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응용 자료

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Abstract

With a burgeoning pipeline of nucleic acid-based therapies, there is a need for improved analytical methods that can quickly confirm the concentration, integrity, and relative abundance of various large nucleic acid species. In particular, anion exchange has significant potential for use as an analytical technique for mRNA concentration and integrity determination, but current methods tend to exhibit high carryover that can make the techniques impractical to implement. With this work, we show that selection of column technology can matter, and that the Gen-Pak™ FAX Weak Anion Exchange Column can serve as a very effective starting point for a new method. Moreover, carryover can be decreased by shortening the analyte's column residence time (1), starting the gradient with comparatively high counter ion concentrations up to 100 mM (2), and by applying an injection approach where the drawn sample is bracketed with modulating salt plugs. This work has also helped us elucidate a relationship between single stranded mRNA denaturation and carryover as well as selectivity and resolution. As mobile phase temperature is brought to approach the melting temperature of self-folding, selectivity increases but so does carryover.

Benefits

- Low carryover between consecutive injections
- Improved recovery for mRNA samples
- Improved repeatability and method robustness

Introduction

In recent years, the biopharmaceutical industry's interest in gene therapy modalities has increased dramatically. Synthetic nucleic acids are providing new forms of vaccination and new means of modulating protein expression. Most recently, it has been *in vitro* transcribed mRNA that has garnered widespread attention.[1,2]

Liquid chromatography (LC) techniques are required to analyze intact mRNA drug substances.[3] Analytical scale separations by ion-pair reversed phase (IP-RP) or size exclusion (SEC) chromatography are often applied and are used to detect heterogeneity or check for molecular integrity.[3]

Despite the fact that large scale anion exchange (AEX) separations (purification) are regularly performed for oligonucleotides and nucleic acids, only a few analytical scale AEX separation methods have been published up to now. The reason for the limited use of AEX for analytical purposes is probably the lack of method robustness, such as poor recovery and high carryover effects which are often observed with large nucleic acids. Bridonneau and co-workers reported that 30 min equilibration in specific conditions and blank runs were required to prevent carryover between AEX chromatographic runs for RNA aptamer purification.[4] Guilherme *et al* proposed some solutions to decrease carryover such as supplementing elution buffers with chelating agents, and/or with denaturing agents (*e.g.* urea, formamide, or isopropanol), to suppress intermolecular interactions.[5] Highly charged cationic molecules (*i.e.* spermidine) have also been proposed as additives and they are believed to have an effect to stabilize compact supercoiled structures, thus improving recoveries.[6] It has been shown recently that a gradient of weak ion-pairing cations can produce intriguing AEX separations of mRNAs.[7] This so-called ion pairing anion exchange “IPAX” method provided improved recovery and selectivity compared to classical salt gradients. In addition to non-specific interactions occurring between the solute ions and the stationary phase ligands, the column hardware material itself can also contribute to poor recovery and high carryover. As it stands, Minkner *et al* considered a recovery of >95% for siRNAs in AEX as an excellent performance, and poor recovery of large mRNAs versus smaller ones has been reported not only for AEX but also for affinity and hydrophobic interaction chromatographic (HIC) separations.[8,9]

With the above considerations in mind, we believe that AEX chromatographic separations could become a relied upon approach for analyzing intact large nucleic samples if improved methods can be established. Here, we propose some efficient solutions to significantly reduce carryover occurring in AEX separations.

Experimental

Sample and Mobile Phase Preparation

ClenCap 5 moU EPO mRNA (length: 858 nucleotide), luciferase (LUC) mRNA (length: 1929 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 injected without further preparation.

Tris-(hydroxymethyl)aminomethane (TRIS), guanidine hydrochloride (Gdn-HCl) and sodium bromide (NaBr) were purchased from Sigma- Aldrich (Buchs, Switzerland). Tris buffer was prepared as 25 mM solution, and its pH was adjusted to ~7.6. This 25 mM Tris buffer was used as mobile phase A. For mobile phase B, 2 M Gdn-HCl or 2 M NaBr was dissolved in 25 mM Tris buffer.

