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Combined Protein A and size exclusion high performance liquid chromatography for the single-step measurement of mAb, aggregates and host cell proteins

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ABSTRACT

Quantification of monoclonal antibody (mAb) monomer, mAb aggregates, and host cell proteins (HCPs) is critical for the optimization of the mAb production process. The present work describes a single high throughput analytical tool capable of tracking the concentration of mAb, mAb aggregate and HCPs in a growing cell culture batch. By combining two analytical HPLC methods, Protein A affinity and size-exclusion chromatography (SEC), it is possible to detect a relative increase or decrease in the concentration of all three entities simultaneously. A comparison of the combined Protein A-SEC assay to SEC alone was performed, demonstrating that it can be useful tool for the quantification of mAb monomer along with trending data for mAb aggregate and HCP. Furthermore, the study shows that the Protein A-SEC method is at least as accurate as other commonly used analytical methods such as ELISA and Bradford.

1. Introduction

The opportunity to develop novel antibody based treatments increases the demand for therapeutic proteins. This in turn places a requirement to produce mAbs more economically by optimizing product quality in the upstream process [1]. To this end, the biopharmaceutical industry has begun adoption of a quality by design (QbD) approach toward high throughput development of mammalian cell lines [2]. For mAb development, the drive is toward higher and higher titer cell lines with minimal compromises to quality. This is of particular importance for the manufacture of biosimilars where cost to manufacture is critical [3]. Optimization of a cell line involves a careful balance of three key quality parameters: the mAb titer, the host cell protein (HCP) content, and the aggregate content. Higher product titers mean less working volume, a smaller footprint, and reduced upfront investment in manufacturing facilities. As cell lines are developed with ever increasing cell density and mAb concentration, HCP concentration increases as well, resulting in an additional burden on downstream processing. To clear the corresponding contaminants and reduce that burden, researchers are developing pretreatment options such as flocculation to be implemented before downstream processing [4]. Such development would benefit from a high throughput analytical tool that is capable of monitoring both HCP and mAb concentration at once.

In addition, mAb aggregates which trigger an immunogenic response in the patient [5] can become prevalent early in cell culture process development. Aggregate formation can be induced by a number of bioreactor conditions such as media components, oxygen levels, pH, and temperature [6,7], suggesting a need for faster, more accurate analytics to monitor, determine, and optimize the variables that contribute to aggregation.

Currently, many techniques exist that can quantify a single protein or group of proteins from cell culture [8] media ranging from colorimetric/immuno-enzymatic assays to chromatographic separation techniques such as affinity [9] and size exclusion chromatography [10–12]. Colorimetric assays such as Bradford [13,14] and BCA [15] are accurate and sensitive enough to detect trace amounts of protein; however, these methods are susceptible to interference by many small molecules found in cell culture media such as salt, detergents, polysaccharides, phenols, and nucleic acids [16–19].

To quantify mAb in a cell culture process containing a complex mix of proteins, an analytical Protein A column is typically used to separate the mAb from the rest of the feed. Protein A binds mAb specifically with high affinity. The elution from a Protein A column normally yields greater than 99% mAb, so the size of the elution peak from the column enables the concentration of mAb

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to be calculated. However, for bioprocess very low levels of HCP are required (<100 parts per million) and despite Protein A being a very productive purification step it cannot normally achieve such low levels of HCP. This is because some HCPs bind non-specifically to the Protein A column and to the mAb. Some of these HCPs are co-eluted from column along with the mAb. If HCP and aggregate quantitation is desired in addition to mAb concentration one would have to employ at least two additional analytical steps: (a) purify over Protein A and collect the eluate to analyze on size exclusion or run directly on size exclusion and perform a background subtraction using depleted Chinese Hamster Ovary (CHO) cell culture supernatant (obtained through Protein A separation); and (b) run a Bradford, BCA, or CHO ELISA to quantify HCP. Both of the outlined techniques contain multiple time-consuming steps that preclude their use for high throughput analysis. The assay described here is a better, simpler and less laborious analytical technique for simultaneous quantitation of two or more proteins that may also be able to quantify any variants or multimers of mAb separable by size-exclusion chromatography. To quantify HCP, mAb and aggregates, an analytical Protein A column was connected in series with a size exclusion column. The columns were operated so that the flow-through, wash, and elution steps in affinity chromatography are continuously loaded on to the size-exclusion column resulting in near-simultaneous quantitation of HCP, aggregates and mAb in a single HPLC run. This technique provides numerous advantages over the traditional methods listed above. (1) It reduces preparation time by eliminating the need to run multiple assays and prepare multiple samples, buffers and other steps associated with those additional assays. (2) It simplifies analysis by eliminating the need to perform background subtraction on any chromatographic trace data. (3) It can be operated using basic HPLC hardware, since no additional valves are necessary in between columns to bypass size-exclusion during elution; and, (4) It enhances analytical capability by allowing researchers to take advantage of size-exclusion to resolve impurities and variants of eluted protein such as aggregates.

