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Article *in* Journal of immunological methods · April 2013

DOI: 10.1016/j.jim.2013.04.008 · Source: PubMed

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Technical note

Coupled affinity and sizing chromatography: A novel in-process analytical tool to measure titer and trend Fc-protein aggregation

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ARTICLE INFO

Article history:
Received 18 January 2013
Received in revised form 12 April 2013
Accepted 15 April 2013
Available online 20 April 2013

Keywords:
Protein aggregation
Affinity chromatography
Size exclusion
Chromatography

ABSTRACT

The expanding use of monoclonal antibodies in the biopharmaceuticals industry has brought the need for new analytical tools. We have developed a coupled affinity and gel-filtration high-performance liquid chromatography method to simultaneously analyze titer and quality of monoclonal antibodies. Before this assay, available analytical methods for protein aggregation required highly purified proteins. This assay can qualitatively describe a protein from a clarified cell culture solution by trending protein aggregation over time while measuring protein titer. It can be used to assess proteins in both early- and late-stage culture due to its dynamic range and sensitivity. This assay is a sensitive technique that overcomes the time limitations of previous approaches. It provides an essential tool to accomplish process optimization.

1. Introduction

There is an increasing need to gain product quality information earlier in biopharmaceutical development. The earliest goals of stable cell line development center around isolating high producing cell lines with acceptable product quality, an often time consuming and expensive process (Birch and Racher, 2006). The measurement of protein aggregation is a critical quality parameter for biopharmaceutical development, as the presence of the self-associated species can have an effect on the clearance, half-life of the therapeutic (Cromwell et al., 2006), and can have an effect on the immunogenic response (Maislos et al., 1988; Rosenberg, 2006). With the uncertainty of the effect of aggregates on the safety and efficacy of the therapeutic, it is advantageous to minimize the presence of high molecular weight species.

Protein aggregation can be caused as early as the cell culture process. Environmental factors such as temperature, pH and dissolved oxygen (Freimuth et al., 1999; Gomez et al., 2012) and medium components (Jing et al., 2012) can all affect product quality and are systematically studied during the development process to limit protein aggregation. The current practice of the pharmaceutical industry is to screen various culture conditions using a "Quality by Design" (QbD) approach (Bhambure et al., 2011) resulting in a large set of outputs (cell culture soups) for analysis to determine the variables which contribute to protein aggregation. Methods exist that measure aggregation of proteins in crude cell culture supernatants (Bondos and Bicknell, 2003), but these techniques can be laborious and expensive to implement.

Although a wide variety of methods are available for the measurement of protein aggregation (Mahler et al., 2009) of purified material, size-exclusion high-performance liquid chromatography (SEC-HPLC) is the long-standing biopharmaceutical standard for quantitation of soluble protein aggregates due to its high throughput capacity, sensitivity and reliability

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http://dx.doi.org/10.1016/j.jim.2013.04.008

(Hjerten and Eriksson, 1984; Hjerten and Mosbach, 1962; Gabrielson et al., 2007; Wehr and Rodriguez-Diaz, 2005). A limitation of the assay however is the need for purified protein for the analysis; the protein load has to be eluted from an earlier column, removing the media components as well as Host Cell Proteins that can interfere with the SEC-HPLC output. This is not only time consuming, but also introduces additional variables which can affect protein aggregation such as harvest and concentration conditions (Bee et al., 2009; Maruyama et al., 2001) or affinity column elution buffer pH (Shukla et al., 2005, 2007).

Here we introduce a method for measuring protein aggregation directly from the cell culture supernatant by the coupling of analytical affinity and size exclusion columns. This is a fast and effective method to report trends of protein aggregation, allowing for decisions affecting downstream processes and upstream approaches to be made earlier in the development process, saving time and resources.

2. Experimental

2.1. Chemicals, standard solutions and protein reagents

Boric acid, sodium sulfate, sulfuric acid, sodium hydroxide and acetic acid were purchased from Alfa Caesar USA (USA). Bi-distilled water and all buffer solutions used in this study were distilled by an Integral 5 water purification system (part no. ZRXQ005T0) and filtered through a BioPak polisher (part number CDUFBI001) both from Millipore USA. If not otherwise stated, a 50 mM borate buffer, 100 mM sodium sulfate adjusted to pH 7.5 with 1 M sodium hydroxide, was used to dilute the references and samples.