For salt plug injections, either mobile phase B or a solution of 2 M NaBr in 10 x strength BioResolve™ CX B Concentrate (p/n: 186009064 <https://www.waters.com/nextgen/global/shop/standards--reagents/186009064-bioresolve-cx-ph-concentrate-b.html>) were used.

LC Conditions

LC system:	ACQUITY™ UPLC™ H-Class PLUS Bio System (quaternary)
Detection:	UV detection at 260 nm
Vials:	Polypropylene Vials (p/n: 186002639)
Column:	Gen-Pak FAX Anion-Exchange Column, 2.5 µm, 4.6 mm x 100 mm (p/n: WAT015490)
Column temperature:	Ambient to 45 °C
Sample temperature:	5 °C
Injection volume:	2.0 µL (sample)

Bracketed injection sequence:	1.0 μL (salt pre-plug) + 2.0 μL (sample) + 1.0 μL (salt post-plug)
Flow rate:	0.6 mL/min
Mobile phase A:	25 mM TRIS in water (pH=7.6)
Mobile phase B:	2 M guanidine-HCl (Gdn-HCl) in 25 mM TRIS (pH=7.6) or 2 M sodium-bromide (NaBr) HCl in 25 mM TRIS (pH=7.6)
Gradient:	Recommended steep gradients for fast separation: - for 2 M Gdn-HCl mobile phase: 0–25%B in 6 min - for 2 M NaBr mobile phase: 15–50%B in 7 min Recommended shallow gradient for higher selectivity: - for 2 M NaBr mobile phase: 12–35%B in 15 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 (*e.g.* 5–10 μg) injections of the sample of interest to condition the active sites of the stationary phase.

Results and Discussion

The phenomenon behind the poor injection repeatability and high carryover effects observed with biomolecules (macromolecules) is often related to non-desired secondary interactions with surfaces and to both inter- and intramolecular interactions occurring in a macromolecular system. Macromolecules in general are surface-active

molecules, which undergo non-specific adsorption when they come into contact with different types of surfaces.[10] This process could be critical as it may cause a loss of solute content or result in aggregation. During an adsorption event, most macromolecules undergo shape changes (conformational changes like unfolding). The area on the adsorbent surface that is occupied by the large macromolecules is often called "the footprint".[11] The footprint usually increases with residence time, which can be referred to as a "spreading process". Footprint related extra-adsorption is usually partially reversible.[12,13] The adsorption kinetics and footprint surface area strongly depend on the solute concentration too.[14] At high analyte concentrations, a surface comes to be occupied in a shorter time and thus the time available for spreading is then shorter as well. This results in a smaller average footprint; however, the adsorbed concentration will be higher. Other parameters such as solvent pH and ionic strength may also impact the size and spreading of the footprint.

Several studies have documented some of these above-mentioned effects.[15–18]

The Effect of Residence Time and Initial Mobile Phase Strength

Preliminary experiments suggested that the length of time an mRNA is allowed to bind to the stationary phase might correlate with carryover.

mRNA has already been confirmed to abide by an on-off (bind and elute) like elution mechanism when separated by AEX.[7] Therefore, it is reasonable to assume that mRNAs are bound at the head of the column and remain motionless until they experience the eluting mobile phase composition of a gradient. Hence, a certain time is available for analyte spreading to occur and for multipoint interactions to form with the stationary phase. One can have the impression that a shorter residence time leads to fewer binding segments and in turn weaker adsorptive interactions and lower carryover.

