Journal of Chromatography A, 1217 (2010) 235–242

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Industrial case study: Evaluation of a mixed-mode resin for selective capture of a human growth factor recombinantly expressed in *E. coli*

Kimberly A. Kaleas, Charles H. Schmelzer, Shelly A. Pizarro*

Genentech, Inc. Process Research and Development, 1 DNA Way, MS 75, South San Francisco, CA 94080, USA

ARTICLE INFO

Article history:
Available online 17 July 2009

Keywords:
Capto MMC
Mixed-mode chromatography
Arginine
Angiogenesis

ABSTRACT

Mixed-mode chromatography resins are gaining popularity as effective purification tools for challenging feedstocks. This study presents the development of an industrial application to selectively capture recombinant human vascular endothelial growth factor (rhVEGF) on Capto MMC from an alkaline feedstock. Capto MMC resin contains a ligand that has the potential to participate in ionic, hydrophobic, and hydrogen boding interactions with proteins and is coupled to a highly cross-linked agarose bead matrix. VEGF is a key growth factor involved in angiogenesis and has therapeutic applications for wound healing. In this process, it is expressed in *Escherichia coli* as inclusion bodies. Solids are harvested from the cell lysate, and the rhVEGF is solubilized and refolded at pH 9.8 in the presence of urea and redox reagents. The unique mixed-mode characteristics of Capto MMC enabled capture of this basic protein with minimal load conditioning and delivered a concentrated pool for downstream processing with >95% yields while reducing host cell protein content to <1.2%. This study explores the impact of loading conditions and residence time on the dynamic binding capacity as well as the development of elution conditions for optimal purification performance. After evaluating various elution buffers, L-arginine HCl was shown to be an effective eluting agent for rhVEGF desorption from the Capto MMC mixed-mode resin since it successfully disrupted the multiple interactions between the resin and rhVEGF. The lab scale effort produced a robust chromatography step that was successfully implemented at commercial manufacturing scale.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Column liquid chromatography is the most prevalent technique used for downstream purification in the biopharmaceutical industry. Several types of adsorptive chromatography are employed to purify a wide variety of biological materials. The most commonly used and extensively studied techniques include affinity-, ion exchange-, and hydrophobic interaction-chromatography. Purification with these techniques relies largely upon a single dominant mode of interaction between the mobile and stationary (immobilized ligand) phase [1]. As the industry evolves new purification tools are being developed to face emerging challenges, including the need for more selective chromatographic media, improved resolution, and/or tolerance of feedstock conditions. Mixed-mode interaction chromatography combines more than one mode of adsorption [1,2] and potentially offers capabilities to accommodate purification needs that single-mode methods cannot.

Recent advancements in synthesizing multifunctional ligands have led to a variety of commercially available resins developed with functional groups to target specific combinations of protein–ligand interactions [3,4]. These resins may provide additional flexibility in designing purification processes because of the ligand's ability to interact with the target molecule through multiple intermolecular forces. Specifically, mixed-mode resins have displayed wider operating ranges in the load phase than traditional ion exchange resins by successfully capturing proteins to a high binding capacity in high ionic strength feedstocks [3–6]. These resins have also been employed in expanded bed adsorption processes to capture the target protein directly from a moderate ionic strength feedstock without the need of dilution or other additions [2], including an extracellular protease [7] and the nattokinase enzyme [8] from microbial fermentations. However, the increased complexity of the protein–ligand interaction may pose additional challenges for method development of the desorption phases. Elution behaviors have been reported to be more challenging and the most successful buffers are pluripotent in nature so that they are able to simultaneously modulate two or more kinds of interactions between a solute and a solid phase [9]. Buffers such as 1 M sodium chloride or 20% ethanol were insufficient to elute the bound protein successfully from a mixed-mode resin while buffers with urea or L-arginine HCl were effective [10,11]; this study further supports these observations. Several papers have also shown that desorption of the target protein from a mixed-mode ligand can be induced with electrostatic charge repulsion while accompanied by a shift in pH

* Corresponding author. Tel.: +1 650 225 5937.
E-mail address: spizarro@gene.com (S.A. Pizarro).

0021-9673/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2009.07.023

[2,3]. After evaluating various elution buffers, L-arginine HCl was shown to be an effective eluting agent for rhVEGF desorption from the Capto MMC mixed-mode resin.

The main driver throughout this study was to develop a commercial process for production of rhVEGF for therapeutic applications. The unique characteristics of mixed-mode resins were well-matched with the molecular properties of rhVEGF and provided an effective solution to a challenging feedstock. VEGF is an important protein in the regulation of the angiogenic cascade [12]. It is a potent and specific endothelial cell mitogen, involved in the development of the vascular system and differentiation of endothelial cells [13,14] and thus has potential for accelerating wound healing [15,16]. It is a homodimeric growth factor and its most abundant and predominantly active form [13,14] consists of two 165-amino acid monomers, each containing 7 pairs of intrachain disulfide bonds, that are covalently linked by 2 interchain disulfide bonds [17]. $VEGF_{165}$ has a molecular weight of ~38 kDa, theoretical p*I* of 8.6, and can be cleaved by plasmin to yield two domains: (1) a receptor-binding domain (residues 1–110, disulfide-linked homodimer) with affinity for the kinase domain receptor (KDR) and the *fms*-like tyrosine kinase receptor (FLT-1) and (2) a heparin-binding domain (residues 111–165) [13,14]. The high electropositivity of the heparin-binding domain suggested that it should have a strong attraction to a negatively charged ligand while the largely hydrophobic nature of the receptor-binding site could be further exploited for selectivity on the mixed-mode resin.

