Application Note

Biopharmaceuticals

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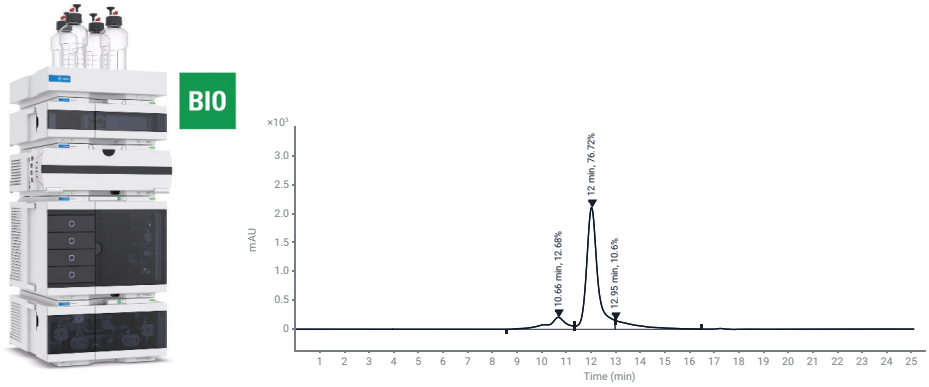
Aggregate Analysis of mRNA Using the Agilent Infinity II Bio LC System and Bio-SEC Columns

mRNA aggregation by SEC-HPLC – USP analytical procedures for mRNA vaccine quality



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Abstract

The United States Pharmacopeia (USP) has proposed quality evaluation methods for managing key quality attributes of mRNA production processes through the Analytical Procedures for mRNA Vaccine Quality Draft guidelines, second edition.[1] The guidelines introduce methods for analyzing mRNA aggregates and provide respective test methods for mRNA drug substances and mRNA drug products. This application note investigates the impact of column and mobile phase selection on the analysis of mRNA aggregates to assess their cohesiveness.

Introduction

mRNA can form secondary structures based on complementary binding between nucleotides, or exist in the form of aggregates. These secondary structures and aggregates not only act as interfering factors that can disrupt the translation of RNA into proteins[2,3], but may also impact the efficiency of formulation into lipid nanoparticles[4]. Therefore, the management of mRNA aggregates is considered a crucial quality assessment criterion from both formulation and efficacy perspectives.

Under low-pH environments, especially below pH 7, nucleic acids can undergo hydrolysis. Thus, for mRNA vaccine production, an appropriate buffering system must be chosen to maintain stability. While phosphate buffers are commonly used in the manufacturing of biopharmaceuticals, concerns arise about potential pH changes at lower temperatures. This is a particular consideration for the typical storage conditions of mRNA vaccines, which involve freezing. Moreover, magnesium (Mg^{2+}) or calcium (Ca^{2+}) ions participate in enzyme-mediated cleavage of mRNA, and the inclusion of EDTA in the mobile phase aids in stabilizing mRNA.[4]

The analytical method for mRNA drug substances, as suggested by USP guidelines, employs a mobile phase with a 150 mM phosphate buffer. In contrast, the method for analyzing mRNA drug products suggests a mobile phase with a 100 mM Tris acetate/2.5 mM EDTA buffer. Under these conditions, considering the influence of salt concentrations in the mobile phase and nucleotide — stainless steel interactions, the evaluation of aggregates requires the use of HPLC with biocompatible materials. Therefore, in this study, the Agilent 1290 Infinity II Bio LC System was used to assess the impact of mobile phase and column selection on the analysis of mRNA aggregates.

Methods

Standards and reagents

The sodium phosphate monobasic, sodium phosphate dibasic, and 10x TAE buffer used in the experiment were purchased from Sigma-Aldrich.