The effect of residence time on carryover has been studied experimentally in a systematic way. A Gdn-HCl gradient of 0–25%B in six minutes was programmed and various initial isocratic holding times (at 0%B) were set prior to the start of the gradient. Namely, 0-, 0.5-, 1-, 2-, 4-, and 8-minute initial isocratic holds were set. EPO and Cas9 mRNA samples were injected and carryover was measured (in %) in the blank injection following the sample injection. Figure 1A shows the obtained carryover as a function of solute binding time. The plot suggests there is indeed a correlation between carryover and binding time. The shorter the binding time, the lower the carryover. As predicted, these experiments suggest that short analytical runs should be applied as a means to limit carryover. When limiting the retention time to about three minute, as low as 4–8% carryover was found in contrast to 10–20% carryover observed with long gradients.

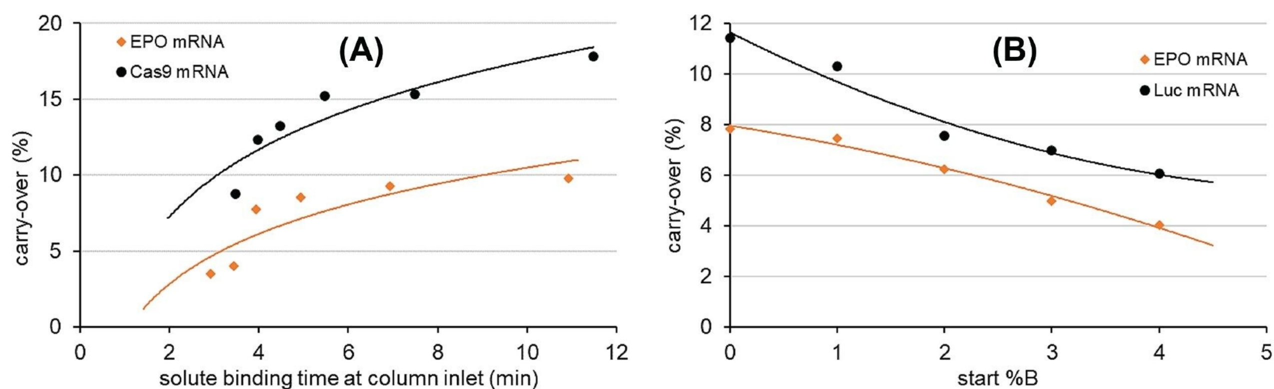

Figure 1. Effect of an mRNA solute's binding (residence) time (A) and of initial mobile phase strength (B) on carryover in AEX. Column: Gen-Pak FAX 100 x 4.6 mm, 2.5 µm Column, mobile phase A: 25 mM TRIS, pH=7.6, mobile phase B: 25 mM TRIS, pH=7.6 + 2 M Gdn-HCl, F=0.6 mL/min, gradient: 0–25% B in six minutes, ambient temperature (~22 °C).

In another experimental setup, we studied the impact of the initial strength of the mobile phase. If solute spreading is assumed to occur on the surface of the stationary phase, one might think that spreading is less significant (or slower) if weaker interactions occur upon initial binding. Therefore, the gradient time was fixed, and the initial %B composition was varied as 0, 1, 2, 3, and 4%B. Figure 1B shows the observed carryover for EPO and Luc mRNAs as a function of starting mobile phase composition. There is an obvious trend, the higher the start %B, the lower the carryover. Starting the gradient from 4% B (~80 mM counterion) instead of 0% B reduced the carryover by a factor 2. This observation suggests that the gradient should start at a reasonably high %B mobile phase composition (*i.e.* 50–100 mM counterion) instead of 0% B.

Please note that both the effect of binding time and of start %B is sample dependent and thus needs to be optimized for the sample of interest.