A particular challenge for this chromatographic capture was the feedstock loading conditions. The rhVEGF was expressed in bacterial inclusion bodies and refolded during recovery at pH 9.8 in a buffer containing 1 M urea and redox reagents. Because mixed-mode resins have been shown to bind proteins from challenging feedstocks, Capto MMC was evaluated for capture of rhVEGF from the refold pool. Fig. 1 shows that the Capto MMC ligand [18] is comprised of multiple functional groups (carboxyl, phenyl, amide) as well as a mercapto group, used for attaching the ligand to the activated matrix [3], which introduces a certain level of thiophilicity. Due to the mixed-mode nature of the Capto MMC ligand, different types of interactions, such as cation exchange, hydrophobic and hydrogen bonding interactions are possible [18,19]. These interactions can work in an independent or cooperative fashion, but strength of individual interactions depends on overall process conditions and characteristics of the target molecule. In this paper, the binding behaviors and challenges for both load and elution conditions will be discussed for the Capto MMC resin and rhVEGF. The trends observed during the load phase development indicates that the interactions between the protein and ligand are driven by more than just electrostatic forces since rhVEGF is binding to the resin at pH values greater than its p*I*. Likewise, the elution phase development results show that a combination of non-covalent forces (ionic, hydrophobic, and hydrogen bonding) are required to successfully elute the rhVEGF from the mixed-mode ligand. The lab scale effort produced a robust chromatography step that was implemented at both pilot and commercial manufacturing scales. The success of this industrial application shows that mixed-mode interaction chromatography is a powerful and scalable purification tool for challenging feedstocks.

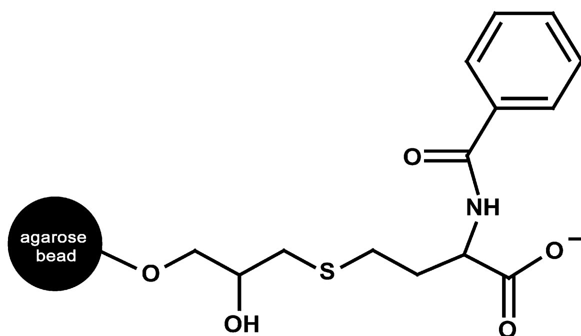

Fig. 1. Structure of the Capto MMC mixed-mode ligand attached to the highly cross-linked agarose matrix [18].

2. Materials and methods

2.1. Feedstocks

rhVEGF is produced by *Escherichia coli* as inclusion bodies and subsequently refolded to achieve its biologically active confirmation. The *E. coli* host strain, 64D1 (W3110 Δ*fhuA* (Δ*tonA*) *ptr3 lacIq lacL8 ompT*Δ(*nmpc-fepE*) Δ*degP ilvG2096* (IlvG+; Val^r) *malE rhaR*), was transformed with a pBR322-based plasmid containing the gene encoding protein. Expression of rhVEGF was controlled by the *phoA* promoter with induction occurring upon the depletion of inorganic phosphate [20]. *E. coli* broth from a 1 kL fermentation was homogenized and rhVEGF-containing inclusion bodies were collected by centrifugation. The refolding buffer contained 2-(N-cyclohexylamino)ethane sulfonic acid (CHES), dithiotreitol (DTT), ethylene diaminetetraacetic acid (EDTA), urea, L-arginine, and L-cysteine, from qualified chemical suppliers Mallindkrodt (Hazelwood, MO) or Ajinomoto USA (Raleigh, NC). The active components of the prepared refold buffer consisted of 2 mM DTT, 15 mM L-cysteine, and 1 M urea. 10 mM CHES and 100 mM L-arginine are present to maintain the buffer pH target upon pellet addition but are not deemed critical reagents for refolding. The refold step was performed at pH 9.8 with constant air sparging and agitation [21] and was processed downstream within 48 h. Prior to capture on the Capto MMC resin, the refold pool was conditioned with 1% (v/v) Triton X-100 (Sigma–Aldrich, St. Louis, MO) and adjusted to the desired load pH with acetic acid. The ionic strength of this material was relatively low (≤5 mS/cm). The adjusted refold pool was clarified by centrifugation and filtration to remove residual solids and submicron particles.

2.2. Mixed-mode chromatography

Capto MMC resin was purchased from GE Healthcare (Uppsala, Sweden). The ligand structure is bound to a highly cross-linked agarose backbone (mean bead 75 μ size) and is shown in Fig. 1. Lab scale chromatography development utilized 0.66 cm diameter Omnifit columns (Sigma–Aldrich, St. Louis, MO) with two bed heights (10, 20 cm) while large scale runs were conducted at a bed height of 20 cm and column diameters of 20 and 63 cm. Lab scale experiments were performed using an ÄKTA Explorer^{TM} 100 Liquid Chromatography system (GE Healthcare, Uppsala, Sweden) and large scale runs used an automated industrial chromatography skid.

To perform an experiment, the column was equilibrated with 25 mM CHES, pH 8.7, for 5 column volumes (CV). The clarified refold pool was adjusted to pH 8.7 with acetic acid, except for some cases where the dynamic binding capacity was examined as a function of load pH and values ranged from 8.5 to 10. The load was followed by a 5 CV wash with equilibration buffer and a second wash phase prior to the elution phase. The examined buffers for the second wash and elution phases are described in the discussion section. The final chromatography buffers were 0.20 M L-arginine HCl and 0.25 M L-arginine in 25 mM HEPES, pH 9.0 (5 CV) for the second wash phase and 0.85 M L-arginine HCl and 0.05 M L-arginine in 25 mM HEPES, pH 7.5 (5 CV) for the elution phase. The column was regenerated with 2 M guanidine HCl in 100 mM Tris, pH 10 (3 CV), and sanitized with 0.5 M NaOH (5 CV). The column was stored in 0.1 M NaOH. Chromatography was performed at room temperature. The

residence time during chromatography was 2.7 min, except where otherwise indicated. All buffer components were of analytical grade and purchased from vendors validated for commercial production.

