On-Column and Post-Column UV Imaging in NanoLC

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Introduction

High resolution separations of macromolecules in nanoLC may be compromised by significant post-column band broadening.

The objective of this work is to document the processes occurring immediately beyond the outlet frit of a capillary column, using high speed, high resolution UV imaging, in order to find optimum detection conditions.

Results: imaging through a packed column

A capillary with 21 cm total length 13.7 cm packed length and 1 cm window length was positioned with the end of the packed bed centred over the detector. Gradient elution was from 72% water 28% ACN with 0.1% TFA to 43% water 57% ACN with 0.1% TFA in 15 min.

Image data (ActiPix D100, 214 nm) were processed using a 4.7 mm on-column detection zone and a 4.0 mm detection zone post-column. Spatial resolution 70 μm (10 binned 7 μm pixels).

Results (Fig. 3) with 16 nL injection,10 mg/mL heat-stressed BSA show

- ***Efficiency greater for on-column detection (Plate height for BSA is 9 μm)***
- ***Sensitivity is greater for post-column detection***

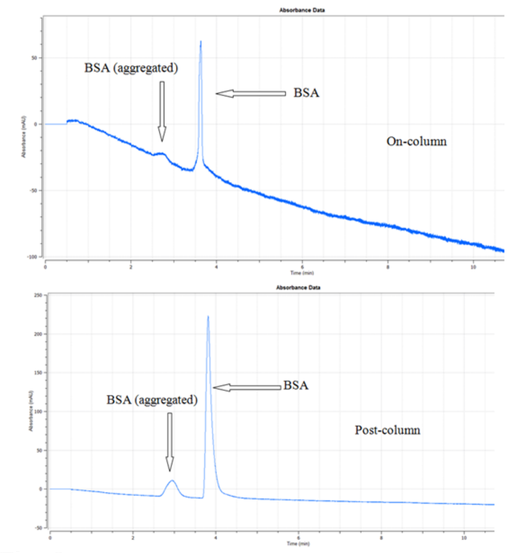

Fig. 3.

Methods

Imaging detection. UV area imaging was carried out with ActiPix D100 and D200 detectors (Paraytec, UK). The new generation D200 detector (Fig. 1) has USB3 output with high speed imaging at frame rates over an order of magnitude greater than the D100. Wavelengths used were 214 nm (pulsed xenon lamp & filter), 255 or 280 nm (UV diode).

Fig. 1. ActiPix D200 detector. Sensor hardware and software in 15x8x2.5 cm housing. 11.26x11.26 mm imaging area with 5.5 μm pixels. USB 3 data output

LC pumps. Gradient runs used a Rheos pump, splitter and manual injector. Isocratic runs used a nano pump / injector [1], kindly loaned by VICI Valco (Fig. 2). This has compact dimensions and attributes well matched to the D200 detector. Fill volume is 35 μL, and injection volume 10 nL.

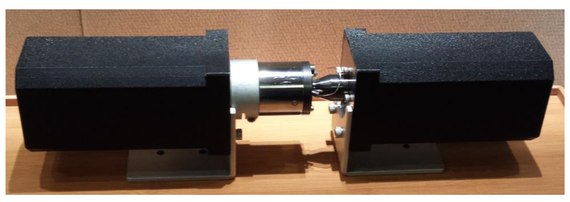

Fig. 2. VICI Valco nanoflow pump / injector

nanoLC columns. These were fabricated with transparent fused silica capillaries (TSU100375, Polymicro) and the "plug and use" methodology described by Zhang and his group [2]. Single particle frits were a gift from Prof. Peter Myers. HALO® Protein C4 bonded silica particles with fused-core® technology were generously provided by Stephanie Schuster of Advanced Materials Technology..

[1] S. Sharma et al., J. Chromatogr. A, 1327, 2014, 80-89.
[2] Z. Xiao et al., J. Chromatogr. A, 1325, 2014, 109-114.
[3] S. A. Schuster et al., J. Chromatogr. A, 1315, 2013, 118-126.

Peak profile and zone distance post-column

Data for BSA processed with 1.0 mm long signal detection and referencing zones spaced at increasing distance from the frit (Fig. 4) show that highest peak efficiency is obtained immediately post-column. Bands then broaden due to Taylor dispersion.

