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The distinctive separation attributes of mixed-mode resins and their application in monoclonal antibody downstream purification process

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ABSTRACT

Increased upstream productivity and the continuous pressure to deliver high quality drug product have resulted in the development of new separation technologies and platform strategies for downstream purification processes of monoclonal antibodies (mAb). In this study, the separation attributes of three mixed-mode resins, Mercapto-Ethyl-Pyridine (MEP) hydrophobic charge induction resin, Capto adhere multi-modal anion exchange resin, and ceramic hydroxyapatite/fluoroapatite (CHT/CFT) resins, were investigated to define their roles in monoclonal antibody purification processes. We demonstrated that the multi-modal nature of ligands on mixed-mode resins allows the separation resolution to be honed, either through a single dominant mechanism or through mix-modal balanced purification strategies. In addition, the three mixed-mode resins present different purification powers for different types of impurities. We also demonstrated that besides enhancing chromatography separation and improve product quality, especially for high molecular weight (HMW) aggregate reduction, mixed-mode resins can also help to improve process efficiency in industrial-scale mAb drug manufacturing. Our results underscore the importance of selecting appropriate chromatography resins during DSP design to obtain the best overall process outcome.

1. Introduction

Increases in upstream mAb production levels result in downstream purification challenges in the scale up efficiency and cost-effectiveness of Protein A (ProA) affinity chromatography based processes [1–4]. The physical constraint of processing increasingly large batches of monoclonal antibodies in existing manufacturing plants demands less sample manipulation and process volume control. It is not preferred and sometimes is not practical to further dilute the large ProA elution pool derived from small ProA column-multiple run capture steps if the subsequent ion exchange column demands lower conductivity for loading [5–7]. Therefore, in-process ultrafiltration/diafiltration (UF/DF) is often added to the process scheme to reduce tank size demand, loading process time, and/or to condition the load for the next ion exchange column. Such in-process UF/DF of ProA elution pools often faces a problem with turbidity which can be either product-related, contaminant-related, or both. The degree of the turbidity problem varies from product to product, and from process to process. In some cases, when in-process UF/DF cannot be developed into a robust process, an alternative approach is required [8–10]. In addition, the increasing emphasis from regulatory authorities on decreasing aggregate levels and improving product purity has influenced the goals of the downstream purification process [11]. ProA affinity and ion exchange separation techniques in the common generic purification platform cannot always handle the purification of all mAb products from discovery pipelines [8,12]. When faced with challenges such as high levels of aggregation from mammalian cell culture, other chromatographic techniques are often necessary to reduce aggregates. Hydrophobic interaction chromatography (HIC) and several newly evolved mixed-mode chromatography techniques have been reported as good options for aggregate reduction [13–17,29]. In a previous study, we have presented the advantages of mixed-mode resins in facilitating process step transitions without complex sample manipulation [18]. In this study, three mixed-mode resins commonly used in mAb DSP, Mercapto-Ethyl-Pyridine (MEP) hydrophobic charge induction resin, Capto adhere multi-modal anion exchange resin, and ceramic hydroxyapatite/fluoroapatite (CHT/CFT) resins, were investigated for their separation attributes and for their distinctive contributions to overall mAb purification process outcomes.

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2. Materials and methods

2.1. Chromatography purification process

The test proteins are three human monoclonal antibodies (hMab) IgG1 selected from Dyax antibody phage libraries. The hMab products were expressed by Chinese Hamster Ovary (CHO) cell lines. CHO fermentation cultures were clarified by depth filtration using Millipore HC pod depth filters (Billerica, MA, USA) and the clarified supernatant materials (Dyax, Cambridge, MA, USA) were subjected to downstream purification by defined processes described below.

Capture step Protein A resin is Mabselect ProA affinity resin from GE Healthcare (Piscataway, NJ, USA). The three mixed-mode resins used in these studies are Hydrophobic Charge Induction (HCIC) resin: MEP (Mercapto-Ethyl-Pyridine) HyperCel from Pall Life Sciences (East Hills, NY, USA); Capto adhere multi-modal anion exchange resin from GE Healthcare (Piscataway, NJ, USA), and Ceramic Hydroxyapatite and Fluoroapatite resins from Bio-Rad Laboratories (Hercules, CA, USA). In addition, other ion exchange resins including Capto Q anion exchange resin from GE; UNO-Q anion exchange resin from Bio-Rad Laboratories; Gigacap Q anion exchange resin from Tosoh Bioscience (Montgomeryville, PA, USA); Poros HS cation exchange and Porous HQ anion exchange resins from Applied Biosystems (Bedford. MA, USA); Fractogel TMAE(M) and TMAEhicap(M) anion exchange resins from EMD Chemicals Inc. (Gibbstown, NJ, USA), were also used in the studies.

All chemical reagents were from Sigma (St. Louis, MO, USA) and J.T. Baker (MallinkrodtBaker, Phillipsburg, NJ, USA) unless otherwise noted. Chromatography separations were operated on AKTA LC systems from GE Healthcare using AP columns from Waters (Millford, MA, USA), XK and BPG100 columns from GE Healthcare.

All chromatography studies used constant residence time across scale up based on capacity study results. Columns were packed to bed heights of 10–20 cm following manufacturer's recommendations and packing efficiency was ensured by preset criteria of HETP (Height Equivalent to a Theoretical Plate) and peak Asymmetry (As) factor. Each chromatographic column separation was operated under the conditions described in the results section or in figure legend. The process yield was measured by analytical ProA-HPLC and A280. Product purity was evaluated by analytical SEC-HPLC and impurity-specific ELISA assays.

2.2. Analytical HPLC

The analytical ProA-HPLC assay was performed using a 30 mm × 2.1 mm Poros ProA column on a Waters 2695 Alliance system. Standard and test samples were injected onto pre-equilibrated ProA column with 50 mM sodium phosphate, 150 mM NaCl, pH 7.0 buffer. IgG in the samples that had bound to the column was eluted by applying an acidic solution, 12 mM hydrochloric acid, 150 mM NaCl, pH 2.0. The amount of sample IgG injected was adjusted to be within the standard curve range of the assay (2–100 μg). The IgG protein concentration in the test sample was determined from the elution peak area using a reference standard curve.