Embracing the Sample Plug With Salt Plugs (Bracketed Injection)

Solvent strength mismatch is a term used to describe a situation where the injection solvent and the mobile phase have different eluent strengths.[19] Solvent strength mismatch is especially problematic when the sample solvent is stronger than the mobile phase composition. Such a situation often results in partial- or total breakthrough effects or at least in peak fronting or splitting.[19] Effects like these have been frequently

encountered in multi-dimensional separations and hydrophilic interaction chromatography (HILIC) analysis. To limit strong solvent effects, the sample can be introduced onto the column by applying a specially programmed injection sequence where the drawn sample is bracketed by a dilutive set of pre and post sample plugs. Such kinds of injection sequences have already been applied to improve separation performance or to limit breakthrough effects.[20,21]

For AEX of mRNAs, where the binding interaction is inherently very strong, the above-mentioned injection sequence approach (normally applied to increase the strength of the initial binding interaction) needs to be inverted. Here, the sample should be injected along with strong solvent plug(s) in order to limit the strength of the initial binding. Therefore, we propose the bracketing of the sample between solvent plugs containing high concentration salt (counterion). In addition to high ionic strength, the pH of the solvent plug can also be adjusted to be close to the p*Ka* of the stationary phase functional groups (*i.e.* pH 10–11 in the case of a weak anion exchanger). Again, the combined effect is intended to limit the strength of the initial adsorption.

Systematic experiments have been performed to identify the most important factors of a sequenced injection and how they affect the carryover of mRNA on an AEX separation. The following factors have been studied: (1) volume of a salt pre-plug, (2) volume of a salt post-plug, (3) volume of the bracketing salt plugs in sum, (4) type of salt employed, including NaCl, $(NH_4)_2SO_4$, Gdn-HCl and NaBr, and (5) the pH of the salt plug.

It was found that the most beneficial sequence is an injection sequence in which the sample plug is bracketed with pre- and post-plugs of 2 M NaBr solution (at pH ~10). Figure 2 shows a schematic view of a bracketed injection. Please note that at pH higher than 10.5–11.0, mRNAs might be denatured, and the base-pairing and base-stacking interactions might be disrupted. At high pH, the intact RNA can thereby be linearized which can lead to spreading, a larger binding footprint, and significantly stronger adsorption. Furthermore, some nucleobases can be deprotonated above pH 10, which would add additional negative charge to the nucleic acid analyte.[22] This is probably the reason why at high pH condition, the retention of mRNAs increases in AEX.[3] Therefore, a too high pH (>11) is not beneficial for use as a solvent plug. Empirically, increased carryover has been observed.

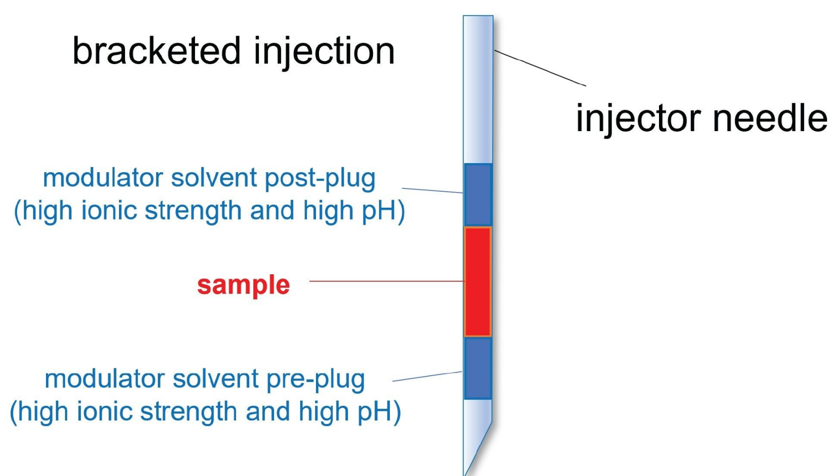

Figure 2. Schematic view of an injection sequence where the drawn sample is bracketed with high ionic strength and high pH modulator plugs. In Empower™ Software, the "auto additions" option helps the user to create any sequence of injection they would like for a Sample Set.[23]