Stock solutions of monoclonal IgG (Novartis) were prepared at a concentration of 2.0 mg/mL in the corresponding buffer (mobile phase A) and injection volumes that were varied ranging from 1 μL to 100 μL of a 2 mg/mL solution were used to establish calibration curves, resulting in amounts of 0.002 to 0.2 mg injected onto the 0.1 mL affinity column (for ALC and ALC–SEC) or 2.75 mL size exclusion column (SEC alone). This IgG was previously validated and measured to be 98% monomer and 2% aggregation products by a Novartis internal drug substance release assay (SEC-HPLC). This standard was used in place of a molecular weight marker due to the fact that the marker would have zero affinity to a protein A column preceding the sizing column in a coupled assay. The presence of aggregate products was confirmed through running of non-reduced agarose gels on purified protein samples due to the absence of a molecular weight marker.

Humanized monoclonal antibodies of IgG1 isotype expressed in various stable CHO cell lines and transient HEK-293 cultures at a 0.5 liter scale were used for the study. Prior to loading onto the HPLC, all cultures were clarified by centrifugation followed by a filtration through a 0.2 μm membrane.

2.2. Methods

2.2.1. Affinity liquid chromatography HPLC (ALC-HPLC)

For the protein A HPLC, mobile phase A (adsorption buffer), consisting of 50 mM borate buffer, 100 mM sodium sulfate, adjusted to pH 7.5 with 1 M sodium hydroxide, was prepared. Further, mobile phase B (elution buffer) consisting of 50 mM borate buffer, 100 mM sodium sulfate, adjusted to pH 2.0 with 1 M sulfuric acid, was prepared. The method was as follows: mobile phase A 0–0.49 min, mobile phase B 0.5–1.19 min, followed by mobile phase A from 1.20 to 3 min to re-equilibrate the protein A cartridge (Life Technologies, part number 2-1001-00, column dimensions 2.1 mm × 30 mm, 0.1 mL column volume) for the next injection at a general flow-rate of 2 mL/min. A detection wavelength of 280 nm and a sample injection volume of 50 μL were used for all samples (excluding standard curve generation, see Section 2.1). The column temperature was kept constant at 25 °C and the sample tray was kept cooled at 10 °C to prevent an early fouling of feed samples. For each sequence, the area under the peak was integrated using the standard curve established as outlined in Section 2.1.

2.2.2. Size-exclusion HPLC (SEC-HPLC)

For the SEC HPLC, mobile phase A (adsorption buffer) consisting of 50 mM borate buffer, 100 mM sodium sulfate, adjusted to pH 7.5 with 1 M sodium hydroxide, was prepared. The instrumental method was as follows: mobile phase A 0–5 min, which allows all of the sample components to elute from the SEC column. Additionally, mobile phase A was kept from 5 to 8 min to re-equilibrate the SEC column (Sepax Technologies part number 213300–7815, 7.8 mm × 150 mm, particle size 3 μm and pore size 300 Å) for the next injection at a general flow-rate of 2 mL/min. A detection wavelength of 280 nm and a sample injection volume of 50 μL were used for all samples (excluding standard curve generation). The column temperature was kept constant at 25 °C and the sample tray was kept cooled at 10 °C. For each sequence, the area under the peaks was integrated using the standard curve established as outlined in Section 2.1.

2.2.3. Coupled affinity and sizing HPLC (ALC–SEC HPLC)

The instrumental set-up for the two columns, protein A and SEC, was performed as follows: the protein A column was connected to the SEC column via a capillary with fittings of the following dimensions; 0.17 I.D. mm × 9 cm. After injection of the sample, all unbound material flows through the protein A and SEC columns with mobile phase A (adsorption buffer). Once the last peak corresponding to the unbound material passes through the SEC-column, the mobile phase B (Elution Buffer) releases the bound proteins (IgGs) from the protein A column and they enter the SEC column. The IgG molecules are now separated based on molecular weight and are directed to the UV-detector where the peak can be analyzed. The instrumental method was as follows: mobile phase A 0–5.49 min, mobile phase B 5.50–6.19 min and kept at mobile phase A from 6.20 to 10 min to re-equilibrate the protein A cartridge for the next injection at a general flow-rate of 2 mL/min. A detection wavelength of 280 nm and a sample injection volume of 50 μL were used for all samples (excluding standard curve generation). The column temperature was kept constant at 25 °C and the sample tray was kept cooled at 10 °C. For each sequence, the area under the peaks was integrated using the standard curve established as outlined in Section 2.1.