The 100 bp DNA ladder and 1 kbp DNA ladder were purchased from Thermo Fisher. Poly(A) (average length 4,831 nucleotides) was purchased from Sigma-Aldrich, CleanCap FLuc mRNA (ORF 1,929 nucleotides, UTR 261 nucleotides) and CleanCap β-galactosidase mRNA (ORF 3420) were purchased from TriLink, and mRNA samples 1 and 2 were provided by the customer.

Instruments

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with sample thermostat
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with Agilent InfinityLab Bio-Inert Quick Connect heat exchanger (G7116-60071)
- Agilent 1290 Infinity II Diode Array Detector (G7117B) with Bio-Inert Max-Light cartridge cell, 60 mm (G5615-60017)

Columns

- Agilent Bio SEC-5 500Å, 7.8 × 300 mm, 5 µm (part number 5190-2531)
- Agilent Bio SEC-5 1000Å, 7.8 × 300 mm, 5 µm (part number 5190-2536)
- Agilent Bio SEC-5 2000Å, 7.8 × 300 mm, 5 µm (part number 5190-2541)

Software

Agilent OpenLab CDS, version 2.7

Mobile phases

150 mM phosphate buffer (pH 7.0) was prepared by dissolving 6.32 grams of sodium phosphate monobasic and 13.8 grams of sodium hydrogen phosphate dibasic in water, and adjusting the volume to 1 L. Additionally, 100 mM Tris acetate/2.5 mM EDTA buffer was prepared by mixing 250 mL of 10x TAE buffer with 750 mL of water and adjusting the volume to 1 L.

Methods

Table 1. Phosphate buffer HPLC analytical conditions.

Parameter	Value
Flow Rate	0.6 mL/min
Column Temperature	25 °C
Injection Volume	5 µL
Sampler Temperature	4 °C
Detector	UV 260 nm
Mobile Phase	150 mM Phosphate buffer
Analysis Time	25 min

Table 2. 100 mM Tris acetate/2.5mM EDTA HPLC analytical conditions.

Parameter	Value
Flow Rate	0.6 mL/min
Column Temperature	40 °C
Injection Volume	5 µL
Sampler Temperature	4 °C
Detector	UV 260 nm
Mobile Phase	100 mM Tris acetate/2.5 mM EDTA
Analysis Time	25 min

Results and discussion

Using the 150 mM phosphate buffer suggested as an aggregate analysis method (Table 1) for mRNA drug substances in the USP mRNA guidelines, the 100 bp DNA ladder and poly(A) (1 mg/mL) were analyzed under SEC-5 500Å, 1000Å, and 2000Å column conditions, respectively. Variations in the resolution of peaks derived from the DNA ladder were observed, depending on the pore size of the column. Additionally, by performing the test on the peak distribution of poly(A), the effective retention time range for SEC was inferred. For mRNA corresponding to DNA sizes less than 300 bp, the 500Å condition was found to be suitable, while mRNA with sizes less than 1,000 bp required the 1000Å condition, and relatively large mRNA corresponding to 2,000 bp was well-suited for the 2000Å column (Figure 1).

On the other hand, using the same standard solution and column with 100 mM Tris acetate/2.5 mM EDTA as the mobile phase (Table 2), the chromatogram obtained appears as follows: compared to the 150 mM phosphate buffer, there was no significant difference in chromatogram patterns, but it was observed that the elution time was slightly shortened and the average molecular weight of each peak was larger (Figure 2).

