Application Note

Biopharma/Pharma

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Wide Pore Polymeric Reversed-Phase PLRP-S Columns for mRNA Analysis

Experimental parameters and comparative analytical outcomes using different molecule sizes

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Abstract

Since the discovery of effective methods of delivery, messenger RNAs (mRNAs) have become increasingly popular in healthcare. These large, complex molecules are designed to allow cells to produce critical proteins and enzymes that can help fight disease. The synthesis of mRNA occurs in several stages: initiation, elongation, editing (processing), and termination. This procedure is very complex, and for this reason it is essential to carry out extensive quality control to ensure that the correct molecule has been created, or to detect the presence of impurities. Impurities might also be the results of product degradation during storage. Liquid chromatography is a well-established separation technique in this regard, but getting an effective analytical separation can prove challenging. In this application note, we explore some of the key parameters when developing an ion-pair reversed-phase (IP-RP) separation of mRNA.

Introduction

mRNA-based therapies are emerging as a safe and efficient alternative to conventional biotherapies in healthcare. These therapeutic mRNA molecules are meticulously designed to deliver specific genetic codes which, upon cellular entry, instruct the production of targeted proteins. This innovative approach has applications across various medical domains, including gene therapy, targeted cancer treatments, infectious diseases, and cardiovascular. These drugs can offer further advantages of high efficiency and ease of prophylactic vaccine production, having proven to be a safe and effective strategy for fighting the spread of COVID-19. However, a significant challenge in this area is the stability of RNA, as these molecules are vulnerable to degradation during various stages of processing, formulation, and storage. Therefore, analytical methods play a pivotal role in evaluating the integrity of RNA molecules. One common degradation pathway involves the fragmentation of large RNA molecules into smaller fragments. Techniques such as anion exchange (AEX), IP-RP, and size exclusion chromatography (SEC) are routinely employed to identify these impurities. Among these techniques, IP-RPLC is frequently favored, offering the highest resolution. IP-RP can offer the ability to characterize a wide range of RNA compounds through modification of chromatographic conditions, including choice of ion-pairing agents, column temperature, and mobile phase composition. Traditional alkyl-bonded silica stationary phases are limited to relatively small pore sizes, rendering them unsuitable for large mRNA molecules due to their size. In contrast, polymeric reversed-phase sorbents, such as the polystyrene/divinylbenzene-based PLRP-S, are more advantageous and can be manufactured in significantly larger pore sizes, including 1,000 and 4,000 Å. In this application note, we investigate the difference in performance of 1,000 and 4,000 Å PLRP-S columns with two model mRNA compounds, Cas9 mRNA and EPO mRNA. Cas9 mRNA encodes the CRISPR-associated protein 9 and is 4521 nucleotides in length; EPO mRNA is only 855 nucleotides long.

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Experimental

Reagents and chemicals

All reagents were HPLC grade or higher.

Instrumentation

A UHPLC instrument with UV detection was used. Method parameters are listed in Table 1.

Sample preparation

Cas9 mRNA and EPO mRNA were obtained from TriLink BioTechnologies and dissolved to 0.5 mg/mL in RNase Free water.

Thermal degradation was performed by heating to 85 °C for 2 hours.

Mobile phase preparation

Mobile phases were prepared from a 1 M TEAA solution. This solution was diluted with suitable solvent. Acetic acid was used to adjust the pH of each solution to pH 7.

Table 1. Method conditions

	HPLC Conditions
Column	(A) Agilent PLRP-S 1,000 Å, 2.1 × 50 mm, 5 µm (B) Agilent PLRP-S 4,000 Å, 2.1 × 50 mm, 5 µm
Mobile Phase	Eluent A: 0.1 M TEAA in water Eluent B: 0.1 M TEAA in 50/50 ACN/water
Flow Rate	0.2 mL/min
Column Temperature	75 °C
Injection Volume	2 µL
Detection	UV, 260 nm
Total Run Time	23 minutes