The analytical SEC-HPLC assay was performed using a 7.8 mm × 30 mm SW3000 column on a Waters 2695 Alliance system. Molecular weight standards, reference IgG standards and test samples were injected onto the pre-equilibrated SEC column using phosphate buffer with 300 mM NaCl at pH 7.0. IgG monomer, together with impurities and contaminants in the samples were separated based on their molecular size. Product purity in a test sample is represented as the ratio of monomer peak area to total peak area at 280 nm.

2.3. Analytical ProA-ELISA and CHO HCP ELISA

The ProA-ELISA was performed with rProtein A^{TM} ELISA kit from Repligen (Waltham, MA, USA). Standard and test samples were diluted with special sample diluent and added to the wells of a pre-coated microtiter plate according to manufacturer's instructions. Protein A in the sample was detected by specific anti-rProtein A antibodies and measured by an enzyme–substrate colorimetric reaction. ELISA assays plates were read on SpectraMax M2 Plate Reader from Molecular Devices (Sunnyvale, CA, USA). Concentrations of rProtein A in each of the test samples were calculated based on the standard curve.

The CHO Host Cell Protein (HCP) ELISA was performed using the CHO HCP ELISA kit F015 from Cygnus Technologies (Southport, NC, USA). Standard and test samples were diluted with the specified sample diluent and incubated 2 h at 37 °C with alkaline phosphatase labeled anti-CHO HCP antibodies. After incubation, samples were added to wells of a microtiter plate that had been pre-coated with anti-CHO antibodies and treated according to manufacturer's instructions. CHO HCP in the sample, detected by specific anti-CHO HCP antibodies, was measured by an enzyme–substrate colorimetric reaction. ELISA plates were read out as described above. The resulting color intensity is proportional to the concentration of CHO HCP. CHO HCP levels in each of test sample were calculated based on the standard curve.

2.4. A280 adsorption and insoluble aggregation index (AI) measurement

Measurement of adsorption was carried on spectrophotometer of DU800 UV–vis from Beckman Coulter (Fullerton, CA, USA) or Cary50 UV–vis from Varian Inc. (Palo Alto, CA, USA). Adsorption at 280 nm was used for IgG concentration quantification, while adsorption at 280 and 350 nm were used for calculation of an insoluble aggregate index (AI) based on the formula below [19]:

$$\mathrm{AI} = \left(\frac{100 \times \mathrm{OD}_{350}}{\mathrm{OD}_{280} - \mathrm{OD}_{350}}\right)$$

2.5. Imaged capillary electrophoresis (iCE)

Samples were prepared with ampholyte from GE Healthcare, methyl cellulose from Convergent, and pI markers from Fluka. After pre-focusing for 1.0 min at 1500 V and focusing for 8.0 min at 3000 V, a UV light absorption image of the entire capillary is captured with a CCD camera of Convergent iCE$_{280}$ Analyzer (Con-

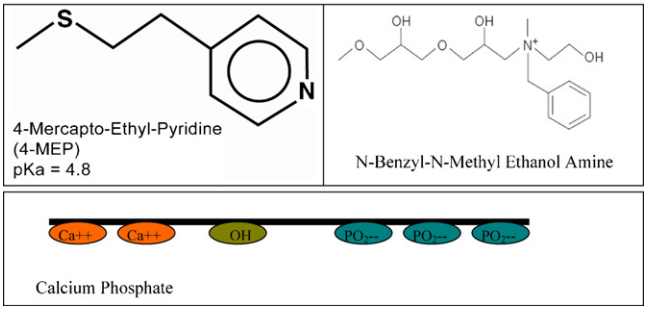

Fig. 1. MEP ligand, 4-Mercapto-Ethyl-Pyridine (A), is a mixed-mode resin with hydrophobic and electrostatic interaction mechanisms. Captoadhere ligand, N-benzyl-N-methyl ethanol amine (B), is a multi-modal strong anion exchanger. The most pronounced functionalities reported are ionic interaction, hydrogen bonding and hydrophobic interaction. Ceramic hydroxyapatite ligand, unique form of calcium phosphate (C), is a mixed-mode resin consisting of electrostatic and metal affinity interaction mechanisms.

vergent Bioscience, Toronto, Canada). The electropherogram is then analyzed using Convergent Biosciences® Software.

3. Results and discussion

3.1. Mixed-mode resin: MEP HyperCel

The separation mechanism on MEP chromatography is based on its pH-dependent ionizable dual-mode hydrophobic ligand design. It is generally understood that in the MEP purification process for a monoclonal antibody, IgG adsorbs to the MEP ligand mainly through hydrophobic interactions under physiological buffer conditions. Desorption from the resin is through electrostatic repulsion between positively charged IgG molecules and ionizable pyridine ring of the ligand when the buffer pH is decreased to close to or below the ligand pK_a of 4.8 (Fig. 1) [20]. Therefore, the common elution strategy for mAb purification processes utilizes the ionic, AEX like characteristics of the MEP resin to elute mAbs by lowering buffer pH [20–23].

We designed a study to assess elution strategies in order to decouple the contribution of hydrophobic and electrostatic interaction based separations when evaluating MEP HyperCel as a post-ProA intermediate purification step. After binding hMab IgG1 product in the neutralized ProA elution pool to the MEP column with a conductivity and pH close to physiological conditions, a wash step with 150 mM sodium acetate, pH 5.8 was applied to remove both the CHO host cell related and product-related impurities prior to IgG product elution. Different elution buffer conditions were

