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REPORT

Integrated flow-through purification for therapeutic monoclonal antibodies processing

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ABSTRACT

An integrated all flow-through technology platform for the purification of therapeutic monoclonal antibodies (mAb), consisting of activated carbon and flow-through cation and anion exchange chromatography steps, can replace a conventional chromatography platform. This new platform was observed to have excellent impurity clearance at high mAb loadings with overall mAb yield exceeding 80%. Robust removal of DNA and host cell protein was demonstrated by activated carbon and a new flow-through cation exchange resin exhibited excellent clearance of mAb aggregate with high monomer recoveries. A ten-fold improvement of mAb loading was achieved compared to a traditional cation exchange resin designed for bind and elute mode. High throughput 96-well plate screening was used for process optimization, focusing on mAb loading and solution conditions. Optimum operating windows for integrated flow-through purification are proposed based on performance characteristics. The combination of an all flow-through polishing process presents significant opportunities for improvements in facility utilization and process economics.

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activated carbon; continuous manufacturing; flow-through chromatography; integrated process; monoclonal antibody; purification

Introduction

Monoclonal antibody (mAb) purification technologies and processes for biopharmaceutical manufacturing have matured substantially since the introduction of platform purification processes based on a common sequence of batch unit operations.[1] Most of these efforts have focused on improving existing process templates through the introduction of high-performance purification technologies. A template process enables rapid process definition and has proven successful for the production of large quantities of mAb.[2]

Current large-scale batch manufacturers, however, still face several challenges, including high operating costs and high capital investment costs for upstream and downstream processing,[3] the speed of both scale-up and transfer,[4] and a large equipment footprint with tank-to-tank liquid transfer.[2] Integrated and continuous manufacturing has been proposed as an advanced processing method to address these challenges.[5]

The potential advantages of integrated processing over a conventional approach include shorter processing times and reduced operator interventions, which may increase operational efficiency. Moreover, the use of smaller equipment and facilities will offer greater flexibility for manufacturing volume. Implementation strategies for continuous processing composed of individual unit operations have been reviewed and discussed,[6-8] including direct chromatography column loading,[9] in-line dilution,[10] multi-column capture[11] and single pass tangential flow filtration.[12] Furthermore, many companies have conducted their own evaluations and feasibility studies for continuous processing.[13,14,15]

In the biopharmaceutical industry, chromatography is a widely used separation and purification technology due to its high resolution. The bioreactor harvest is followed by at least three unit operations, including primary capture, secondary purification, and final polishing. The majority of purification processes for mAbs involve capture with a Protein A-based chromatography media, which results in a high degree of purity and recovery in a single step. Continuous processing comprising high-density perfusion cell culture and a directly coupled continuous capture chromatography can be successfully implemented as a universal biomanufacturing platform obviating the bottleneck of bioprocess scaling.[13]

The purification and polishing steps generally incorporate cation and anion exchange chromatography, although hydrophobic interaction chromatography, mixed mode chromatography or hydroxyapatite chromatography may be used.[16] These steps provide additional viral, host cell protein (HCP) and DNA clearance, and they remove aggregates, unwanted product variant species and other minor contaminants.[17] Each chromatography step, either cation exchange or anion exchange, can be performed in bind and elute or flow-through mode, depending upon the physicochemical properties of the target protein and impurities. Continuous multi-column chromatography can be applied using either chromatographic mode, but is more frequently used with cation exchange mode for mAbs, where the media volumes required for processing are primarily dependent upon the product binding capacity of the media as opposed to the adsorption of trace impurities, as in the case of anion exchange chromatography. The flow-through concept can lead to a further increase in productivity for

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feedstocks in which the impurity is less abundant than the product. For example, anion exchange chromatography is well known for acidic impurity removal in mAb processing,[18-20] and hydrophobic interaction chromatography can be an effective step for aggregate clearance and host cell protein reduction.[21] Mixed mode chromatography has also been applied in the bioprocess industry.[22] Furthermore, a fully connected flow-through purification system could eliminate several intermediate pool tanks and significantly reduce capital investment through a single processing skid approach.[23] Zhang et al. connected two flow-through chromatography steps and successfully demonstrated a pool-less concept without intermediate product hold tanks.[24]

As described here, two media, activated carbon and a new flow-through cation exchange chromatography (CEX), were employed in flow-through mode in addition to flow-through anion exchange chromatography (AEX). Activated carbon is a porous material with a high surface area that adsorbs molecules through non-covalent interactions. The use of activated carbon to remove small molecules from protein solutions has been previously described by Marsh and Rodriguez-Reinoso.[25] Activated carbon has also been applied to the purification of higher molecular weight mAbs, where it was found that lower molecular weight proteinaceous impurities can be efficiently removed with minimal impact on mAb yield.[26]

Operating CEX chromatography in the flow-through mode under solution conditions that that strongly binding both the monomer and aggregate can be categorized as frontal separation,[27] where as a series of fronts, where the least retained mAb monomer is obtained from a fast breakthrough followed by the breakthrough of the more retained species. Suda et al. reported mAb aggregate clearance using flow-through operation on CEX resins.[28] However, the weaker binding of impurities and narrow operating window of pH and conductivity was one of the disadvantages of this mode. A specific resin should be guided by fit and compatibility with the overall flow-through train. A new CEX media has been developed with an optimized surface chemistry that enables selective flow-through aggregate (more retained species) removal from monomer mAb solutions (least retained species). Application data show robust aggregate removal with high selectivity and binding capacity for aggregates, while simultaneously providing high monomer product yield at practical process loadings in flow-through mode.[29] Proof-of-concept studies using combinations of these media have demonstrated high product yields with a reduction in impurity levels that are below targets.[30,31] Thus, a combination of flow-through polishing media may offer a robust, simple, and viable process alternative to the bind and elute options that are currently available for impurity and aggregate clearance.[30] A cost-of-goods analysis demonstrated that the combined process advantages exceed those provided by each operation separately.[32]

In this study, we evaluated the use of integrated flow-through technologies for mAb purification as a replacement for a conventional bind and elute chromatography platform. The flow-through technologies were designed to intensify the purification process with higher chromatography loadings, leading to a reduction of the resin volume while maintaining good selectivity of impurities with satisfactory yield. To adapt the approach for flow-through polishing, strategic case studies of the combination of flow-through technologies were evaluated (Fig. 1). A brief analysis of the sequence order of activated carbon, AEX, and flow-through CEX with optimized ligand density for selective aggregate clearance[29] were performed based on impurities clearance and mAb recovery. Process optimization was performed by design of experiments (DOE) evaluation with the small scale screening of loading conditions.