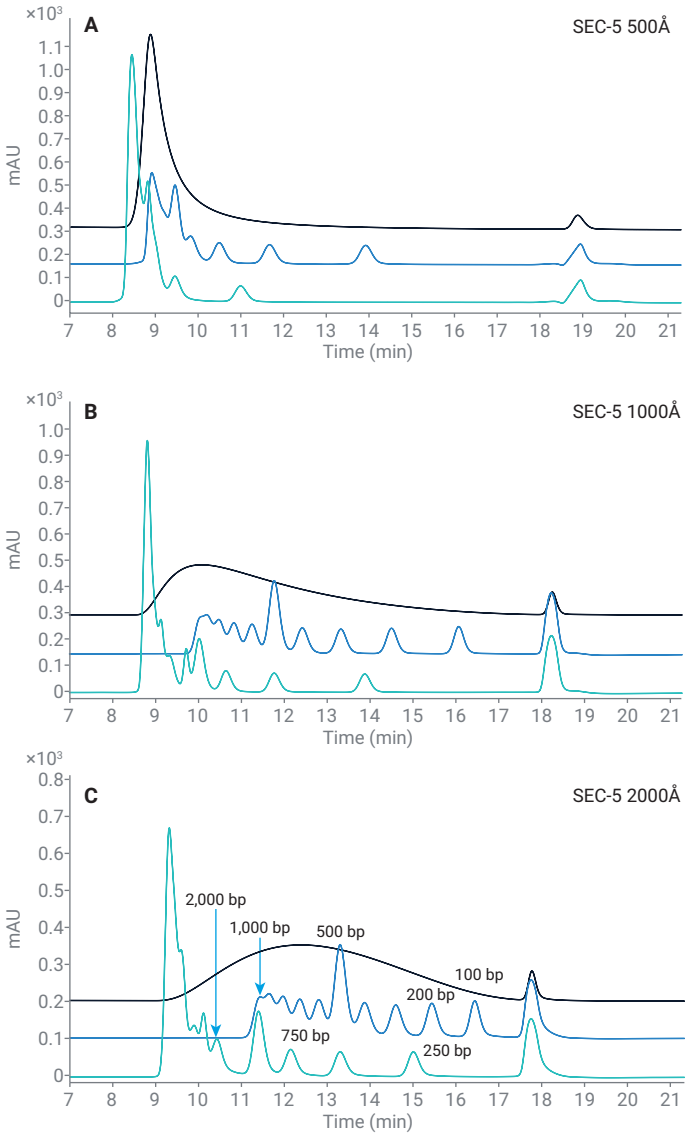


Figure 1. Chromatograms of (A) poly(A), (B) 100 bp DNA ladder, and (C) 1 kbp DNA ladder by column dimension under 150 mM phosphate buffer conditions (poly(A) (black), 100 bp DNA ladder (blue), and 1 kbp DNA ladder (green) from the top down in each chromatogram).