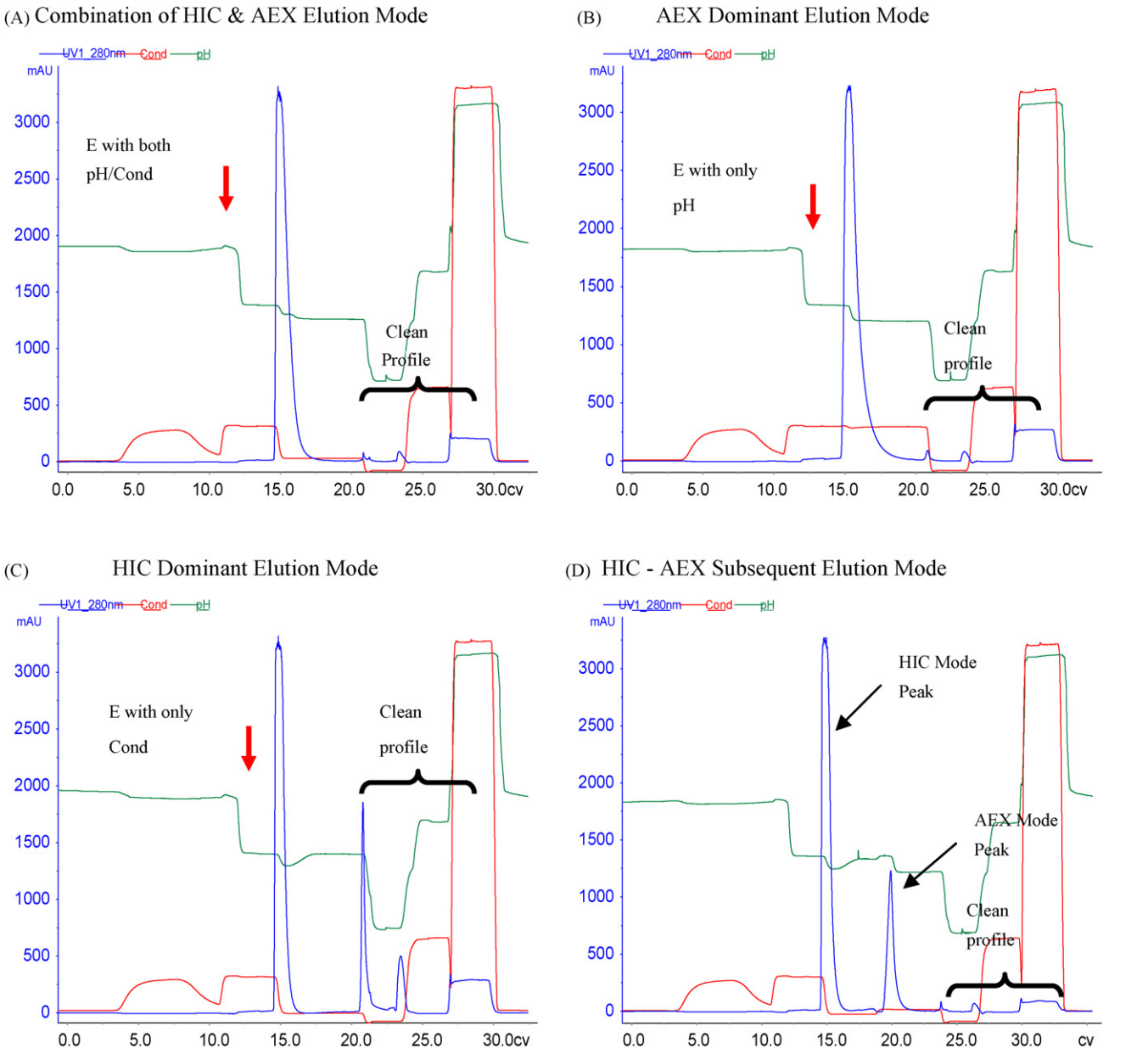

Fig. 2. All chromatography runs were conducted with a fixed residence time of 3 min using 1 cm diameter columns packed at bed heights of approximately 10 cm on an AKTA chromatography system. Neutralized ProA elution pool containing the hMab IgG1 at pH 7.4 and conductivity of 10–12 mS/cm was directly loaded on pre-equilibrated MEP HyperCel column by 100 mM Tris buffer at pH 7.6. After washing the column with equilibration buffer followed by a sodium acetate wash buffer (150 mM) at pH 5.8 and conductivity of 10 mS/cm, different sodium acetate elution buffers were used to desorb IgG product from the resin: Panel A, elution by reducing both conductivity and pH; Panel B, elution by decreasing pH only; Panel C, elution by decreasing conductivity only; Panel D, sequential elution by decreasing conductivity first followed by lowering buffer pH.

Table 1
Product quality analysis for HIC vs. AEX elution peak derived from sequential elution of MEP HyperCel post-ProA purification step.

	Load	Sequential mode elution	
		1st HIC mode peak	2nd AEX mode peak
Yield		80%	10%
HMW by SEC-HPLC	2.90	1.03%	5.59%
ProA (ppm)	9.18	3.34	8.83
CHO HCP (ppm)	95.73	45.81	184.1
iCE (acidic peak%)	33.8	31.8	45.5
iCE (main peak%)	61.8	62.5	49.6
iCE (basic peak%)	7.5	6.4	7.4
Bioactivity		1.99	1.79

Note: Product pool derived from AEX mode elution strategy including both peaks contains 1.48% HMW by SEC-HPLC, 3.68 ppm leached ProA and 51.76 ppm CHO HCP with 86% step yield.

then designed to differentiate the HIC and AEX mechanisms. This was achieved by desorbing the protein from the resin with the combination of HIC and AEX mechanisms by decreasing both the wash buffer pH and conductivity simultaneously (control elution, Fig. 2A) or with AEX dominant mechanism by decreasing the buffer pH only (Fig. 2B) or with HIC dominant mechanism by decreasing the buffer conductivity only (Fig. 2C). It was interesting that elution using the AEX dominant mechanism (decreasing buffer pH only) produces an elution profile that is similar to that obtained using the control elution strategy (decreasing both buffer pH and ionic strength). Under conditions where the HIC dominant elution buffer was used, the cleaning peak was increased considerably.

A two step elution experiment with different mechanisms was then conducted by first using the HIC dominant mechanism elution buffer and then following with the AEX dominant mechanism elution protocol. As expected, two well resolved elution peaks were observed and the post cleaning peak was similar to that found in the control run profile (Fig. 2D). Analytical testing showed that the second AEX elution peak was enriched for various impurities including aggregates, CHO host cell proteins, and more acidic variants (Table 1). Thus, by using the HIC dominant mechanism elution strategy we are able to improve the separation resolution and obtain better product quality from MEP mixed-mode chromatographic step of purification.

