2006

Studying the exclusion effect in commercial cation exchange resins

Joumana Zeid
San Jose State University

Follow this and additional works at: https://scholarworks.sjsu.edu/etd_theses

Recommended Citation

Zeid, Joumana, "Studying the exclusion effect in commercial cation exchange resins" (2006). *Master's Theses*. 2986.

DOI: <https://doi.org/10.31979/etd.8kyx-hcej>
https://scholarworks.sjsu.edu/etd_theses/2986

This Thesis is brought to you for free and open access by the Master's Theses and Graduate Research at SJSU ScholarWorks. It has been accepted for inclusion in Master's Theses by an authorized administrator of SJSU ScholarWorks. For more information, please contact scholarworks@sjsu.edu.

**STUDYING THE EXCLUSION EFFECT IN COMMERCIAL CATION
EXCHANGE RESINS**

A Thesis

Presented to

The Faculty of the Department of Chemical and Materials Engineering

San Jose State University

In Partial Fulfillment

Of the Requirements for the Degree

Masters of Science

by

Joumana Zeid

August 2006

UMI Number: 1438601

INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

UMI Microform 1438601

Copyright 2007 by ProQuest Information and Learning Company.

All rights reserved. This microform edition is protected against unauthorized copying under Title 17, United States Code.

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

© 2006

Joumana Zeid

ALL RIGHTS RESERVED

APPROVED FOR THE DEPARTMENT OF CHEMICAL AND
MATERIALS ENGINEERING

Dr. Claire Komives, Associate Professor

Dr. Joseph Pesek, Professor

Chithkala Harinarayan, Senior Engineer
Genentech, Inc.

APPROVED FOR THE UNIVERSITY

ABSTRACT

STUDYING THE EXCLUSION EFFECT COMMERCIAL CATION EXCHANGE RESINS

by Joumana Zeid

The aim of this project was to characterize the exclusion mechanism previously documented in cation exchange resins at Genentech, Inc. through dynamic binding capacities. This project further studied the effect of the exclusion mechanism on purity and yields. To complete the project, SP SepharoseTM Fast Flow (SP FF) and SP SepharoseTM XL (SP XL) resins were tested with two proteins, Mab1 and Mab2.

The presence of the exclusion regime was reconfirmed for both resins with both proteins. Purity was also systematically assessed within both the equilibrium and the exclusion regime. It was has shown that CHOP levels, in ng/ml, in the elution pools follow the dynamic binding capacities. Furthermore, when comparing the two resins investigated, a trade-off between capacity and purity was observed. Higher capacities were obtained with SP XL while SP FF achieved higher CHOP clearance.

ACKNOWLEDGMENTS

I would like to acknowledge my adviser, Dr. Claire Komives, for her supervision and my committee members for their advice and direction. I would also like to thank Chithkala Harinarayan and Robert van Reis for their guidance and continuous probing. Furthermore, I would like to thank Dominique Ziech, Mandakini Sharma, Jean Luo, and Roshan Shrestha for their help and patience.

Table of Contents

List of Figures.....	viii
List of Tables.....	x
CHAPTER 1 INTRODUCTION.....	1
1.1 Biotechnology.....	1
1.2 Biopharmaceutical Manufacturing.....	1
1.3 Ion Exchange Chromatography.....	2
1.4 Exclusion Phenomena.....	4
1.5 Significance.....	6
CHAPTER 2 LITERATURE REVIEW.....	8
2.1 Historical Development.....	8
2.2 Importance of Optimization.....	8
2.3 Critical Parameters Investigated.....	9
2.4 Resin Performance Comparison.....	10
2.5 Resin Performance Trends.....	15
2.6 Summary.....	19
CHAPTER 3 RESEARCH OBJECTIVES.....	21
3.1 Research Objectives.....	21
CHAPTER 4 RESEARCH APPROACH.....	22
4.1 Materials and Methods.....	22
4.2 Experimental Design.....	24
4.3 Analysis and Calculations.....	26

