Increased UV-sensitivity In Combination With Novel WCX Column Separation For Better Detectability Of Charge State Variants Of Biotherapeutic Proteins

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Introduction

The structural integrity of proteins is affected by many parameters such as pH, buffering ions, ionic strength, co-factors and more.

Therefore, the production process of biologicals like monoclonal antibodies (mAbs) has to be tightly controlled in order to avoid truncation and aggregation, and to obtain the correct glycosylation pattern.

Proper control of physico-chemical and other protein expression parameters is of high importance to prevent immunogenicity and to warrant high efficacy of the applied drug.

QA/QC of the biological during all clinical phases is therefore crucial and detailed characterization is required to fulfill safety requirements of regulatory agencies.

One commonly applied assay is the identification of charged variants of recombinant mAbs which could arise from de-amidation or cleavage of N-terminal lysyl residues during production of the protein drug .
For this task often HPLC is used, preferably weak cation exchange chromatography.

Major limitation of IEX compared to RP-chromatography is the lack of resolution. It remains a difficult analytical task to separate acidic and basic variants closely eluting to the main peak.

Identification of low level amounts of charge state variants requires high sensitivity UV detection. Here, we demonstrate an up to 10-fold gain in sensitivity for protein analysis when using the Agilent 1260 Infinity DAD in combination with a bio-inert high sensitivity flow cell in comparison to a bio-inert standard DAD SL 10mm cell. With a novel Agilent polymeric WCX column designed for the separation of mAbs we demonstrate that lower level of impurities can be detected more reliably even in difficult to resolve samples.

This technology will provide a valuable tool to efficiently characterize protein modifications and to provide valuable feedback for the safety of newly developed biologicals.

Experimental

The new Agilent 1260 Infinity Bio-inert HPLC Solution was used for this study (Fig.1). It offers a flexible solution for high performance analytics as well as small scale purification for all types of chromatography in a bioinert environment. With a stainless steel free 600 bar quaternary pump and a complete metal free sample path and a large set of compatible detectors it combines robustness, reliability, low surface activity, and high and low pressure capability for most HPLC purposes.

Instrumentation:

The Agilent 1260 Infinity Bio-inert LC system consisted of

1. 1260 Infinity Bio-inert Quat-Pump 600bar with built-in degasser (G5611A)
2. 1260 Infinity Bio-inert HP Autosampler 600bar with PEEK/ ceramic valve, PEEK needle seat and ceramic needle (G5667A)
3 1290 Infinity thermostatted column compartment (G1316C) with Bio-inert click-in Solvent Heater 9µl (G5616-60050)
4.1 Diode array detector 1260 Infinity (G4212B) with Bio-inert Max-Light flow cell (G5615-60017), 60 mm path length
4.2 Diode array detector 1200 DAD SL (G1315C) with Bio-inert 10mm flow cell (G5615-60022).
5. Titanium capillaries from the pump to the metering device (0.17 mm i.d.), metal-coated PEEK capillaries from the metering device to the column (0.17 mm i.d.), PEEK tubing from column to detector and PP from detector to waste. System operation/data analysis with Agilent Chem-station instrument control software (Rev. B.04.02 SP1)
6. Weak cation exchange column Agilent Bio Mab NP10 in PEEK housing.

Fig. 1: Agilent 1260 Infinity Bio-inert HPLC

Protein samples:

RNAse and aprotinin were purchased from GE Healthcare (Freiburg, Germany), monoclonal anti-FLAG antibody (F3165) was purchased from Sigma-Aldrich (Munich, Germany), therapeutic protein P128 was kindly provided by Gangagen Biotechnology. Anti-FLAG and P128 were desalted into the corresponding mobile phase A using mini spin columns (Pierce, Rockford, US)

Chemicals:

All chemicals for preparing running buffers were purchased from Sigma-Aldrich (Munich, Germany) or VWR international (Darmstadt, Germany).

Running conditions: as indicated.

Bio-inert Max-Light flow cell

The Agilent 1260 Infinity DAD (G4212B) features an optical design based on the Agilent Max-Light cartridge cell with optofluidic waveguides (Fig. 2). This cell technology uses the principle of total internal reflection along a noncoated fused silica capillary to increase the light transmission at a very low internal volume.

Use of this technology achieves a new level of sensitivity without the decreased resolution that can be caused by cell dispersion effects.

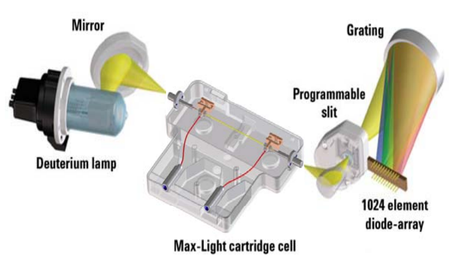


Fig. 2: Agilent 1260 Infinity DAD light path with Max-Light cartridge

Results and Discussion

Since the 10 x sensitivity gain of the Max-Light cell has already been shown for small molecule analysis (Agilent Publication Number 5990-5326EN) we evaluated its performance in protein chromatography and analysis.