The ratio of modulating salt plug volume to sample plug volume needs to be optimized to find the lowest carryover whilst avoiding sample breakthrough. Figure 3 shows the change in EPO mRNA's carryover and recovery as a function of modulating plug to sample volume ratio (V_{mod} / V_{sample}), which can be used in a case where the volume of the pre- and post-plugs are identical. The figure shows that carryover decreases and recovery increases until (Vmod / Vsample) reaches a value of ~1.2–1.3. Beyond this "limit" value, a fraction of the injected sample volume is taken by the strong modulating plug and a partial breakthrough peak appears on the chromatogram. If (V_{mod} / V_{sample}) ≥2 then the entire mRNA peak elutes at the column's dead time (total breakthrough). When setting a (V_{mod} / V_{sample}) ≈1–1.2, as low as 2–3% carryover can be reached instead of 10–20% which is often observed without salt plug modulation. Please note that the ideal (V_{mod} / V_{sample}) ratio might depend on the sample, injector apparatus, system volumes, mobile phase, and column. It needs to be optimized individually for each method setup. Setting (V_{mod} / V_{sample}) ≈1 seems to be a good starting point. As an example, if 2 µL of mRNA sample is going to be injected then a good start is to program a sequence with a 1 µL modulator pre-plug + 2 µL sample + 1 µL modulator post-plug.

