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 biologics 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 drug.

Transfer 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 tryptide residues during production of the protein drug.

For this task often HPLC is used, preferably capillary electrophoresis 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 related to the main protein.

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 1200 Infinity DAD in combination with a bio-inert high sensitivity flow cell in comparison to a bio-inert standard DAD 10 mm 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 protein modifications and provides a valuable feedback for the safety of newly developed biologics.

Experimental

The new Agilent 1200 Infinity Bio-inert HPLC Solution was used for this study (Fig. 1). It offers a flexible solution for high performance analysis as well as small scale economical for all types of chromatography in a biotech environment.

With a stainless steel free DAD barrier pump and a complete metal free sample path and a large set of compatible detection it combines robustness, reliability, low surface activity, and high and low pressure capability for most HPLC purposes.

Instrumentation:

The Agilent 1200 Infinity Bio-inert LC system consisted of:

1. 1200 Infinity Bio-inert Quat-Pump 600bar with built-in degasser (G5611A)
2. 1200 Infinity Bio-inert HP Autosampler 600bar with PEEK/ ceramic valve, PEEK needle seat and ceramic:needle (G5626A)
3. 1200 Infinity Thermostatted column compartment (G1315E) with Bio-inert click-in Sorbent Heater 9µl (G1616-60050)
4. 4.0-Diode array detector 1200 Infinity (G4212B) with Bio-inert Max Light flow cell (G0516-60117), 40 mm path length
5. 4.0-Diode array detector 1200 DAD SL (G1315C) with Bio-inert 10mm flow cell (G4816-60112J)
6. 4.0-Titanium capillaries from the pump to the metering device (0.17 mm i.d., metal-coated) 0.032 mm 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)
7. 4.0-Wet-ion exchange column Agilent Bio-HP NP10 in PEEK housing.

Protein samples:

RNaseA and aprotinin were purchased from GE Healthcare (Freiburg, Germany), monoclonal anti–FLAG antibody (FS165) was purchased from Sigma-Aldrich (Munich, Germany), therapeutic protein P128 was kindly provided by Gangang 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 1200 Infinity DAD (G4212B) features an optical design based on the Agilent Max-Light cartridge with optofluidic waveguides (Fig. 2). This cell technology uses the principle of total internal reflection along a nanofused 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.

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 5995-5126EN) 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 1200 Infinity Bio-inert LC system with either a standard DAD SL (G1315C) with a bio-inert 10mm flow cell or a 1200 DAD (G4212B) with a bio-inert Max-Light 10mm flow cell.

Detector settings were kept identical.

Aprotinin (0.5 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) elutes in a single peak with some contaminations which could only be detected as low humps with the standard DAD 10 mm cell (Fig. 1, inset). Calculating the signal to noise ratios leads to a 7.7 higher sensitivity with the Max-Light cell.

The biotherapeutic protein P128 (26.6 kDa, pi: 9.76) was tested according 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 (‘550M5, pi n.d.) against the FLAG cetapetide (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 sensitivity could be calculated for the Max-Light cell compared to the standard DAD 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, nonchromogenics etc.) and the use of aqueous, salt containing buffers in biochromatography.

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 proteins when using the Bio-inert Max-light flow cell together with our new 1260 Infinity Bio-inert HPLC System and Bio-mab columns.

Fig. 1: Agilent 1200 Infinity Bio-inert HPLC

Fig. 2: Agilent 1200 Infinity DAD light path with blue light cartridge

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 MOPS, pH 6.5, B: A + 0.3 M 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 (25mg/ml) was run with Eluent A: 20mM NaPh, pH 6.0; B: A + 3 M NaCl; gradient: 30min from 10 to 35 % B; injection volume: 3µl. All proteins were run at 1.0ml/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.

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