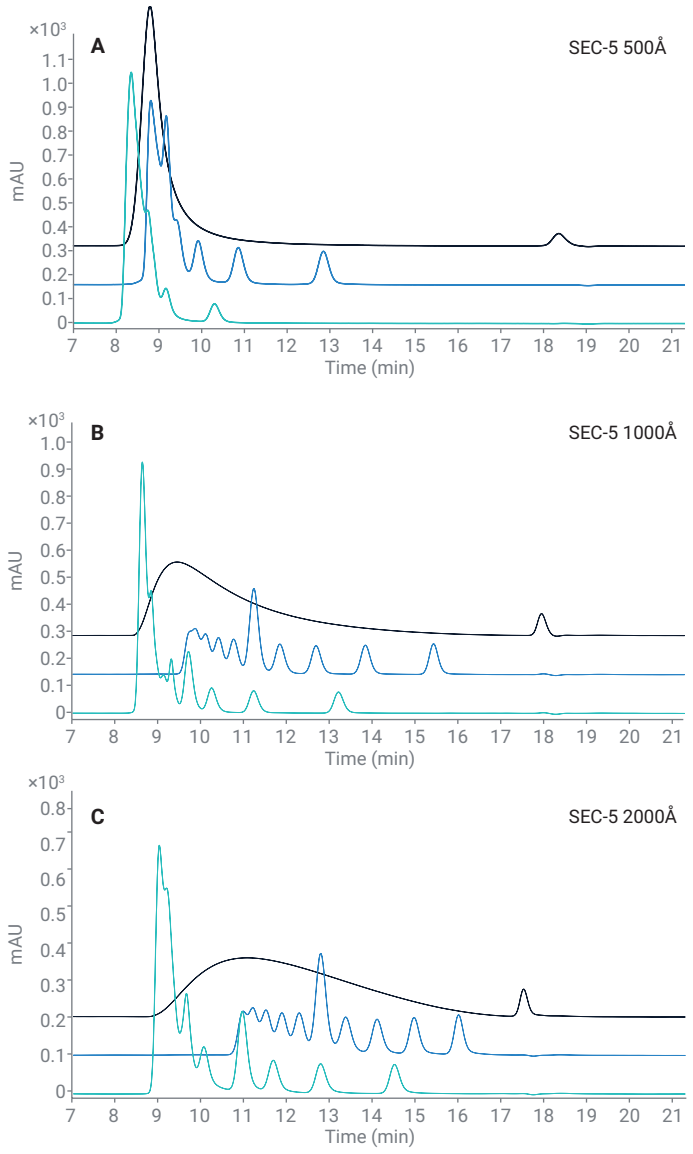


Figure 2. Chromatograms of (A) poly(A), (B) 100 bp DNA ladder, and (C) 1 kbp DNA ladder under 100 mM Tris acetate/2.5 mM EDTA conditions and various column pore sizes (poly(A) (black), 100 bp DNA ladder (blue), and 1 kbp DNA ladder (green) from the top down in each chromatogram).

However, for the evaluation of mRNA aggregates, column selection must also consider the size of the aggregates. Approximately 2,000 nt of CleanCap FLuc mRNA was analyzed under each condition, and the results were compared. The column calibration information from the OpenLab CDS 2.7 GPC add-on was used to estimate the size of mRNA by converting it based on the number of base pairs in the DNA ladder. When comparing the size using the 100 bp DNA ladder and 1 kbp DNA ladder as references, the peak value corresponded to a size of 422 bp (Figures 3 and 4). It was observed that not only the target mRNA, but also the peak fronting corresponding to aggregates were all encompassed within the range of the column.

Double-stranded DNA has a relatively simple three-dimensional structure, forming a long linear structure through complementary binding, while single-stranded RNA, representing a complex three-dimensional structure, exhibits secondary structures through localized complementary binding. The retention time based on molecular size in size exclusion chromatography is directly influenced by the hydrodynamic radius (Rh), and the branching structure of RNA due to complementary binding affects Rh. Therefore, the elution time of single-stranded RNA may vary based on the structure of RNA compared to dsDNA. Furthermore, the diversity of secondary structures and the length distribution of poly(A) contribute to the characteristic of having a broad peak width.

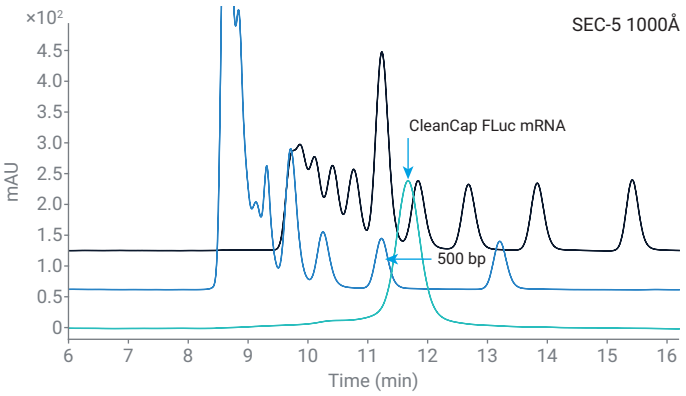
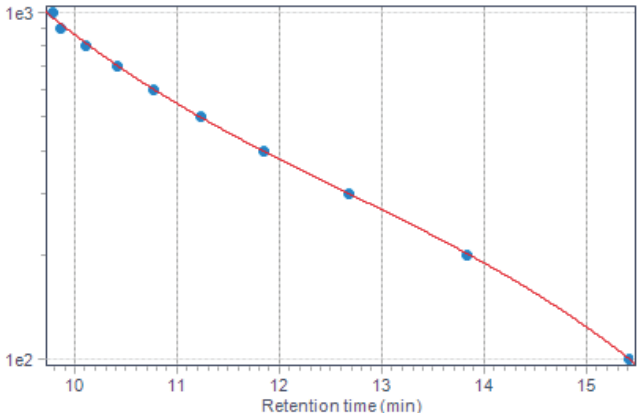