2.2.4. Preparative-grade chromatography

All of the samples used in this study were humanized monoclonal antibodies of IgG1 isotype allowing purification by MabSelect Sure resin (GE Healthcare) using gravity columns containing 1 mL of resin. The column was equilibrated with 10 mL 1× dPBS, and then 400 mL of the clarified harvest was slowly loaded over the course of 2 h. After washing with 20 mL of 1× dPBS, the protein was eluted with 5 mL 50 mM citrate pH 3.5. The eluate was collected and neutralized to pH 7 with 1 M tris pH 9 and 0.2 μm filtered. Protein concentration of purified samples was measured by recording absorbance at 280 nm using a UV/VIS spectral-photometer Nanodrop 1000 from ThermoScientific (USA)

3. Results and discussion

3.1. Interpretation of chromatograms

Following injection of the cell culture supernatant, all components which have no affinity to protein A directly enter the SEC column and are separated by their hydrodynamic radius. The UV signal from 0 to 5.5 min in Fig. 1A shows the flow-through consisting of Host Cell Proteins, DNA and media components. After 5.5 min, the UV trace reaches baseline level and the IgG1 captured on the protein A column is eluted by changing to mobile phase B (Elution buffer). Consequently the IgG is also separated by its size in the SEC column. Typically,

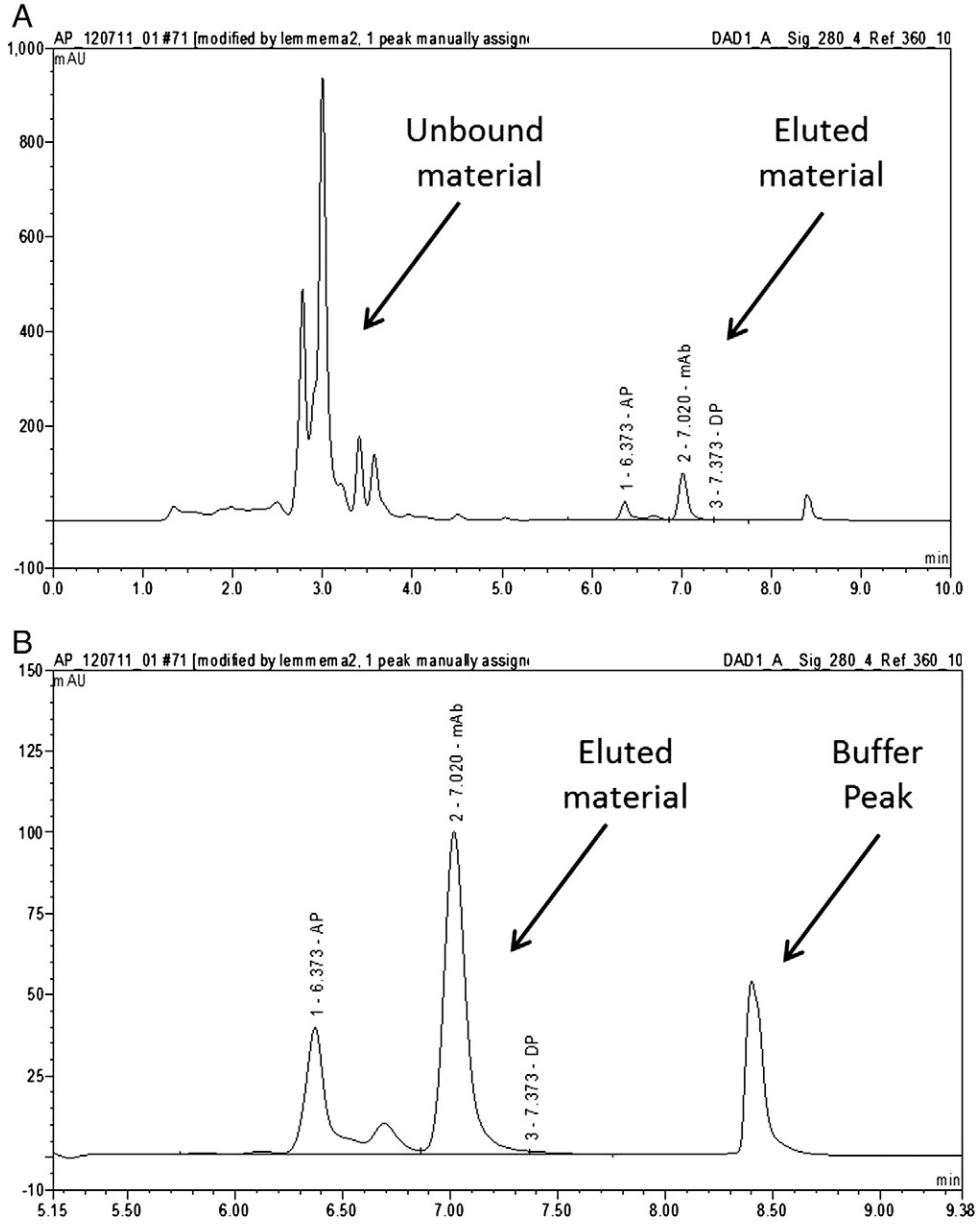

Fig. 1. A. Chromatogram of a cell culture supernatant containing IgG1 analyzed with ALC–SEC. B. Zoom of panel A shows integration of IgG aggregation products, monomer and degradation products.

aggregation products (AP), monomer (mAb) and degradation products (DP) elute from the SEC column between 6.0 and 7.5 min as shown in Fig. 1B. The buffer peak is system dependent and its retention time can be determined with a blank injection. For the system used to develop this method, the buffer peak appears at 8.5 min as shown in Fig. 1B.

3.2. Linear calibration of ALC, SEC and ALC–SEC

As shown in Fig. 2, a seven point calibration measuring protein concentration utilizing a purified stock monoclonal IgG (Section 2.1) was established for each assay, ranging from 0.002 to 0.2 mg total injection amount. The three linear calibrations in Fig. 2 do not show any significant deviations from each other. All further analysis concerning determination of concentration is based on those standard curves. Note that for ALC the total area under the IgG peak was used for calibration; for the calibration of SEC and ALC–SEC the following equation was utilized.