Results

Impurity clearance

The major impurities in a mAb feedstream include HCP, DNA, high molecular weight (HMW) mAb aggregates and low molecular weight (LMW) degradation products. The post-Protein A purification of mAbs was achieved using four different downstream templates (Fig. 2). The reduction of mAb-related HMW aggregates was attained using the new flow-through CEX1 step, exceeding 98% mAb monomer recovery in all cases. The AEX step contributed to a slight LMW removal for mAb-A. When the mAb dimer and multimer were distinguished, the dimer (HMW2) was significantly reduced by the CEX1 step. Reduction of the mAb-B multimer (HMW1) was primarily achieved by pH adjustment, but was completely removed by activated carbon and AEX, although the effect of pH adjustment observed for mAb-B was more significant than mAb-A.

HCP and DNA concentrations resulting from each of the polishing steps are shown in Fig. 3. Purification without activated carbon displayed a modest decrease in the DNA and HCP content. However, a reduction of DNA and HCP was facilitated by the activated carbon. Soluble impurities of DNA and HCP were precipitated upon pH adjustment as shown in Fig. 3 for mAb-B (mAb-A was not measured), which are

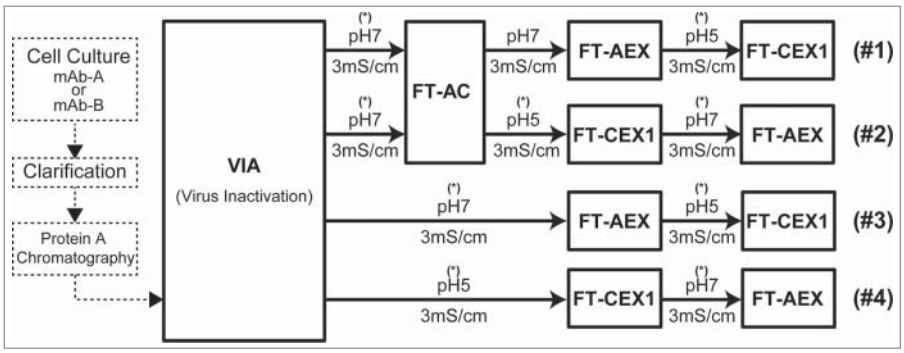

Figure 1. Test matrix of purification strategies for flow-through technologies. (*) Sample conditions were adjusted by buffer dilution and/or pH adjustment.

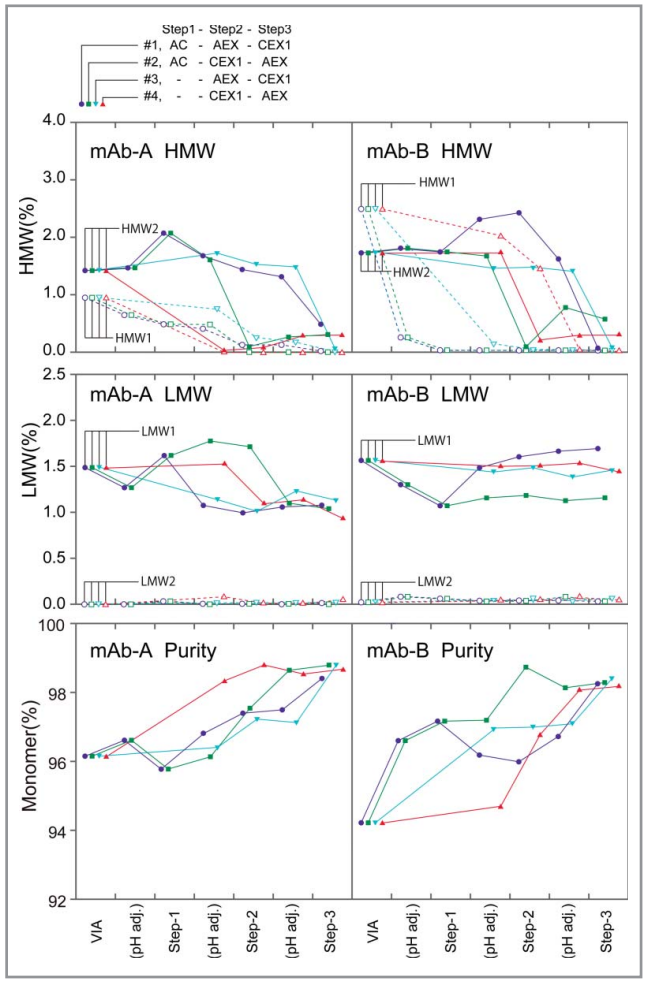

Figure 2. Plots of % monomer purity and mAb related impurities (LMW1, LMW2, HMW1 and HMW2) throughout the entire flow-through downstream purification, where HMW1 and HMW2 refer to the mAb multimer and dimer, respectively. Lines are meant to guide the eye.

subsequently captured by the activated carbon. Since the adjustment of pH and conductivity followed by filtration using 0.2 micron Millex® also showed a complementary impurity reduction, the phenomena of precipitation of impurities might be helpful to flow-through polishing. As a result, the change from low pH viral-inactivated pooled protein-A chromatography eluates (VIA) to pH 7 with 3 mS/cm conductivity had significant concentration reductions of DNA (99.3% to 99.4%), HCP (72% to 73%) and HMW1 (90% to 95%).