2. Experimental

2.1. Chemicals, standard solutions and protein reagents

Glycine, sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, sodium perchlorate, phosphate buffered saline, sodium chloride, acetic acid, sodium hydroxide, tris, para-aminobenzoic acid, HPLC grade ethanol, sodium azide, thyroglobulin, ovalbumin, ribonuclease A were purchased from Sigma–Aldrich. Bovine serum albumin and Bradford protein assay reagent were purchased Thermo Fisher Scientific. HCP ELISA kit (#F550) was purchased from Cygnus Technologies (North Carolina, USA). Cell supernatant containing humanized monoclonal antibody (h-IgG1) supplied from a stable, 20 L scale, in-house CHO cell expression system was used for this study. Stock solutions of monoclonal antibody were purified in-house.

2.2. Methods

2.2.1. Preparative scale affinity chromatography

Pure mAb was prepared from CHO cell culture supernatant using MabSelect SuRe resin (GE Healthcare) on an ÄKTA avant. The depleted CHO cell culture supernatant (the flow through of the column) was collected, pooled, sterile filtered, and stored along with the purified mAb at −20 °C.

2.2.2. Protein A affinity chromatography HPLC (Protein A-HPLC)

Loading buffer A was prepared at 25 mM phosphate pH 7.2 + 300 mM NaCl for Protein A HPLC. For elution buffer B, a 10 mM glycine buffer + 300 mM NaCl solution was adjusted to pH 3.0 using 1 M HCl. The instrument method was as follows: loading buffer A 0–1.5 min, elution buffer B 1.5–3.5 min, followed by loading buffer A from 3.5 to 5 min to re-equilibrate the Protein A column (Life Technologies, #1-5024-12, column dimensions 2.1 × 30 mm, 0.1 mL) at a flow rate of 1 mL/min. A detection wavelength of 280 nm and an injection volume of 20 μL was employed for all samples including standards.

2.2.3. Size-exclusion HPLC

For SEC HPLC, a mobile phase consisting of 20 mM sodium phosphate pH 6.7 + 0.5 M sodium perchlorate was used. A TSKgel GW3000xL size exclusion column (part no. 08541, Tosoh Biosciences) was used to perform SEC. The instrument method consisted of running mobile phase buffer through the column using a detection wavelength of 280 nm and an injection volume of 20 μL for all samples including standards.

2.2.4. Protein A-HPLC coupled with size-exclusion HPLC

A POROS A20® Protein A column (part no. 1-5024-12, Life Technologies) was used in combination with a TSKgel GW3000xL size exclusion column (part no. 08541, Tosoh Biosciences). The columns were coupled to one another with the Protein A column preceding the size-exclusion column. A Shimadzu HPLC system comprising of a degasser, a pump, one UV detector, a control module, an autosampler system with cooler, and LC Solutions software was used. The assay was performed using a 1 mL/min flow rate and 20 μL injection volume. Monobasic sodium phosphate buffer (25 mM, pH 7.2) + 300 mM NaCl was used as a loading buffer A. Glycine buffer (10 mM, pH 3.0) + 300 mM NaCl was used as an elution buffer. Run time varied between 35–45 minutes depending on length of elution chosen. The instrument method was as follows: loading buffer A 0–15 minutes, elution buffer B from 15–20 minutes to elute bound mAb and aggregates from the Protein A column and pass through the size exclusion column, loading buffer A 20–35 minutes to complete the run and re-equilibrate the column for the next run.

2.2.5. Tangential flow ultrafiltration

Protein sample concentration was performed using tangential flow filtration (TFF). The TFF comprised 4 × Pall T02 10 kDa molecular weight cutoff (MWCO) Omega ultrafiltration cassettes connected in parallel. Eight liters of mAb-depleted CHO cell culture supernatant were concentrated to twenty times the initial volume.

2.2.6. CHO protein ELISA method for HCP quantification

The concentrated CHO cell culture supernatant feed samples were quantified for HCP concentration using an HCP ELISA (Cygnus Technologies #F550). Samples were quantified in triplicate using a dilution factor of 1:20,000, 1:40,000, and 1:80,000 for load samples and a dilution factor of 1:10, 1:100, and 1:1000 for the permeate.

2.2.7. Bradford method for total protein analysis

Purified stock mAb (1 mg/mL) and concentrated CHO cell culture supernatant feed samples were quantified in triplicate using a dilution factor of 1:8, 1:10, and 1:16. The corresponding absorbance of 1 mg/mL purified mAb (extinction coefficient = 1.45) was subtracted from each of the prepared CHO cell culture supernatant feed samples in order to calculate the trend in HCP concentration.

3. Results and discussion

3.1. Separation of mAb from contaminants in CHO cell culture using Protein A-SEC HPLC

The Protein A and size exclusion columns were connected in series during operation so that the sample flow-through, wash, and elution steps required for affinity chromatography are entering the