2.3. Measurement of dynamic binding capacity

The dynamic binding capacity (DBC) of the Capto MMC resin at 1% breakthrough for rhVEGF was determined as a function of either residence time or load pH. Using clarified refold pool (pH 9.8) the DBC was studied as a function of residence time, testing 1, 2, and 6 min. To examine the effect of load pH on DBC, the refold pool was adjusted to pH 8.5, 9.0 and 9.5 with acetic acid or pH 10 with sodium hydroxide. Each pH adjusted refold pool was applied to the column using a 1 min residence time. During the load phase, flow through fractions were collected every 0.5 CV and analyzed by CEX-HPLC to discriminate low breakthrough concentrations of rhVEGF from over-whelming contaminant loads. The sensitivity of the CEX-HPLC assay allowed for breakthrough determinations at levels <5%. Dynamic binding capacities were calculated according to Eq. (1):

$$\mathrm{DBC}_{x\%\mathrm{BT}} = \frac{(V_{x\%\mathrm{BT}} - V_0) \times C_0}{V_c} \quad (1)$$

where $\mathrm{DBC}_{x\%\mathrm{BT}}$ is the dynamic binding capacity (mg/mL), $V_{x\%\mathrm{BT}}$ is the volume of protein loaded at x% breakthrough (mL), V_0 is the hold-up volume of the system (mL), C_0 is the load protein concentration (mg/mL), and V_c is the column volume (mL).

2.4. Assays

The amount of properly folded rhVEGF in the Capto MMC load and pool was determined by an analytical cation exchange (CEX) HPLC assay. The CEX-HPLC column, e.g., SP-5PW (TSK gel SP-5PW, 7.5 mm × 75 mm, 10 μ, by Tosoh Biosciences LLC, Tokyo, Japan) is equilibrated at 40 °C in 50 mM sodium phosphate pH 7.5. At a flow rate of 1 mL/min the column is eluted using a linear gradient from 30–50% buffer B (50 mM sodium phosphate, 2 M sodium chloride pH 7.5) over 20 min. The eluant absorbance is monitored at 214 nm. A linear curve between peak area and concentration is made using purified rhVEGF (generated and characterized in-house) to determine the sample rhVEGF concentration.

Reverse phase HPLC (rpHPLC) was used to assess the product quality by determining the amount of product variants in the chromatography pool. Capto MMC elution pools were injected directly onto the rpHPLC column. The rpHPLC column, e.g., Jupiter C18 column (4.6 mm × 250 mm, 5 μ, by Phenomenex, Torrance, CA) is equilibrated at 60 °C in 0.2% trifluoroacetic acid (TFA) and eluted using a linear gradient of 25–45% acetonitrile containing 0.2% TFA over 20 min (flow rate = 1 mL/min). The eluant absorbance was monitored at 214 nm and results are reported as main peak percent.

The *E. coli* host cell protein (ECP) and DNA concentrations were determined by two internally developed assays. The ECP content was measured by a multi-product sandwich enzyme-linked immunosorbent assay (ELISA) and the DNA content was measured by a TaqMan PCR *E. coli* DNA assay.

3. Results and discussion

The main goals for the development of the rhVEGF capture step were to find a resin compatible with the refold pool with minimal adjustment for loading, and to produce a concentrated and stable elution pool for further downstream purification. Economic drivers for capacity and manufacturability (i.e. process time, tank volumes, buffer preparation and composition, etc.) were also a consideration for the commercial application during decision points in the process development. The implementation of the Capto MMC mixed-mode resin resulted in an efficient and robust unit operation that effectively accommodated the challenges of an atypical feedstock to produce an elution pool that was high in yield and compatible with downstream chromatography. The discussion of the purification strategy development is divided into the following topics: (1) identification of the optimal load conditions for maximum capacity and process fit; (2) selection of the elution and wash buffers; and (3) process scale-up and robustness.

3.1. Effect of residence time and load pH on dynamic binding capacity

The refolding pool contained 1 M urea at pH 9.8 and low ionic strength (≤5 mS/cm) therefore compatibility for direct capture on single-mode resins was limited without pool adjustment. Once it was established that Capto MMC was able to directly capture the rhVEGF from the clarified refold pool, our development approach was focused on exploring the protein binding characteristics to maximize resin capacity and satisfy the economic driver for a commercial manufacturing scale process. In particular, the DBC of refolded rhVEGF was investigated as a function of residence time and load pH. When clarified refold pool at pH 9.8 was applied to the column, a DBC of 11 g rhVEGF/L resin was obtained with a 1 min residence time. Evaluating longer exposure times with this feedstock showed that the maximum DBC is obtained at the longest residence time examined (6 min) while the greatest relative gain was observed in going from 1 to 2 min. Specifically, the $\mathrm{DBC}_{1\%\mathrm{BT}}$ increased by 26% from 1 to 2 min of exposure and only 22% in going from 2 to 6 min, indicating an area of diminishing returns at residence times greater than 2 min. These results resemble trends observed on traditional ion exchange media with a porous-based matrix, where the DBC increases at longer residence times due to an increase of intraparticle mass transport [22–24].

Continuing to use a 1 min residence time, the DBC was then studied as a function of load pH and it was observed that the DBC increased as the load pH decreased. The maximum DBC was reached at the lowest pH value examined of pH 8.5, and the greatest increase in DBC was observed in adjusting the load pH from pH 10 to 9.5 (Fig. 2). Even though the trend appears similar to traditional cation exchange chromatography, all experiments were completed at pH values equal to or greater than the molecule's p*I*, when the protein's net charge is neutral or negative. Three factors may play a role in this unique protein–ligand interaction.

First, rhVEGF contains a highly positively charged pocket near its carboxyl-terminal region for binding heparin. This concentration of positive charges may promote local electrostatic interactions despite the net negative charge of the protein at pH 8.5–10 and the negative charge of the carboxylic functional group in the ligand. The heparin-binding region is thought to remain as an intact pocket with a locally positive charge at most pH values. Peptide mapping of this carboxylic domain reveals that it is very basic in nature with an estimated p*I* of ∼11.8 and an amino-acid sequence made up of ∼22% arginine and lysine residues [13]. However, it should also be noted that when the rhVEGF refold pool was applied to a cation exchange resin, SP Sepharose XL containing a sulfopropyl ligand, similar binding behavior above the molecule's p*I* was not observed. In fact, comparable DBC was obtained with an optimal load pH that was ∼2 pH units below the protein's p*I* (data not shown). This illustrates that the electrostatic interactions of the heparin-binding site alone is not the only factor responsible for protein binding to the Capto MMC resin at higher load pH values. Although a molecule's p*I* is often used as a guiding property for binding interactions in traditional ion exchange media [25,26], it cannot by itself be predictive in mixed-mode interactions.

