FAST AND HIGH RECOVERY ANALYTICAL CHARACTERIZATION OF mRNAs USING WEAK ANION EXCHANGE AND ION PAIRING REVERSED PHASE CHROMATOGRAPHY

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INTRODUCTION

To fully harness the potential of mRNA as a new modality therapeutic, there is a need for more analytical characterization tools that can tie together insights on stability, drug design, and structure function relationships.

intact mRNA chromatography

AAAAA

Shortage of high resolution, fast and high recovery methods[1]

SEC

IP-RP

AEX

Here, we present developments of IP-RP and AEX chromatographic separations that focus on speed and recovery, and as such can facilitate R&D and quality control work in the mRNA-based drugs development.

ION PAIRING REVERSED PHASE (IP-RP)

We have been interested in developing a fast IP-RP mRNA analysis using a short column format with **minimal sample consumption**, while providing **maximum information.** We started our investigation with established conditions, in which a strong ion pairing system is used to characterize vaccine mRNA and its impurities[2]. It is known that under such conditions the retention is driven by oligonucleotide length[3], therefore we used RNA ladders to verify that analyzed RNA sample elutes in the correct size range using ACQUITY™ BEH™ C18 Column. *Figure 1* shows the separation of single stranded RNA ladders, in which acceptable resolution is achieved for nucleic acids <2000 nt, making it suitable for EPO mRNA (858 nt) analysis; however, in order to achieve confident detection as much as **1 µg of mRNA** had to be injected – only then the characteristic, intrinsic pattern of two peaks (i, ii) could be discerned in a **30 min method** that also required a lengthy equilibration period. Lowering the amount of injected material led to insufficient amount of signal (inset), while optimization of the gradient produced complex baselines. We reasoned that by applying lower amount of the ion pairing agent we will be able to reduce the background noise – increase the sensitivity and shorten the analysis time (together with the equilibration time).