Figure 3. Chromatogram of CleanCap FLuc mRNA under 100 mM Tris acetate/2.5 mM EDTA + SEC-5 1000Å condition (100 bp DNA ladder (black), 1 kbp DNA ladder (blue), and CleanCap Fluc mRNA (green) from the top).



Curve fit

Order of curve fit	3
Curve fit equation	$y = -0.00380656x^3 + 0.144361x^2 - 1.96903x + 11.9933$

Curve fit statistics

Residual sum of squares	0.000448
Coefficient of determination	0.999509
Linear correlation coefficient	−0.998317
Corrected sum of squares	0.912110
Standard Y error estimate	0.008641

Figure 4. Example of length conversion of retention time using the Agilent OpenLab CDS GPC add-on based on 100 bp DNA ladder results (calibration curve horizontal axis: retention time, vertical axis: length (bp)).

When analyzing RNA aggregates, it is essential to choose a column that considers both the measurable size range of the column and the distribution range of the aggregates. Analyzing samples that cover the full exclusion and full permeation ranges of the column, such as poly(A) with a broad distribution or a 1 kbp DNA ladder, allows for the assessment of the column's measurable range.

Additionally, it is crucial to identify analysis conditions that facilitate the easy confirmation of aggregates. In the case of CleanCap FLuc mRNA, when analyzed under SEC-5 1000Å conditions with 100 mM Tris acetate/2.5 mM EDTA (Table 2), approximately 8.5% of aggregates were observed (Figure 5). However, obtaining satisfactory resolution between the target mRNA and aggregates proved to be challenging under SEC-5 2000Å conditions (Figure 5).

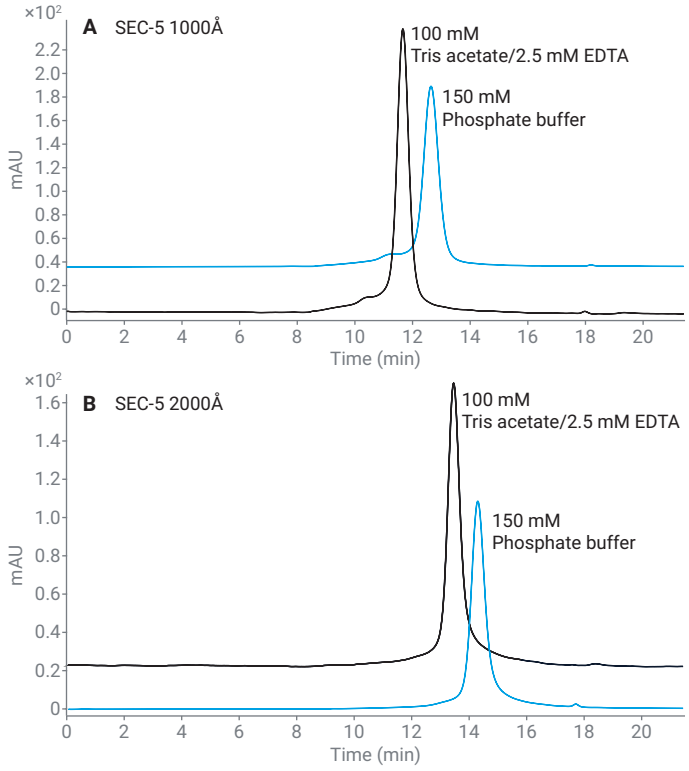


Figure 5. Chromatograms of CleanCap FLuc mRNA under various conditions.