The second and third factors of the rhVEGF structure that may promote protein–ligand binding are hydrophobic and hydrogen

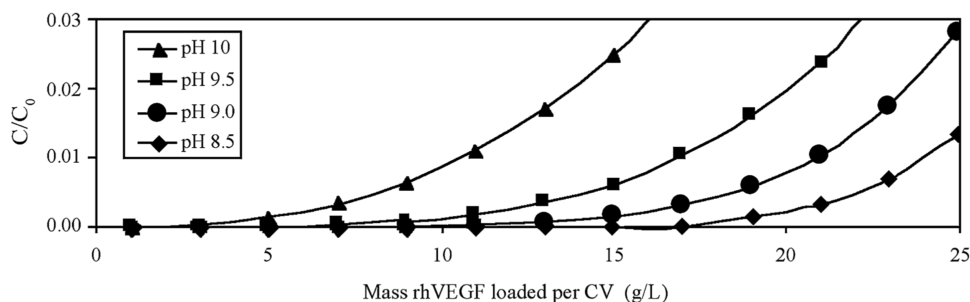

Fig. 2. rhVEGF breakthrough curves at various load pH values on the Capto MMC. Normalized eluent concentration (C/C_0) versus amount loaded (g rhVEGF/L resin).

bonding interactions. It has been observed in other heparin-binding molecules that hydrogen bonding characteristics were a large component of the receptor–ligand binding in addition to electrostatic interactions [27]. The crystal structure of this protein has revealed that the VEGF receptor-binding face to Flt-1 (domain 2) is rather flat, consisting of a several-stranded beta sheet conformation with a high percentage of leucine and isoleucine residues [14]. Intermolecular contacts at this interface are strong and predominately driven by hydrophobic interactions. Specific polar interactions were also identified and these hydrogen bonds were found to be stable and to occur between charged side chains [12]. Ultimately, it is probable that these three forces (electrostatic, hydrophobic, and hydrogen bonds) are not exclusive of each other but rather all may be occurring simultaneously and are responsible for the binding interaction between rhVEGF and Capto MMC. This phenomenon, where the protein interacts with the ligand by multipoint attachment, supports the idea that mixed-mode resins may be more tolerant of loading conditions because protein binding is not reliant on a single-mode attachment.

Operational tradeoffs must be considered to select the final load parameters for a commercial manufacturing scale process. Process considerations such as minimizing load processing time, curtailing potential product degradation, and equipment limitations must be weighed against achieving the maximum DBC. For this process the fastest flow rate was selected to decrease process time and minimize potential product oxidation. The load phase had a residence time of 2.7 min and was completed in ~11 h (15 kL refold). To maximize the DBC, the refold pool was adjusted to pH 8.7 ± 0.1 using a concentrated acetic acid solution that led to a volume increase of 1.5%. The refold pool pH was restricted to greater than pH 8.6 because rhVEGF precipitation occurred at values lower than pH 8.5, causing a potential loss of product. The robustness of the refold pool titration was not a concern as the slope is shallow and reproducible at all scales. Optimization of the upstream steps (i.e. fermentation, inclusion body collection, refold) combined with the target Capto MMC loading parameters described above led to a final load density of ≤30 g rhVEGF/L of resin.

3.2. Development of rhVEGF elution conditions

A number of chromatography experiments were conducted to find a viable elution buffer to provide a good recovery yield and be suitable for use in a commercial process. Table 1 shows a list of the salt-based and organic buffers tested and the resulting rhVEGF recoveries. Further evidence to reinforce the idea of multipoint attachment between rhVEGF and the Capto MMC resin was found during the elution phase development as no single component buffer was able to fully desorb the protein. Elution gradients employing buffers of increasing ionic strength and varying pH to target the disruption of ion exchange interactions, or organic solvents known to specifically disrupt hydrophobic interactions were unsuccessful. Salt-based buffers produced only a small elution peak with extensive trailing that recovered less than 10% rhVEGF. Similar results were reported by Girot et al. [11] where 1 M sodium chloride was insufficient to elute the bound protein when using MBI HyperCel resin to capture an IgG monoclonal antibody (mAb) and by Arakawa et al. [10] when using MEP HyperCel to capture a mAb and Fc-fusion protein. As in HIC, it is possible that strong retention at high salt concentrations reflects enhanced hydrophobic interactions thus preventing the release of rhVEGF from Capto MMC. Buffers made from either organic solvents, urea, or L-arginine were individually tested using a step elution and each produced little to no elution peak, recovering less than 5% rhVEGF and indicating that protein binding is not simply due to hydrophobic interactions.

Table 1
Evaluation of elution buffers for rhVEGF desorption on the Capto MMC.

Mechanism	Elution buffer component (s)[a]	pH range	Conductivity (mmho)	rhVEGF yield (%)
IEX	1 M sodium acetate	6.5–12	50	0.00
	1 M TMAC + 1 M sodium chloride	8.0	85	
	1 M ammonium chloride	9.5	100	<10
	1.5 M sodium chloride	7.0–9.5	120	
HIC	20% propylene glycol	8.0	<2	<5
	20% ethanol	8.0	<2	
	6 M urea	6.5–7.0	<2	
	0.75 M L-arginine	7.5	10	
IEX + HIC	20% propylene glycol + 1.5 M sodium chloride	8.0	65	50–60
	20% ethanol + 1.5 M sodium chloride	8.0	70	
	6 M urea + 1.5 M sodium chloride	6.5–7.0	78	>90
	1 M L-arginine HCl	6.5–7.5	43	
	0.75 M L-arginine + 0.45–0.75 M sodium chloride	9.0–9.5	43–58	

The column was equilibrated with 5 CV of HEPES buffer, pH 7.5. Clarified refold pool was loaded to approximately 20 mg rhVEGF/mL of resin. The elution was completed using a step for the HIC buffers and gradient elution for all others. The residence time was 2.3 min.