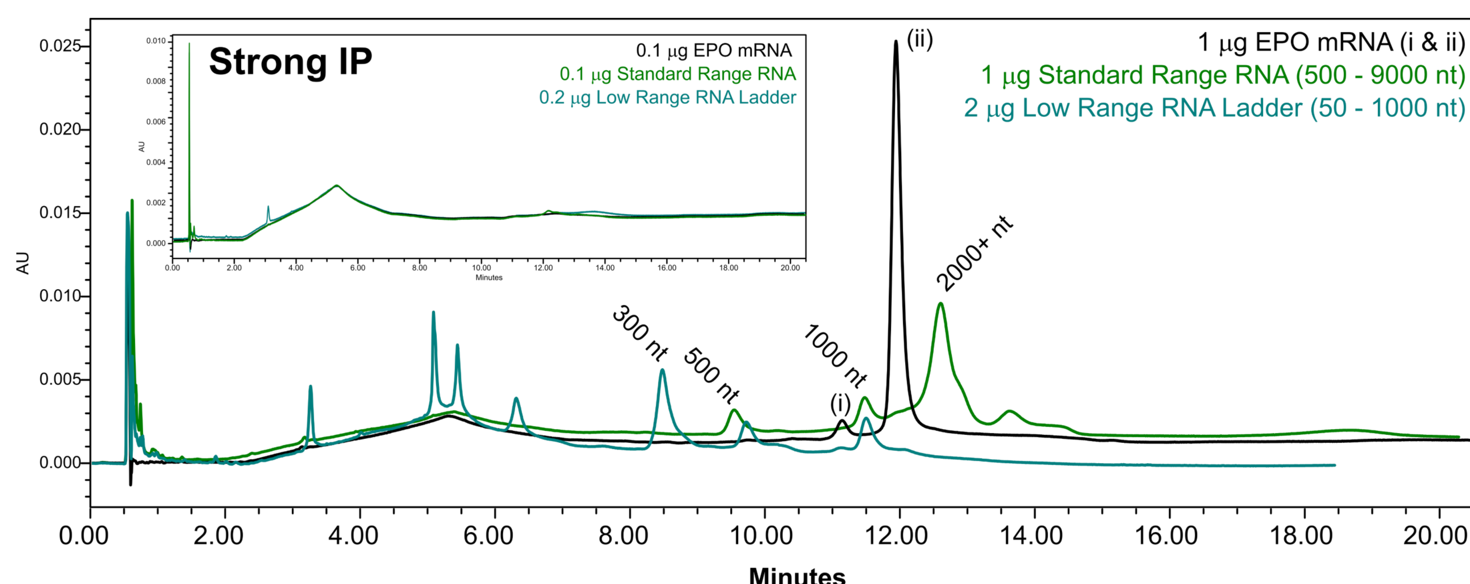
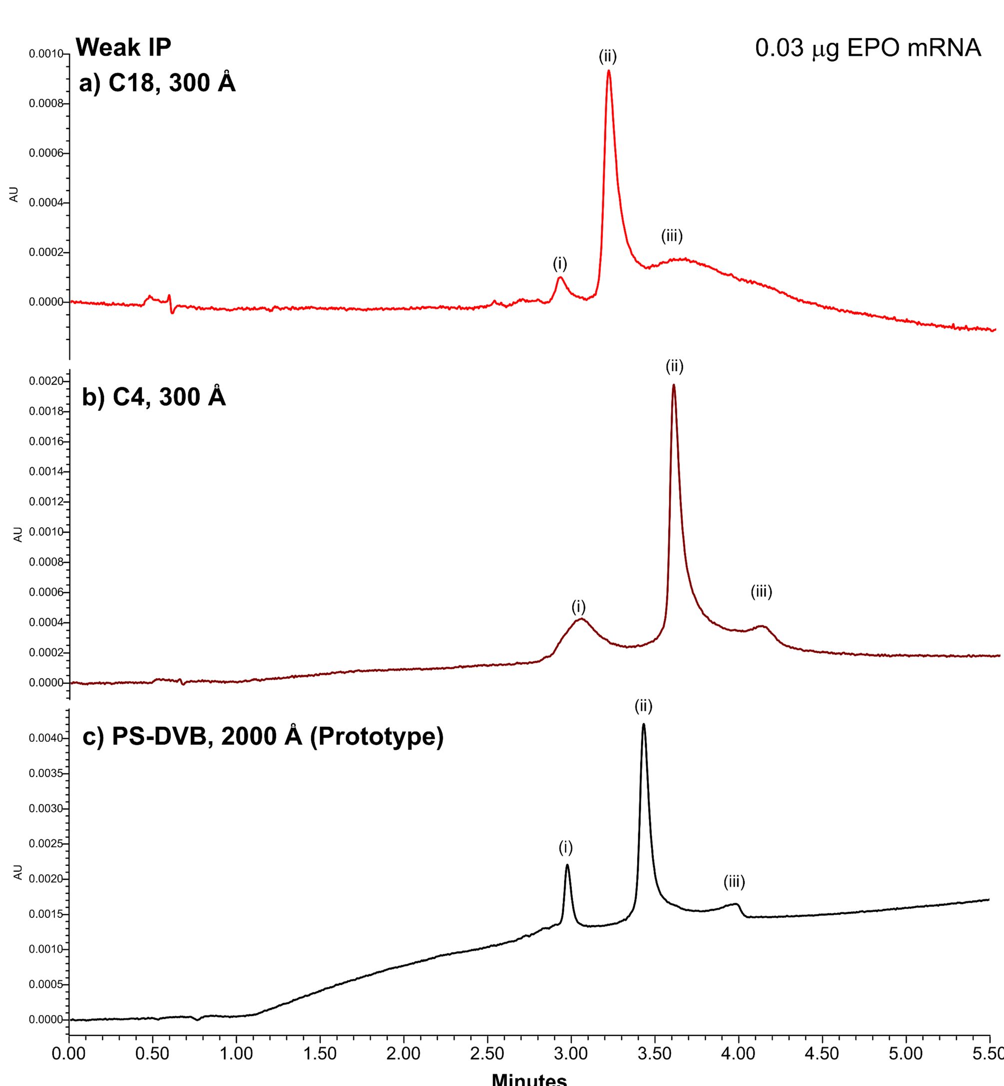


Figure 1. A suitability test of the ACQUITY BEH C18 Column for the analysis of large RNAs using standard ladders and an EPO mRNA sample with a strong ion pairing mobile phase. Gradient[2]: resolving part: 4.5 – 20 min; 50 - 60% B.



