Improvement of Empty|Full Resolution By Implementation of Novel Eluents Using PATfix

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Introduction

In every adeno associated virus (AAV) downstream process the key steps are the enrichment of full capsids and the in-process empty/full analytics. A common approach for enrichment of full capsids is liquid chromatography using ion exchange or multimodal chemistries, which based on particles' surface charge differences, enables separation of full (F) capsids and product related impurities. Removal of impurities is an important step as they may pose a serious health risk, as well as deteriorate the economics of the production process.

A PATfix® triple detector set in combination with an CIMac™ AAVEmpty/Full analytical column by Sartorius BIA Separations was used to determine E/F ratio. The column is a strong anion exchanger which exploits capsid charge differences for separation when employing an ascending salt gradient. Usually, NaCl with the addition of 2 mM magnesium is used, as the presence of magnesium has been shown to improve the peak shape and the removal of empty capsids. According to the Hofmeister series salts have different impact on non-covalent interactions among capsid themselves and between columns and capsids. For example, antichaotrope salts were shown to improve the empty/full capsid ratio, possibly by inducing the hydrophobic interactions between them. Quaternary alkyl ammonium salts (QAAS) are known to have antichaotropic properties and they differ in the substituents (Figure 1). Wang et al. reported that QAASs produced a better empty/full separation than NaCl. This poster presents a screening of different eluents for empty/full analytics. Beside NaCl as a reference salt, two QAAS were chosen : tetramethylammonium chloride- TMAC and a possible nonhazardous alternative choline chloride, which is bifunctional and contains both a quaternary ammonium and hydroxyl groups. The influence of magnesium on E/F separation was also tested.

Figure 1: Structural formulas of two quaternary alkyl ammonium salts, TMAC and choline chloride.

1. Experimental approach

Sample preparation

AAV 2/8 serotype sample, previously captured by cation exchange chromatography (CIMmultus™ SO3), was diluted in Buffer A loading buffer and loaded on CIMac™ AAV full/empty-0.1 Analytical Column at 3.66E+11 vg per column. Initial analytics of sample showed following values:

- Titer ddPCR: 7.32E+12vg/mL
- Titer ELISA: 2.69E+13 vp/mL

For this experiment PATfix® system was used. It is powered by PATfix® software, a comprehensive, user-friendly toolbox for automated analysis of chromatographic data sets.

PATfix® system Detectors:

- Conductivity and pH
- Absorbance (260nm, 280 nm), 50 mm UV cell
- Fluorescence (Ex/Em: 280/348 nm)
- Light scattering (angle: 90°)

Buffer composition (similar salt/conductivity gradient):

- Buffer A : 20 mM BTP + 0/2 mM MgCl$_2$ + 1 % sorbitol + 0.1 % poloxamer pH 9.0
- Buffer B: 20 mM BTP + 0/2 mM MgCl$_2$ + 400 mM NaCl + 1 % sorbitol + 0.1 % poloxamer pH 9.0 or
  20 mM BTP + 0/2 mM MgCl$_2$ + 450 mM TMAC + 1 % sorbitol + 0.1 % poloxamer pH 9.0 or
  20 mM BTP + 0/2 mM MgCl$_2$ + 400 mM choline chloride + 1 % sorbitol + 0.1 % poloxamer pH 9.0
- Buffer C: 1 M NaOH + 2 M NaCl
- Buffer D: 1 M CH$_3$COONH$_4$

The resolution between peaks is based on fluorescence detector with the standard equation: $R = 2\ t_F - t_E / w_F + w_E$, where t_F and t_E are retention times and w_F and w_E are peak widths at baseline of full and empty AAV capsids. Percentage of empty and full (% E and %F) was also obtained considering tryptophan fluorescence (Figure 2).

