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Two-stage chromatographic separation of aggregates for monoclonal antibody therapeutics

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ABSTRACT

Aggregates of monoclonal antibody (mAb) therapeutics, due to their perceived impact on immunogenicity, are recognized as a critical quality attribute by the regulatory authorities as well as the industry. Hence, removal of aggregates is a key objective of bioprocessing. At present, this is achieved by a combination of two or more orthogonal chromatographic steps with possible modalities of ion exchange, hydrophobic interaction and mixed mode. A two-stage chromatographic purification process consisting of ion-exchange and hydrophobic interaction modes is proposed in this paper for effective and efficient control of aggregates for a mAb therapeutic. The proposed scheme does not require any intermediate processing of the process stream. Further, baseline separation is achieved for monomer and aggregates resulting in robust performance. This was possible because the proposed operational scheme allowed for an addition of selectivities of the two chromatography modes *vs.* the traditional two column scheme where part of the separation of aggregates achieved by the first column is lost upon pooling. The proposed process scheme yielded improved separation of aggregates (0% *vs.* 1–2%) at >95% recovery and reduced overall process time (6 h *vs.* 14 h) for a typical application. Further, clearance of host cell proteins was also shown to have improved with the suggested process scheme. Successful implementation of the proposed scheme has been demonstrated for two different monoclonal antibody therapeutic products.

1. Introduction

Monoclonal antibodies (mAbs) constitute an increasingly larger share of biotech therapeutics for diseases like cancer, rheumatoid arthritis, psoriasis and multiple sclerosis [1,2]. Due to the complexity of these products and the large amounts that need to be produced, last two decades have witnessed major efforts toward improving the quality of these products and reducing the cost of manufacturing [3,4]. These products are typically expressed in mammalian cell culture followed by a multistep purification process such that the different steps work on different separation mechanisms and thus together are able to provide the product which meets the stringent purity levels expected of therapeutic products.

Downstream processing of mAbs is generally based on a platform process which includes Protein A affinity chromatography as a capture step, followed by one or more polishing steps [5]. The latter could be an ion exchange or hydrophobic interaction or mixed mode chromatography. The primary impurities that require clearance during bioprocessing are the host cell proteins (HCP), host cell DNA, and aggregates [6]. Protein aggregation is an undesirable event that occurs during manufacture of therapeutic mAbs [7,8]. As mentioned above, presence of even trace amounts of aggregates is considered to be undesirable due to the concern of adverse immunological response [9].

Cation exchange chromatography (CEX) is one of the polishing steps used for achieving effective removal of protein aggregates for mAbs. It involves binding of a protein under pre-equilibrated low salt condition followed by a buffer wash and elution of product either by step or linear addition of salt or by altering the pH [10]. Product related impurities such as mAb aggregates can be more effectively separated by linear salt gradient. Aggregate species in the CEX elution pool generally elute out in the later fractions due to their high avidity compared to the monomer species [11,13]. To get the desired purity, elution can be fractionated and pooled according to the target aggregate content. Alternatively, elution of aggregate species can be controlled by changing from a linear gradient to constant salt at the concentration where elution of the aggregate species is expected. Key factors that impact aggregate clearance on CEX include, resin ligand chemistry,

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sample loading, binding and elution conditions, and pooling criteria [11–15].

Hydrophobic interaction chromatography (HIC) is another major mode of chromatography for achieving effective clearance of aggregates [16–18]. More recently, HIC membrane adsorbers have also been successfully used for clearance of these aggregates [19]. Protein binding in HIC is achieved under high salt condition and elution is performed by step or linear gradient reduction in salt concentration. Removal of protein aggregates in HIC is also achieved in flow-through mode in addition to bind elute mode, where the salt condition is just enough to bind the more hydrophobic variant while the product flows through. In this mode, high protein load capacity can be achieved. However, addition of high concentrations of salt to the product to promote protein binding in HIC can be detrimental to the product itself [20]. Process parameters and raw material attributes that have been known to impact the clearance in HIC include resin ligand, lyotropic salts used in binding and pH [17].

Almost always, both CEX and HIC steps are required to get the desired clearance of aggregates. The two steps are generally performed one after the other, thus requiring more process time. In this paper, we propose use of a two-stage chromatography process for highly efficient and high throughput separation of aggregates for mAb therapeutic products. This has been achieved by selecting operating conditions such that the elution from the CEX step, operated in a bind and elute mode, can be directly loaded on the HIC column with the HIC column operated in the flow through mode connected in series. The proposed configuration results in optimal and robust removal of the aggregate species.

2. Materials and methods

2.1. Instruments and materials

2.1.1. mAb samples

Two human IgG1 mAbs (mAb A and mAb B) were donated from a major producer of biotherapeutics. These were produced from CHO cell lines. The pI was 8.2–8.5 for mAb A and 7.7–8.2 for mAb B. Both the mAbs has a molecular weight of 150 kDa. The feed material consisted of neutralized Protein A elute from different batches pooled together. Feed concentrations were in the range of 10–20 mg/mL.