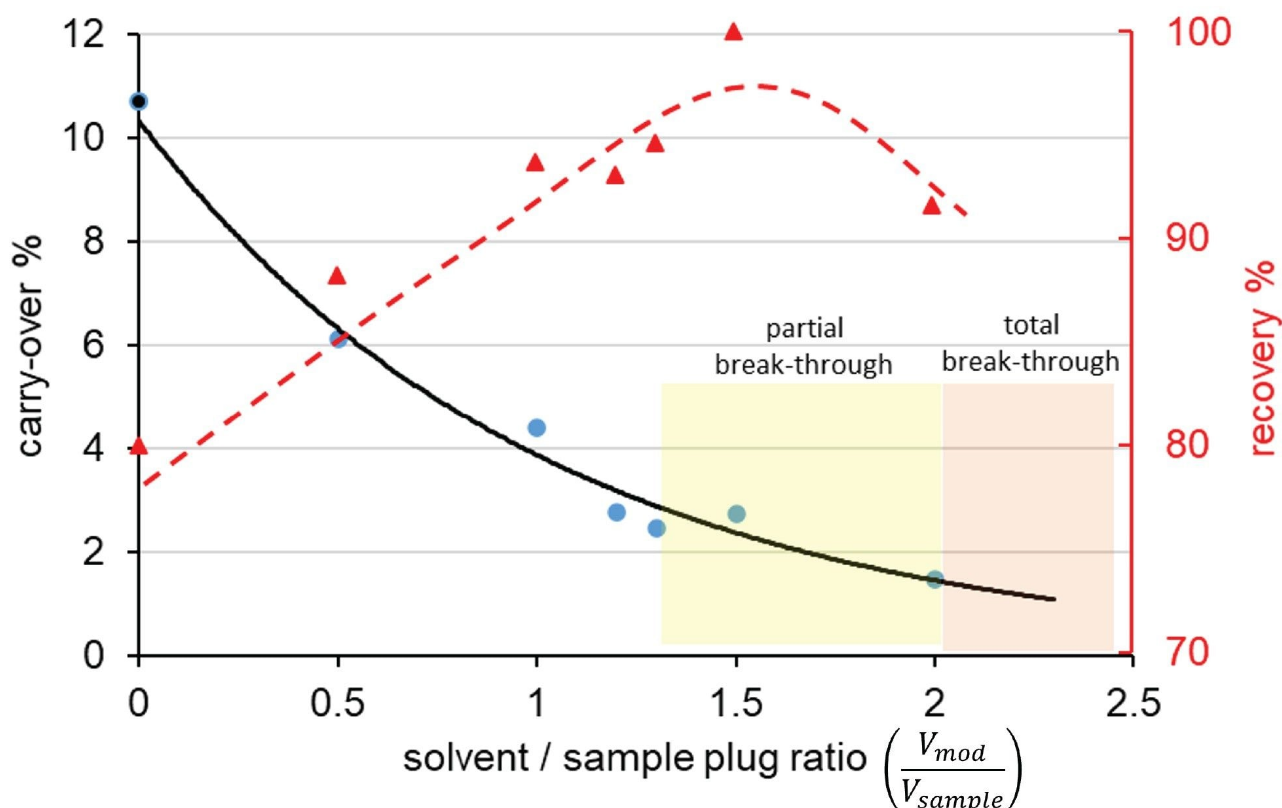

Figure 3. The effect of modulator plug to sample volume ratio on carryover (black curve) and recovery (red curve). Column: Gen-Pak FAX 100 x 4.6 mm, 2.5 µm Column, mobile phase A: 25 mM TRIS, pH=7.6, mobile phase B:25 mM TRIS, pH=7.6 + 2 M NaBr, F=0.6 mL/min, gradient: 15–50% B in seven minutes, ambient temperature (~22 °C). Sample: EPO mRNA (2 µL injected), modulator plug: mobile phase B.

Column (Stationary Phase) Dependent Carryover

Various stationary phases have been studied including both weak and strong AEX phases. A minor difference was found, it seems that weak exchangers in general result in slightly lower carryover. However, it should be noted that carryover will always be sample and condition dependent. Difference can be observed even between weak ion exchangers which is probably related to differences in ligand density, ligand accessibility (morphology) and the possibility of additional interactions, like H-bonding.

Figure 4 shows a comparison between a Gen-Pak FAX Weak Anion-Exchange Column and a monolithic weak

anion-exchange column using the same optimized bracketed injection for both columns. The Gen-Pak FAX Column exhibited significantly lower carryover and comparable resolution with some instances of significantly improved peak sharpness.

