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Applikationsbericht

Methods for the Anion Exchange Chromatographic Analysis of mRNAs

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Abstract

mRNA-based therapeutics and vaccines are a relatively new class of biopharmaceuticals that have demonstrated significant growth and potential for a variety of biotechnology applications. Methods for analyzing mRNA samples, particularly under less denaturing conditions, are needed to evaluate their heterogeneity. In this application note, we demonstrate that using an anion exchange column between 30–50 °C at neutral pH might be suitable for this type of analysis. Both ion pairing and classical salt gradient conditions can be applied. The effect of temperature on the anion exchange column is discussed and how it might be utilized to detect conformational changes to the mRNA molecules.

Benefits

- Fast, quantitative analysis of mRNA samples
- With the applied conditions, the primary structure and sequence of mRNA is preserved, and thus its intact heterogeneity can be analyzed
- Retention, peak shape, and recovery can be tuned by optimizing column temperature
- Both ion pairing and classical salt gradient conditions can be applied

Introduction

Use of messenger RNA (mRNA) for therapeutic and prophylactic applications is now in full stride with mRNA vaccines having been successfully used to vaccinate millions of people against SARS-CoV-2. An mRNA is a transcript form of a DNA gene sequence. Within the main body of a cell it joins up with ribosomes (a cell organelle), which then translate the nucleotide sequence of the mRNA into an amino acid sequence, or a protein. mRNA therapeutics and vaccines now representing a major new class of advanced therapy medicinal products (ATMPs). With these advances in medicine, there is a need for sensitive and informative analytical methods to characterize the biophysical properties of mRNA drug amd vaccine candidates. In current practice, the reference method is ion-pair reversed-phase liquid chromatography (IP-RP) performed at elevated temperature and in presence of an organic co-solvent.[1–4] Another common practice is based on the use of capillary gel electrophoresis (CGE).[5]

Anion exchange (AEX) chromatographic separations could become a relied upon approach for analyzing intact mRNA if improved methods can be established. Very few analytical scale anion exchange separations for mRNA have been published on. To date, one of the highest resolution ion exchange methods reported separation with a pellicular particle stationary phase and either a neutral pH sodium chloride or sodium perchlorate salt gradient combined with a 60 °C separation, or an inordinately high pH 12 salt gradient separation performed at 10 °C.[6] These separation conditions may not be ideal for preserving mRNA and optimally profiling their intact heterogeneity. The main reason for applying such relatively harsh conditions is that longer oligoribonucleotides (*e.g.* >50 nucleotides in length) exhibit self-structure due to short intramolecular interactions and a wide array of accessible base-stacked conformations.[7] Their self-structure may lead to the presence of several conformers. Denaturing conditions, like high pH, elevated temperature, and the presence of organic cosolvent, can aid a separation by disrupting base-pairing and base-stacking interactions. Under certain conditions, an mRNA can thereby be linearized such that there is a chance to separate and elute sharper chromatographic peaks.

Herein, we propose an alternative, less denaturing technique for mRNA ion exchange LC analysis. A salt gradient ion-exchange separation is performed yet the salt that is chosen is selected from a series of options that can also impart an ion pairing effect. In this experiment, the salt that is employed for gradient elution is tetramethylammonium chloride and the separation is combined with a column temperature of 40 °C. Besides ion pairing salts, common salt gradients such as NaCl, KCl, or $NaClO_4$ can also be used to perform a simple ion-displacement based separation, but it is seen that a higher column temperature of 60 °C might be needed to achieve high resolution separations.

Experimental

Sample and Mobile-Phase Preparation

EPO mRNA (length: 858 nucleotide) and Cas9 mRNA (length: 4521 nucleotide) were purchased from TriLink Biotechnologies (San Diego, CA, USA). Samples were diluted to 25 µg/mL in water and directly injected without further preparation.