Indeed, *Figure 2a* shows that switching to a weaker ion pairing system, resulted in a **9 min** method and good sensitivity using **30x less material (c. 30 ng)**. The change of the retention mechanism (higher influence of hydrophobic interactions under weak ion pairing)[3] resulted in the appearance of a third peak (iii). We found that its tailing can be reduced by using different stationary phases (*Figure 2b&c*). Moreover, 300Å RP columns were compared to a, more commonly used for large nucleic acids, large pore DVB column which yielded a similar result but with sharper peaks, indicating general suitability of the method for analysis of mRNA of this size.

*Figure 2. A weak ion pairing IP-RP analysis of EPO mRNA using different columns and fast 5 min gradient a) ACQUITY BEH C18 Column, 20 – 40% B b) ACQUITY BEH C4 Column, 10 – 40% B c) Prototype PS-DVB, 10 – 40% B with **30 ng injection** of the sample.*

METHODS

Samples

mRNA samples (erythropoietin, EPO - 5moU, 858 nt; luciferase, LUC, 1929 nt; and Cas9, 4521 nt) from TriLink Biotechnologies. ssRNA ladders (Low Range: 50 – 1000 nt and Standard Range 500 – 9000 nt) from New England Biolabs. The samples were diluted with water (0.025 - 0.1 µg/µL for mRNA and around 1.5 µg/µL for ladders) and the required volume injected. Weak ion pairing (Weak IP): triethylamine (TEA) buffered with hexafluoroisopropanol (HFIP) while, strong ion pairing (strong IP) employed additionally dibutylamine (DBA) pairing and acetic acid (AcOH) buffering.

System and columns

All measurements on ACQUITY UPLC™ H-Class Bio BSM System detecting at 260 nm with following columns:
IP-RP: ACQUITY Premier BEH C18 Column (50 x 2.1 mm, 300 Å, 1.7 µm, Waters), ACQUITY Premier BEH C4 Column (50 x 2.1 mm, 300 Å, 1.7 µm, Waters), Prototype PS-DVB (50 x 2.1 mm, 2000 Å, 3 µm, Waters),
IEX: Gen-Pak FAX Anion-Exchange Column (100 x 4.6 mm, non-porous, 2.5 µm, Waters).

Mobile phases and conditions:

IP-RP: column at 70 °C, flow rate: 0.25 mL/min, IEX: column at ambient temperature (≈ 22 °C), flow rate: 0.6 mL/min

Mobile phase	A	B
Weak IP	7 mM TEA + 50 mM HFIP, pH = 8.5	A in 20% MeCN
Strong IP	100 mM TEA + 50 mM DBA, 150 mM AcOH, pH = 8.5	A in 50% MeCN
AEX	25 mM TRIS, pH = 7.6	A + 2 M Guanidine hydrochloride

ANION EXCHANGE CHROMATOGRAPHY (AEX)

AEX analyses of biomolecules often suffers from **poor injection repeatability and high carry-over effects** related to non-desired secondary interactions with surfaces.

During an adsorption event, the area that is occupied by macromolecules is often called *the footprint*[4]. The footprint usually increases with the residence time, which can be referred to as a "spreading process" – illustrated in the *Figure 3*. Footprint related extra-adsorption is usually only partially reversible and might be responsible for experienced problems[5]. Therefore, one can have the impression that a shorter residence time leads to fewer multipoint interactions with the stationary phase and hence lower carry-over.

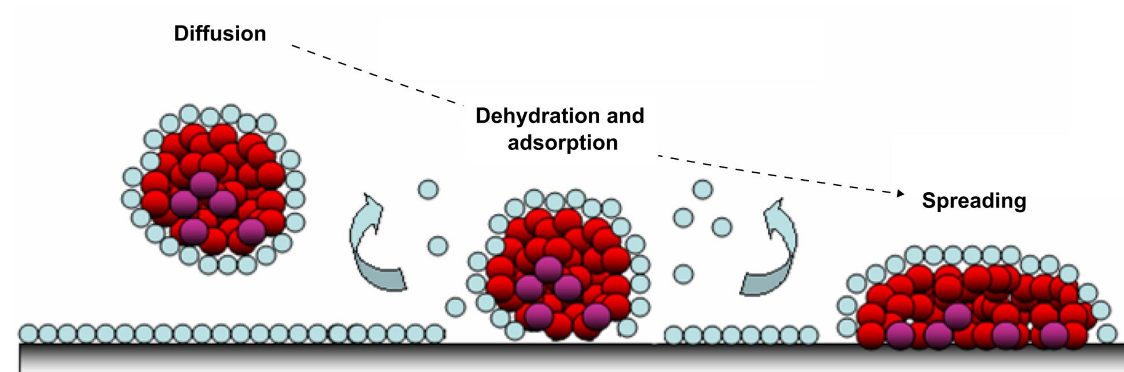


Figure 3. Illustration of a possible mechanism for increased macromolecules retention leading to carry-over and analytical complications.(Adapted from Ref[6])