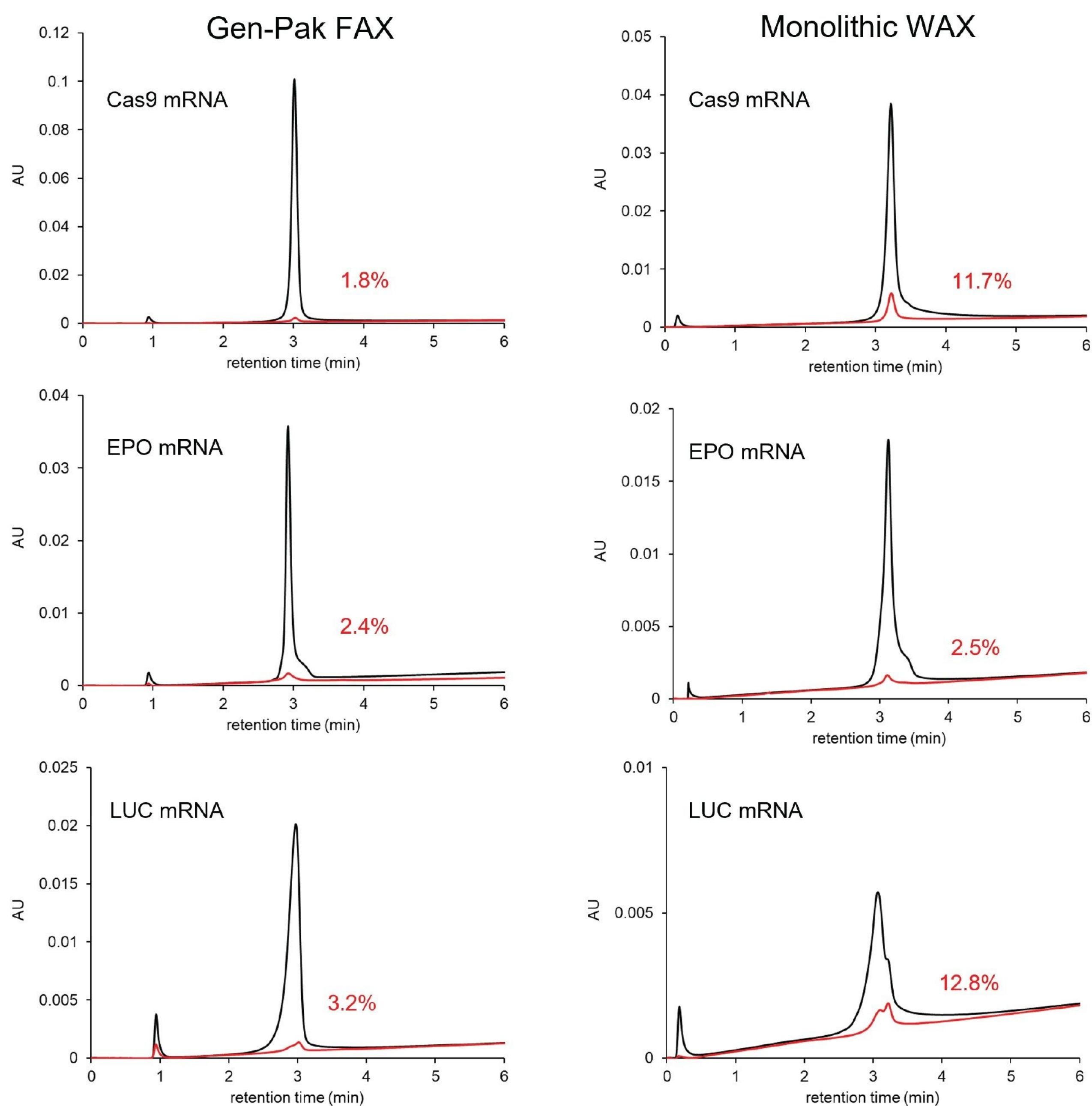

Figure 4. Comparison of carryover observed on two columns. Columns: Gen-Pak FAX 100 x 4.6 mm, 2.5 µm Column (left), and monolithic WAX 4.95 x 5.2 mm (right). Gradient conditions: same as for Figure 3, modulator solvent plug: 2 M NaBr in pH 10.2 buffer. Bracketing injection: 1 µL modulator pre-plug + 2 µL sample + 1 µL modulator post-plug. The red numbers expressed in % correspond to the carryover % observed in blank injection

following the sample injection.

Temperature Effects

It has been reported that in AEX chromatography, increasing temperature resulted in fewer and more defined peaks for RNA samples.[24] This was attributed to diminished secondary structure. Moreover, an increase in retention was also observed at higher temperatures, which can also be explained by the loss of self-structure. Recently, we found that temperature also has a huge impact on mRNA recovery and carryover in AEX.[7] Changes in carryover due to temperature effects are difficult to predict, since many parameters might change the strength of the interactions between the mRNAs, the aqueous mobile phase, and the stationary phase. In the presence of large amounts of salt, solvophobic effects, salting-out, salting-in, dehydration of the mRNA and structural rearrangements might all occur to some varying degrees.[7] Therefore, we were interested to study the effect of temperature for bracketed injections. Figure 5 shows the chromatograms obtained when injecting EPO and Cas9 mRNA samples at ambient versus elevated temperatures (T = 35 and 45 °C). Carryover seems to increase with temperature which is in-line with observed retention increases (suggesting stronger binding at elevated temperature). As such, when considering sample carryover, ambient temperature experiments are preferred. However, peak shape, selectivity and separation profiles significantly change with temperature, and there appears to be some corresponding advantageous effects on resolution of sample components. Ultimately, it may be useful to set up two methods, one operated at ambient conditions and another set for running an elevated column temperature. The method with ambient (low) temperatures might be suitable for content/concentration determining measurements while the elevated temperature method can be valuable to investigate biophysical properties and the chemical heterogeneity of the mRNA.

