Protein A Affinity Capture followed by AdvanceBio SEC Aggregation Analysis using the Agilent Infinity Series 2D-LC System

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Introduction

Protein biopharmaceuticals such as monoclonal antibodies and recombinant proteins are currently in widespread use for the treatment of various life-threatening diseases including cancer and autoimmune diseases. Protein therapeutics have a complexity far exceeding that of small molecule drugs, hence, unraveling this complexity represents an analytical challenge. During the development and lifetime of these molecules, an in depth characterization is required.[1]

Protein A is an immunoglobulin-Fc (IgG) receptor found in the cell wall of *Staphylococcus aureus.* It has strong affinity for polyclonal and monoclonal IgGs like human IgG 1, IgG 2, and IgG 4, in addition to IgGs from some other species such as rabbits and some mouse IgGs. Immobilized Protein A is commonly used for preparative and process scale purifications of IgG. At the analytical scale, the Agilent Bio-Monolith Protein A HPLC Column can be used for fast quantification and small scale purification of IgGs in complex mixtures or pure samples.[2, 3]

Size Exclusion Chromatography is the method of choice for quantification of aggregates in Bio therapeutic samples. Protein A Capture followed by size exclusion chromatography for aggregation analysis yields more accurate characterization as purer sample is analyzed. With the Agilent 2D LC System, this analysis can be done with one injection. Protein A capture is performed in the 1st dimension. Eluted IgG is trapped in the multiple heart cutting loops and eluted in the 2nd dimension onto an Agilent AdvanceBio SEC sizing column for accurate aggregate analysis.

Experimental

The 2DLC system used in this study consists of Agilent 1260 BioLC as 1st dimension LC. The BioLC is coupled with an Agilent 2DLC quick change valve, a multiple heart-cutting valve (with twelve factory pre-installed 40ul sample loops) and an Agilent 1290 binary pump for 2nd dimension LC. Diode Array detection is employed in the 2nd dimension The instrument modules used are shown in Figure 1.
Agilent OpenLab with 2DLC add-on software is used to configure the system, setup 2DLC methods and control the 2DLC data acquisition (Figure 2).

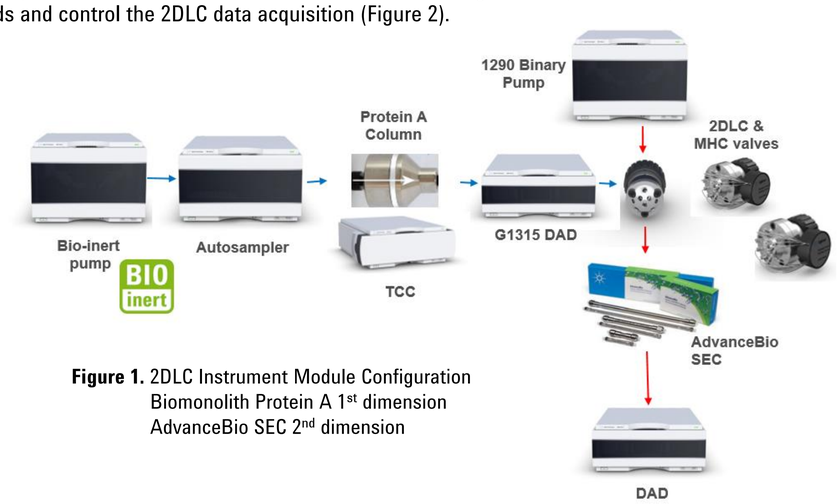

Figure 1. 2DLC Instrument Module Configuration
Biomonolith Protein A 1st dimension
AdvanceBio SEC 2nd dimension

