APPLICATION NOTE

AN1616: SEC-MALS Method for Characterizing mRNA Biophysical Attributes

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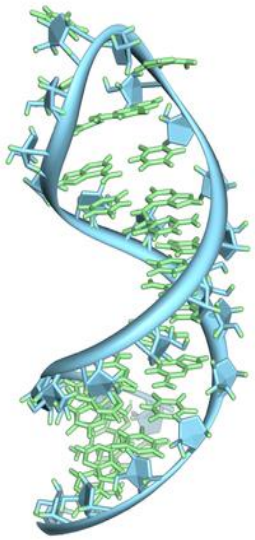
Summary

An SEC-MALS method incorporating DAWN and Optilab detectors is developed to quantify a number of biophysical attributes of mRNA. These attributes include molecular weight, percentage of aggregates, R_g, R_h, and R_g/R_h. This method can be readily adopted to further study how these attributes are being affected by mRNA preparation, formulation and storage conditions.

Introduction



RNA hairpin loop (Wikipedia)

RNA therapeutics is a rapidly growing field that encompasses a wide variety of biologically inspired molecules. Messenger ribonucleic acid (mRNA) is a transient biopolymer responsible for converting genetic information stored in DNA into functional proteins[1]. Over the past decade, mRNA has emerged as an increasingly desirable alternative to traditional protein-based therapies; mRNA molecules with the appropriate sequence, synthetic modifications, and delivery vehicle are much more likely to lead to the production of properly folded and post-translationally modified proteins within the human body. Furthermore, unlike DNA-based gene therapy, mRNA drugs pose minimal risks of genome integration and reduce long-term safety concerns. Considering these benefits, mRNA has the potential to revolutionize the biologicals industry, providing a versatile platform for vaccine development, immuno-oncology, and protein replacement therapies[2].

Despite its apparent advantages, mRNA therapeutic development is in its early stages, and clinical success will rely heavily on the elucidation of mRNA attributes using high-quality analytical tools. Given the intrinsic dynamics of RNA molecules, 3D structural information is particularly difficult to obtain. Therefore, complementary techniques that can provide insight into size, oligomeric state, and conformation would be invaluable for understanding the biochemical and biophysical properties of mRNA.

High-performance size-exclusion chromatography (SEC) has been widely used in protein research and protein therapeutic development with applications including purification and molecular weight (MW) estimation, and aggregation quantification. Its application can be further extended to include accurate MW determination and shape estimation when light scattering (LS) detectors, namely multi-angle light scattering (MALS) and online dynamic light scattering (DLS), are added[3].

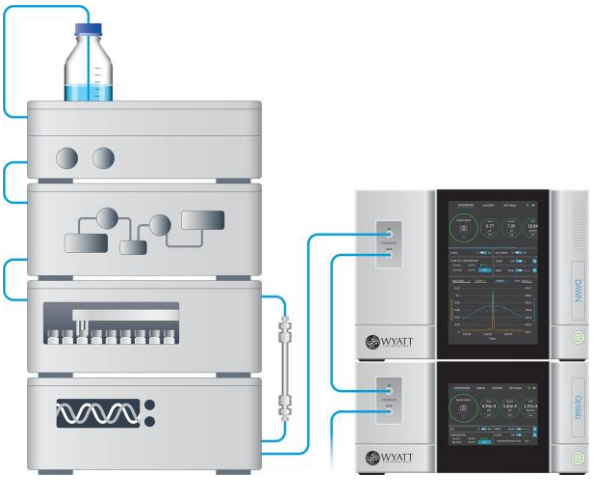
A DAWN multi-angle light scattering detector, equipped with internal WyattQELS DLS module.

Commonly used in protein analysis, characterization by SEC-MALS is particularly important for RNA molecules, especially the large RNA molecules like mRNA. The SEC-MALS method can provide biophysical and structural information that are otherwise difficult to obtain given the large size and high charge density of mRNA.

In order to develop an mRNA molecule into a drug product, properties like MW, size, and conformation need to be well characterized and monitored throughout the development cycle. In this note we will discuss the use of an SEC-MALS method for comprehensive mRNA characterization, employing two commonly used tool constructs: RNA for human erythropoietin (EPO), a hormone that plays a key role in the production of red blood cells; and firefly luciferase (fLuc), a bioluminescent protein whose expression levels can be easily quantified *in vivo*[4].