The overall mAb purity with impurity clearance in the final pool are summarized in Table 1. All combinations in this study utilizing all flow-through purification technologies provided excellent impurity clearance, and achieved sufficient purification performance for therapeutic mAbs specification.

mAb yield

The overall process yields of mAb-A and mAb-B for each of the four purification trains evaluated are shown in Fig. 4(a). The overall process yields were in the acceptable range of 80% to 86%. Although the presence of activated carbon resulted in a lower overall yield than when it was absent, all process permutations met downstream purification targets for both mAbs.

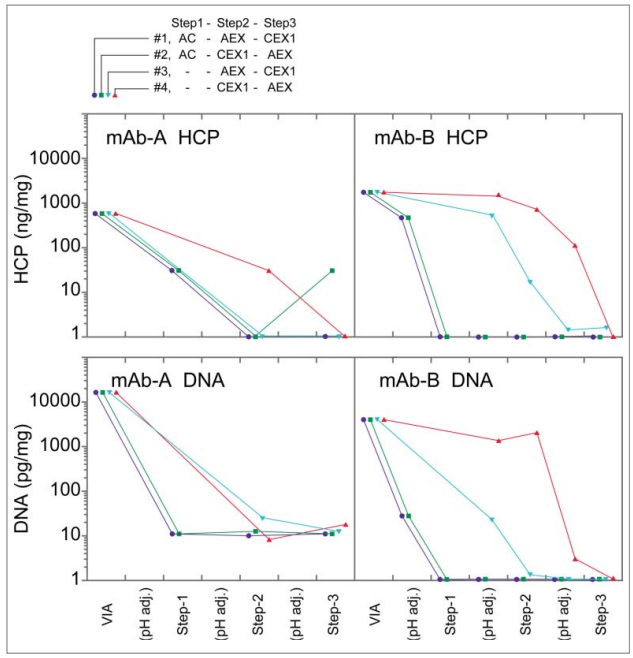

Figure 3. HCP and DNA concentrations as a function of the flow-through process proximity for mAb-A and mAb-B.

Comparing step recoveries shown in Fig. 4(b), both activated carbon and AEX demonstrated effective recoveries of 94% to 97%. The mAb recoveries of the flow-through CEX1 step were consistently lower at 88% to 91%. This is expected from the limited loading of 1000 mg / mL resin used for the feasibility test, and can be improved from the optimization of loading (as discussed below).

Breakthrough curves

The dynamic breakthrough curves of HCP and DNA for activated carbon are shown in Fig. 5(a). A slight HCP breakthrough of 1.9% was detected when the loading of 620 mg mAb/mL resin was achieved, but the breakthrough profile of DNA remained at low levels at the same loading. This observation suggests that there is a difference in the binding capacity of activated carbon in terms of different impurity components. Comparatively, a mAb breakthrough of 97% was reached at 207 mg mAb/mL resin loading (data not shown here), and a cumulative total mAb recovery of 90% was achieved at 311 mg mAb/mL resin.

As shown in Fig. 5(b), AEX demonstrated no clear DNA breakthrough and similar mAb recovery to that of activated carbon. HCP concentrations, however, increase immediately with lower loading volumes, indicating that activated carbon provides a much better clearance of HCP than AEX chromatography for this mAb.

The removal of aggregates by flow-through cation exchange chromatography was evaluated as a breakthrough curve for both monomer and HMW as shown in Fig 5(c). All monomer breakthrough curves rapidly saturated at the same loading of 400 mg/mL resin. There is a trade-off on the protein loading between CEX performance with respect to mAb yield, with faster breakthrough and aggregate removal with slower breakthrough. Monomer purities varied with the HMW breakthrough. The concentration of mAb multimer (HMW1) from the direct VIA

Table 1. Summary of final pool purity as a function of process applied.

Protein	Process step	Concentration (mg/mL)	DNA (pg/mg)	HCP (ng/mg)	HMW1 (%)	HMW2 (%)	Monomer (%)	LMW1 (%)	LMW2 (%)
mAb-A	#1 AC-AEX-CEX1	2.55	< 11.8	< 0.4	0.02	0.49	98.4	1.08	0.01
	#2 AC-CEX1-AEX	2.67	< 11.2	31.0	0.00	0.31	98.6	1.04	0.01
	#3 AEX-CEX1	2.43	< 12.3	< 0.4	0.00	0.07	98.8	1.13	0.02
	#4 CEX1-AEX	1.78	< 16.8	< 0.6	0.00	0.36	98.6	0.94	0.06
mAb-B	#1 AC-AEX-CEX1	3.95	< 0.76	< 0.25	0.00	0.07	98.2	1.68	0.03
	#2 AC-CEX1-AEX	3.06	< 0.98	< 0.33	0.00	0.57	98.2	1.16	0.03
	#3 AEX-CEX1	3.11	1.10	2.00	0.00	0.06	98.5	1.44	0.04
	#4 CEX1-AEX	3.60	0.70	< 0.28	0.00	0.29	98.2	1.44	0.04

feed increased with loading volume; however, some reduction was clearly observed at an early loading volume. In contrast to the breakthrough profile of HMW1, the effluent concentration of HMW2 from CEX1 resin was significantly lower than that of the feed and slightly increased with the loading volume. Interestingly, mAb-B dimer (HMW2) exhibited higher clearance than the mAb multimer (HMW1) in all process configurations. A comparison of the breakthrough curves of the two types of CEX resins shows there was no reduction of HMW1 with the CEX2 resin and a faster breakthrough of HMW2 was observed clearly at lower loading volumes.