2. Results – Screening of different elution salts

The results illustrated bellow were obtained using different elution salts and different concentrations of magnesium.
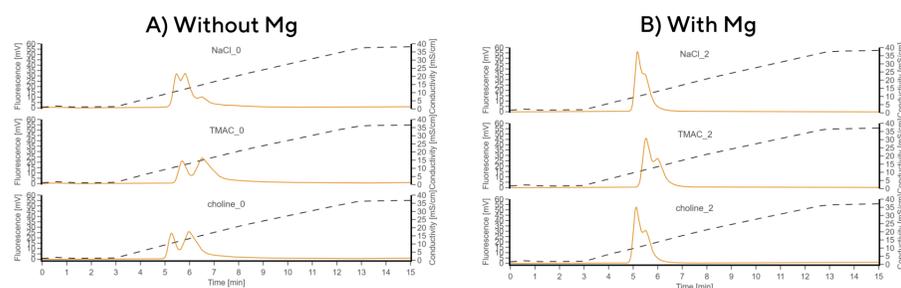
A) Without Mg
B) With Mg

Figure 2: Stacked chromatograms representing fluorescence signal versus time over a 100 CV gradient of 0%– 100% elution salt are shown (from top to bottom: NaCl, TMAC, choline chloride). A comparison of E/F separations profiles using mobile phases without (A) and with (B) 2 mM MgCl$_2$

(A)Each chromatogram shows two to three overlapping peaks. The first and third (unresolved in case of TMAC and choline chloride) for empty capsids and the second peak for full capsids. A higher E/F resolution is observed in QAAS, but there is a third unresolved peak.

(B) Each chromatogram shows two overlapping peaks. The first for empty capsids and the second peak for full capsids. Addition of magnesium to the buffers results in decrease of resolution and a uniform profile for all elution salts. Moreover, the intensity of the empty peak increases and the peaks become narrower.
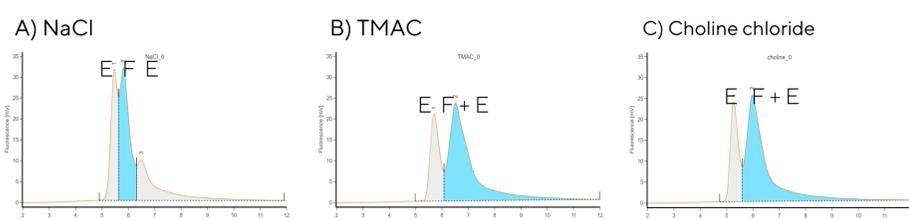
A) NaCl
B) TMAC
C) Choline chloride

Figure 3: Chromatograms representing peak integration(from left to right: NaCl, TMAC, choline chloride). No magnesium was present, so each chromatogram shows two to three overlapping peaks. The first and third (unresolved for B and C) for empty capsids and the second peak (blue color) for full capsids.
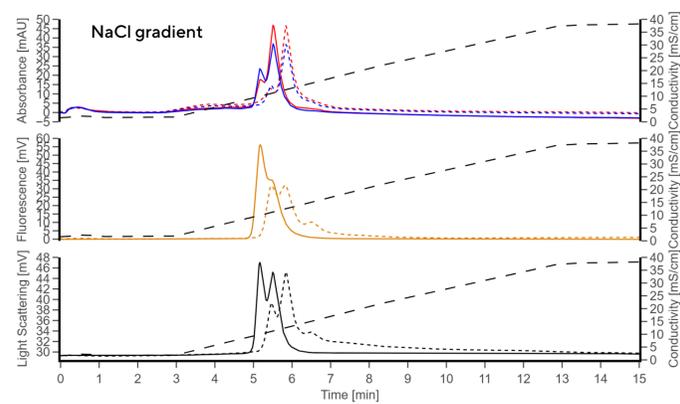