CHAPTER 5 EXPERIMENTAL RESULTS	29
5.1 Completed Experimental Matrix	29
5.2 Result Variability	30
5.3 Resin Performance Results	31
5.3.1 Mass Balance and Percent Yields	31
5.3.2 Dynamic Binding Capacities	32
5.3.3 Impurity Clearance	38
CHAPTER 6 DISCUSSION	44
CHAPTER 7 CONCLUSIONS	50
CHAPTER 8 FUTURE STUDY	52
REFERENCES	55

List of Figures

Figure 1. An illustration of protein separation using cation exchange chromatography...	3
Figure 2. Dynamic binding capacity as a function of solution conductivity	5
Figure 3. Dynamic binding capacity as a function of solution pH	6
Figure 4. Capacity factor as a function of the conductivity of the mobile phase.....	17
Figure 5. Dynamic binding capacity as a function of solution pH	18
Figure 6. Confocal images recorded after 5 minutes by A. Ljunglöf at GE Healthcare...	19
Figure 7. Dynamic binding capacity at 10% BT in mg/ml as a function of conductivity in mS/cm for SP XL and pH = 4 (Mab1 ■, Mab2 □).....	33
Figure 8. Dynamic binding capacity at 10% BT in mg/ml as a function of conductivity in mS/cm for SP XL and pH = 5 (Mab1 ■, Mab2 □).....	33
Figure 9. Dynamic binding capacity at 10% BT in mg/ml as a function of conductivity in mS/cm for SP XL and pH = 6 (Mab1 ■, Mab2 □).....	34
Figure 10. Dynamic binding capacity at 10% BT in mg/ml as a function of conductivity in mS/cm for SP FF and pH = 4 (Mab1 ■, Mab2 □).....	36
Figure 11. Dynamic binding capacity at 10% BT in mg/ml as a function of conductivity in mS/cm for SP FF and pH = 5 (Mab1 ■, Mab2 □).....	36
Figure 12. Dynamic binding capacity at 10% BT in mg/ml as a function of conductivity in mS/cm for SP FF and pH = 6 (Mab1 ■, Mab2 □).....	37
Figure 13. Dynamic binding capacity at 1% BT in mg/ml (SP XL ■, SP FF □) and CHOP concentration in ng/ml (SP XL ♦, SP FF ◇) for Mab 1 at pH = 4 as a function of conductivity in mS/cm	39
Figure 14. Dynamic binding capacity at 1% BT in mg/ml (SP XL ■, SP FF □) and CHOP concentration in ng/ml (SP XL ♦, SP FF ◇) for Mab 1 at pH = 5 as a function of conductivity in mS/cm	40
Figure 15. Dynamic binding capacity at 1% BT in mg/ml (SP XL ■, SP FF □) and CHOP concentration in ng/ml (SP XL ♦, SP FF ◇) for Mab 1 at pH = 6 as a function of conductivity in mS/cm	40

Figure 16. Dynamic binding capacity at 1% BT in mg/ml (SP XL ■, SP FF □) and CHOP concentration in ng/ml (SP XL ◆, SP FF ◇) for Mab 2 at pH = 4 as a function of conductivity in mS/cm41

Figure 17. Dynamic binding capacity at 1% BT in mg/ml (SP XL ■, SP FF □) and CHOP concentration in ng/ml (SP XL ◆, SP FF ◇) for Mab 2 at pH = 5 as a function of conductivity in mS/cm41

Figure 18. Dynamic binding capacity at 1% BT in mg/ml (SP XL ■, SP FF □) and CHOP concentration in ng/ml (SP XL ◆, SP FF ◇) for Mab 2 at pH = 6 as a function of conductivity in mS/cm42

Figure 19. A comparison between the equilibrium and dynamic capacity as a function of conductivity for SP XL at pH = 553