Similar reduction of interactions can be achieved if the solute is injected at higher ionic strength. These hypotheses were studied experimentally for mRNA samples and a weak anion exchange column.

RESIDENCE TIME

The residence time was controlled by varying the initial isocratic hold (as mRNAs follow the on-off like elution mechanism[7]). EPO and Cas9 mRNA samples were then eluted with a salt gradient, and the carryover was measured (as %area) in the following blank injection. *Figure 4* shows the obtained carryover as a function of solute binding time suggesting proportional correlation between the parameters and reinforcing the idea that short analytical runs should be applied to limit the carry-over.

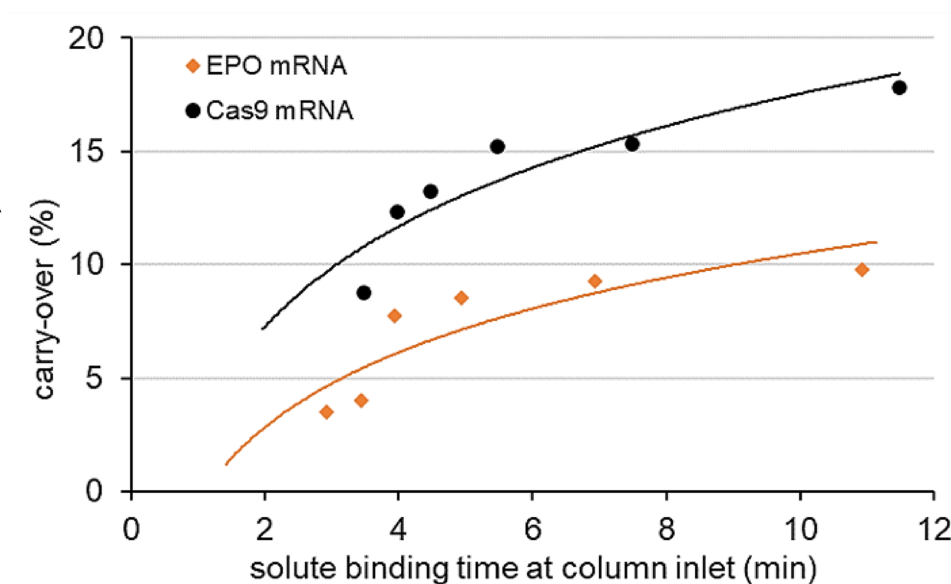


Figure 4. Effect of an mRNA solute's binding (residence) time on the carry-over in AEX. Gradient: 0 – 25% B in 6 min after a 100%A variable time isocratic hold.

When minimizing the retention time to **~3 minutes**, as low as **4 – 8% carry-over** was found, in contrast to 10 – 20% carry-over observed with long gradients.

INITIAL STRENGTH OF THE GRADIENT

Another parameter which may reduce macromolecular spreading is the initial strength of the mobile phase that weakens the interactions occurring during the initial binding. With a fixed gradient time the initial %B composition was varied. *Figure 5* shows the results for EPO and Luc mRNAs and reveals an inverse proportional correlation as the higher the starting %B, the lower the observed carry-over.