Materials and Methods

EPO and *fLuc* mRNA were synthesized by *in vitro* transcription. The purified mRNA products were quantified by UV absorbance. SEC was carried out on an industry standard HPLC system with a Wyatt WTC050S5 column using PBS (50 mM phosphate, 50 mM NaCl, and 250 ppm NaN_3 at pH 6.8) as the mobile phase. A relatively high flow rate of 0.8 mL/min was used here to shorten run time and increase assay throughput.



A complete SEC-MALS system comprises the HPLC including the UV detector, SEC column(s), MALS and RI detectors. Control, data acquisition and analysis are performed by ASTRA.

The effluent was detected by a variable UV detector at 260 nm for mRNA molecules and 280 nm for proteins, followed by a DAWN® MALS detector equipped with a WyattQELS™ embedded DLS module, and an Optilab® differential refractive index (RI) detector. ASTRA® software was used for HPLC control, data collection, and data analysis. A *dn/dc* value of 0.172 mL/g was used for mRNAs and 0.185 mL/g was used for proteins.

Results and Discussion

Small amounts of large MW species were observed in both *EPO* and *fLuc* mRNA samples, adequately separated from the main population, as seen in the UV and light scattering (LS) chromatograms presented in Figure 1. Since LS intensity is proportional to the product of concentration and MW, the LS signal is particularly useful for detecting small quantities of large MW species, compared to UV or refractive index (RI) detection.

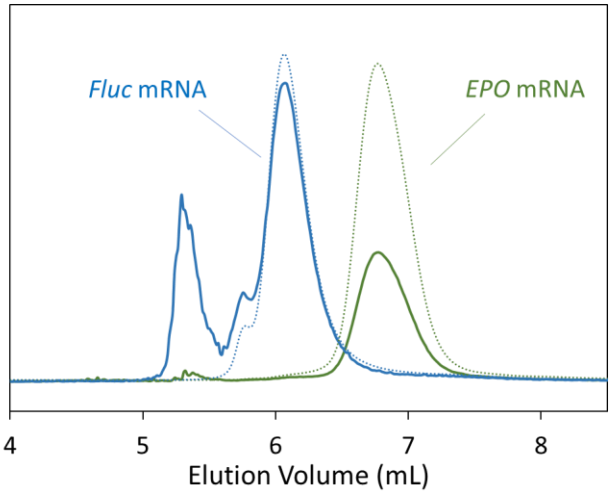


Figure 1. Chromatograms of *fLuc* (blue) and *EPO* (green) mRNA obtained from 90° LS (solid) and UV at 260 nm (dashed).

Using the hardware configuration detailed in the Materials and Methods section, we can simultaneously measure MW and two types of radius:

- radius of gyration (R_g), using the MALS detector, and
- hydrodynamic radius (R_h), using the online DLS detector.

The properties that are measured, including MW, R_g, R_h, R_g/R_h, and UV extinction coefficient at 260 nm of the main peak, as well as percentage of aggregate, are summarized in Table 1. It should be noted that the

method is highly reproducible, and all of the attributes were measured within a single SEC-MALS experiment, requiring less than 15 minutes.

The MW values of the main peak of each mRNA determined from MALS-RI analysis are in near-perfect agreement with the theoretical values for *EPO* and *fLuc* mRNA (273 kDa and 619 kDa, respectively). The results confirm that the mRNAs used in this study remained intact throughout the production, storage, and transportation.

Table 1. Summary of measured attributes of *EPO* and *fLuc* mRNA.

	MW [kDa]	Agg[a] [%]	R_g [nm]	R_h [nm]	R_g/R_h	ε[b]
EPO	272 ± 1	4.8	15 ± 1	12+/-1	1.2	22.8
fLuc	622 ± 1	2.6	20 ± 1	17+/-1	1.2	22.4

[a] Agg is the percentage of aggregate by weight

[b] ε is the extinction coefficient at 260 nm in mL·mg^{-1}cm^{-1}

This level of detail provided by SEC-MALS cannot be revealed by the traditional SEC-UV method of column calibration. Column calibration using globular protein standards typically overestimates the molar mass of RNA molecules by a factor of a few times due to the drastic difference in compactness between a globular protein and an RNA molecule[5]. Even if polynucleotides of various lengths are used as the calibration standards, the calibration method can provide, at its best, a rough approximation of the actual molar mass of the RNA molecules, given the low resolution of the SEC separation.