Under optimal elution conditions, MEP HyperCel proved to be a very powerful intermediate purification tool applicable to the post-ProA affinity capture step (Table 2). In this example, the MEP column step was found to reduce HMW levels by 90%, HCP levels by 98% while maintaining a good overall product yield of 80%.

In addition to its high separation power, the HIC dominant mechanism elution strategy has also enhanced MEP HyperCel's bridging role in DSP process design. MEP HyperCel is able to tolerate a relatively wide range of conductivity of feedstock materials. This load conductivity tolerance, combined with the low conductivity of the MEP HyperCel elution product pool, means that MEP HyperCel can effectively bridge the ProA capture step to subsequent chromatographic process steps that require low conductivity for loading without the need for further dilution or in-process UF/DF [18]. Even the direct transition to CHT that usually requires very low conductivity for loading now becomes possible.

Table 2
Product quality of MEP HyperCel purification post-ProA purification step.

Loading (mg IgG/ml resin)[a]	16
Step yield	80%
HMW in load by SEC-HPLC	10.07%
HMW in elution by SEC-HPLC	1.04%
HMW reduction%	90%
CHO HCP in load (ppm)	106.82 ppm
CHO HCP in elution (ppm)	1.98 ppm
CHO HCP reduction%	98%

[a] Optimal loading is defined as 80% of 5% breakthrough.

MEP HyperCel was initially designed with the intention of capturing IgG from clarified mammalian cell culture supernatant [24–26]. However, despite being more affordable per gram of resin than Protein A, the lower binding capacity and selectivity together with undefined regulatory history have prevented MEP HyperCel from being a commonly used alternative to ProA affinity chromatography in mAb manufacturing [27,18]. In special cases where hMab products are prone to aggregation in the ProA purification process, alterations in downstream purification steps can provide significant benefits over the conventional ProA based platform processes. Table 3 shows a case, using a Dyax IgG1 hMab, in which alternatives to ProA affinity chromatography may be desirable as the product capture step. The table lists the values of the insoluble aggregation index measurements for the elution pools obtained from three primary capture chromatography steps. The generic capture ProA step, which uses sodium citrate buffer at pH 3.2 for elution, results in a high turbidity, cloudy product pool and subsequent filtration yielded significant product loss. In testing non-ProA capture methods, we found that two chromatographic capture methods (mixed-mode MEP and cation exchange) provided significant reductions in the levels of insoluble aggregates. The investigation of capture step process conditions including elution buffer components, elution buffer pH, inclusion of excipients in elution buffers, elution product pool concentrations, and leached ProA levels, identified high hydrophobicity of the hMab molecule and leached ProA as two critical factors causing insoluble aggregation in the ProA capture step process (data not shown). Thus, a non-ProA based DSP was necessary for successful purification of this hMab product. Although CEX and MEP HyperCel can both capture the IgG1 product from clarified CHO culture supernatants, MEP HyperCel has an advantage over CEX in large scale manufacture in that it does not require dilution of the feedstock. On the other hand, CEX possesses a much higher capacity and also has better defined clearing and regeneration procedure, which could be advantageous for the capture step. If CEX is chosen as the capture step, MEP HyperCel may still be incorporated into the process as a powerful polishing step for this product because its low salt process conditions allow the antibody to remain in solution through its purification.

3.2. Mixed-mode resin: Capto adhere

Capto adhere is a multi-modal strong anion exchange resin developed by GE Healthcare. Its ligand, N-benzyl-methyl ethanol amine, contains anion exchange, hydrophobic and hydrogen bonding interaction groups (Fig. 1) [28,29]. We designed a D-optimal 4 factor by two level (loading pH 7–8.5 and conductivity 3–12 mS/cm, IgG loading 20–125 mg/ml resin, and column operating residence time 1–8 min) DOE study to compare separation attributes of six conventional AEX resins with Capto adhere. Results show that Capto adhere demonstrated significant benefits in both aggregate and leached ProA removal while there are no statistically significant differences among the six conventional AEX resins themselves (Fig. 3). Since the true measurement of AEX loading capacity in flow-through mode is its ability to bind various impurities and contaminants. For process operation practical reasons we used milligrams of hMab product per ml of resin to define AEX process performance while monitoring the breakthrough of various impurities (Fig. 4A and B). Aggregate reduction performance by the Capto adhere resin operated in flow-through mode as a polishing step was confirmed by an aggregate breakthrough curve comparison study. Capto adhere presented substantially more binding capacity for aggregates than conventional AEX resin.

Table 3
Insoluble aggregate index of elution pool derived from capture step.

	ProA step	MEP HyperCel step	CEX step
Elution buffer	NaCitrate, pH 3.2	NaAcetate, pH 4.5	$NaPO_4$, pH 6.5
Insoluble aggregate index	18.2	3.5	2.9

When CHO host cell protein (HCP) removal was evaluated, it was found that the level of CHO HCP is relatively higher at both the front and back parts of the flow-through peak of Capto adhere. The CHO HCP breakthrough curve comparison study reveals that, unlike traditional AEX resin which has typical binding breakthrough curve, Capto adhere presents a "V shape" breakthrough curve (Fig. 4B). This indicates that the multi-modal interaction features of the ligand design influences the clearance of diverse populations of CHO host cell proteins by Capto adhere across the flow-through separation. SEC-HPLC also shows that the low molecular weight (LMW) species of impurities are enriched at the front arm of flow-through peak while high molecular weight (HMW) species are breakthrough later. The mechanism of the "V" shape curve is unclear, and whether the phenomena is universal to Capto adhere needs further investigation. The performance of Capto adhere can be enhanced by making cut at both the front and end of the flow-through peak for better product purity with some sacrifice of yield. However, "peak cutting" may present extra challenge in manufacturing setting.