Table 2. Gradient profile

Time	%A	%B
0	85	15
15	60	40
17	0	100
20	0	100
20.5	85	15
23	85	15

Results and discussion

Initially, mobile phases containing 0.1 M TEAA in water and 0.1 M TEAA in 25% acetonitrile/water to elute mRNA samples were tested. However, these analytical conditions yielded unsatisfactory peak shapes, displaying fronting phenomena and significant peak width, even at a column temperature of 75 °C. As a result, modifications were made by increasing the acetonitrile concentration to 50% v/v in mobile phase B and incorporating a clean-up step at the end of the gradient. Interactions between nucleic acids and TEAA ions render this complex hydrophobic, improving its interaction with the column's stationary phase. Acetonitrile is an additive that makes it possible to reduce the polarity of the mobile phase, improving the nucleic acid ion-pairing fragments' desorption from the column. The separation process is size-based: as the acetonitrile percentage rises, larger nucleic acids are able to desorb. The separation gradient runs from 15% to 40% B with a flow rate at 0.2 mL/min (345 cm/h linear velocity) and column temperature at 75 °C.

Figures 1a and 1b illustrate the elution profile of Cas9 mRNA on the PLRP-S 4,000 Å and PLRP-S 1,000 Å column, respectively, using conditions described above. The larger-pore-size (4,000 Å) column enables improved separation between intact mRNA and impurities. To assess the method and column performances, Cas9 mRNA and EPO mRNA samples were heated for 2 hours at 85 °C. The heat-treated Cas9 mRNA elutes earlier than native Cas9 and small peaks eluting before the main peak, indicating the presence of smaller species, are observed. A decrease in the peak area of the main peak, as well as an increase of the peak area of the impurities, are also observed

A similar pattern is observed with the EPO mRNA (Figure 2a and 2b). The 4,000 Å column exhibits superior separation and peak resolution compared to the PLRP-S 1,000 Å column, for both the native and heat-treated EPO mRNA samples. Heat treatment does not appear to have changed the retention time of the main peak, but additional earlier-eluting impurities are present, and these are more clearly defined with the wider-pore-size PLRP-S 4,000 Å column. Heat treatment also led to a decrease in the peak area of the main peak, and an increase of the peak area of the impurities. One notable feature of this approach was the significantly short run time (15 min) and dramatically narrow peak widths. This method demonstrated the separation of various lengths of mRNA using the PLRP-S column.

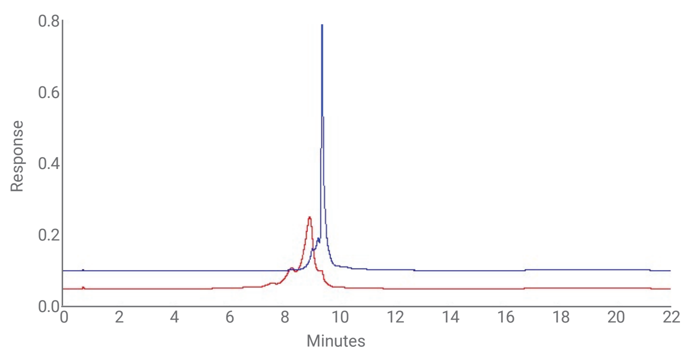

Figure 1a. Cas9 mRNA (blue: native; red: heat treated) on the Agilent PLRP-S 4.000 Å column.

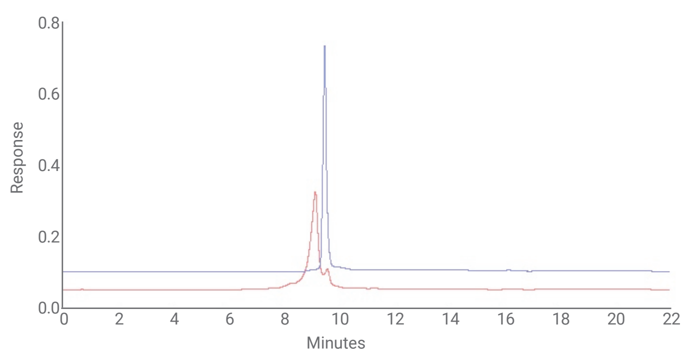

Figure 1b. Cas9 mRNA (blue: native; red: heat treated) on the Agilent PLRP-S 1,000 Å column.