List of Tables

Table 1. Static and dynamic capacity results for cation exchange resins compared to dynamic capacities provided by suppliers	11
Table 2. Static and dynamic capacity results for anion exchange resins compared to dynamic capacities provided by suppliers	11
Table 3. Summary of comparison of characteristics of SP FF versus SP XL resin	22
Table 4. Net Charge of Mab1 and Mab2 as a function of pH	23
Table 5. Chromatography run conditions	24
Table 6. Proposed experimental matrix	25
Table 7. The conductivity and pH values tested on SP FF and SP XL using two proteins, Mab1 and Mab2.....	29
Table 8. Percent error in the dynamic binding capacities obtained via replicate runs	31
Table 9. The critical dynamic binding capacities in mg/ml at10% BT, 1%BT, and based on elution for SP XL.....	35
Table 10. The critical dynamic binding capacities in mg/ml at10% BT, 1%BT, and based on elution for SP FF	38
Table 11. CHOP concentrations in ppm in the elution pool at the critical dynamic binding capacities	42

CHAPTER 1 INTRODUCTION

1.1 Biotechnology

Biotechnology is a growing industry which is based on acquiring biological knowledge and employing it to develop new industrial techniques, processes, and products [1]. This technology has been applied within a wide array of industries including health, agricultural, food processing, as well as resource management. The health industry sector encompasses gene therapy, medical devices, and biopharmaceuticals with biopharmaceuticals being the focus of this research [2].

Biopharmaceuticals are protein based drugs that are made to meet significant unmet medical needs [1]. The manufacture of those drugs involves bacterial and mammalian cells. Much of the pharmaceutical industry resources are devoted to developing new generation therapeutics from proteins produced by recombinant DNA techniques.

1.2 Biopharmaceutical Manufacturing

Specifically within the biopharmaceutical industry, protein manufacturing includes various unit operations and techniques including cell culture, harvest, purification, and formulation [3]. Purification processes utilize many techniques which include, but are not limited to, centrifugation, ultrafiltration, crystallization, and

chromatography. Given that in the biopharmaceutical industry purity standards include that the final product has less than 1 virus particle per million doses and the host cell protein is in the part per million range, the purification processes are crucial.

Hence, developing and optimizing various steps within protein purification are becoming increasingly necessary areas for industrial research. One of the most common unit operations used is chromatography which includes affinity, hydrophobic interaction, and ion exchange chromatography. Ion exchange chromatography will be further discussed [3].

1.3 Ion Exchange Chromatography

Ion exchange chromatography is a purification technique that separates molecules based on differences in their ionic charges and can be divided into cation and anion exchange chromatography [4]. Proteins are initially bound to the ion exchange resin at low conductivity (a measure of ionic concentration) conditions and are then eluted by introducing a change in pH (a logarithmic scale of hydrogen ion concentration) or a high conductivity where the latter is more common [4,5]. Figure 1 summarizes the principles and main phases of ion exchange chromatography, specifically cation exchange chromatography [5].

Cation exchange chromatography will be the focus of this research. In cation exchange chromatography a positively charged protein binds to a negatively charged resin [4]. This chromatography technique is widely used in biopharmaceutical

manufacturing. With the increasing demand for biopharmaceuticals and limited manufacturing facilities, optimizing cation exchange chromatography, and purification processes in general, has become of greater importance. Therefore, a lot of effort has been put into better understanding cation exchange chromatography to enable efficient optimization.

Figure 1. An illustration of protein separation using cation exchange chromatography [5]. The column is initially equilibrated with a low salt buffer. Next, the protein load material is applied to the column. Then, elution is accomplished by increasing the salt concentration in the mobile phase causing the proteins to elute in an order that depends on their net surface charge.

First, a few terms will be defined as a reference for the discussion that follows.

Dynamic binding capacity is the amount of protein that binds to the resin. It is reported as mass of protein per volume of resin and is determined by continually supplying a protein feed to the chromatography column until a predetermined percent breakthrough is achieved [6]. Breakthrough values, such as 1%, 10%, or 50%, are a fraction of 100%

breakthrough where the protein concentration in the exit stream is equivalent to that in the feed. A given resin will have different binding capacities for different proteins.