2.1.2. Preparative chromatography

Äkta Explorer (GE Healthcare Life Sciences, Uppsala, Sweden) with UV and conductometric detectors with a fraction collector attached was used for performing process chromatography. Samples were injected using sample loops and the data was collected using Unicorn software (GE Healthcare Life Sciences, Uppsala, Sweden).

2.1.3. Columns and membrane

Glass columns (Tricorn, GE Healthcare Life Sciences, Uppsala, Sweden) with I.D. = 0.5 cm, L = 20 cm and 10 cm were packed with different stationary phases. Poros XS (PXS), 50 μm and Poros HS (PHS), 50 μm, from Applied Biosystems, Capto Impress SP(CI), 39–42 μm from GE Healthcare, Ceramic HyperD S (CS), 50 μm from Pall Life Sciences, and Fractogel S (FS) and Fractogel COO^- (FC) from Millipore were used for CEX. Phenyl Sepharose (90 μm), Capto Butyl (75 μm), and Capto Phenyl (75 μm) from GE Healthcare (Uppsala, Sweden) were used for HIC. Sartobind Phenyl (3 mL) membrane adsorber was obtained from Sartorius AG, Germany, for performing HIC membrane chromatography.

2.1.4. Chemicals

Acetic acid, sodium acetate anhydrous, sodium chloride, sodium dihydrogenphosphate, disodium hydrogen phosphate, sodium sulfate, potassium di-hydrogenphosphate, dipotassium hydrogen phosphate, citric acid, sodium citrate, HEPES buffer were purchased from Merck, Germany, and Merck, India. For preparative chromatography, chemicals were of analytical grade and for analytical chromatography, of HPLC grade.

2.1.5. Analysis

Ultra performance liquid chromatography (UPLC) (ACQUITY UPLC Waters corporation, Milford USA) consisting of an autosampler, a column compartment, and a PDA detector was used. Data collection and analysis was performed using Empower 2 software. Super SW3000 TSKGel (Tosoh Bioscience LLC, Tokyo, Japan) of I.D. = 0.4 cm, L = 30 cm, and particle size = 5 μm was used for analysis of HMW content by size exclusion chromatography (SEC). UV 280 absorbance was used to measure sample concentration *via* Spectra Max (Molecular Devices, Sunnyvale, USA).

Samples were analyzed for HCP content using microtiter plate immunoenzymatic assay (ELISA – catalog no. F015) purchased from Cygnus Technologies, Southport, NC, USA.

2.2. Methods

2.2.1. Cation exchange chromatography (CEX)

CEX was performed using a manually packed column of 20 cm bed height and a column volume (CV) of 4 mL. Prior to sample loading, the column was equilibrated with the buffer to obtain the desired pH and conductivity. For equilibration, 15 mM sodium phosphate buffer ($NaPO_4$) was used for pH range of 6.0–7.5 and 50 mM sodium acetate buffer (NaOAc) was used for pH range of 5.0–5.5. The column was loaded with mAb after adjusting the sample to the desired pH using phosphoric acid (for pH 6.0–7.0) or acetic acid (for pH 5.0–5.5). Conductivity was adjusted to that of the equilibration buffer either by diluting with buffer or by adding salt. Sample loading was done for a maximum of 30 mg/mL of the resin. The wash step was performed with the equilibration buffer if the elution and binding pH were same. If the elution and binding pH were different then wash was performed at pH same as of the elution buffer but without any salt. Elution was performed by forming a gradient from equilibration buffer by linear addition of salt. Eluted intermediate was collected in 2 mL fractions (maximum–minimum: 50–50 mAu as per UV 280) and analyzed for concentration using UV 280 and for aggregate content using SEC. The column was regenerated and sanitized with 1 M NaCl and 0.5 M NaOH, respectively, after every run.

Experimental parameters that were examined in this study included elution pH (5.0–7.5), linear gradients (3.6, 5.5 and 11.2 mM/CV), elution salts (NaCl, KCl and Na_2SO_4) at three pH values and CEX resin media listed above. The flow rate was kept at 300 cm/h for all runs. All the experiments were performed at ambient temperature.

2.2.2. Hydrophobic interaction chromatography (HIC)

2.2.2.1. Column chromatography. A manually packed column of 20 cm bed height and CV of 4 mL was used for initial optimization. Prior to sample loading, the column was equilibrated with buffer to obtain the desired pH and conductivity. For equilibration, 15 mM $NaPO_4$ buffer was used for pH range of 6–7.5 and 50 mM NaOAc buffer was used for pH range of 5.0–5.5. The equilibration buffer and sample also contained lyotropic salts (phosphate, citrate and sulphates) of concentration greater than 0.2 M. Sample loading was kept at a maximum of 25 mg/mL of the resin and the wash step was performed with the equilibration buffer. Elution was performed by forming a gradient of equilibration buffer by linear removal of salt. The elution was collected in 2 mL fractions and analyzed for concentration using UV 280 and for aggregate content using SEC. The