LC Conditions

Condition A (Classical Salt Gradient AEX)

LC system:	ACQUITY UPLC H-Class PLUS Bio (quaternary)
Detection:	UV detection at 260 nm
Vials:	Polypropylene Vials (P/N 186002639)
Column:	Protein-Pak High Res Q, 5 µm, 4.6 x 100 mm (P/N 186004931)
Column temp.:	60 °C
Sample temp.:	5 °C
Injection volume:	5.0 µL (sample)
Flow rate:	0.6 mL/min
Mobile phase A:	25 mM TRIS in water (pH=7.6)
Mobile phase B:	2 M Sodium chloride (NaCl) in 25 mM TRIS (pH=7.6)

Gradient:	Recommended gradient steepness: 4–5 ΔB%/min *e.g.* 20–70%B in 12 min

Condition B (Ion Pair AEX)

LC system:	ACQUITY UPLC H-Class PLUS Bio (quaternary)
Detection:	UV detection at 260 nm
Vials:	Polypropylene Vials (P/N 186002639)
Column:	Protein-Pak High Res Q, 5 µm, 4.6 x 100 mm (P/N 186004931)
Column temp.:	40 °C
Sample temp.:	5 °C
Injection volume:	5.0 µL (sample)
Flow rate:	0.6 mL/min
Mobile phase A:	25 mM TRIS in water (pH = 7.6)
Mobile phase B:	3 M Tetramethylammonium chloride (TMAC) in 25 mM TRIS (pH = 7.6)
Gradient:	Recommended gradient steepness: 4–5 ΔB%/min *e.g.* 60–100%B in 10 min

Column Conditioning

Equilibrate the column with a minimum of 20–50 column volumes of the mobile phase to be used. Then perform a few consecutive (3–4) high mass load injections of the sample of interest to condition the active sites of the stationary phase. Before starting the quantitative analysis, make sure that the relative standard deviation of peak areas from the consecutive injections are sufficiently low (*e.g.* RSD <2%).

Results and Discussion

Classical Salt Gradient AEX

We selected two samples (Cas9 and EPO mRNA) as representative examples to demonstrate the applicability of a non-porous quaternary amine anion exchanger for the analysis of mRNA. A Tris buffered mobile phase was applied along with an NaCl salt gradient and column temperatures ranging from 30 up to 60 °C. With these conditions, it can be seen that the highest resolution separations were achieved at 60 °C (Figure 1). It was also with the 60 °C column temperature that peak tailing was minimized.