Furthermore, the critical dynamic binding capacity is the capacity at the intersection of the equilibrium and the exclusion regime. The conductivity at which the critical dynamic binding capacity occurs is referred to as the critical conductivity.

1.4 Exclusion Phenomena

Previously in literature, when discussing the change in binding capacity of an ion exchange resin with respect to changes in conductivity of the mobile phase, it has been documented that the dynamic binding capacity decreases as conductivity increases. This trend has been attributed to the change in the equilibrium binding isotherm and will be referred to as the equilibrium regime [7]. In the equilibrium regime, as conductivity increases, charge shielding around the protein increases [8]. In ion exchange chromatography, this translates to a decrease in capacity due to decreased electrostatic binding between the protein and resin.

However, C. Harinarayan et al., at Genentech, Inc., discovered that two different performance regimes occur, equilibrium and exclusion [9]. In the exclusion mechanism, an opposite trend was observed where an increase in conductivity resulted in an increase in capacity as demonstrated in Figure 2. This trend will be referred to as the exclusion regime.

In the exclusion mechanism, as proteins start adsorbing to the resin particles, subsequent proteins encounter repulsion forces from similarly charged molecules before adsorbing to the resin [9]. The more charged the proteins, the greater the repulsive forces and hence, more exclusion is observed. As the conductivity of the mobile phase increases, ionic shielding surrounding the proteins also increases. The presence of the ionic shielding results in the exclusion mechanism being less prominent. Hence, the ability of the protein to adsorb to the resin is enhanced as the conductivity increases within the exclusion regime.

In addition, proteins diffusing into the pores of the resin encounter steric hindrance from proteins already bound to the resin [9]. Hence, the exclusion effect is a combination of both charge exclusion and steric hindrance. So, resin capacity will initially increase as conductivity increases until a maximum is achieved after which equilibrium behavior dominates.

Figure 2. Dynamic binding capacity as a function of solution conductivity.

Along with conductivity, pH is a critical factor in this exclusion mechanism. At a low pH, proteins are further away from their isoelectric point and hence, are more highly charged [9]. This results in increased repulsion between similarly charged proteins. This increased repulsion decreases the adsorption of proteins to the resin. Hence, the exclusion mechanism is more significant at lower pH values. This shift in behavior with changing pH as well as conductivity is illustrated in Figure 3.

Figure 3. Dynamic binding capacity as a function of solution pH.

1.5 Significance

Previous studies that investigated this exclusion mechanism within commercially available resins examined dynamic binding capacity as a function of pH and conductivity. The aim of this project was to further characterize the exclusion mechanism through dynamic binding capacities and yields. Furthermore, this project

studied the effect of the exclusion mechanism on purity. A set of experiments were designed to examine resin capacity and purity performance at industrially applicable conditions. The conditions under which the exclusion mechanism occurred were determined and purity was assessed.

Being able to better understand the resin performance within both the equilibrium and exclusion regimes is an important process development tool. Thorough understanding of cation exchange chromatography trends is crucial for developing robust and efficient biopharmaceutical manufacturing processes.

CHAPTER 2 LITERATURE REVIEW

2.1 Historical Development

In 1947, Spedding and Powell performed research at Iowa State that utilized ion exchange chromatography for separation of earth metals [10]. Later, in the 1950s, further development was completed by Kraus and Nelson where they separated metal ions via anion chromatography. Since then, investigations by various groups continued with major developments achieved in 1975 by Small et al. marking the introduction of modern ion exchange chromatography [11]. Small and coworkers were able to design a system that integrates novel resins and a detector based on a conductivity cell to separate an ion of interest from a background eluant.

Today, ion exchange chromatography has evolved into an efficient and robust purification technique [8]. Also, recently developed commercial resins are chemically tolerant to impurities present in feed stocks, such as urea [12]. All of the above factors have resulted in the wide range of applications that employ ion exchange chromatography including extracting albumin from plasma and purification of monoclonal antibodies for use in biopharmaceuticals.