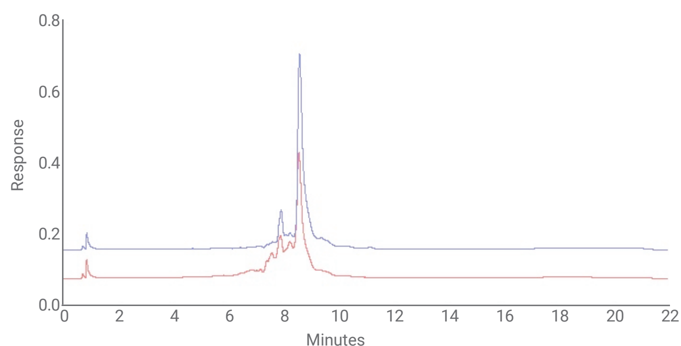

Figure 2a. EPO mRNA (blue: native; red: heat treated) on the Agilent PLRP-S 4,000 Å column.

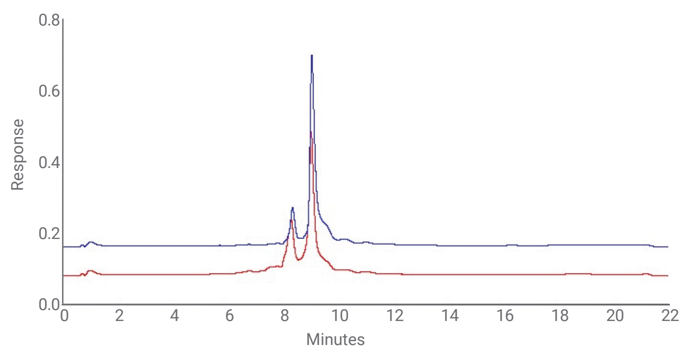

Figure 2b. EPO mRNA (blue: native; red: heat treated) on the Agilent PLRP-S 1,000 Å column.

However, despite the improved mass transfer of the 4,000 Å column, it is not uncommon to find a small but acceptable amount of material is retained and appears as carryover in a subsequent injection. Carryover appears to be slightly more pronounced with larger mRNA but lower than 2.5% (Figure 3a and 3b). It can also be affected by the amount injected, analysis conditions (gradient steepness, flow rate, temperature) and whether the gradient profile includes a high organic clean-up portion.

Conclusion

An IP-RP method was developed for purity determination of mRNA samples. Good elution and separation for mRNA samples of various lengths were obtained in a short run time and with limited carryover. The PLRP-S polymeric materials were an ideal choice, providing the best pore size for the mRNA under investigation. More specifically, using the Agilent PLRP-S 4000 Å column, high resolution and peak separation for both native and heated sample were easily obtained.

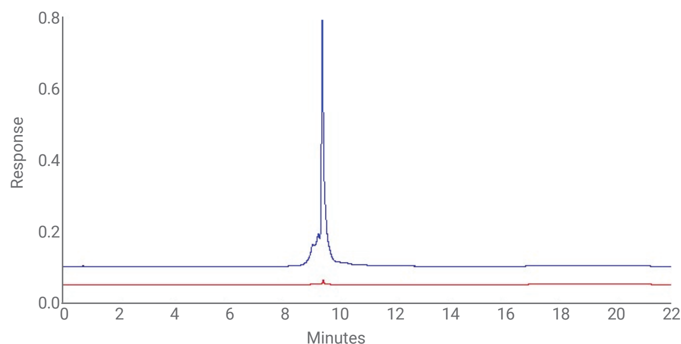

Figure 3a. Cas9 mRNA (blue: native; red: carry over in subsequent blank) on the Agilent PLRP-S 4,000 Å column.

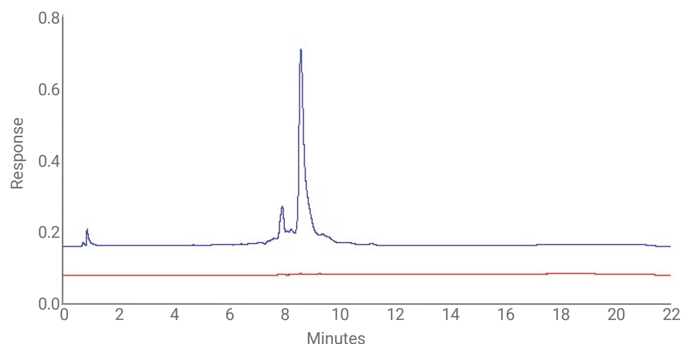

Figure 3b. EPO mRNA (blue: native; red: carry over in subsequent blank) on the Agilent PLRP-S 4,000 Å column.

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DE39329943

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Published in the USA, March 20, 2024
5994-7199EN

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