[a] All elution buffers contain 25 mM HEPES.

Ultimately, the most successful approach used buffers that combined sodium chloride with agents that disrupt hydrogen bonding or hydrophobic interactions such as organic solvents, urea, and L-arginine. In these conditions rhVEGF eluted with significantly higher yields and remained biologically active, indicating conservation of its native conformation despite the potential denaturing influence of high concentrations of solvents. The combination of sodium chloride and either ethanol or propylene glycol, where each component by itself was minimally effective, improved the elution behavior. The elution peak shape was sharpened and yields increased to 50–60% rhVEGF. A similar effect was observed with the combination of 6 M urea with sodium chloride as well as high concentrations of L-arginine HCl. These last two buffers produced a sharp, compact elution peak resulting in greater than 90% yield. These results make clear that the interaction between rhVEGF and the Capto MMC ligand has multiple components that must be simultaneously disrupted to successfully release the protein. Similarly, Arakawa et al. [10] has reported that the mixed-mode MEP HyperCel resin binds proteins through both polar and hydrophobic interactions with some contribution of electrostatic interaction. Their experiments found that 20% ethanol was ineffective at eluting both mAb and protein contaminants; however, 1–3 M urea without an inorganic component and 1 M L-arginine HCl effectively eluted bound proteins [10].

Interestingly, not all combinations of an organic solvent and salt species result in similar elution profiles and protein recovery. Specifically, ethanol and propylene glycol elution buffers appear less effective at disrupting the protein–ligand interactions of rhVEGF and Capto MMC than urea or L-arginine. All four compounds are capable of competing with hydrophobic interactions and hydrogen bonds between a bound solute (protein) and a mixed-mode ligand, although to different degrees. Propylene glycol and ethanol can participate in hydrogen bonding through their hydroxyl groups but to a lesser degree than the carbonyl group present in urea and the guanidium group in arginine. The higher effectiveness of urea and L-arginine as eluting agents could simply reflect their higher efficiency for suspending hydrophobic interactions, but strongly suggests that their ability to disrupt hydrogen bonds is the key to effective desorption of rhVEGF.

Having established that the two most promising elution buffers for this application were urea with sodium chloride or L-arginine HCl, further investigation was undertaken to examine the protein desorption mechanisms in more depth. Specifically, the minimum buffer concentration for optimal protein recovery was determined and the impurity profiles were compared. A step-wise gradient was conducted for each elution buffer. The L-arginine HCl buffer experiment consisted of five steps from 0 to 1.25 M in 0.25 M increments (Fig. 3a) while the 6 M urea with 1.5 M sodium chloride experiment maintained a constant urea concentration while increasing the sodium chloride concentration in increments of 0.25 M up to 1.5 M (Fig. 4a). Each peak was evaluated for protein recovery, host cell protein content, and protein quality by rpHPLC (Figs. 3b and 4b). As shown in Figs. 3a and 4a, five distinct elution peaks were observed for both elution buffers, peaks A–E eluted during the L-arginine HCl elution from 0.25 to 1.25 M while peaks F–J eluted during the urea/salt elution from 0 to 1.0 M sodium chloride. The majority of rhVEGF was detected in two peaks for both elution buffers (B and C; H and I). It is noted that better separation of host cell proteins and rhVEGF is achieved with L-arginine HCl than the urea/salt buffer. Higher ECP concentrations are obtained in peaks D and E ($\geq$1 M L-arginine HCl) after the majority of rhVEGF has eluted while ECPs co-eluted with rhVEGF throughout peaks F–J. Analysis by rpHPLC of all rhVEGF-containing peaks showed that the quality of the refolded product remained constant and that no differentiation of product variants occurs with elution buffer species or concentration.

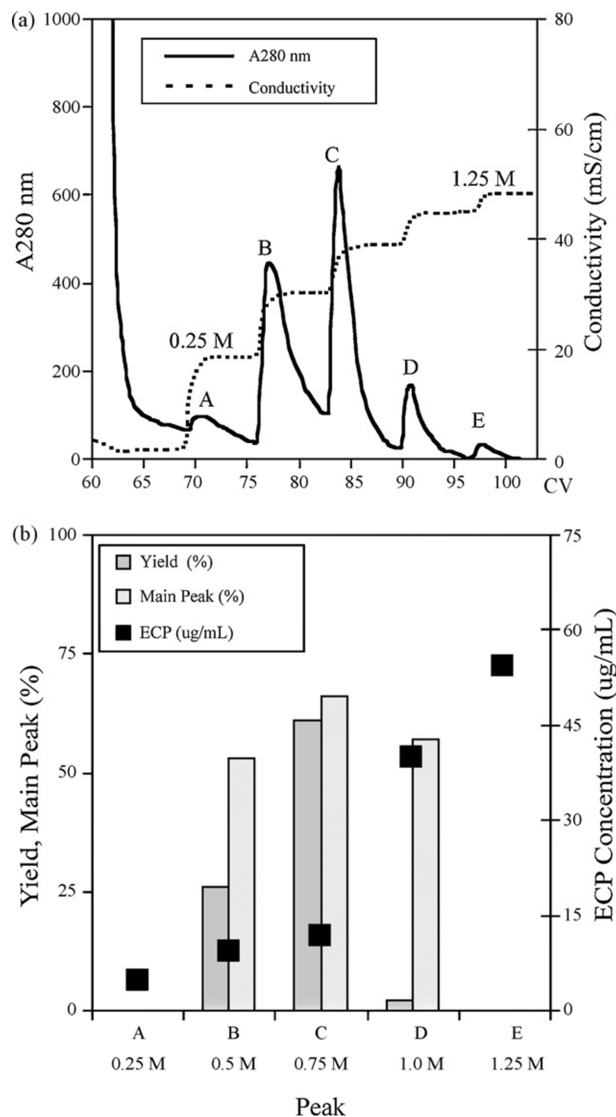

Fig. 3. Resulting (A) chromatographic separation elution profile of rhVEGF using a Capto MMC column and (B) peak analysis. The elution phase consisted of steps from 0 to 1.25 M L-arginine HCl (pH 7.5) in 0.25 M increments.