Analysis of loading conditions

Optimization of the flow-through activated carbon and CEX1 chromatography steps should account for differences in the breakthrough concentrations of the mAb monomer and impurities as a function of solution conditions (i.e., solution pH and conductivity). A full multi-factorial DOE screening was designed to evaluate the effects of solution pH, conductivity and mass loadings on mAb recovery, monomer purity, and the reduction of HCP and DNA.

Results from the DOE screening study for activated carbon are shown in Fig. 6. For both mAbs evaluated, mass loadings of at least 1000 mg mAb/mL resin were required to achieve ~ 98% mAb recovery, but often at the expense of purity. As expected for activated carbon,[33] solution pH had little effect on mAb recovery, but impurity clearance varied as a function of pH and the mAb evaluated. For example, HCP clearance with mAb-A increased with decreasing pH while DNA clearance was unaffected. For mAb-B, however, both HCP and DNA binding increased with increasing solution pH. The effect of conductivity on HCP clearance was again observed to be mAb dependent, whereby mAb-A was insensitive and mAb-B exhibited improvements in HCP clearance with decreasing conductivity. The activated carbon results show that with appropriate loading conditions, high levels of impurity clearance may be possible even at 1500 mg mAb/mL loadings.

The DOE results of flow-through CEX1 are shown in Fig. 7. Similar to the trends observed for activated carbon, mAb recovery increased with increasing loadings, where recoveries of ~ 95% are possible at loadings of 1500 mg mAb/mL resin, with little impact on HCP or DNA clearance. As expected, increasing the solution conductivity had a negative impact on monomer purity as a function of loading, where conductivity at or below 4 mS/cm is necessary for both mAbs evaluated. In contrast to conductivity, the optimum pH condition for the removal of aggregate, HCP, and DNA was found to be mAb dependent, with pH 5.5 to pH 6.0 being optimal for mAb-A and pH 6.5 to pH 7.0 for mAb-B.

Discussions

Here, we described an innovative downstream process utilizing all flow-through purification technologies consisting of activated carbon and flow-through anion and cation exchange chromatography steps for the purification of two mAbs. Holistic development of a flow-through process was demonstrated on the combination of flow-through technologies. All combinations resulted in processes for two mAbs that provided good impurity clearance combined with high overall mAb yield. The

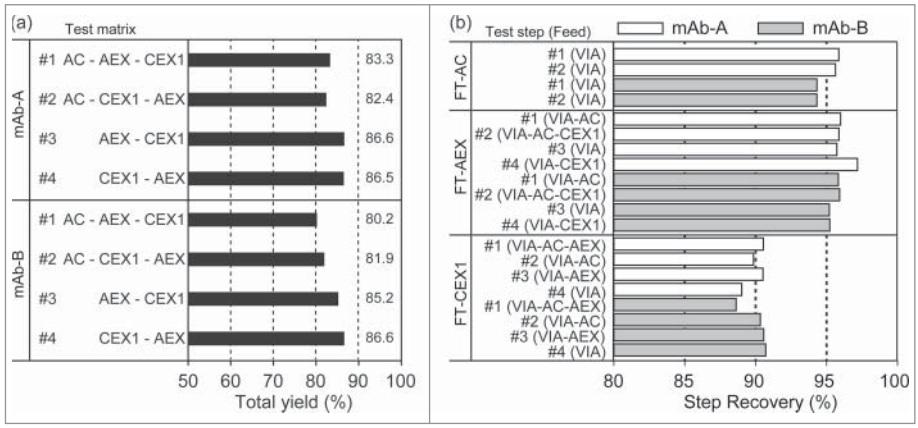

Figure 4. Total yield and step recovery. (a) Total mAb yield for the various flow-through downstream purification trains. (b) MAb step recovery as a function of the different unit operations applied, where the previous steps are shown in the left of bar. See text for a complete description of the different technologies evaluated.