We tested 2 standard proteins, aprotinin and RNase A, one monoclonal anti-FLAG antibody and one biotherapeutic protein on an Agilent Bio Mab NP10 column using the Agilent 1260 Infinity Bio-inert LC system with either a standard DAD SL (G1315C) with a bio-inert 10mm flow cell or a 1260 DAD (G4112B) with a bio-inert Max-Light 60mm flow cell. Detector settings were kept identical.

Aprotinin (6.5 kDa, pI: 10.5) is eluting in a single peak with a pronounced shoulder on the basic side and two minor contaminations on the acidic side of the main peak which are only detectable with the Max-Light cell (Fig. 3). Comparing signal to noise ratios of both detectors give a sensitivity gain of the Max-Light cell of almost 10.

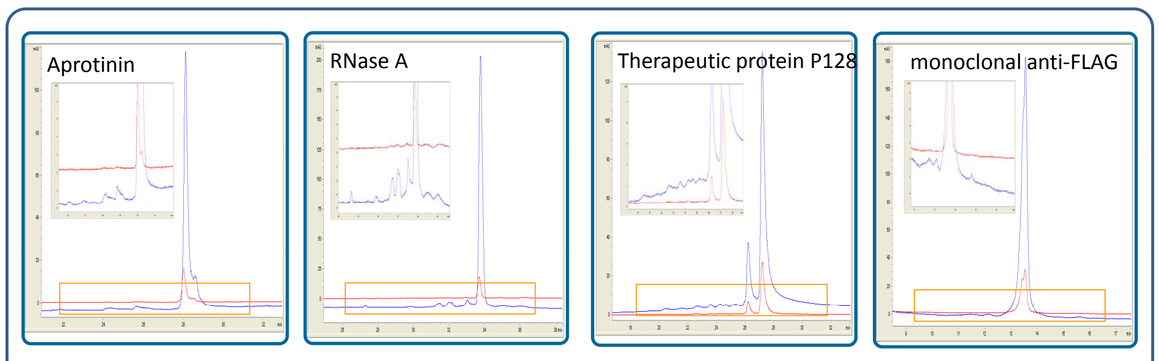
RNase A (13.7 kDa, pI: 9.45) elute in a single peak with some contaminations which could only be detected as low humps with the standard DAD SL 10mm cell (Fig. 3, inset). Calculating the signal to noise ratios leads to a 7.7 higher sensitivity with the Max-Light cell.

The biotherapeutic protein P128 (26.5 kDa, pI 9.73) was tested accoding to the marker proteins. Two main peaks are eluting under the applied conditions (Fig. 3). Additional species could be detected with the Max-Light cell which are barely detectable with the standard 10mm cell. Calculated sensitivity gain for the Max-Light cell is 7.2.

The 4th protein tested is a monoclonal antibody (~150kDa, pI: n.d.) against the FLAG octapeptide (Fig. 3). It elutes in a broad peak with a pronounce shoulder on the acidic side of the main peak. Some minor impurities are detectable with the Max-Light cell.

A 6.5-fold higher sensitivty could be calculated for the Max-Light cell compared to the standard DAD SL 10mm flow cell.

The slightly lower signal to noise ratio compared to similar experiments conducted with low molecular weight compounds is due to a more complex sample composition (salts, additives, inhomogeneities etc.) and the use of aequous, salt containing buffers in biochromatography.



Aprotinin	1260 DAD Max-Light	DAD SL
Height (mAU)	120.5	15.7
Noise (PtoP)	0.1902	0.2364
Signal to Noise	633.5	66.4
Factor	9.5	

RNase A	1260 DAD Max-Light	DAD SL
Height (mAU)	209.6	17.3
Noise (PtoP)	0.4391	0.2772
Signal to Noise	477.3	62.4
Factor	7.7	

Therapeutic protein P128	1260 DAD Max-Light	DAD SL
Height (mAU)	130.4	26.6
Noise (PtoP)	0,1013	0,1495
Signal to Noise	1287	178
Factor	7.2	

monoclonal anti-FLAG antibody	1260 DAD Max-Light	DAD SL
Height (mAU)	182.9	30.5
Noise (PtoP)	0.3204	0.3460
Signal to Noise	570.9	88.2
Factor	6.5	

Fig. 3: WCX of protein samples. Aprotinin (5mg/ml), RNase A (5mg/ml) and monoclonal anti-FLAG (3.8mg/ml) were separated with Eluent A: 20mM MES, pH 6.5; B: A + 0.3M NaCl; gradient: 30min from 0 to 100 % B; injection volumes were 1µl for aprotinin and anti-FLAG and 0.5µl for RNase A. Therapeutic protein P128 (2mg/ml) was run with Eluent A: 20mM NaPi, pH 6.0; B: A + 1M NaCl; gradient: 35min from 10 to 35 % B; injection volume: 2µl. All proteins were run at 0.5ml/min flow rate, detection: 280nm. WCX column used was an Agilent Bio Mab NP10, 4.6x250 in a PEEK housing.

Orange box indicate the zoom region of the inset.

Conclusions

In former experiments and technical notes we could demonstrate a more than 10-fold sensitivity gain for small molecular weight compounds when comparing Max-Light DAD with standard DAD 10mm flow cells.

The data we show here demonstrate that an up to 10-fold increase in sensitivity could also be reached in biochromatography with globular protein samples using the Bio-inert Max-Light flow cell together with our new 1260 Infinity Bio-inert HPLC system and Bio Mab columns