When Capto adhere is operated in a flow-through mode, its process binding conditions and outcomes are greatly influenced by the specific physical–chemical properties of the individual mAb product [27]. Thus for Capto adhere, it is hard to determine *a priori* what the optimal loading capacity would be based on an impurity breakthrough study because the process yield is also related to the protein loading mass. The front of the product A280 flow-through peak often appears half a column volume later under optimal impurity binding conditions. Lower loading can cause a significant loss of yield, while a higher loading could easily be beyond the best impurity binding range. In order to obtain the desired process outcomes, a DOE study is essential [27]. Therefore, unlike the MEP elution strategy described above, Capto adhere needs to be optimized using a mixed-mode balanced strategy on loading pH and conductivity to obtain the best process conditions.

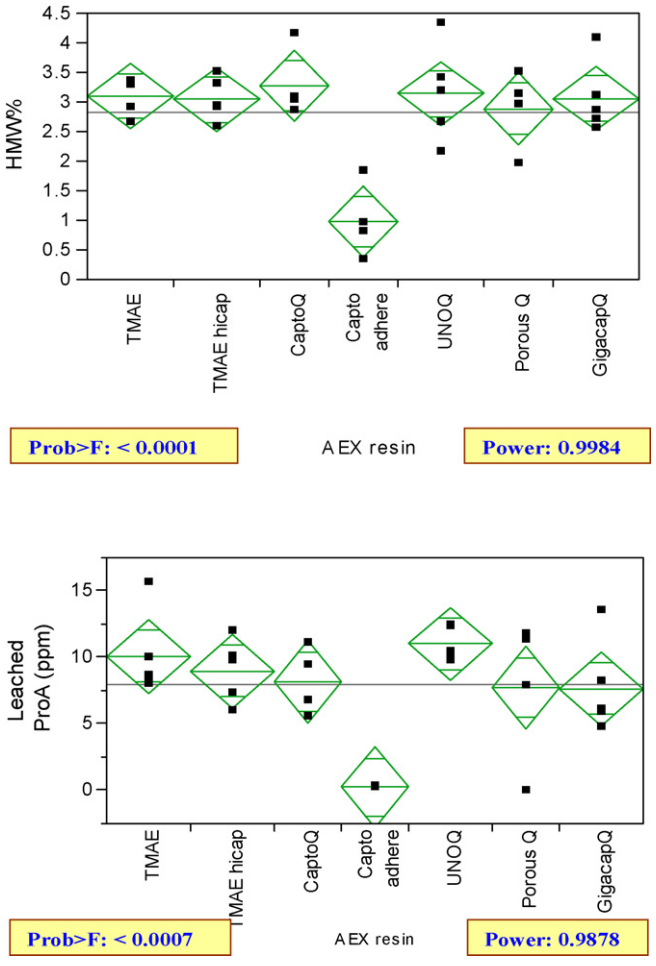

Fig. 3. DOE study was designed to compare 6 conventional AEX resins with Capto adhere. All chromatography runs were conducted as flow-through mode using 1 cm diameter columns packed at bed heights of approximately 10 cm on an AKTA chromatography system. Collected product pool were subjected for testing by a set of analytical assays to assess process yield and product purity include measurement of absorbance at 280 nm, analytical SEC-HPLC, CHO HCP ELISA, and rProteinA detection ELISA. The ANOVA analysis for the relative effectiveness of impurity clearance among different AEX resins was conducted with JMP software. Only aggregate reduction and leached Protein A removal present significant difference among test resins shown in the current figure. Note: For leached ProA-ELISA assay all samples from Capto adhere runs have leached Protein A level below the limit quantification and the values given by calculation were 0.2, 0.2, 0.2, and 0.31 ppm, which appear overlapped on chart.

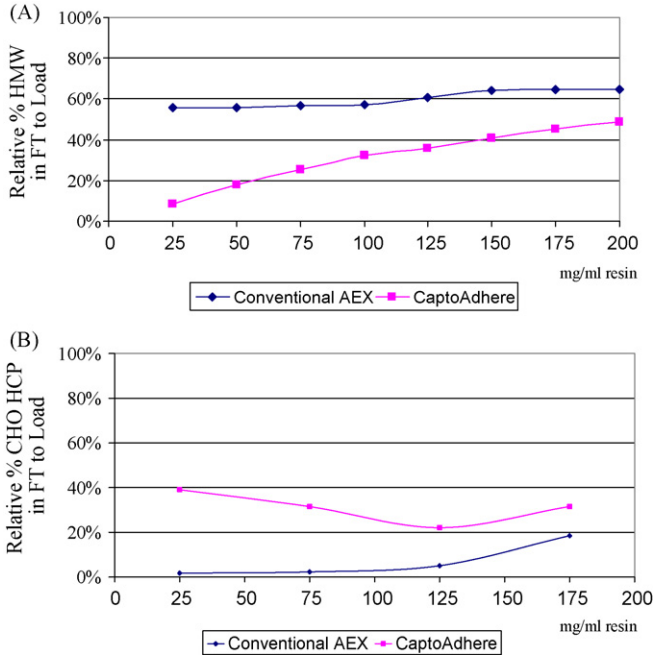

Fig. 4. The AEX runs were carried out as flow-through mode using 1 cm diameter columns packed at bed heights of approximately 10 cm on an AKTA chromatography system. Each resin evaluation was conducted at a pre-determined optimal pH and conductivity loading condition based on DOE study with a fixed residence time of 4 min (loading condition is 100 mM Tris at pH 8.5 for conventional AEX and 100 mM sodium phosphate at pH 7.6 for Capto adhere). As hMab IgG1 loading increased, flow-through samples were taken at defined loading points and subjected to analysis. The relative percentage of HMW aggregates of flow-through samples to load determined by analytical SEC-HPLC (A) and CHO HCP level determined by CHO HCP ELISA (B), is plotted against hMab IgG1 mg/ml resin loading for AEX resin performance assessment with regard to HMW aggregate and CHO HCP binding capability.

Table 4
Product quality of AEX post-ProA purification step.