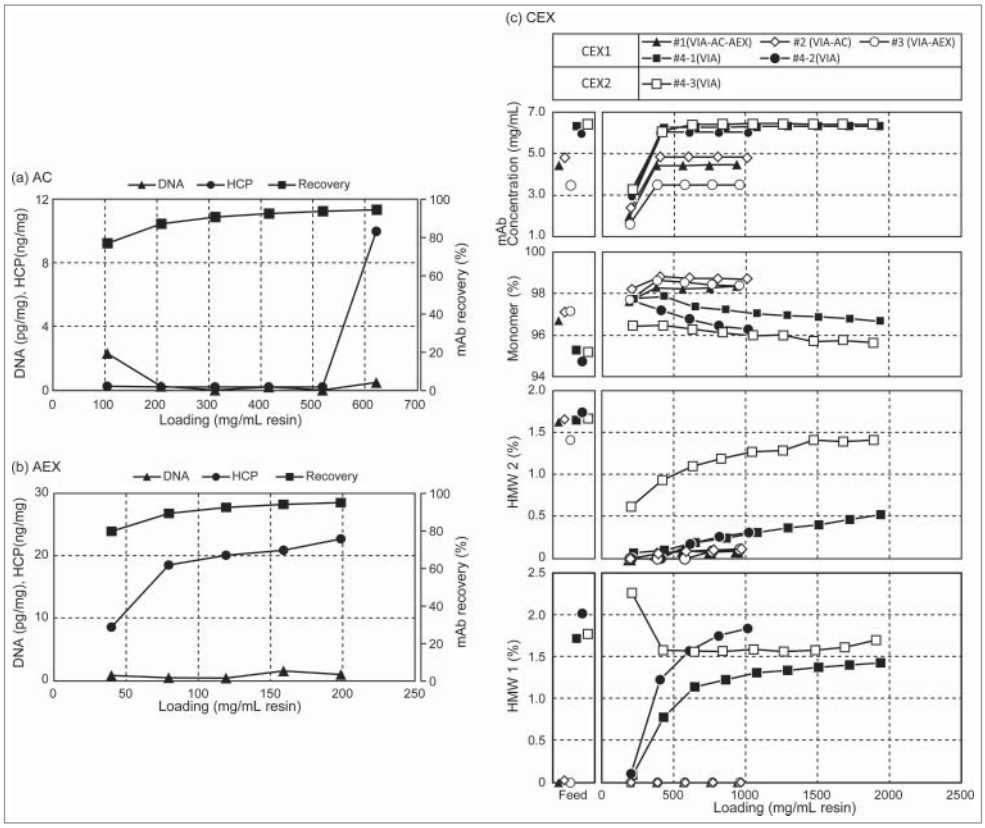

Figure 5. Change in the concentration of impurities. (a) DNA and HCP effluent from AC (#1 and #2) as a function of mAb-B loading. DNA feed = 28 pg/mg, HCP feed = 512 ng/mg. (b) DNA and HCP effluent from AEX (#3) as a function of mAb-B loading. DNA feed = 23 pg/mg, HCP feed = 537 ng/mg. (c) Breakthrough concentrations of mAb concentration, Monomer, HMW1 and HMW2 species from CEX1 (CEX prototype) and CEX2 (SP Sepharose® Fast Flow) as a function of mAb-B loading.

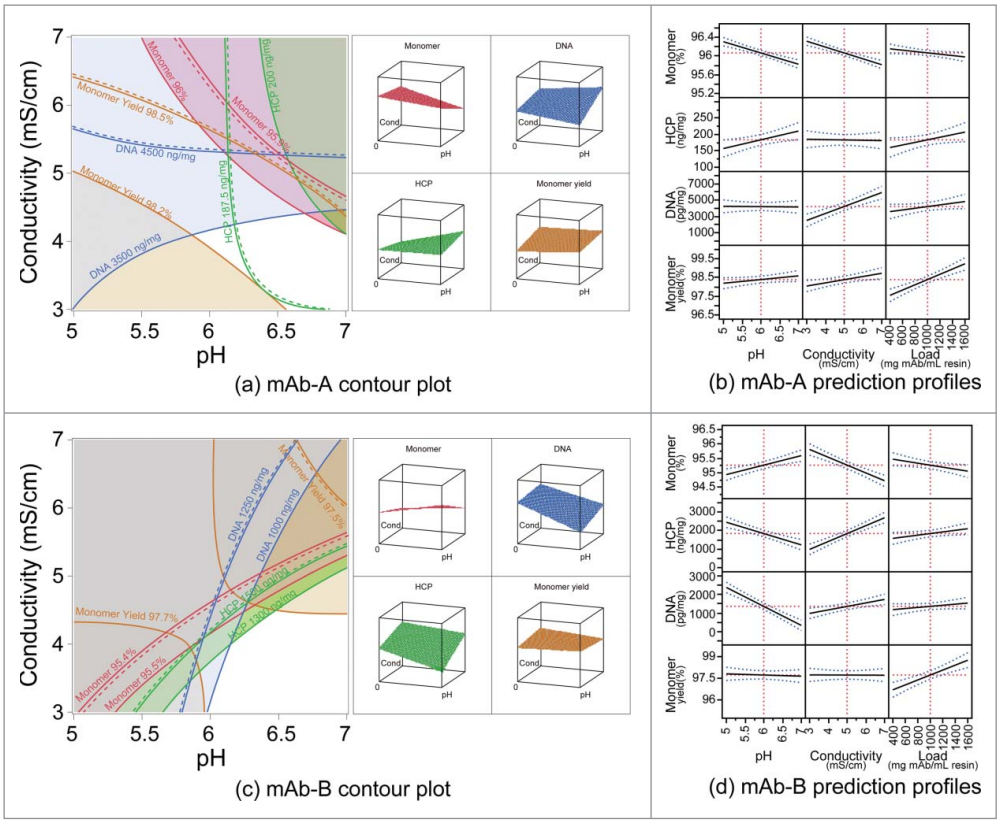

Figure 6. Operating condition profiles from DOE screening for AC. The effect of three input parameters (pH, conductivity, mAb loading) was evaluated for four response parameters: monomer yield, DNA, HCP, % monomer. Both response contour plots for pH and conductivity (a) and (c) were plotted at the loading of 1000 mg mAb/mL resin.

