto both test columns, satisfactory resolution of oligomeric and monomeric BSA species was achieved with the GTxResolve Premier SEC 1000 Å Column while more pronounced co-elution was observed with the manufacturer A column. Subsequently, normalization, alignment, band broadening, and data analysis was performed with ASTRA™ Software. These results are shown in Figure 2. Both columns yielded a main peak with a weight-average molar mass (M_w) within error of the expected BSA monomer molar mass (66.4 kDa). However, less precise measurements were obtained with the manufacturer A column, due to the inferior separation and MALS signal-to-noise and tailing of the BSA peak. A larger macromolecule such as an antibody would be more suitable for use with the manufacturer A column, but its molecular properties (hydrodynamic radius, extinction coefficient, molar mass and presence of aggregates) would need to be orthogonally established and its use as a MALS validation standard would need to be validated.

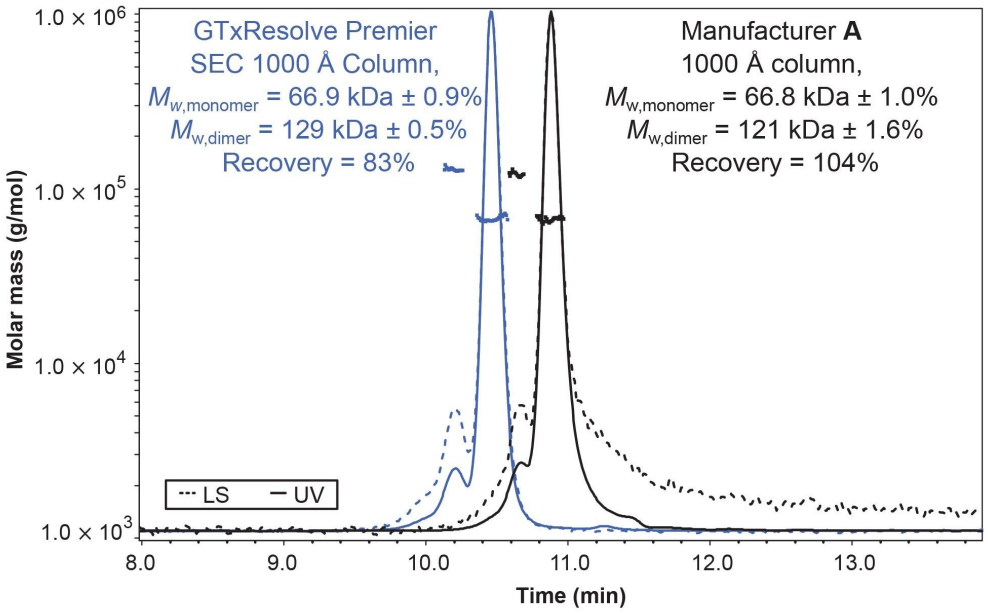

Figure 2. SEC-UV-MALS measurement of BSA on GTxResolve Premier SEC 1000 Å 3 µm Column (blue) and manufacturer A 1000 Å 2.7 µm (black) column with measured weight-average molar mass (M_w) of monomer and dimer species and total recovered mass (assuming 50 µg as 100% recovery), ASTRA Software calculated uncertainties.

Measurement of Limited Amount mRNA Sample

Finally, a minimal quantity of mRNA sample (1 µg) was measured on both test columns. This sample amount is relatively low. However, new therapeutic applications of mRNA are producing sample-limited testing scenarios, as is the case with personalized neoantigen therapies. As is, this 1 µg quantity of mRNA could be accurately analyzed using the GTxResolve Premier SEC 1000 Å Column. Light scattering chromatograms for Cas9 mRNA are provided in Figure 3. Single digit precision was obtained for the monomer and dimer masses of Cas9 mRNA, as well as a determination of the monomer's radius of gyration. Each of these measured values were confirmed to be within 15% difference of predicted values. MALS measurements with the manufacturer A column provided lower confidence measurements and measured values up to 50% different than predicted values. The accuracy of this analysis, as performed with the GTxResolve Premier SEC 1000 Å Column, is sufficient for high-throughput screening of candidate molecules. For comprehensive characterization of a sample including quantification of the relative amounts of any oligomers and aggregates, a different protocol should be followed with higher amounts

of sample being injected and an optimized mobile phase used. The details of such protocol can be found in another Application Note, where 50 mM Tris buffer pH 7.5 and 250 mM ammonium chloride is applied.[3]