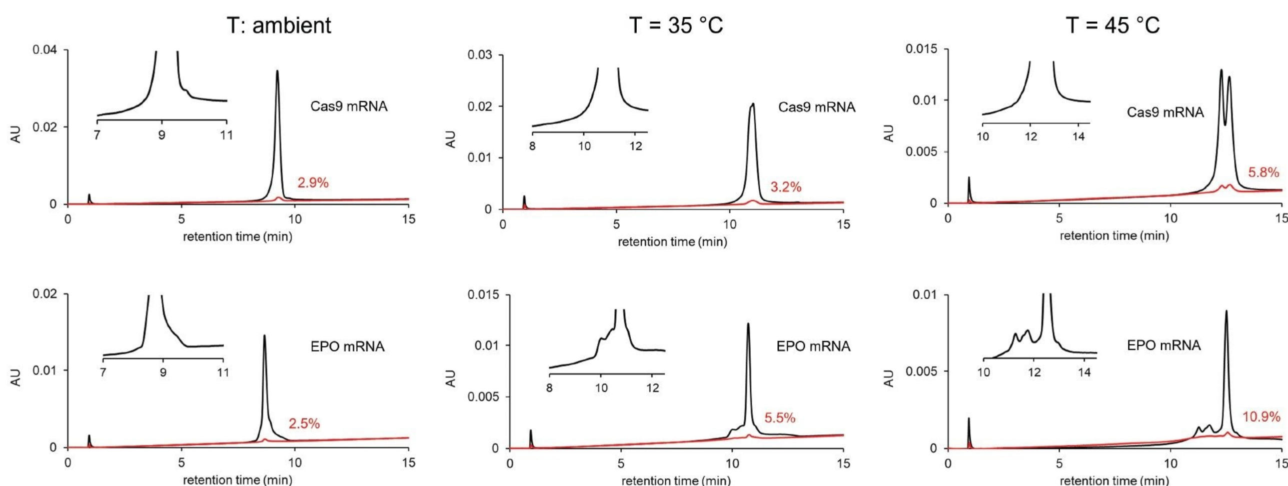

Figure 5. The effect of temperature on the chromatographic profile (selectivity) and carryover. Column: Gen-Pak FAX 100 x 4.6 mm, 2.5 µm Column, mobile phase A: 25 mM TRIS, pH=7.6, mobile phase B:25 mM TRIS, pH=7.6 + 2 M NaBr, F=0.6 mL/min, gradient: 12–35% B in 15 min (shallow gradient), modulator solvent plug: mobile phase B. Bracketing injection: 1 µL modulator pre-plug + 2 µL sample + 1 µL modulator post-plug. Temperature: ambient (left), 35 °C (middle) and 45 °C (right).

Conclusion

High carryover and low recovery are known to occur in anion exchange analyses of large nucleic acids. Here, we propose some new method considerations to reduce carryover and thus to improve method robustness. Through multiple rounds of past investigation, the Gen-Pak FAX Column has been confirmed to give some of the most effective separations of large, single stranded nucleic acid samples. It provides a reliable starting point for implementing new methods.

It has been found that a short analysis time is favorable in terms of carryover. It is also helpful to start the gradient with a relatively high eluent strength.

However, the most valuable approach is to apply a specially programmed sample injection in which the sample is bracketed with so-called "modulator" plugs. These plugs contain high concentrations of salt and are buffered to have a pH of approximately ten. This bracketed injection helps reduce the strength of solute binding at the

head of the column thus improving recovery and carryover. The volume and the ratio of the modulator plug needs to be optimized for each individual method setup. In this study, a ratio for (V_{mod} / V_{sample}) of approximately 1 proved to be quite effective. By using this novel bracketed injection mode with a Gen-Pak FAX Column, we have been able to reduce the carryover of large mRNAs to ~2%, in contrast to the 10–20% carryover which has often observed with conventional AEX methods.

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