The online MALS detector, on the other hand, permits the accurate molar mass measurement of both mRNAs *and* proteins, regardless of their compactness and potential non-ideal column interaction. Figure 2 presents chromatograms and molar masses from MALS of the two mRNAs studied here plus a protein, thyroglobulin, and the aggregates of all three. The *fLuc* mRNA has a similar molar mass as thyroglobulin yet it eluted at a much earlier elution volume due to its less compact conformation. It is also seen from Figure 2 that the molar mass across the main peak of the mRNA samples is constant, suggesting the main population in both samples is a pure monomer.

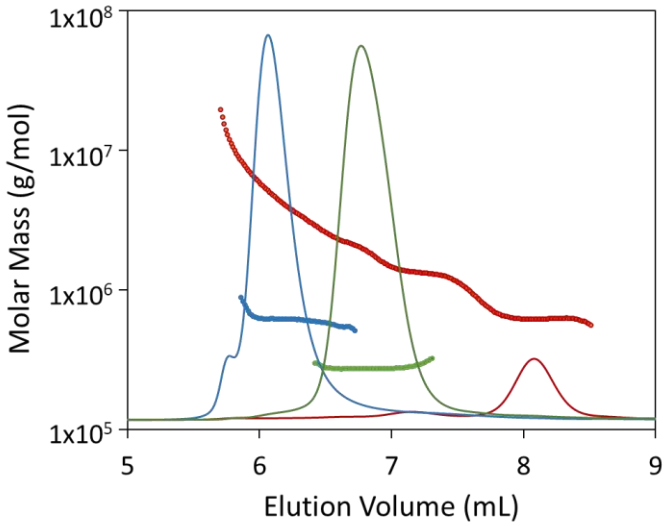


Figure 2. Molar mass of *fLuc* mRNA (blue), *EPO* mRNA (green) and thyroglobulin (red) samples, superimposed on UV chromatograms acquired at 260 nm.

In addition to molar mass, online LS detectors can measure other biophysical properties like size and conformation which may impact delivery strategy. Hydrodynamic radii of the two mRNAs and thyroglobulin versus elution volume are depicted in Figure 3.

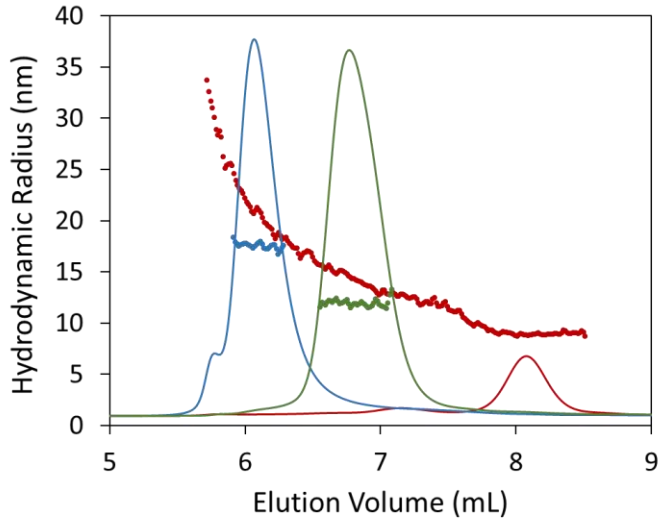


Figure 3. Hydrodynamic radius (R_h) from online DLS for *fLuc* mRNA (blue), *EPO* mRNA (green) and thyroglobulin (red) are plotted against elution volume, superimposed on UV chromatograms at 260 nm.