A small study was undertaken to understand L-arginine HCl's dominant mode of protein desorption by exploring the impact of ionic strength and L-arginine concentration. Two desorption phases were completed per run, where the first buffer contained 0.5 M L-arginine HCl in the absence or presence of sodium acetate (0.6 M) to increase the buffer ionic strength while the second buffer contained 1 M L-arginine HCl. The run without sodium acetate confirms the results from the previous study where 0.5 M L-arginine HCl was not sufficient to fully desorb rhVEGF (Fig. 3a). When the 0.5 M L-arginine HCl buffer was supplemented with sodium acetate to reach a comparable conductivity to the 1 M L-arginine HCl buffer, the resulting chromatogram was identical to the run without acetate (Fig. 5). In short, the increase in conductivity alone did not significantly alter the elution peak shape nor adjust the relative rhVEGF yield of the 0.5 M L-arginine HCl buffer. Higher levels of L-arginine are necessary to disrupt the hydrophobic and polar interactions (i.e. hydrogen bonding) to effectively release this molecule from the Capto MMC resin.

To make a final decision for the elution buffer composition, the factors considered included process performance (i.e. selectivity and yield) as well as manufacturability, such as buffer makeup, com-

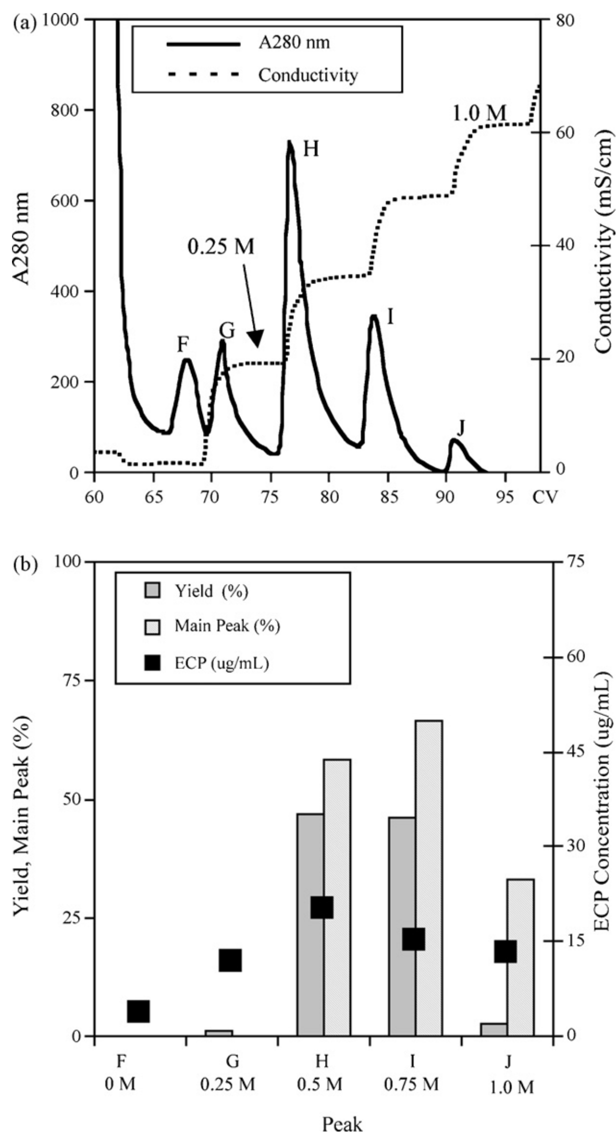

Fig. 4. Resulting (A) chromatographic separation elution profile of rhVEGF using a Capto MMC column and (B) peak analysis. The elution phase maintained a constant 6 M urea concentration while increasing the sodium chloride concentration in increments of 0.25 M up to 1.5 M (pH 7.5). No elution peaks were observed >1.0 M NaCl.

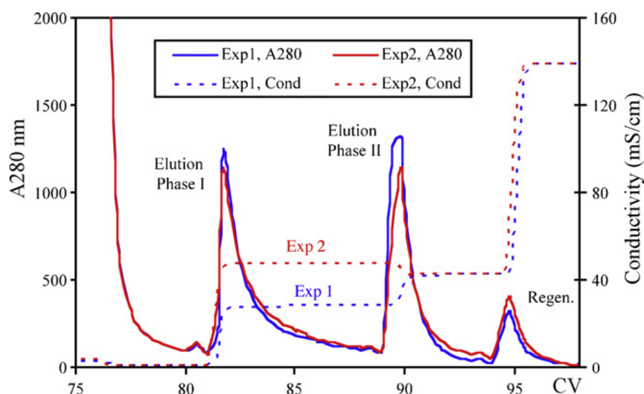

Fig. 5. Comparison of elution profiles of rhVEGF from Capto MMC using two-step elution phases. For the first elution phase, experiment run 1 used 0.5 M L-arginine HCl (28 mS/cm) while experiment run 2 used 0.5 M L-arginine HCl with 0.6 M sodium acetate (47 mS/cm). For the second elution phase, both runs used 1 M L-arginine HCl (43 mS/cm). All buffers were at pH 7.5 and contain 25 mM HEPES.

Table 2
Evaluation of elution buffer molarity.

Elution buffer[a]		Elution pool		
L-Arginine HCl	Conductivity	Volume	rhVEGF yield	ECP
(M)	(mmho)	(CV)	(%)	(ng/mg)
0.7	36	5.5	83	950
0.8	38	3.4	97	2,720
0.9	41	2.9	93	2,090
1.0	43	2.6	94	2,180

Clarified refold pool was loaded to approximately 30 g rhVEGF/L of resin. The column was washed 3 CV of 0.45 M L-arginine, pH 9, for the second wash phase. The residence time was 2 min.