Column	GTxResolve Premier SEC 1000 Å Column	Manufacturer A	Expected
$M_{w,\,monomer}$ (MDa)	1.7 MDa ± 2%	1 MDa ± 10%	1.5 MDa
$M_{w,dimer}$ (MDa)	3.5 MDa ± 3%	2 MDa ± 13%	3.0 MDa
Recovery (%)	78%	166%	80–100%
Radius of gyration (nm)	29.3 nm ± 8%	n/d	n/a

Table 1. Comparison of SEC-MALS measured and expected results for molecular mass and size of species present in a Cas9 mRNA sample using limited amount of sample.[4]

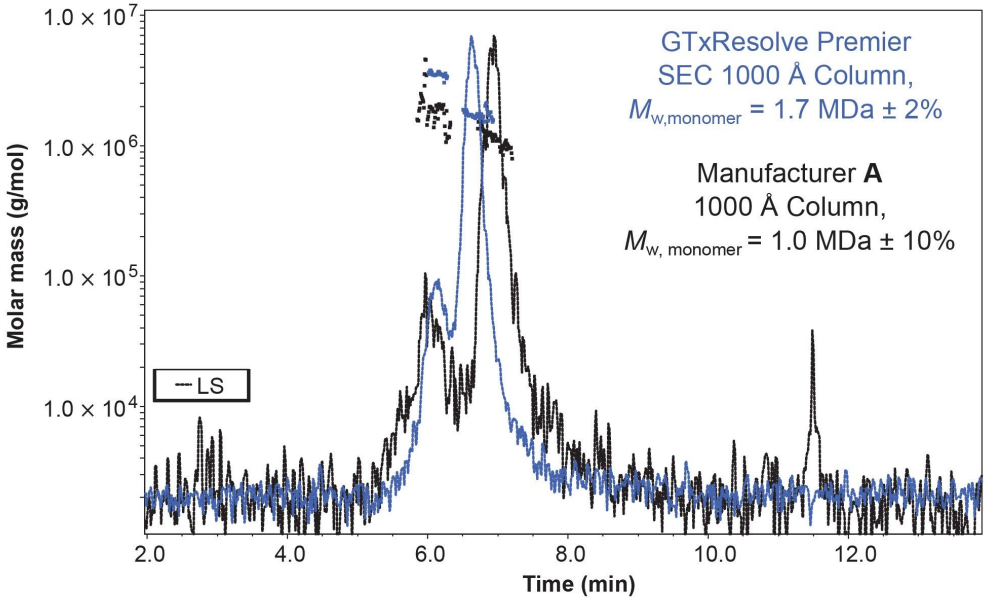

Figure 3. SEC-UV-MALS measurement of Cas9 mRNA on GTxResolve Premier SEC 1000 Å Column (blue) and manufacturer A 1000 Å (black) column with measured weight-average molar masses (M_w) of monomer and dimer species.

Conclusion

SEC-MALS using an optimally designed widepore column is expected to play an increasingly important role in the development of nucleic acid therapeutics. In this Application Note, we show how SEC-MALS can be used for cursory screening of mRNA samples available in limited quantity. We compared the performance of two different columns, highlighting that certain column design considerations such as low particle shedding, crucial for MALS readiness, as well as broad pore size distribution, allowing standardized validation procedures, proved enabling for this type of analysis. A deeper look into particle and chemical design considerations was recently published in the Journal of Separation Science.[5]

References

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