	Conventional AEX	Capto adhere
Loading (mg IgG/ml resin)	100	105
Load material	UFDF ret pool (post-ProA)	Neutralized ProA E pool
Step yield	92%	73%
HMW in load by SEC-HPLC	12.8%	10.5%
HMW in flow through by SEC-HPLC	11.44%	2.33%
HMW reduction%	10.6%	77.8%
CHO HCP in load (ppm)	114.28 ppm	121.06 ppm
CHO HCP in flow through (ppm)	18.98 ppm	26.47 ppm
CHO HCP reduction%	83.4%	78.1%

Table 4 presents an example of the performance comparison of the traditional AEX resin and the mixed-mode AEX resin Capto adhere, both operated as a polishing step immediately following the ProA capture step in the context of DSP. Post ProA capture step, the traditional AEX step requires in-process UF/DF process to prepare the ProA eluate for loading in order to avoid dilution. In contrast, Capto adhere can use the neutralized ProA elution pool directly. Both AEX purification runs were conducted in a flow-through mode. With the two resins tested at similar loadings (~100 mg hMab/ml resin), Capto adhere achieves a much stronger reduction of HMW aggregates, dimer in particular, while both resins show similar reductions in CHO HCP levels.

3.3. Mixed-mode resin: CHT

Ceramic hydroxyapatite (CHT) is a resin in which both the ligand and matrix are constructed with same chemical material, calcium phosphate. It is a crystalline material consisting of Ca^{2+} and PO_4^{3-} groups in the lattice [30,31]. CHT is categorized as a mixed-mode resin based on its two functional groups: positive charged calcium ions (C-site) and negative charged phosphate groups (P-sites). The electrostatic interaction of both C- and P-sites with charged moieties of protein surface, and the metal affinity attraction between carboxyl groups on the protein and C-sites on CHT support are the two major characterized separation mechanisms of the resin (Fig. 1) [30,31]. The application of CHT to therapeutic protein purification was initially recognized due to the exceptional aggregation reduction power of the resin when conventional chromatography methods had previously failed [16,17]. Since then, CHT has been gradually incorporated into industrial-scale processes for biologics manufacturing including mAb products. Because CHT is sensitive to low pH, which causes calcium phosphate to dissolve, ceramic fluoroapatite (CFT) was developed to improve resin pH tolerance [30]. Two different strategies have been reported for CHT separation including a sodium chloride salt gradient (cation exchange dominant mechanism) and sodium phosphate (mix-mode balanced mechanism) elution methods [16,17,30–32].

We first screened the binding capacity of four different resins: CHT Type I and II, CFT Type I and II, and found that the CFT type I resin showed a distinctive shallow breakthrough curve indicating mass transport restriction (Fig. 5). From the elution strategy study we found that the sodium phosphate (mixed-mode) approach was easier to adapt to a step elution method than was the sodium chloride elution approach. Therefore, a mixed-mode balanced elution methodology was used for all subsequent separation resolution studies. A resolution study on the rest of three resins demonstrated that CHT-II and CFT-II behave similarly and have better resolution than CHT-I at their optimal operating conditions. However, CHT-I showed higher binding capacity and was relatively more robust in operating process parameters (Table 5). An interesting observation during a DSP process train study was that CHT-I performance was very consistent under the same DSP train, but varied considerably with changes in feedstock derived from different platforms (Table 6). Our analysis revealed that although all three mixed-mode resins effectively resolve aggregates, the different mixed-mode chromatographic steps have a unique capability to remove different species of HMW aggregate impurities. As shown by SEC analysis (Fig. 6), the ProA elution product pool contains three populations of high molecular weight aggregate species defined as HMW-peaks A–C (Fig. 6A). Conventional AEX was effective in removing the highest molecular weight impurities (peak labeled as HMW-A in Fig. 6B), while conventional CEX was able to partially reduce all three species (Fig. 6C). However, the three mixed-mode resins all show better separation resolution than do the conventional ion exchange resins. The mixed-mode resin MEP HyperCel, when used as a post-ProA intermediate purification step, was effective at reducing all three impurity peaks HMW-A–C, and especially was able to abolish the HMW-A and -C species (Fig. 6D). Capto adhere, which was also used as a post-ProA intermediate purification step, effectively reduced the high molecular weight peaks (HMW-A and -B), while having little effect on HMW-C (Fig. 6E). CHT, which was used as a final polishing step, was demonstrated

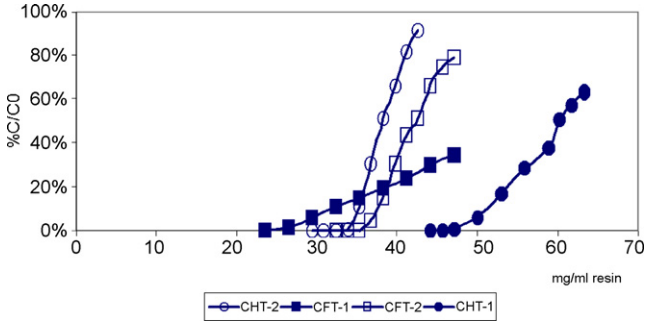

Fig. 5. Neutralized ProA eluate pool of hMab IgG1 was buffer exchanged using a UF/DF system to 10 mM sodium phosphate buffer at pH 6.5 to prepare the loading material for the CHT/CFT resin binding capacity study. The study was carried out using 0.5 cm diameter columns with packed bed heights of approximately 10 cm on an AKTA chromatography system at a constant residence time of 3 min. Breakthrough curves are plotted as $\%[C/C_0]$ against IgG loaded in mg/ml resin (C is IgG concentration in flow-through fraction and C_0 is IgG concentration in the load).

Table 5
HMW reduction of CHT/CFT resins at optimal operation condition.

	CHT-I	CHT-II	CFT-II
Loading (mg IgG/ml resin)[a]	38	25	28
HMW reduction % by SEC-HPLC	56%	80%	75%

[a] Optimal loading is defined as 80% of 5% breakthrough.

Table 6
CHT performance variation as final polishing step in DSP platform study.