When approximately 3,500 nt of CleanCap β-galactosidase mRNA was analyzed using 100 mM Tris acetate/2.5 mM EDTA conditions (Table 2), the SEC-5 1000Å column showed good results, with an aggregate ratio of approximately 24.9% (Figure 6).

Similar-sized mRNA of samples 1 and 2 exhibited optimal test results in the previous condition, screening under the SEC-5 2000Å column and the mobile phase condition of 100 mM Tris acetate/2.5 mM EDTA (Figure 7).The evaluation of the target mRNA was also suitable for the SEC-5 1000Å column; however, considering the range at which aggregates are eluted and the column's range, the SEC-5 2000 Å column was more appropriate. In this setup, approximately 12.7% of aggregates were observed for sample 1, and about 9.6% for sample 2.

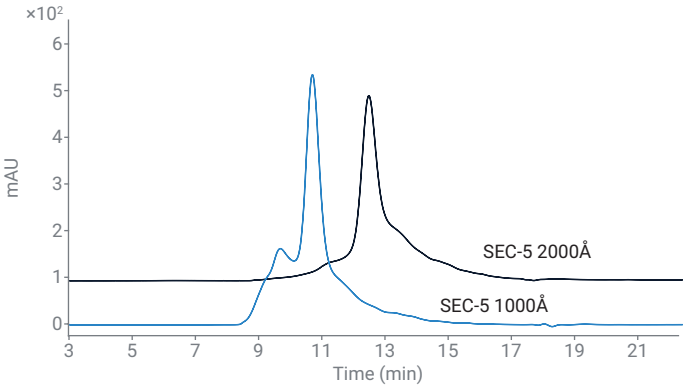


Figure 6. Chromatogram of CleanCap β-galactosidase mRNA under the different pore size column conditions.