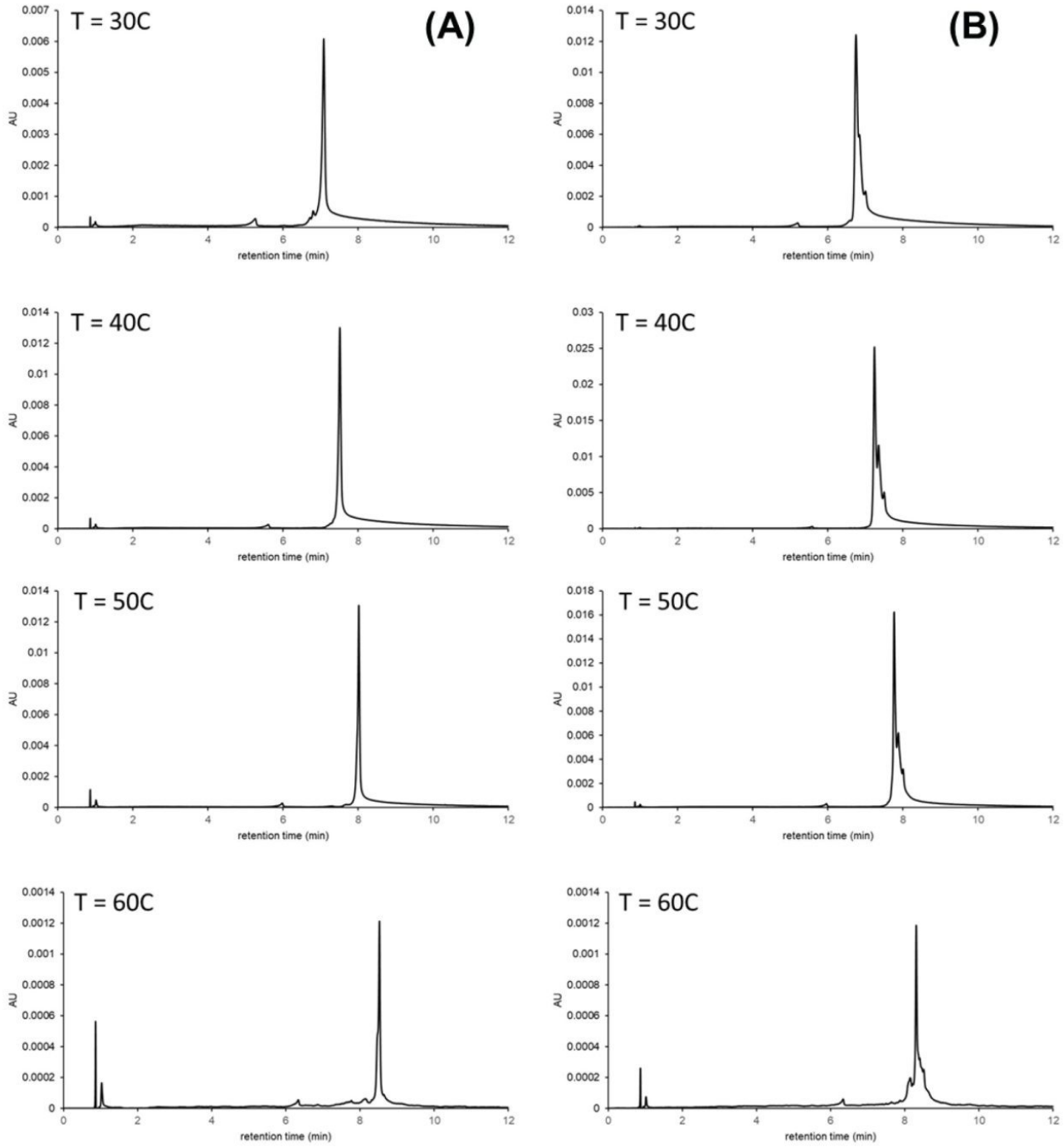

Figure 1. Ion exchange separations of Cas9 mRNA (A) and EPO mRNA (B) using an anion exchange column, a Tris buffered mobile phase, and sodium chloride gradient combined with a series of different column temperatures ranging from 30 to 60 °C.

Ion Pair AEX

Alkyl ammonium salts have already been used as mobile phase additives for the separation of DNA fragments.

[8–10] An interesting aspect of using alkyl ammonium salts versus the commonly used NaCl or KCl is that it enables the elution of nucleic acid fragments mostly in the order of increasing length (size). Moreover, tetramethylammonium chloride as a mobile phase salt has been recently applied for the separation of empty and full capsids of adeno associated virus.[11]

In a second example, the potential benefits of an alternative mobile phase were explored, wherein Cas9 and EPO mRNA samples were separated with a Tris buffered mobile phase and salt gradient based on tetramethylammonium chloride, and again with column temperatures ranging from 30 up to 60 °C. With these separations, it can be seen that the highest resolution separations were achieved at a lower temperature around approximately 40 °C. It is at 40 °C that baseline resolution of two acidic variants could be observed in the EPO mRNA sample. This resolution could not be achieved with the NaCl gradient no matter the temperature employed. This example thus shows that unique results can be obtained upon applying a tetramethylammonium chloride gradient along with a neutral pH mobile phase and a column temperature of no greater than 50 °C. Using this method, a high-resolution separation is achieved that we believe reflects more accurately the integrity of the mRNA sample (Figure 2). While additional work is needed, it might be that tetramethylammonium chloride salt gradient combined with less than or equal to 60 °C separation temperature is particularly well suited to the analysis of single stranded nucleic acids.