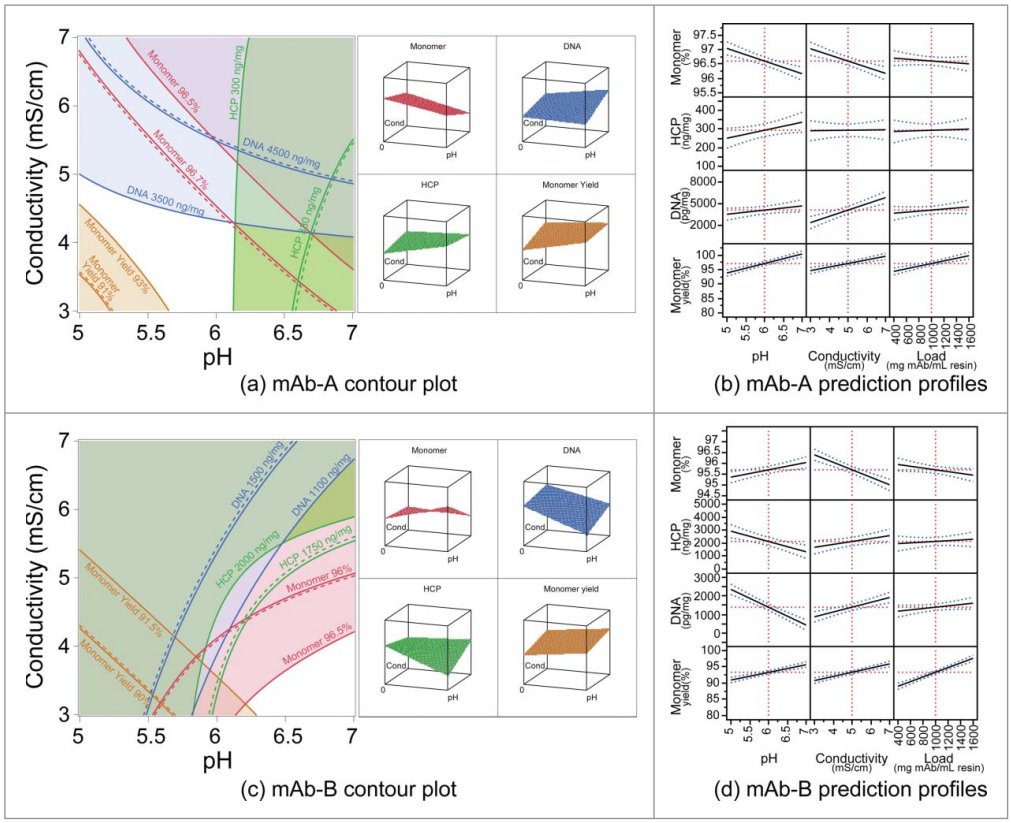

Figure 7. Operating condition profiles from DOE screening for CEX1. The effect of three input parameters (pH, conductivity, mAb loading) was evaluated for four response parameters: monomer yield, DNA, HCP, % monomer. Both response contour plots for pH and conductivity (a) and (c) were plotted at the loading of 1000 mg mAb/mL resin.

overall mAb purity with impurity clearance compared favorably with a typical mAb purification process.[34]

Activated carbon exhibited robust DNA and HCP removal as the first unit operation step, and may intensify the subsequent anion exchanger to reduce the impurity burden. A greater than 3 $\log_{10}$ DNA clearance for both mAbs was observed on activated carbon, while HCP removal varied between the two mAbs tested. Differences in HCP populations could account for the differences in HCP reductions across activated carbon for these two mAbs.[26] From a difference in the binding of activated carbon in terms of different impurity components (Fig. 5(a)), the size exclusion property of activated carbon proved to be reliable for the reduction of HCP and DNA as well as for mAb recovery, indicating that protein loading is one of the critical process parameters for successful application of an activated carbon containing process. The effects of pH and conductivity on HCP clearance were observed to be mAb dependent. This observation may be a result of differences in HCP isoelectric points,[26] as well as the difference of HCP concentration and populations that ultimately manifest as differences in the adsorption isotherms.[35,36] As suggested, binding capacity for a highly charged HCP may be reduced by protein-protein electrostatic repulsion at low conductivities such that an optimum HCP clearance condition may be defined with combinations of pH and conductivity.[26] A "sweet spot" of around pH 6 to pH 7 and conductivity below 4 mS/cm was defined from DOE screening for both mAbs.

AEX chromatography was observed to consistently facilitate the removal of HCP and DNA, and the magnitude varied as a function of process position and mAb. From the similar mAb recovery of activated carbon and AEX (Fig. 4(b)), the decision to include the activated carbon step should be determined from the balance between impurity clearance and mAb recovery. The breakthrough results of AEX indicate lower clearance of HCP (Fig. 5(b)). The robust clearance of HCP and DNA is easily achieved from the combination of activated carbon and AEX. Furthermore, the required subsequent AEX media volume can be reduced by a complementary activated carbon step. This is observed in Fig. 3 from the step-by-step reduction of HCP in mAb-A by the combination of activated carbon and AEX.

A new flow-through cation exchange resin (CEX1) provided excellent clearance of mAb aggregate at very high product loadings, leveraging the competitive binding behavior of strongly bound aggregate with the faster breakthrough of monomer (Fig. 5). The selectivity with the optimized surface chemistry of CEX1 indicates that the function of specific aggregate removal with CEX1 plays a role in dimer removal. The results of the DOE also indicated that the mass loading exerted a negative trend in monomer purity with an increase in monomer yield, as expected (Fig. 7). Resin productivity, defined as the amount of protein purified per unit column volume, can be expected to increase relative to the bind and elute mode as a result of the combination of media used here. CEX1 exhibited better mAb dimer removal than CEX2 (Fig. 5) and a differentiated superior capacity of HMW2 that is approximately 10 times compared to a current cation exchange resin designed for use in bind and elute mode. The DOE optimization indicates this mass loading is 15 – 50 times higher than the loading of bind and elute cation exchangers, which typically achieve about 30 – 100 mg mAb/mL resin.[37] As a result, process compression can be achieved with reduced chromatography media volumes with higher loading, and easy flow-through operation focused on aggregate removal.

Frontal separation between monomer and aggregate on a cation exchange resin is achieved by the competitive binding kinetics from the selectivity of components.[38] Gradual replacement of the pre-adsorbed monomer by the HMWs[39] was not observed in this loading study (Fig. 5). However, the effluent value of HMW1 of CEX2 exceeded the inlet concentration during the early loading stage. It might be due to the competition binding equilibrium between the slower binding, but more strongly held HMW2, and the faster binding, but weakly bound HMW1, that results in the formation of multi-component fronts of monomer and HMW including HCP.

Due to slight increases in the levels of HMW species observed when AEX is placed as the third step, with respect to the mAbs tested here, CEX1 should be placed at the position of final polishing. These results also suggest that the order of the various polishing steps should be investigated as part of a complete process development.

Optimization of these technologies requires tuning of buffer conditions as well as mAb loading to consider connected flow-through operation. It is estimated that the "sweet spot" for CEX1 should be influenced by the p*I* of the mAb (Fig. 7). The observed pH optima trend for each mAb is consistent with the estimated p*I* of the molecules, where the lower p*I* mAb-A also requires a lower pH for optimal aggregate clearance on the flow-through CEX1 media.