Figure 4: Overlay of chromatograms using NaCl as the elution salt, with Mg (full)/without Mg(dotted line). Three visible peaks without the addition of Mg.
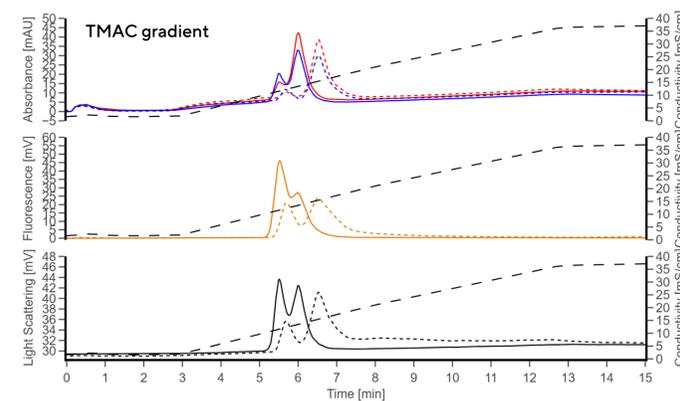

Figure 5: Overlay of chromatograms using TMAC as the elution salt, with Mg (full)/ without Mg(dotted line). Third peak is unresolved without the addition of Mg. Higher elution conductivities.
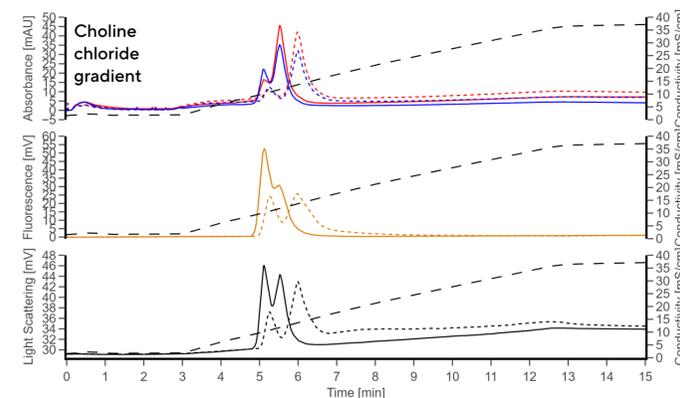

Figure 6: Overlay of chromatograms using choline chloride as the elution salt, with Mg (full)/without Mg(dotted line). Third peak is unresolved without the addition of Mg. Similar profiles but lower elution conductivities in comparison to TMAC.

As shown in Figures 2-6 PATfix® software enables us simple sample and screening conditions comparison as well as simple value read outs. Three different elution salts (NaCl, TMAC, choline chloride) and two different concentrations of magnesium (0 and 2 mM) were screened for the separation of full and empty AAV 2/8 capsids. Figures 2 and 4-6 show the corresponding chromatograms.

Addition of magnesium to the buffers lowers the resolution (Table 1) but improves the elution profiles for all elution salts (Figure 2). That means only two peaks, the left for empty, the right for full. Opposite to NaCl quaternary amino acid salts (TMAC, choline chloride) without magnesium result in broader, unresolved peaks (Figure 3). These discrepancies are also visible in Table 1 and Figure 3, where accurate integration was not possible due to unresolved peaks. These salts however show better resolution compared to NaCl (Table1). The reason for it is they possibly, as antichaotropes, stabilize hydrophobic interactions between empty and full virus capsids. Finally, we showed that choline chloride is an excellent nonhazardous substitute for TMAC and is worth considering for preparative runs.

	Elution salt	Addition of Mg	Resolution	%F area FLD
1	NaCl	no	0.58	45.4
2	NaCl	yes	0.53	45.1
3	TMAC	no	0.87	N/A**
4	TMAC	yes	0.64	44.1
5	CholineCl	no	0.88	N/A**
6	CholineCl	yes	0.61	43.3

Table 1: Shows the influence of different elution salts on the resolution and E/F ratio. In two cases ** accurate integration was not possible due to unresolved peaks.

3. Conclusion

- AAV sample was characterized using three different elution salts on a CIMac™ AAV full/empty-0.1 column.
- The presence of MgCl$_2$ decreased the resolution and the number of peaks resolved.
- Despite the lower resolution, using NaCl shows comparable %F in case of magnesium.
- Mg is needed for accurate E/F ratio determination when using quaternary ammonium salts.
- Quaternary ammonium salts as antichaotropes result in better resolution, due to stabilizing hydrophobic interactions between viral capsids.
- We showed that choline chloride has similar values as TMAC but is nonhazardous and is therefore useful for preparative runs.