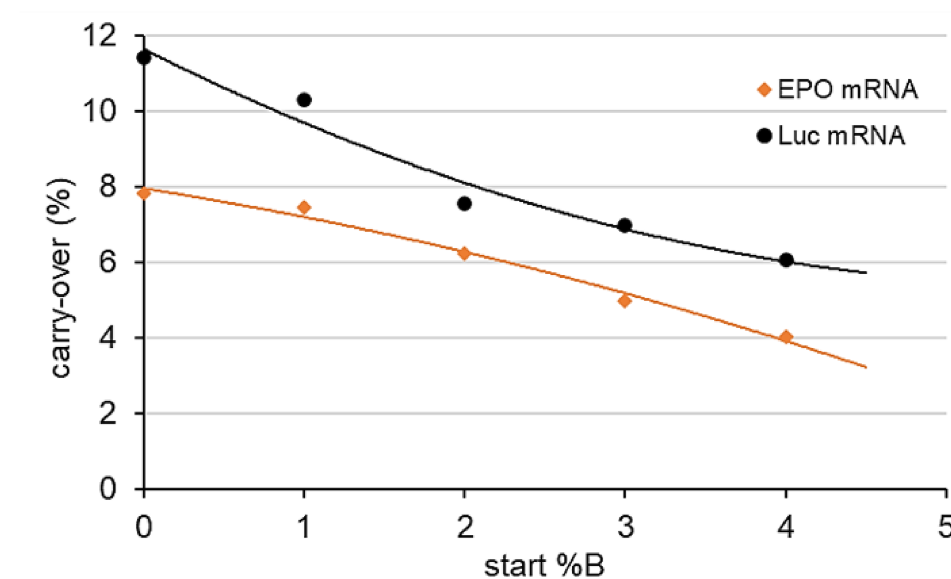


Figure 5. Effect of the initial mobile phase strength on the carry-over of mRNAs in AEX. Variable gradient: *x* – 25% B in 6 - 10 min adjusted to maintain a similar retention time.

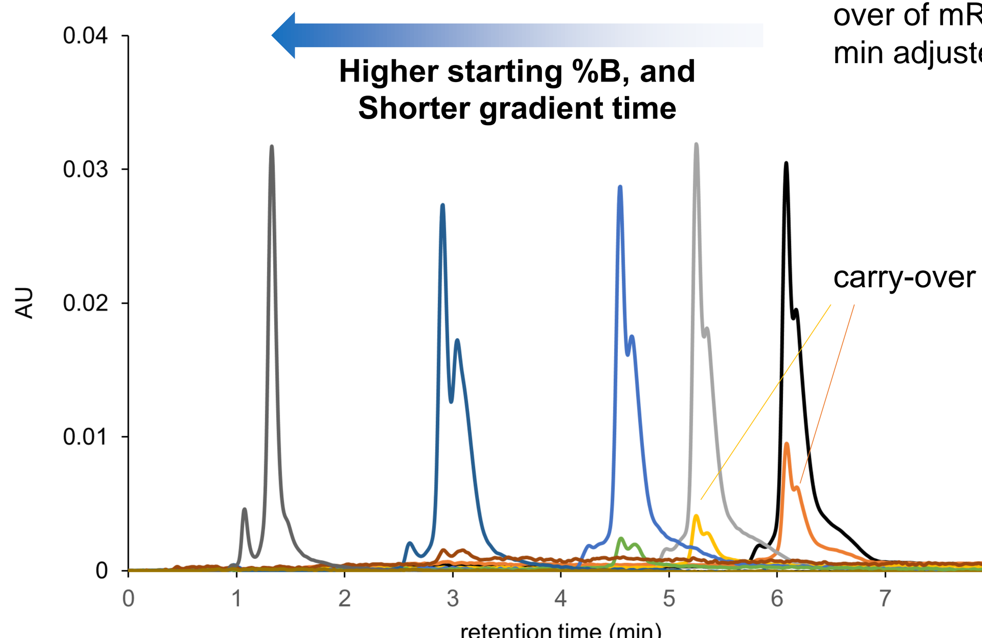


Figure 6. Weak anion exchange chromatography for EPO mRNAs with increasing starting B% and decreasing gradient times illustrating the reduction in carry-over in the subsequent blank injections.

Starting the gradient at 4%B (~80 mM counterion) instead of 0%B **reduced the carry-over by a factor of 2**. This observation suggests that the gradient should start **at a reasonably high %B** mobile phase composition (often 50-100 mM counterion), which needs to be optimized for every sample to avoid break-through effects.

CONCLUSION

- **For a fast and high recovery IP-RP separation of large nucleic acids, weak ion pairing system are preferred and they can be matched with a variety of columns**
- **For reduced carry-over and increased recovery of mRNAs during AEX it is preferable to minimize the residence time and start with an adequate level of the strong eluent**

References

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