DSP platform	The number of runs	Step yield%	HMW reduction%
Generic platform	4	82–87%	67–72%
4 different platforms	4	79–90%	42.8–78.3%

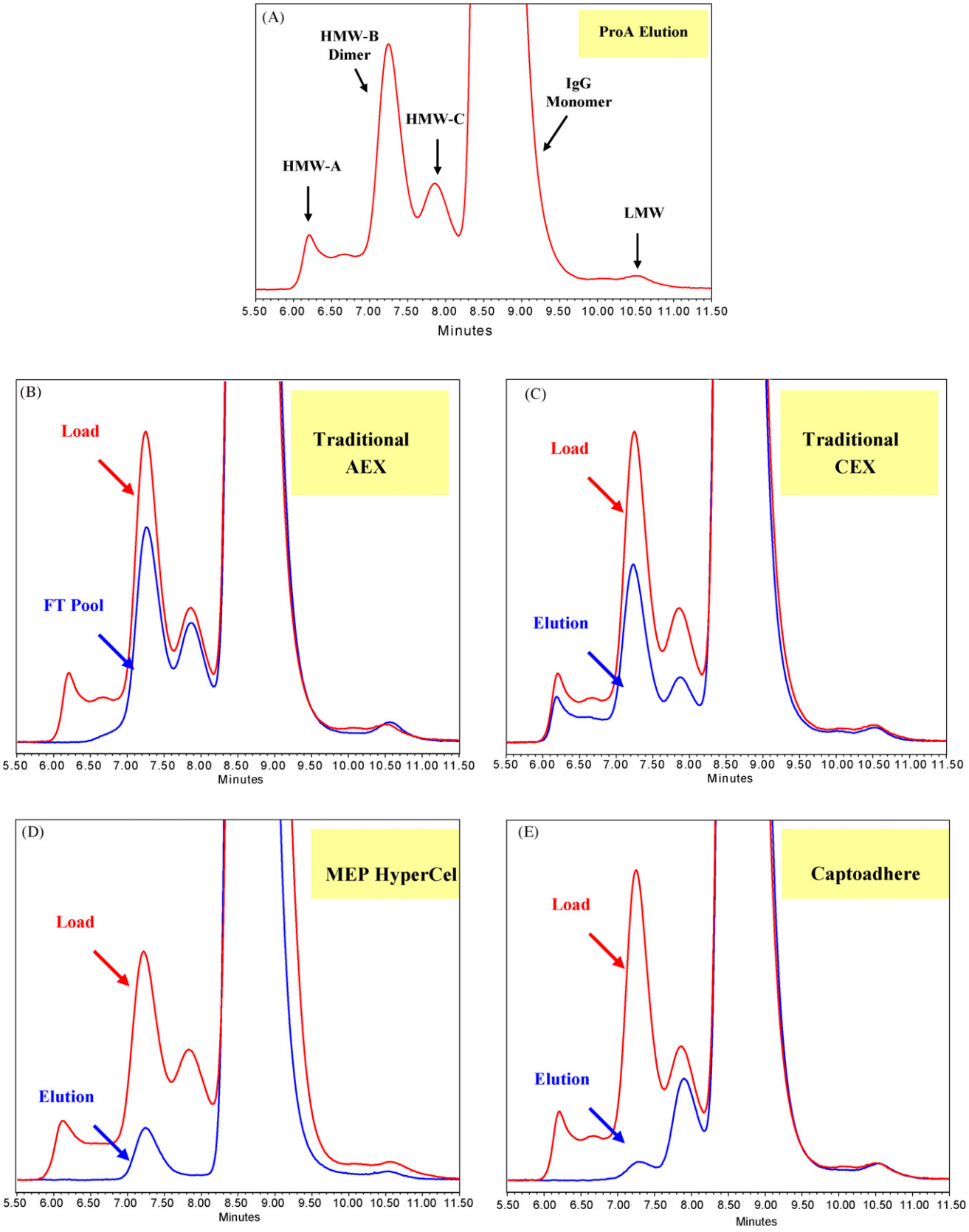

Fig. 6. Comparison of three mix-mode resins for their selective resolution of aggregate species removal. Analytical SEC-HPLC results show ProA elution pool of hMab IgG1 contains three populations of high molecular weight (HMW) aggregates (A). SEC chromatograms in (B)–(F) show both load (red) and elution/flow-through (blue) product pool for each chromatographic purification step. Different species of HMW impurity peaks were removed based on separation resolution of the individual chromatograph technique under the test conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

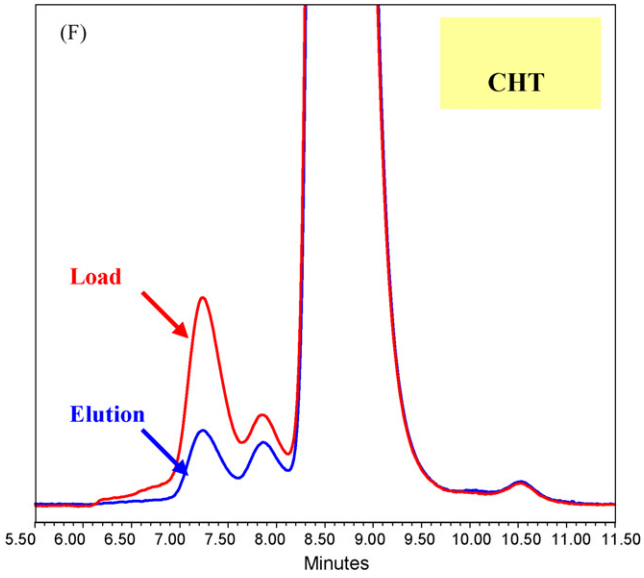

Fig. 6. (*Continued*).

to be capable of further decreasing the levels of both HMW-B and -C (Fig. 6F).

These findings underscore the need to intelligently place the individual purification blocks especially the mixed-mode resins in DSP design in order to achieve maximum product quality. In our case, one of the hMab IgG1 products contained relatively high levels of soluble aggregates after ProA capture step. The generic ProA-UF/DF-AEX-CEX-VF-UF/DF platform failed to deliver product having sufficiently low levels of impurities, particularly HMW, to meet our manufacturing specification. In addition, post-ProA in-process UF/DF resulted in a serious turbidity problem for which it was difficult to develop a robust process to address. By switching to a ProA-MEP-CHT DSP platform we were able to eliminate the in-process UF/DF step and deliver a high quality drug product.

