Fluorescence, light scattering and UV absorbance detector calibration plots of empty and full AAV2/8 capsid mixtures

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INTRODUCTION

AAV vector lots are generally a heterogeneous mixture of empty particles (i.e. do not contain DNA) and full particles (i.e. contain DNA). Different spectrometric based methods can be used to establish the ratio between full and empty AAV particles, but accurate evaluation of empty/full ratio is often obstructed due to complex spectroscopic behavior of empty and full AAV particles, such as poor separation and impurity overlapping. An approach that takes difference in physical-chemical properties between empty and full capsids into account overcomes limitations of spectrometric based evaluation of empty and full AAV particle ratio.

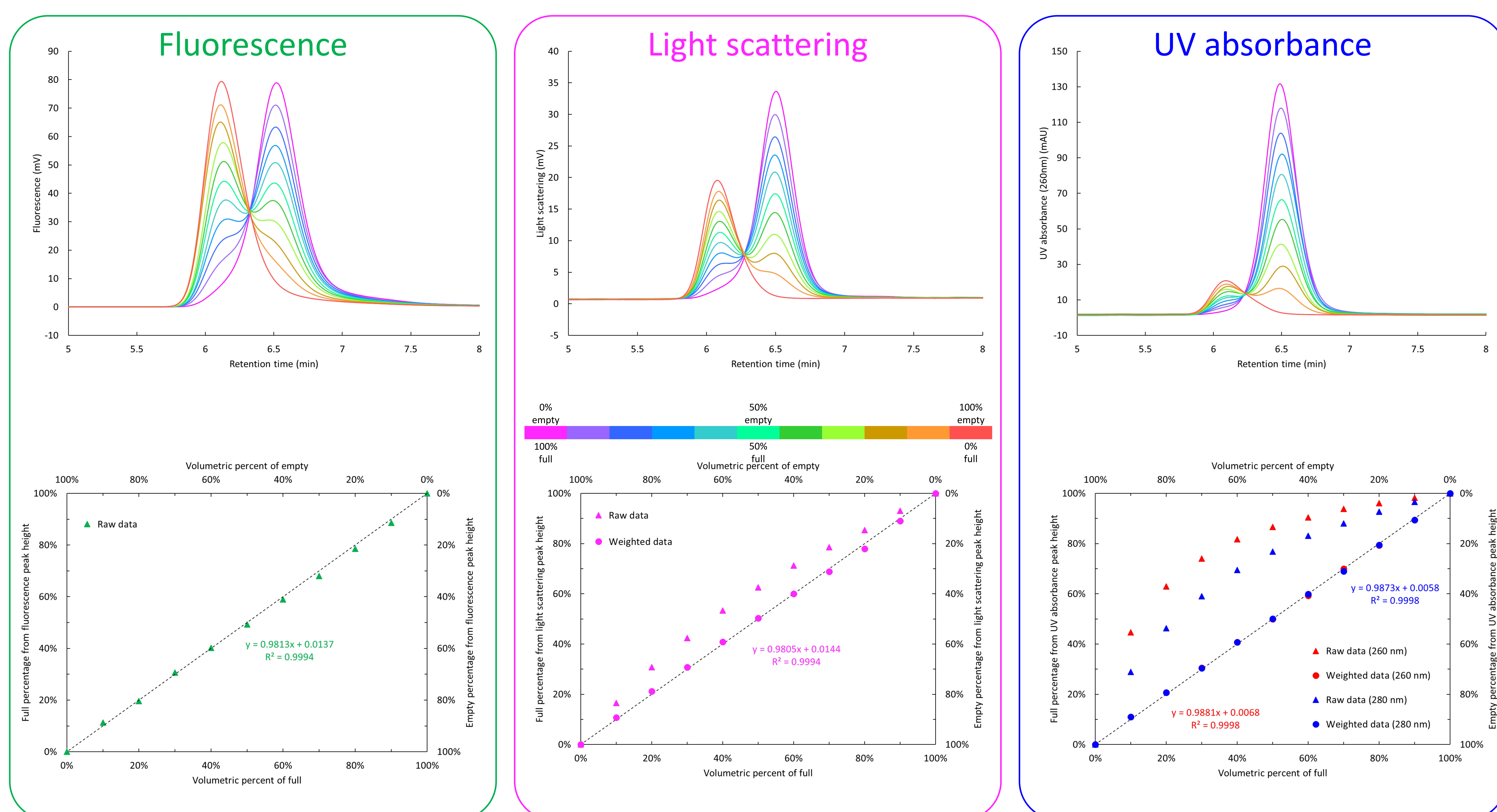
Chromatographic separation of empty and full AAV2/8 capsids was achieved on the CIMac^{TM} AAV full/empty analytical column (strong anion exchanger, QA – quaternary amine chemistry) with the PATfix^{TM} HPLC system using a linear NaCl gradient at pH 9.0. Signal response from three different detectors connected in series was analyzed: fluorescence (excitation 280 nm, emission 348 nm), light scattering (90° angle, LS) and UV absorbance (260 nm and 280 nm).