The results of this feasibility study reflect the presence of a library of optimum operating windows that must be aligned to maximize the performance of the complete flow-through process. The true flow-through evaluations that were described here were performed at pH 5 and pH 7 only, though in-line dilution from VIA and also in-line pH adjustment between pH 5 and pH 7 are mandatory to connect them (2–3 times dilution or 1-3% addition of pH adjuster were used in this study). While the DOE suggests that a complete connected train operating at low conductivity and pH 7 throughout may be practical for mAb-B, mAb-A can be applied with pH 6, at least, resulting in an even further simplified post-Protein A polishing process without in-line pH adjustment.

The application of the three technologies evaluated here may be used to replace the traditional batch polishing process, resulting in reduced media volume requirements, especially for the CEX step, and substantial simplification of the polishing process. Furthermore, these technologies could be expected to provide significant cost savings compared to current processes.[31] Finally, these new technologies could be applied to future template processes providing higher productivity through shorter process times with the potential to enable production with a single processing skid. The benefits of such single skid operations can result in further process integration between unit operations without intermediate tanks, as well as enable continuous processing with further flexibility in compact facilities.

Materials and methods

Media

Activated carbon (AC) powder (Merck KGaA, Darmstadt, Germany prototype product) found in Millistak+® CR40 devices[33] was packed to a bed volume of 1 mL in an OmniFit® (Diba Industries, Inc., Danbury, CT, USA) chromatography column (10 mmϕ x 13 mmH) with deionized water. A 0.2 mL MediaScout® MiniChrom column (5 mmΦ x 10 mmH) packed with CEX prototype[29] (surface grafted rigid hydrophilic polyvinyl-ether polymer with S-function group, mean particle size = 50 μm, reduced ligand density was optimized to maximize mAb dimmer selectivity) provided by Merck KGaA (Darmstadt, Germany), and an Eshmuno® Q 1 mL MiniChrom (Merck KGaA, Darmstadt, Germany 1250650001) column were used for flow-through experiments as cation exchange (CEX1) media and anion exchange (AEX) media, respectively. A Tricon™ column 0.4 mL (5 mmΦ x 20 mmH) SP Sepharose® Fast Flow (GE Healthcare, Buckinghamshire, UK) was used as the comparison of flow-through cation exchange performance (CEX2). Bulk media of each adsorber (i.e., AC, CEX1, and AEX) were used for static binding experiments within 96-well plates (AcroPrep™ Advance 96 Filter Plate, Pall, Cat. No. PN8184).

Buffers for flow-through and 96-well plate studies

Sodium acetate (Cat. No. 1.37012.5000, Merck KGaA, Darmstadt, Germany), Tris-HCl (Cat. No. 016–17453, Wako, Osaka, Japan), acetic acid (Cat. No. 17114–08, Kanto Chemical, Tokyo, Japan), Tris (Cat. No.41101–08, Kanto Chemical), sodium chloride (Cat. No. 38129–08, Kanto Chemical), and sodium hydroxide (10M, Cat. No. 37901–08, Kanto Chemical), were used to prepare buffers of specific pH and concentrations. All media were evaluated at pH 5 or pH 6 and were performed with a background buffer of 25 mM sodium acetate adjusted to pH 5 or pH 6 using either 1 M acetic acid (pH 2.5) or 2 M Tris base (pH 11), while all media evaluated at pH 7 were performed with 25 mM Tris-HCl adjusted to pH 7.

Reagent for HPLC

Disodium hydrogenphosphate (Cat. No. 197–02865, Wako), potassium dihydrogenphosphate (Cat. No. 28721–55, Nacalai Tesque, Kyoto, Japan), hydrochloric acid (1 M, Cat. No. 083–01095, Wako), IPA (Cat. No. 166–04831, Wako), 2-amino-2-hydroxymethyl-1,3-propanediol (Cat. No. 207–06275, Wako), and phosphoric acid (Cat. No. 167–02166, Wako) were used for IgG quantitative analysis by HPLC. Sodium dihydrogenphosphate dihydrate (Cat. No. 37239–00, Kanto Chemical), phosphoric acid (Cat. No. 167–02166, Wako), and disodium hydrogenphosphate dodecahydrate (Cat. No. 37240–00, Kanto Chemical) were used for size-exclusion chromatography (SEC). Sodium chloride (Cat. No. 191–01665, Wako) and methanol (Cat. No. 25183–3B, Kanto Chemical) were also used as common analytical reagents.

Model mAb solutions

Two mAbs were obtained as a frozen stock of VIA, produced by Astellas Pharm. Inc. The properties of the model mAbs in this study are shown in Table 2. The starting mAb-containing material was prepared by first thawing the frozen stock in a room temperature water bath followed by solution adjustment of pH and conductivity. The conductivity of the model mAb

Table 2. List of in-house mAb VIA feeds used in this study.

Protein	Subtype	pI*
mAb-A	IgG1	7.66
mAb-B	IgG1	8.16

*pI (Isoelectric point) as calculated from the primary amino acid sequence (Genetyx, Tokyo, Japan).

solutions was adjusted by dilution with water until the measured conductivity was equivalent to or below 3 mS/cm. The model mAb solutions were adjusted to pH 7 for both activated carbon and AEX media, and pH 5 for the CEX media by either 1 M acetic acid (pH 2.5) or 2 M Tris base (pH 11). After pH adjustment, all protein solutions were filtered using a 0.2 mm Millex® filter (Merck KGaA, Darmstadt, Germany) prior to use.

Methods

Flow-through procedures

All experiments were performed on the fully automated liquid chromatography system, ÄKTA® Avant 25 (GE Healthcare, Buckinghamshire, UK). Fig. 1 shows the feasibility study matrix used to evaluate the position of each unit operation. In total, four purification strategies (Fig. 1 #1 - #4) were compared in flow-through mode where each unit operation was performed in batch with pre-adjustment of pH condition. Two activated carbon columns were used in-series (#1 and #2). Both AEX and CEX resin volume was adjusted to get enough feed for the next step by connecting two columns in-series (AEX for #1 and #3, CEX for #2 and #4). The details of the mAb solution compositions are shown in Table 3.