[a] All buffers contain 25 mM HEPES, pH 7.5.

patibility with downstream chromatography, and pool stability. In these operational respects, the L-arginine HCl buffer was superior to the urea/salt buffer. A well-defined zone was discovered between 0.8 and 1.0 M L-arginine HCl that gave consistently reproducible results. In this zone, yields were greater than 90% and impurities were cleared to acceptable limits (Table 2). When starting load ECP concentrations were ≥500,000 ng/mg the host cell protein content was reduced by ≥150-fold. The final elution buffer streamlined the buffer makeup by utilizing the acid and base conjugates so that pH adjustment would be unnecessary, 0.85 M L-arginine HCl, 0.05 M L-arginine in a background of 25 mM HEPES (pH 7.5, 41 mS/cm). An elution buffer pH of 7.5 was selected to streamline load conditioning required for the second chromatography step. Lab scale experiments confirmed a robust range for Capto MMC elution from pH 6.5–9.0 (data not shown).

3.3. *Optimizing impurity removal through a wash step*

The goal of implementing a pre-elution wash phase was to selectively remove host cell impurities from the column while keeping the rhVEGF bound to the resin. Data from the elution buffer development was used to select a limited subset of buffers for further investigation as a pre-elution wash buffer. In addition to their effectiveness as wash buffers we considered manufacturing issues such as ease of preparation, disposal, and corrosiveness. Buffers composed of L-arginine and L-arginine HCl were found to be the best candidates. As shown in Fig. 6, both removed a large amount of protein during the wash phase, however, 0.75 M L-arginine (pH 9.0) prematurely eluted ∼5% rhVEGF. A combination of L-arginine and L-arginine HCl embodied the best process and manufacturability characteristics. The final wash buffer of 0.25 M L-arginine, 0.20 M L-arginine HCl, 25 mM HEPES (pH 9.0) reduced ECP concentrations in the pool by approximately 25%.

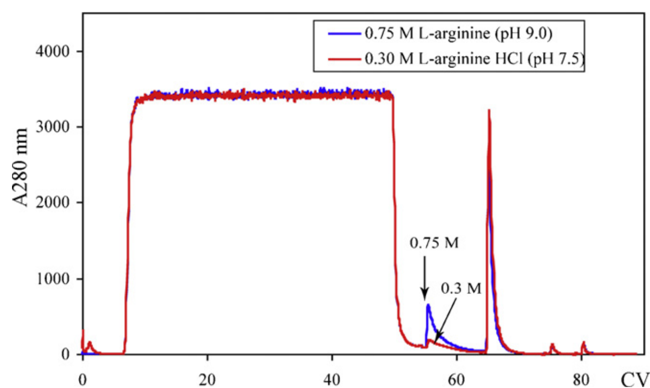

Fig. 6. Comparison of Capto MMC washes.

Table 3
Summary of the purification of rhVEGF at lab, pilot, and manufacturing-scales.

Diameter	Load		Elution pool			
(cm)	Feedstock	Density	Yield	ECP	DNA	Volume
		(g/L)	(%)	(ng/mg)	(ng/mg)	(CV)
0.66	A	26	81	1,710	<0.001	2.3
	B	19	99	11,100	0.14	2.1
	B	30	91	4,130	0.11	2.2
	C	30	95	5,480	1.58	2.0
	D	21	96	1,940	0.01	1.6
	E	30	88	1,910	0.13	1.6
	F[a]	26	99	2,170	0.04	1.3
20	A	26	97	2,290	0.90	2.4
	B	18	100	12,100	2.6	2.5
	C	31	89	4,780	2.0	2.3
63	D	21	100	3,670	0.06	1.8
	E	29	98	2,350	0.26	1.9
	F[a]	26	95	3,240	0.02	1.4

[a] Elution flow rate decreased 75% during elution phase due to high pressure on pool inlet filter. Lab scale run performed to mimic manufacturing process.

3.4. Process scale-up and robustness

The Capto MMC chromatographic process developed at lab scale was successfully implemented at both pilot and manufacturing scales. To maintain the process integrity, the bed height (20 cm), residence time (2.7 min), and elution pooling parameters (0.5 OD start to 0.7 OD end) were held constant for all three scales while varying the column diameter [28]. For each feedstock tested, all scales showed similar chromatograms, impurity profiles, and protein recovery demonstrating process scalability (Table 3). Regardless of significant differences in feedstock ECP concentration, the Capto MMC step produced pool ECP concentrations consistently <5500 ng/mg. The elution pool volume appears to have a dependence on the process scale and the feedstock. For example, elution pool volumes at the pilot plant scale with a 20 cm column diameter were 12% larger than obtained at lab scale (0.66 cm dia). Manufacturing scale elution pools with a 63 cm column diameter were 13% larger than achieved at lab scale. Pool volume differences may be attributed to column packing differences and/or skid variations, including automation, pipe configurations, and buffer mixing within line hold-up volumes, etc. [28,29]. Feedstock differences also appear to impact the elution pool volume. Although all fermentations were completed at 1 kL scale, runs D–F resulted in different upstream performance (i.e. rhVEGF titer) and displayed smaller elution volumes than feedstocks A–C. At lab scale this resulted in a variation of 28% in pool size. In all cases, the elution peak is compact and less than 2.5 CVs which is an acceptable and manageable range at large scale, reducing the conditioned load volume by greater than 75-fold. Feedstock B was noted to have a higher impurity level at lower load densities, however, this was consistent between scales and was cleared downstream to target levels. A robust load density range was established from 18 to 31 g rhVEGF/L resin.

Additional lab scale studies of multiple resin lots demonstrated that the mixed-mode resin was reliable, producing consistent chromatograms and comparable elution pools at target operating conditions. Preliminary lab scale column lifetime studies tested up to 50 cycles with no adverse effects on column performance and confirmed effective cleaning procedures that reduced the total carryover protein and DNA to well below acceptable levels (data not shown).