Empty and full capsids were mixed in predefined volumetric ratios from pure empty and pure full AAV capsids. Calibration plots for empty and full AAV capsid mixtures were constructed based on the chromatogram peak heights (*H*) from mixtures of empty and full AAV samples.

To prevent overestimation of AAV capsid population, correction factors (*f*) for UV absorbance and light scattering were implemented. The correction factor for UV response and LS response takes into account the difference in extinction coefficients and molar masses, respectively, of empty and full AAV capsids. To calculate percentage of full capsids with correction factor taken into account the following formula was derived:

$$Full\ (\%) = \frac{H_{\text{Full}}}{H_{\text{Full}} + H_{\text{Empty}} \cdot f}$$

RESULT



Fluorescence data shows linear correlation of raw data throughout the whole mixture range, indicating that fluorescence detection of empty and full capsids gives a straightforward way to determine empty/full ratio simply by calculating the ratio of corresponding peak heights for empty and full AAV particles.

Direct comparison of peak heights for light scattering and UV detectors does not provide linear correlation, but gives an overestimate for full AAV capsid concentration. Overestimate is particularly obvious for UV detector. The linearity of response could be achieved by implementing specific correction factors into the spectrometric model.

Light scattering intensity at specific component concentration depends on the physical size of the molecule and its molar mass. Assuming the same particle size of empty and full AAV particles, only the molar mass could be considered a variable, therefore, a correction factor, which accounted for differences in molar masses between empty and full AAV particles was implemented.

Full AAV particle includes a protein capsid containing single stranded DNA (ssDNA), which contributes to the molar mass of the particle. For light scattering, the correction factor is defined as a full/empty molar mass ratio.

Similar to light scattering characterization, a correction factor is also needed to predict the empty/full ratio of particles with a UV detector. UV response is dependent on molar extinction coefficients of empty and full AAV capsids. In this case, the correction factor is defined as full/empty molar extinction coefficient ratio.

The correction factors for light scattering and UV absorbance were reverse calculated by fitting the above equation to the fluorescence data. The fitted *f* value for light scattering was 1.65. For UV absorbance at 260 nm and 280 nm, the fitted values were 6.5 and 3.3, respectively.

CONCLUSIONS

- Linear correlation throughout the whole mixture range is achieved with the fluorescence detector (raw data), light scattering detector (weighted data) and UV absorbance detector (weighted data).
- Empty/full ratio could be calculated directly from fluorescence signal peak heights without any data correction. Non-corrected (raw) peak heights from UV absorbance and light scattering signals considerably overestimate the amount of full AAV particles.
- For light scattering, a correction factor that accounts for the differences in molar masses between empty and full AAV particles is needed.
- For UV absorbance, a similar correction factor that accounts for the differences in molar extinction coefficients between empty and full AAV particles is also needed.
- Actual purity of initial samples estimated from fluorescence peak height was 91% empty for empty and 92% full for full AAV sample. Cryo-TEM analysis of the same samples showed only 85% empty for empty and 73% full for full AAV sample.
- For this experiment, certain assumptions were made: no co-eluting proteins or other impurities were present at the retention time of the AAV and there were no damaged or partially-filled capsids present, only a binary mixture of empty and full AAV capsids.

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