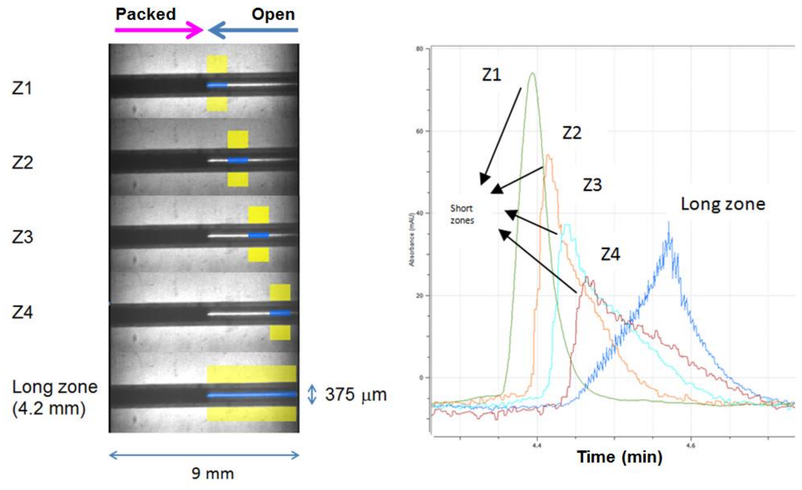

Fig. 4. D100 image data, column length 8.6 cm, mobile phase water:acetonitrile 50:50 % v/v with 0.1% TFA. Sample BSA 10 mg/mL.

- ***Efficiency post-column best using 1.0 mm zone immediately post frit***

Noise as a function of frame rate

Whereas the D100 is typically restricted to data acquisition at 2 Hz in image mode, the D200 detector can run at frame rates up to 2 orders of magnitude higher. Fig. 5 shows the effect of change of frame rate from 2 to 20 Hz. RMS noise decreases by a factor 2.5 (220 to 90 μAU).

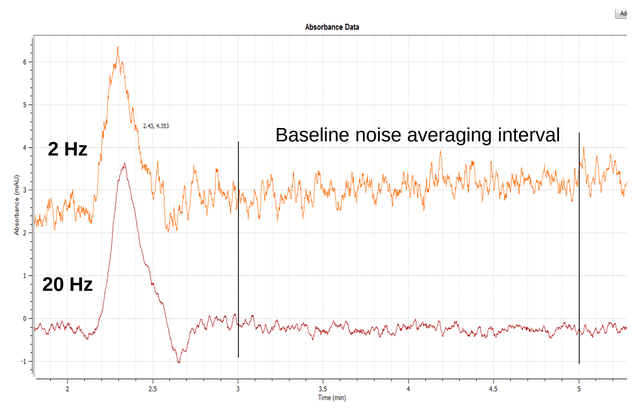

Fig. 5. D200 data as a function of frame rate, 1 s time constant. Sample: 10 nL 0.2 mg/mL BSA. Mobile phase: water:acetonitrile 40:60 % v/v with 0.1% TFA.

- ***Noise decreases and sensitivity increases on increasing frame rate***
- ***Next steps - testing at 200 Hz***

NanoLC of proteins

Imaging detection with isocratic separation of BSA and chymotrysinogen (Fig. 6) shows baseline noise 84 μAU RMS. The retained peak has amplitude 12 mAU, peak efficiency 4x10^4, plate height 5 μm.

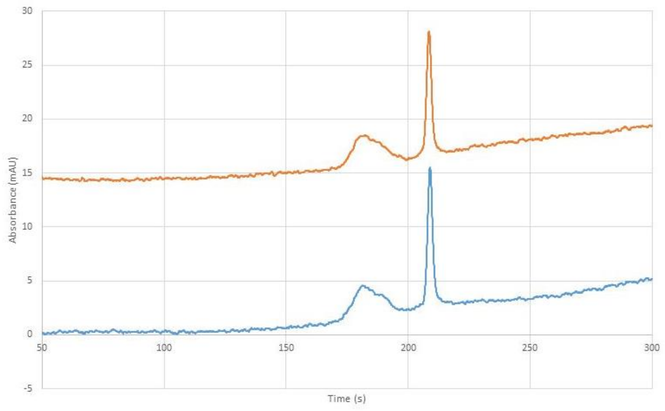

Fig. 6. 2 runs with sample 10 nL BSA & chymotrypsinogen (0.2 mg/mL each) in water:acetonitrile 10:90 % v/v with 0.1% TFA**.** Stationary phase: HALO® Protein C4, 100 μm i.d. column packed length 21.6 cm. Mobile phase: water:acetonitrile 50:50 % v/v with 0.1% TFA. Wavelength 214 nm. D200 data acquisition rate 20 Hz, 1.0 mm detection zone immediately post frit. Spatial resolution 44 μm.

- ***Protein separation with high sensitivity using ActiPix D200 imaging detection***

Conclusions

Imaging UV detection across 100 micron i.d. nanoLC capillary columns with single-particle frits & chromatography of intact proteins show

- **Narrow bands immediately post-column: best signal only within first 1 mm**
- **Distance dependence readily visualised and quantified**
- **Advantage of sensitivity increase with new generation D200 detector & high frame rate**
- **Sensitivity: < 10 fmol for proteins**
- **Highly compatible with compact nanoflow pump / injector**
- **Advantage in working with high performance nano columns: no column − transfer line − detector connectors!**
- **Excellent potential for nanoLC of proteins, & rapid method development for bioseparations**