Two activated carbon columns and an AEX column were equilibrated with Tris-HCl pH 7 at a residence time of 1 min. All column loadings were performed at 3-minute residence time (i.e., 0.33 mL/min for #2 #4, 0.66mL/min for #1 #3). The activated carbon column was loaded to a mass loading of 600 mg mAb/mL media while fractionating the effluent every 100 mg mAb/mL media loading. The AEX column was loaded to 200 mg mAb/mL resin with fractionation of the effluent every 40 mg mAb /mL resin loading.

The CEX1 columns were equilibrated with the pH 5 acetate buffer (residence time = 3 min, i.e., 0.07 mL/min for #1 #3, and 0.14mL/min for #2 #4). Loading was carried out with a mAb-containing solution of pH 5 and 3 mS/cm at the same flow condition of equilibrium (residence time = 3 min) to achieve the loading of ~ 1000 mg mAb /mL resin, while collecting fractions every 200 mg mAb/mL resin loading. A direct comparison of CEX1 and CEX2 was performed by loading by to 1900 mg mAb/mL resin at the same residence time.

96-well plate study

DOE screening was conducted to identify the optimum impurity static binding capacities of the activated carbon and CEX1. The equilibrated activated carbon and CEX1 resin was distributed into a 96-well plate with 10 μL resin/well. The media was washed with the appropriate equilibration buffer at 500 μL/well followed by 1 minute of shaking. The buffer was removed by centrifugation (Himac Compact Centrifuges, CF16RXII, HITACHI) at 500 x g for five minutes. Washing with equilibration buffer was repeated for a total of three washes. Next, the solution of mAb (see Table 3 detailing mAb solution compositions) was introduced at 800 μL/well, mixed briefly using a microplate shaker for 20 minutes, and centrifuged to remove liquid that was then collected into a collection plate. To achieve the target mAb loadings, the loading step was repeated and followed by a buffer flush after loading the target volume as performed for equilibration. Static capacity experiments were performed at combinations of three pH values (i.e., pH 5, pH 6 and pH 7) and three different conductivities (i.e., 3, 5 and 7 mS/cm). Three target mAb loadings were performed at 500, 1000, and 1500 mg mAb /mL resin. All data processing with respect to recovery and purity of mAb calculated based on the amount in the supernatants, residual DNA, and residual HCP were analyzed by Prediction profiler function on the cross-effects of pH, conductivity and loading from standard least squares model in JMP® 11 (SAS Institute Inc., Cary, NC, USA) software.

HPLC - IgG concentration

MAb concentrations were analyzed by HPLC-Protein A affinity chromatography using a POROS® A/20 affinity column (Life Technologies Japan Ltd, Tokyo, Cat. No. 1-5024-12) with a Shimadzu Prominence system with detection at 280 nm (Shimadzu corp., Kyoto, Japan). The binding buffer was a combination of 9.4 mM potassium phosphate buffer and 0.6 mM sodium phosphate buffer, 150 mM NaCl, and 5% IPA (pH 7.9), and the elution buffer was 12 mM HCl, 125 mM NaCl, and 5% IPA (pH 2.0). The sample volume applied was 15 μL for samples at < 1 mg mAb/mL and 3 μL at >= 1 mg mAb/mL. All steps were performed at a constant flow rate of 1.5 mL/min. MAb concentration was calculated based on a concentration calibration curve obtained using a known reference mAb material from 0.05 to 1 mg/mL and 1 to 20 mg/mL for low and high concentration, respectively.

HPLC – Size exclusion chromatography

Analytical SEC was performed using a TOSOH TSKgel® G3000SWXL column (5 μm, 7.8 mm ID x 300 mm, Tosoh

Table 3. Summary of mAb concentrations and impurities used in flow-through study and 96-well plate study.

Study	Protein	Concentration (mg/mL)	DNA (pg/mg)	HCP (ng/mg)	HMW1 (%)	HMW2 (%)	Monomer (%)	LMW1 (%)	LMW2 (%)
Flow-through study	mAb-A	8.32	17117	782	0.95	1.42	96.1	1.49	0.00
	mAb-B	10.9	3985	1888	2.47	1.72	94.2	1.57	0.03
96-well plate study	mAb-A	9.33	8552	885	1.44	1.35	95.9	1.23	0.09
	mAb-B	10.94	4047	2191	1.22	1.86	95.4	1.44	0.11

Corp. Cat. No. 08541) with a Shimadzu Prominence / Nexera X2 system with detection at 280 nm. The mobile phases for mAbs were 50 mM sodium phosphate buffer solution (pH 6.8) with 0.3 M NaCl for mAb-A and 20 mM phosphate buffer solution (pH 6.5) with 1 M NaCl for mAb-B. A constant flow rate was set at 0.5 mL/min. The sample volume applied was 10 μL. The concentration of intact mAb in unknown samples was determined using a standard calibration curve generated with the purified antibody.

HCP ELISA

HCP was detected using a commercial microtiter plate ELISA method (Cygnus Technologies, CHO HCP ELISA kit, 3G, Cat. No. F550). Samples were diluted with sample diluent buffer (Cygnus Technologies, Cat. No. 1028) and analyzed according to the manufacturer's standard assay protocol with a plate spectrophotometer (Perkin Elmer Japan, Wallac, EnVision^{TM}, 2104 Multilabel Reader) used for detection.

DNA analysis

The residual host cell DNA was measured using quantitative PCR (qPCR). The DNA was extracted using QIAamp DNA Mini Kit (250) (QIAGEN, Cat. No. 51306). Amplification reactions were carried out on a 7500 fast real-time PCR system (Applied Biosystems). The samples were analyzed and compared with a standard curve generated with known amounts of single-stranded DNA (resDNASEQ Quantitative CHO DNA Kits, Life Technologies Japan, Cat. No. 4403965), ranging from 3 to 300000 pg/mL.

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