4. Concluding remarks

This industrial case study presents an example of how mixed-mode interaction chromatography can effectively meet the challenges of an atypical feedstock to produce a unit operation that is suitable as a commercial process. The unique functionality of the Capto MMC mixed-mode ligand showed significant advantage over single-mode media as rhVEGF was captured from a refold pool at pH 8.5–10 and low ionic strength (conductivity $\leq$5 mS/cm) with minimal load conditioning. This illustrates the ability of mixed-mode resins to expand the loading operating space beyond traditional methods and enhance manufacturability. Development of optimal load and elution conditions at lab scale resulted in a robust and scalable chromatography step that delivered comparable recovery and purity at pilot and manufacturing scales. When performed at target conditions, the Capto MMC purification step removes host cell protein levels to <1.2% and reduces *E. coli* DNA to <10 ng/mg with yields >95%. The purification step is completed in ~13 h and reduces the refold pool volume by 75-fold. Although method development with mixed-mode resins is further challenged by the ability of any process variable to affect multiple kinds of interactions; thorough development of these resins and understanding of the target molecule may result in practical utility for many applications and streamline future development efforts [9]. In the present case, rhVEGF adsorption and desorption conditions from Capto MMC were found to be due to a combination of electrostatic, hydrophobic, and hydrogen bonding interactions. This proved advantageous in being able to capture rhVEGF at pH values greater than its p*I* and in developing a robust and simple elution buffer that met process performance targets as well as providing ease of operation in commercial manufacturing.

Acknowledgements

We are grateful to many people and groups at Genentech, Inc. that enabled this study—a select few are noted here. Ailen Sanchez and Rachel Adams for supplying feedstock; Susan Gruerman as well as Analytical Operations for assay support; Philip Chung and the pilot plant recovery facility for implementation at pilot scale; and the SSF Bacterial Production Operations teams for implementation at manufacturing scale.

References

[1] L.W. McLaughlin, Chem. Rev. 89 (1989) 309.
[2] D. Gao, D.Q. Lin, S.J. Yao, J. Chromatogr. B 859 (2007) 16.
[3] B.L. Johansson, M. Belew, S. Eriksson, G. Glad, O. Lind, J.L. Maloisel, N. Norrman, J. Chromatogr. A 1016 (2003) 35.
[4] B.L. Johansson, M. Belew, S. Eriksson, G. Glad, O. Lind, J.L. Maloisel, N. Norrman, J. Chromatogr. A 1016 (2003) 21.
[5] P. Li, G. Xiu, V.G. Mata, C.A. Grande, A.E. Rodrigues, Biotechnol. Bioeng. 94 (2006) 1155.
[6] S.C. Burton, N.W. Haggarty, D.R. Harding, Biotechnol. Bioeng. 56 (1997) 45.
[7] G.E. Hamilton, F. Luechau, S.C. Burton, A. Lyddiatt, J. Biotechnol. 79 (2000) 103.
[8] M.-H. Lu, D.-Q. Lin, Y.-C. Wu, J.-X. Yun, L.-H. Mei, S.-J. Yao, Biotechnol. Bioprocess Eng. 10 (2005) 128.
[9] P. Gagnon, Curr. Pharm. Biotechnol. 10 (2009) 434.
[10] T. Arakawa, Y. Kita, H. Sato, D. Ejima, Protein Expr. Purif. 63 (2009) 158.
[11] P. Girot, E. Averty, I. Flayeux, E. Boschetti, J. Chromatogr. B 808 (2004) 25.
[12] B.A. Horta, J.J. Cirino, R.B. de Alencastro, J. Mol. Graph Model 26 (2008) 1091.
[13] R.G. Keck, L. Berleau, R. Harris, B.A. Keyt, Arch. Biochem. Biophys. 344 (1997) 103.
[14] C. Wiesmann, G. Fuh, H.W. Christinger, C. Eigenbrot, J.A. Wells, A.M. de Vos, Cell 91 (1997) 695.
[15] L.F. Brown, K.T. Yeo, B. Berse, T.K. Yeo, D.R. Senger, H.F. Dvorak, L. van de Water, J. Exp. Med. 176 (1992) 1375.
[16] N.N. Nissen, P.J. Polverini, A.E. Koch, M.V. Volin, R.L. Gamelli, L.A. DiPietro, Am. J. Pathol. 152 (1998) 1445.
[17] N. Ferrara, K.A. Houck, L.B. Jakeman, J. Winer, D.W. Leung, J. Cell. Biochem. 47 (1991) 211.
[18] GE Healthcare, Data File 11-035-45AA (2005).
[19] J.-L. Maloisel, PDA Emerging Manufacturing and Quality Control Technologies Global Conference, San Diego, CA, 2007.
[20] M.W. Laird, G.C. Sampey, K. Johnson, D. Zukauskas, J. Pierre, J.S. Hong, B.A. Cooksey, Y. Li, O. Galperina, J.D. Karwoski, R.N. Burke, Protein Expr. Purif. 39 (2005) 237.
[21] S.A. Pizarro, A. Sanchez, C.H. Schmelzer, US Patent application 20,080,125,580 (2008).

[22] M.C. Stone, G. Carta, J. Chromatogr. A 1146 (2007) 202.
[23] R. Hahn, M. Panzer, E. Hansen, J. Mollerup, A. Jungbauer, Sep. Sci. Technol. 37 (2002) 1545.
[24] R. Hahn, R. Schlegel, A. Jungbauer, J. Chromatogr. B 790 (2003) 35.
[25] F. Dismer, J. Hubbuch, J. Chromatogr. A 1149 (2007) 312.
[26] F. Dismer, M. Petzold, J. Hubbuch, J. Chromatogr. A 1194 (2008) 11.
[27] L.D. Thompson, M.W. Pantoliano, B.A. Springer, Biochemistry 33 (1994) 3831.
[28] A. Rathore, A. Velayudhan, BioPharm Int. 16 (2003) 34.
[29] M. Aleman, E. Noa, A. Tamayo, M. Dubed, S. Padilla, G. Alvarez, D. Geada, L. Dorta, L. Navea, T. Gonzalez, B. Reyes, E.G. Fernandez, R. Valdes, BioPharm Int. 20 (2007) 45.