$$\text{Total Area} = \text{Area AP} + \text{Area Main} + \text{Area DP}$$

Reporting of AP, monomer and DPs was performed for SEC and ALC–SEC only; results are reported in relative area and calculated as follows: e.g. for AP

$$\text{Relative Area AP [\%]} = ((\text{Area AP})/(\text{Area AP} + \text{Area Main} + \text{Area DP})) \times 100.$$

As shown in Fig. 2, all three calibration curves have high linearity. Based on the range studied, AP/DP products were measurable (the limit of detection) at the lowest concentration measured for all three methods (0.002 mg). The limit of quantification for the three methods varies, with the ALC having the lowest limit at 0.002 mg (the lowest point on the tested calibration) and the SEC and ALC–SEC showing constant and accurate relative area content (including AP and DP) beginning at 0.01 mg injected.

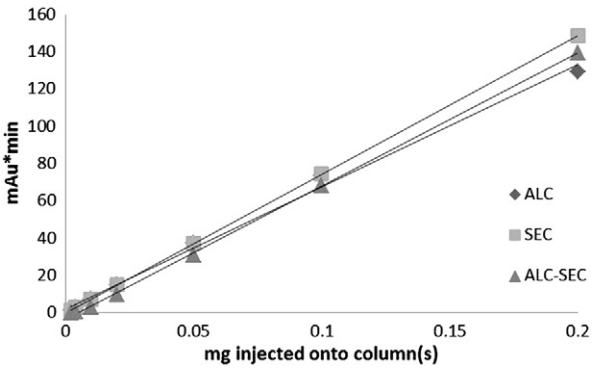

Fig. 2. Standard curves of protein injections (mg injected onto column) utilizing purified monoclonal IgG (Novartis stock solution, Section 2.1) as measured by three different techniques: ALC, SEC and coupled ALC–SEC. All samples showed high linearity — ALC: $y = 654.95x + 2.049$, $R^2 = 0.9951$; SEC: $y = 743.66x + 0.0848$, $R^2 = 1$; ALC–SEC: $y = 713.27x - 3.3614$, $R^2 = 0.9995$.

3.3. Comparability of concentration determination by ALC vs. ALC–SEC

The concentration of cell culture supernatants was measured by ALC and ALC–SEC. The comparison of the results obtained by both assays is shown in Fig. 3. In general, the data shows similar trends and a high R^2 (0.99) which indicates a strong correlation between the compared assays, with ALC–SEC reading approximately 5% lower concentration than ALC alone. (Measurement of protein concentration of purified antibody samples also showed a high R^2 value when the two methods were compared, however this data is not shown outside of the standard curve generation in Fig. 2). Differences in titer measurements (±5%) between the two methods are primarily seen in low concentration samples (<0.1 mg/mL) due to potential AP Peaks not being detected or broadened due to limit of detection of the HPLC, resulting in a lower measurement of the concentration.