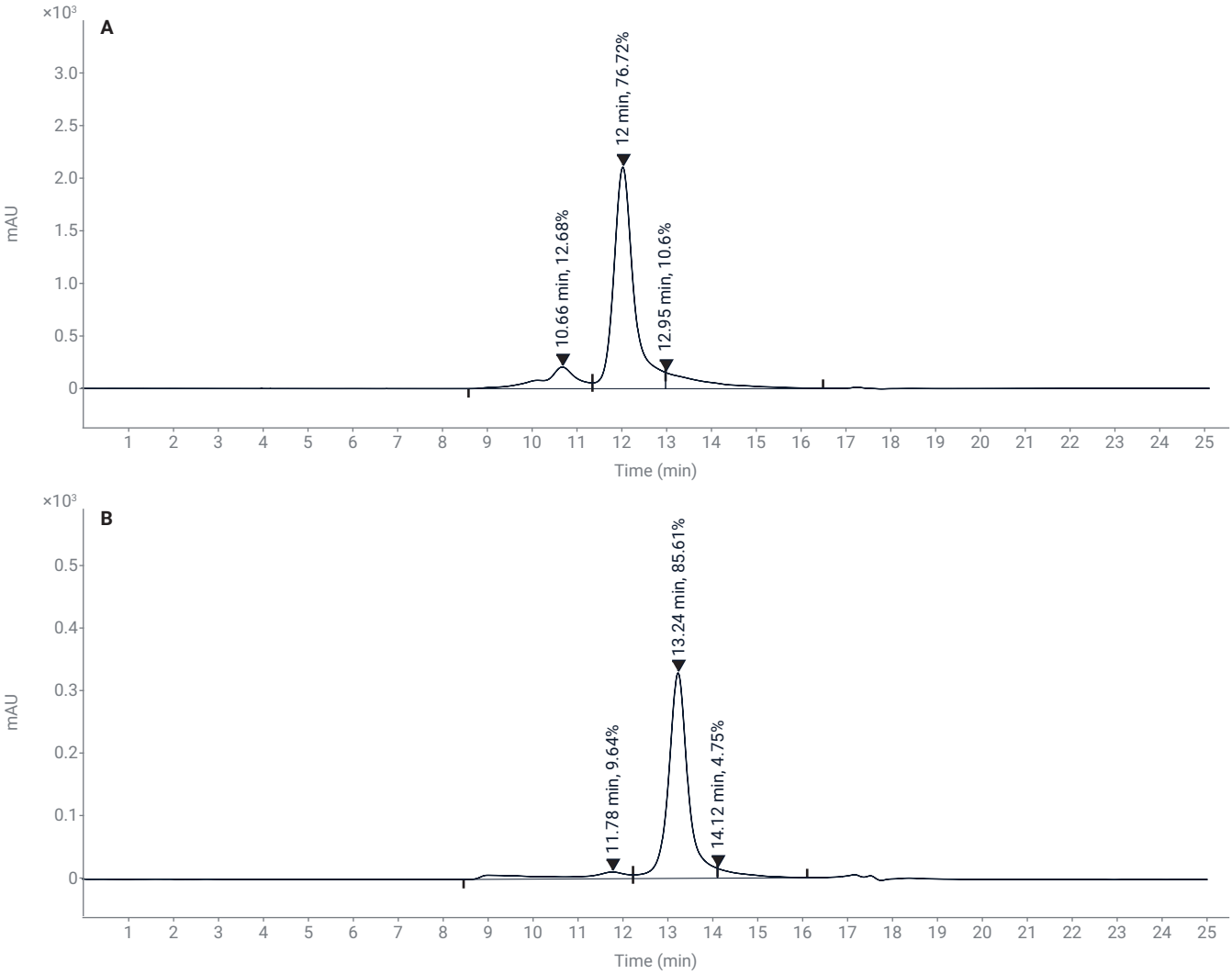


Figure 7. Aggregate analysis results of mRNA, (A) sample 1 and (B) sample 2.

Conclusion

The analysis of mRNA aggregates was performed using the Agilent 1290 Infinity II Bio LC, guided by the USP Analytical Procedures for mRNA Vaccine Quality Draft guidelines, second edition. The selection of an appropriate mobile phase and column was crucial based on the size and characteristics of mRNA. The measurable range of the column was verified using ladder standards. mRNA, with its diverse structural isoforms and poly(A) distribution, exhibited a broader peak width compared to the DNA ladder. Therefore, optimal separation conditions induced by the mobile phase and column were essential for clear differentiation from aggregates. Additionally, column selection should consider the range that covers the distribution of aggregates.

The tests indicated that for the evaluation of mRNA aggregates, a 1000Å column is suitable for mRNA of approximately 2,000 nt, while a 2000Å column is suitable for mRNA below 4,000 nt. However, considering exceptional cases like CleanCap β-galactosidase mRNA, it is necessary to establish optimal conditions through column screening.

The Agilent 1290 Infinity II Bio LC System, with its completely iron-free flow path, is optimally suited for the conditions used in size exclusion chromatography — avoiding potential corrosive damage to the system.

References

1. USP. Analytical Procedures for mRNA Vaccine Quality Draft Guidelines: 2nd Edition. **2023**.
2. Park, C.; Chen, X.; Yang, J. R.; Zhang, J. Differential Requirements for mRNA Folding Partially Explain Why Highly Expressed Proteins Evolve Slowly. *PNAS*, **2013**, *110(8)*, E678–E686.
3. Borodavka, A.; Singaram, S. W.; Stockley, P. G.; Gelbart, W. M.; Ben-Shaul, A.; Tuma, R. Sizes of Long RNA Molecules are Determined by the Branching Patterns of Their Secondary Structures. *Biophys. J.* **2016**, *111(10)*, 2077–2085.
4. Cheng, F.; Wang, Y.; Bai, Y.; Liang, Z.; Mao, Q.; Liu, D.; Wu, X.; Xu, M. Research Advances on the Stability of mRNA Vaccines. *Viruses* **2023**, *15(3)*, 668.

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