4. Conclusion

Mixed-mode chromatography has become one of the major branches of the chromatography separation field. Although mixed-mode mechanisms may be involved in every chromatographic separation through protein interaction with ligand, linker and matrix, the resins commonly referred to as mixed-mode resins are usually those with designed multi-modal operating ligands [17,32]. Systematic studies were designed to investigate the value of mixed-mode resins in the monoclonal antibody purification process. We discovered that by operating the HCIC resin MEP HyperCel through a HIC dominant elution strategy, MEP HyperCel can not only considerably increase the impurity reduction, but also directly connect to subsequent chromatographic steps including CHT without the requirement for complicated sample manipulation. With this separation strategy, MEP HyperCel was demonstrated to have excellent properties in removing a high molecular weight impurity peak (HMW-peak C) with a retention time that falls between monomer and dimer in analytical SEC-HPLC. The characterization of the peak C material is on-going. In addition, we also found that use of non-ProA chromatography resins such as MEP HyperCel can reduce insoluble aggregate formation in those cases where mAb products are negatively impacted by the generic platform ProA capture process. Through a comparison study with traditional AEX resins, we highlight the unique separation features of Capto adhere. It not only provides an aggregate reduction benefit, in particular for dimers, but also gives a different CHO HCP binding pattern and removal mechanism to allow improved product pool through appropriate peak cutting. In the study of CHT, four different types of CHT/CFT resins were compared, with CHT-I found to be a powerful tool for impurity removal, including aggregates. CHT can lower both dimer and HMW-peak C but the reduction is not as significant as MEP HyperCel for peak C or Capto adhere for dimer peak under the test conditions. Therefore, intelligently selecting appropriate chromatography resins during the design of the DSP process is critical to obtain the best overall process outcome. Our new platform design of ProA-MEP-CHT is able to respond to the challenge of high levels of aggregation, where the generic platform failed to deliver a drug product that met specification. This new DSP design eliminates in-process UF/DF and presents high reduction resolution for all three types of aggregates.

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References

[1] U. Gottschalk, Suppl. Biopharm. Int. (June) (2006) 8.
[2] G. Jagschies, A. Gronberg, T. Bjorkman, K. Lacki, H.J. Johansson, Suppl. Biopharm. Int. (June) (2006) 10.
[3] U. Gottschalk, Suppl. Biopharm. Int. (March) (2009) 7.
[4] M. Trexler-Schmidt, S. Sze-Khoo, A.R. Cothran, B.Q. Thai, A. Sargis, B. Lebreton, B. Kelley, G.S. Blank, Suppl. Biopharm. Int. (March) (2009) 8.
[5] S. Hober, K. Nord, M. Linhult, J. Chromatogr. B 848 (2007) 40.
[6] K. Swinnen, A. Krul, I. Van Goidsenhoven, N. Van Tichelt, A. Roosen, K. Van Houdt, J. Chromatogr. B 848 (2007) 97.
[7] S. Aldington, J. Bonnerjea, J. Chromatogr. B 848 (2007) 64.
[8] A. Shukla, B. Hubbard, T. Tressel, S. Guhan, D. Low, J. Chromatogr. B 848 (2007) 28.
[9] J. Chen, October Presentation on IBC International Bioprocessing Exhibition and Conference, Boston, USA, 2007.
[10] Y. Zhang, November Presentation on Wilbio International Filtration Biological Products Conference 2008, Santa Barbara, CA, USA, 2008.
[11] B. Demeule, R. Gurny, T. Arvinte, Eur. J. Pharm. Biopharm. 62 (2006) 121.
[12] J. Glynn, T. hagerty, T. Pabst, G. Annathur, K. Thomas, P. Johnson, N. Ramasubramanyan, P. Mensah, Suppl. Biopharm. Int. (March) (2009) 16.
[13] F. Li, J.X. Zhou, X. Yang, T. Tressel, B. Lee, BioProcess. J. (Winter) (2006) 16.

[14] S. Franklin, BioProcess Int. (May) (2004) 56.
[15] W. Schwartz, J. Jiao, J. Ford, D. Conrad, J. Hamel, P. Santambien, L. Bradbury, T. Robin, BioProcess. J. (September/October) (2004) 53.
[16] P. Gagnon, P. Ng, C. Aberrin, J. Zhen, J. He, H. Mekosh, L. Cummings, R. Richieri, S. Zaidi, BioProcess Int. 4 (2002) 50.
[17] P. Gagnon, in: U. Gottschalk (Ed.), Purification of Monoclonal Antibodies by Mixed-Mode Chromatography, in Process Scale Purification of Antibodies, John Wiley and Sons, New York, 2009, p. 125.
[18] J. Chen, J. Tetrault, A. Ley, J. Chromatogr. A 1177 (2008) 272.
[19] W. Wang, Y.J. Wang, D.Q. Wang, Int. J. Pharm. 347 (2008) 31.
[20] Pall Life Sciences, MEP resin instruction manual.
[21] J. Spencer, E. Boschetti, S. Bengio, BioProcess. J. (May/June) (2003) 67.
[22] G.T. Weatherly, A. Bouvier, D.D. Lydiard, J. Chapline, I. Henderson, J.L. Schrimsher, S.R. Shepard, J. Chromatogr. A 952 (2002) 99.
[23] A. Lees, A. Topping, A. Razzaq, K. reiter, A. Acosta, BioProcess Int. (February) (2009) 42.
[24] W. Schwartz, D. Judd, M. Wysocki, L. Guerrier, E. Birck-Wilson, E. Boschetti, J. Chromatogr. A 908 (2001) 251.
[25] E. Boschetti, Trends Biotechnol. 20 (2002) 333.
[26] G.M. Ferreira, J. Dembecki, K. Patel, A. Arunakumari, BioPharm. Int. (May) (2007) 32.
[27] S. Ghose, B. Hubbard, S. Cramer, Biotechnol. Prog. 21 (2005) 498.
[28] GE Healthcare Inc., Capto adhere Instruction manual.
[29] K. Eriksson, A. Ljunglof, G. Rodrigo, E. Brekkan, BioProcess Int. (Febrauary) (2009) 52.
[30] Bio-Rad laboratory, CHT/CFT Instruction manual.
[31] P. Ng, A. Cohen, P. Gagnon, Gene Eng. News 26 (2006) 14.
[32] M. Rios, Pharmceut. Technol. (May) (2007) 40.