Both R_g and R_h are insightful as their ratio is correlated to the conformation of a macromolecule. *EPO* and *fLuc* mRNA monomer showed an R_g : R_h ratio of approximately 1.2 (see Table 1), consistent with a random coil conformation. A random coil structure is much less

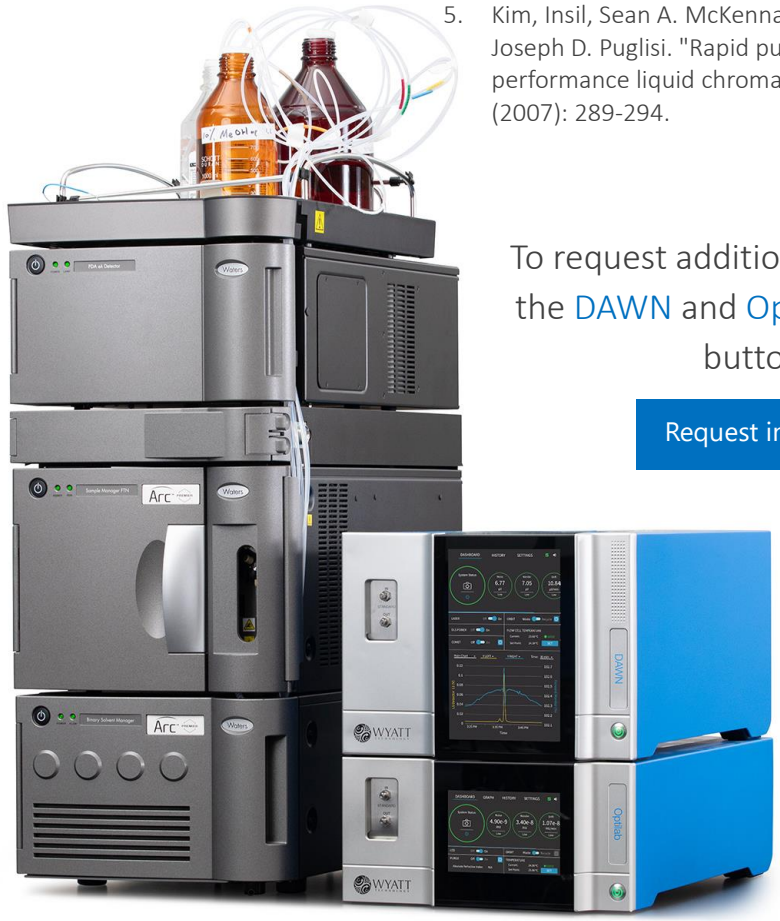
compact than the globular conformation typical of proteins which has an R_g : R_h ratio of 0.77.

Conclusions

The SEC-MALS method reported in this note provides a convenient and quick, yet powerful tool for measuring the biophysical attributes of mRNA molecules. The method can also be used to study the effects of different ions, ionic strength, pH, and other buffer conditions on mRNA biophysical properties and stability. These attributes are crucial in understanding any large RNA molecules, especially when developed as a therapeutic product.

References

1. Cross, Ryan. “Can mRNA disrupt the drug industry?” *Chemical & Engineering News* **96**, 35 (2018).
2. Jain, R., Frederick, J. P., Huang, E. Y., Burke, K. E., Mau-ger, D. M., Andrianova, E. A., Farlow, S. J., Siddiqui, S., Pimentel, J., Cheung-Ong, K., McKinney, K. M., Köhrer, C., Moore, M. J., Chakraborty, T. “MicroRNAs Enable mRNA Therapeutics to Selectively Program Cancer Cells to Self-Destruct.” *Nucleic Acid Therapeutics* **28**(5) (2018): 285-296.
3. Wyatt, P. J. Light scattering and the absolute characterization of macromolecules. *Anal Chim Acta* **272** (1993).
4. Thess, A., Grund, S., Mui, B. L., Hope, M. J., Baumhof, P., Fotin-Mleczek, M., Schlake, T. “Sequence-engineered mRNA without chemical nucleoside nodifications ena-bles an effective protein therapy in large animals.” *Mol Ther* **23**(9) (2015): 1456-1464.
5. Kim, Insil, Sean A. McKenna, Elisabetta Viani Puglisi, and Joseph D. Puglisi. "Rapid purification of RNAs using fast performance liquid chromatography (FPLC)." *RNA* **13**(2) (2007): 289-294.



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