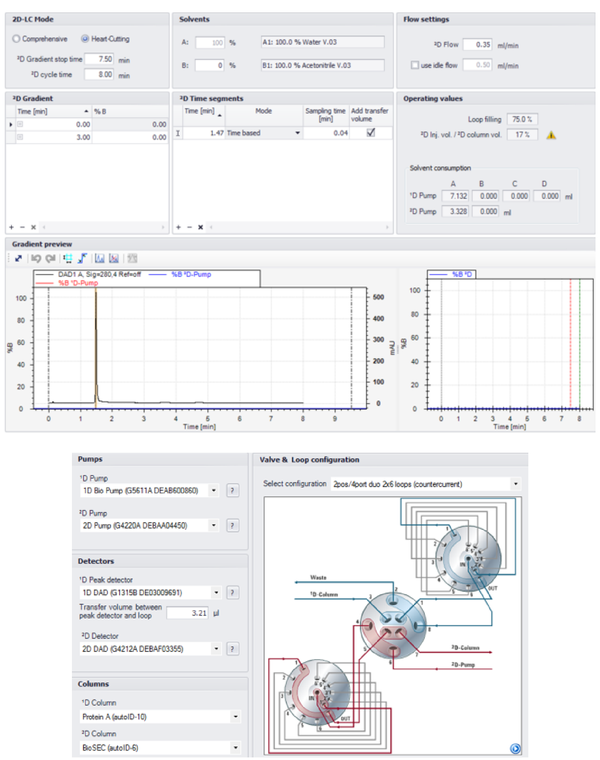

Figure 2. Configuring 2DLC system with 2DLC add-on software and setting up 2nd gradient and multiple time cuts with a reference 1st chromatogram along with overlaid 1st D gradient. The method setup dialog allowing easy setup of multiple time times/timing events.

Results and Discussion

This 2D separation served to speed up the aggregate analysis of monoclonal antibodies. By combining two steps in the aggregation characterization, we are able to conduct the entire analysis with one injection. Thus, removing the fraction collection step all together. A typical Protein A affinity capture separation is show in (Figure 3). The sample was captured in Phosphate Buffered Saline and eluted with 500mM Acetic Acid (Aqueous).
When utilized as a 1st dimension in a 2D analysis, the size exclusion chromatographic step can follow immediately after release from the Protein A (Figure 4).

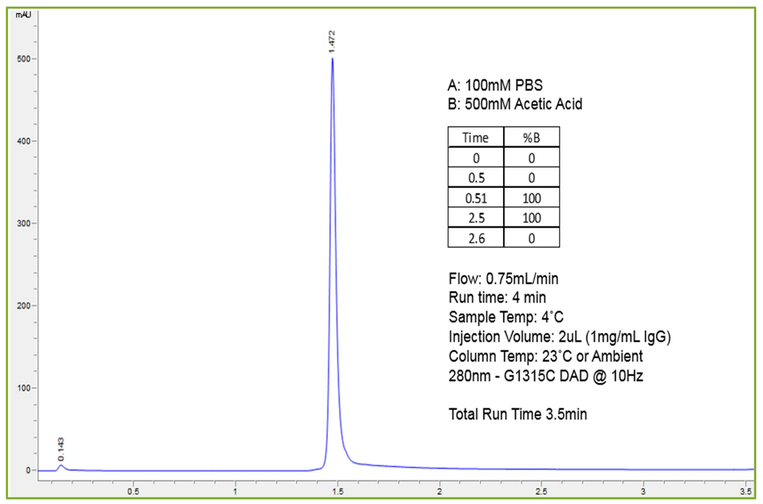

Figure 3. Protein A Affinity Capture separation of IgG

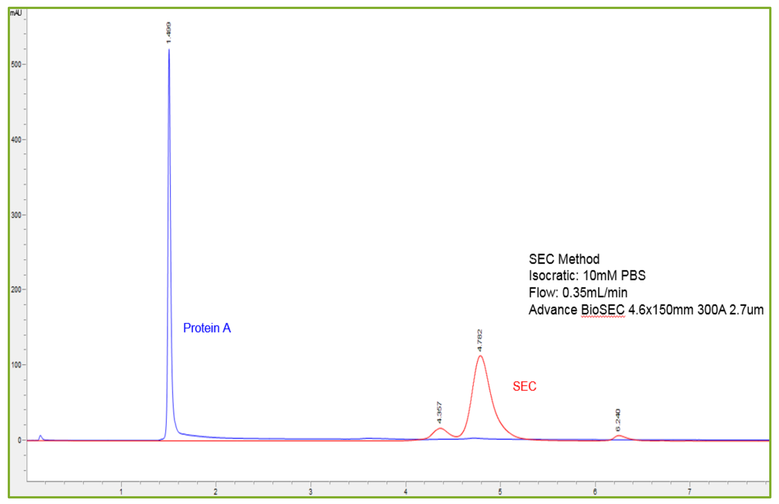

Figure 4. Protein A Affinity Capture and size exclusion separation of IgG

After demonstrating the feasibility of coupling Protein A and SEC techniques together in one analysis with the Agilent Infinity 2D LC System, we set out to test SEC detection of different aggregation concentrations using the 2D method. The IgG standard was heat stressed in a hot water bath for 24 hours at 65˚C. This resulted in a larger concentration of aggregates (Figures 5 & 6). We can see clear resolution of aggregates from the monomer and dimer species in Figure 6.