3.4. Comparability of aggregation determination by SEC vs. ALC–SEC

The aggregation products of multiple samples were measured by SEC and ALC–SEC. Note that for this comparison protein A purified material (via gravity columns, Section 2.2.4) was utilized. The comparison of the results obtained by both assays is shown in Fig. 4. In general, the data shows similar trends and a high R^2 (0.97) which indicates a strong correlation between the compared assays. However, the absolute aggregation levels measured by ALC–SEC show a lower relative amount of aggregates compared to SEC. It is noteworthy to keep in mind that the sample in ALC–SEC enters the SEC column at a low pH due to the elution conditions used to elute the IgG from the ALC column. It has been shown (Ahmed and Saunders, 2010; Compton and Kreilgaard, 1994; Stulik et al., 2003) that if the pH of the mobile phase is lower than ~5.0 (and more specifically, the pI of the protein) a significant drop of resolution of the SEC column is observed. Furthermore, different IgGs were utilized to generate the correlation, thus it is difficult to make a general assumption. Overall, the precise determination of high molecular weight species percentage by SEC cannot be replaced by ALC–SEC. However, monitoring of the presence or absence of aggregates in cell culture supernatant is possible and thus an

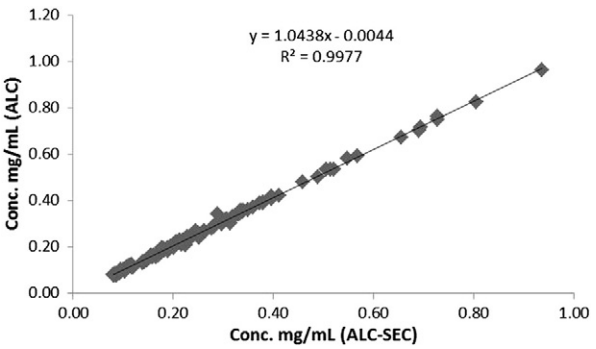

Fig. 3. Comparison of mAb concentration (mg/mL) measurement using ALC chromatography and coupled ALC–SEC chromatography of unpurified cell culture supernatants.

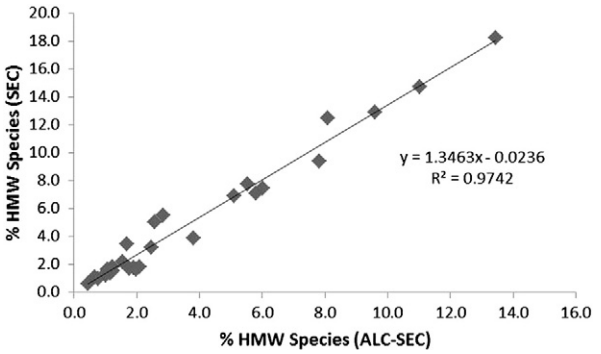

Fig. 4. Comparison of HMW species measurement using SEC chromatography and coupled ALC–SEC chromatography of purified (via gravity protein A columns) mAb solutions.

early trending of the material quality can be achieved. The presence of multiple peaks preceding the monomer peak suggests unique species, by nature of the mechanism of SEC chromatography. Due to the absence of a molecular weight marker, the authors do not qualify the nature of these species. For the species to have eluted off of the preceding affinity column, they must be a variant of an IgG, and since they are unique from the monomer, it can be concluded that they are larger multimers of the IgG monomer.

3.5. Conclusion

The primary benefit to a trending application such as the coupled ALC–SEC is the saving of time. Previous studies (Horak et al., 2010a, 2010b) have employed a coupled ALC–SEC for the rapid screening of affinity sorbents and media impurities, however many possibilities exist for its application for measuring and trending the causes of protein aggregation. Traditional methods to measure protein aggregation require an initial capture chromatography step to remove cell culture impurities, which may include long loading times, column washes and regeneration. From this eluted material the protein aggregation is measured through analytical sizing chromatography. These steps are avoided in the ALC–SEC method, allowing for information to be gathered earlier and quicker in the protein production process by the measurement of quality directly from the cell culture supernatant. This approach will serve as a valuable tool for the trending of protein quality, allowing for earlier decisions to be made in downstream processing and provides future opportunities for high throughput process development.

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