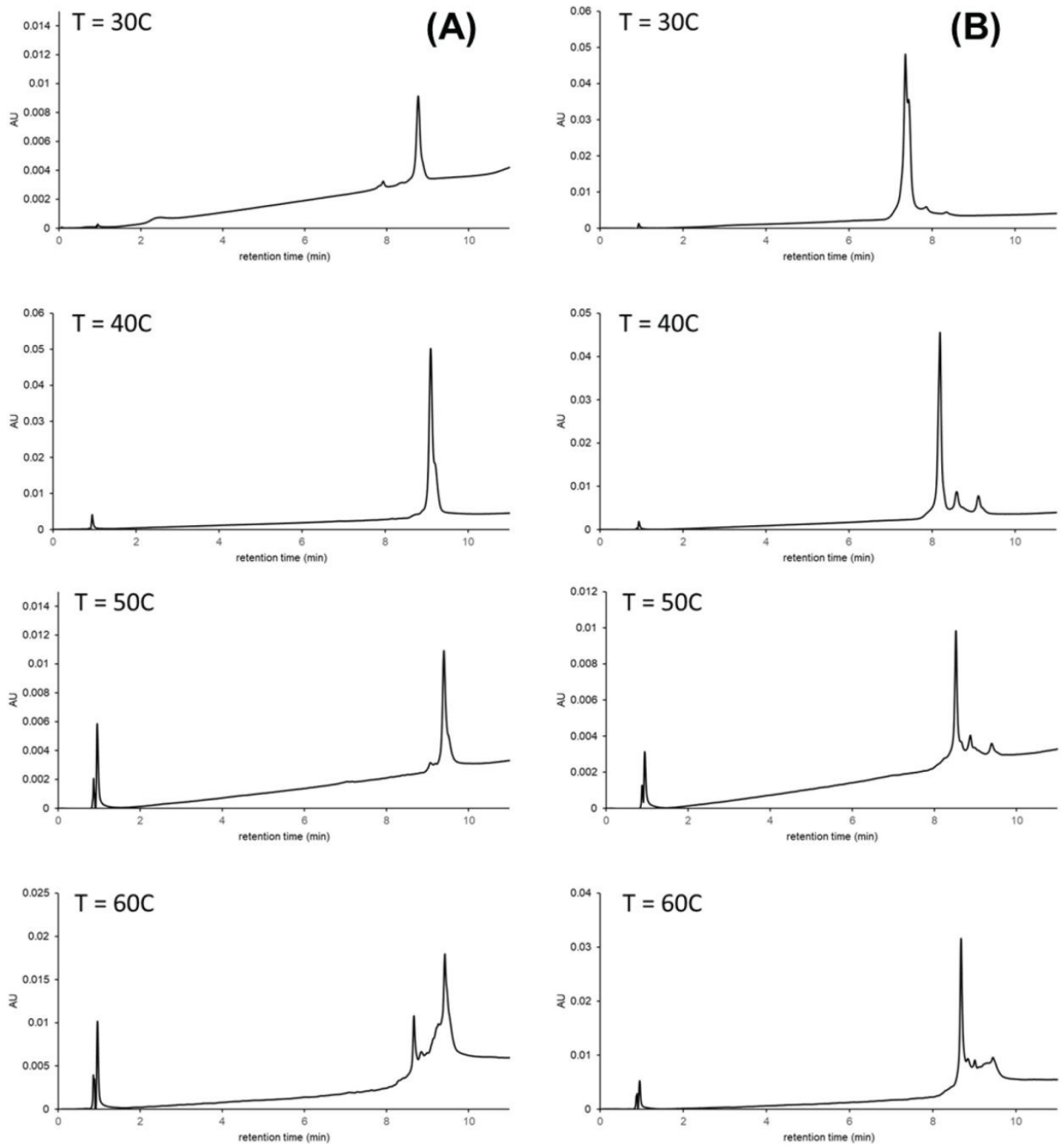

Figure 2. Ion exchange separations of Cas9 mRNA (A) and EPO mRNA (B) using an anion exchange column, a Tris buffered mobile phase and tetramethyl ammonium chloride gradient combined with a series of different column temperatures ranging from 30 to 60 °C.

Conclusion

mRNA sequences are translated by ribosomes into to proteins, and they represent a new class of advanced therapy medicinal products. There is a need for sensitive and informative analytical methods to characterize, monitor and release mRNA drug candidates. Among several analytical techniques, IP-RPLC is one of the most well-established reference methods. In current practice, harsh conditions are applied for IP-RPLC which may not be ideal for investigations into mRNA self-structure, or when studying their intrinsic heterogeneity. Here, we propose a less-denaturing anion-exchange method as an alternative to IP-RPLC. The Protein-Pak High Res Q anion exchanger column offers appropriate peak shape and selectivity for mRNA samples in both classical salt gradient and ion pairing modes of separation. Two methods are proposed, one is based on running a sodium chloride gradient while the other is based on use of a tetramethylammonium chloride gradient. We found that a 4–5 ΔB%/min gradient steepness at 0.5–0.6 mL/min flow rate gives a good compromise between resolution and analysis time. For most mRNA samples, a 40–50 ΔB% gradient range was sufficient and can easily be adjusted for any mRNA. In addition to gradient steepness and ΔB% range, the column temperature was found to be an important method variable as it impacts recovery, retention, and peak shape. Temperature changes the self-structure of mRNAs. As such, it might be useful to test the effects of column temperature on chromatographic values, including retention and recovery, and to thereby make unique observations of an mRNA molecule's folding and self-structure.

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