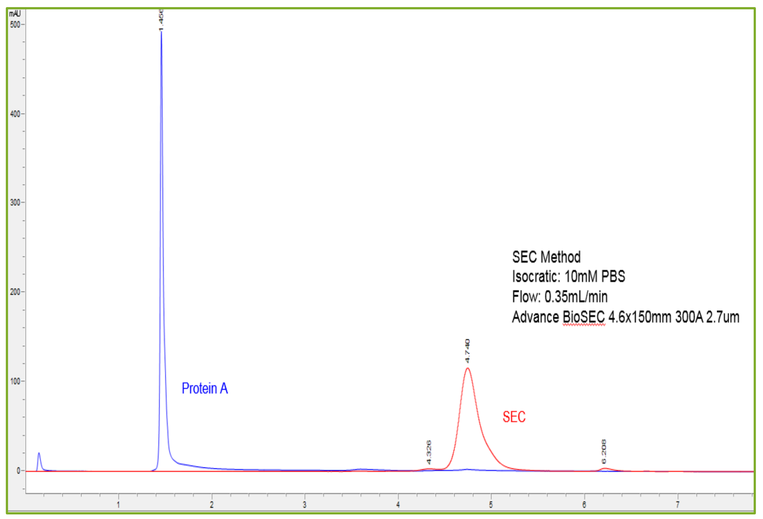

Figure 5. Protein A Affinity Capture and size exclusion separation of heat stressed IgG

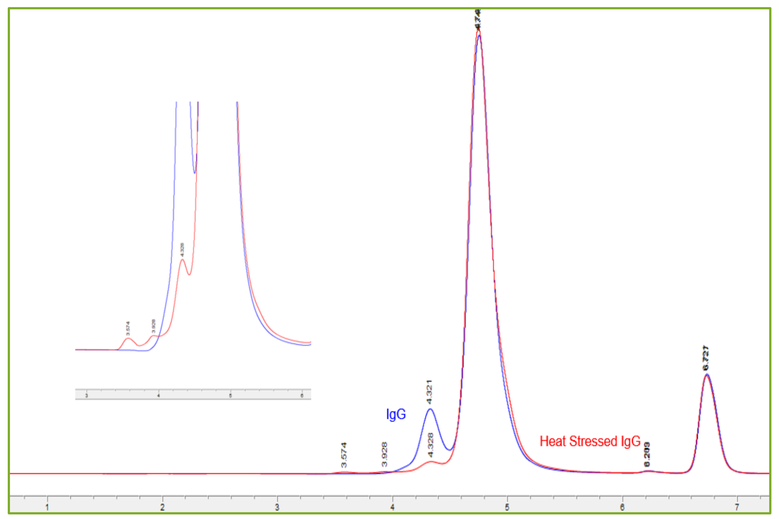

Figure 6. 2D size exclusion separation overlay of IgG and heat stressed IgG

Sample: IgG Standard (from Sigma *P#I9640*)

First Dimension Separation: **Protein A Affinity Capture with UV detection**
- Agilent Biomonolith Protein A (P#5069-3639) at room temperature.
- Flow rate is 0.75 mL/min

Mobile Phases & Gradient
- A-100mM PBS
- B: 500mM Acetic Acid

Time	%B
0	0
0.5	0
0.51	100
2.5	100
2.6	0

Second Dimension: **Size Exclusion Chromatography**
- Agilent AdvanceBio SEC 300A 2.7um (P# PL1580-3301), at 25°C
- Mobile phases: 10mM PBS pH 7.4 Isocratic @ 0.35 ml/min

2DLC Mode: **Multiple Heart Cutting**

Conclusions

- The Agilent 2D LC system offers a versatile and efficient way to determine mAb titer and aggregation in one single method, negating the need for fraction collection and multiple injections
- Agilent Biomonolith Protein A columns used as the first dimension of a 2D LC workflow are well suited for the analytical scale purification of mAbs for titer determination and as an initial purification step prior to aggregation analysis

The new AdvanceBio SEC 300 Å SEC column shows excellent resolution of aggregate species from monomer to allow accurate evaluation as the second dimension in a 2D LC workflow

References
1. Analysis of Monoclonal Antibody Digests with the Agilent 1290 Infinity 2D-LC Solution *Agilent Technologies Application Note*, publication number 5991-2880EN, **2013**.

2. Rapid Human Polyclonal IgG Quantification Using the Agilent Bio-Monolith Protein A HPLC Column, *Agilent Technologies Application Note*, publication number 5989-9733EN, **2008**. 3.Agilent Bio-Monolith Protein A Monitors Monoclonal Antibody Titer from Cell Cultures, *Agilent Technologies, Inc. Application Note*, publication number 5991-2990EN, **2014**.