2.2 Importance of Optimization

As the applications for ion exchange chromatography expand, the need for higher capacities required to develop economical processes is growing as well. Hence, process optimization has become more essential. In ion exchange chromatography, optimization includes examining resin performance by determining resin capacity, resolution, and achieved purity, all of which are related to the resin properties and performance [13]. Resolution is a measure of how well different proteins are separated and is defined by the distance between various protein peaks on the chromatogram.

Given the importance of understanding resin capacity and performance, research aimed at characterizing and comparing various resins is presented and discussed next. The presentation will cover both anion and cation exchange resins to provide a comprehensive overview.

2.3 Critical Parameters Investigated

Many papers have been published comparing various cation and anion exchange resins while varying different operating parameters. Performance is then evaluated by studying various characteristics including static capacity [14,15,17], dynamic capacity [14-17], elution dependence on pH [20], pressure drop [20], compressibility [11,18,21], stability [20], resin base matrix [12], and operating flow rate [14-19].

Other factors, discussed briefly by E. Boschetti, include parameters that govern resolution and hence describe how effective the protein separation is under a given set of

conditions [13]. Some of the factors addressed by Boschetti include pH, conductivity, flow rate, column length as well as elution gradient slope and shape.

2.4 Resin Performance Comparison

With regard to resin performance comparisons, one of the more comprehensive resin reviews was performed by Arne et al. [14,15,16,17]. Within this research, the properties and performance of various anion and cation exchange resins were determined and reported. The data presented includes static capacity and dynamic capacity at 10% and 50% breakthrough which were compared to dynamic capacities (DC) obtained from resin suppliers. The dynamic capacities were also determined at different flow rates. A sample of the results reported is shown in Table 1 and Table 2 for cation and anion exchange resins respectively where DC is dynamic capacity, BT is breakthrough, PI is the protein's isoelectric point, and Lysozyme, BSA and Anti-F VII are the proteins used within the studies to determine dynamic capacities [14,15].

For determining the dynamic capacity for cation exchange resins, the experiments were conducted in a buffer system of 16.7mM HEPES ($C_8H_{18}O_4N_2S$) + 16.7mM MES ($C_6H_{13}NO_4S$) + 16.7mM sodium acetate at pH 5.5 [14]. For examining anion exchange resins, the conditions used included a 25mM Bis-Tris-propane + 25mM Tris at pH 8 [16]. For all resins, the high and low flow rates were determined by calculating 25% and 75% of the manufacturer's maximum recommended flow rate, respectively [14,16].

Table 1. Static and dynamic capacity results for cation exchange resins compared to dynamic capacities provided by suppliers.

	DC, %BT, Anti-FVII Mab (mg/ml) Anti-FVII PI = 6.5				DC, %BT, Lysozyme (mg/ml) Lysozyme PI = 11.0				DC by Supplier, Lysozyme (mg/ml)	Static Capacity, Lysozyme (mg/ml)
Resin	Low flow-rate		High flow-rate		Low flow-rate		High flow-rate			
	10%	50%	10%	50%	10%	50%	10%	50%		
SP Sepharose XL	-	-	-	-	188	210	180	210	> 160	337
SP Sepharose BB	13	29	7	23	92	103	47	72	-	138
Poros 50 HS	27	38	23	35	43	59	36	55	55	63
Source 30S	1	4	1	4	79	82	77	80	> 80	76
Toyopearl SP 550c	39	47	25	38	107	124	69	95	80 - 120	139
Toyopearl SP 650c	~0	~0.1	~0	~0.1	29	33	28	33	35 - 55	41
TSKGel SP- 5PW-HR20	-	-	-	-	28	39	28	39	20 - 40	41

Table 2. Static and dynamic capacity results for anion exchange resins compared to dynamic capacities provided by suppliers.

	DC, %BT, Anti-FVII Mab (mg/ml) Anti-FVII PI = 6.5				DC, %BT, BSA (mg/ml) BSA PI = 4.8				DC by Supplier, BSA (mg/ml)	Static Capacity, BSA (mg/ml)
Resin	Low flow-rate		High flow-rate		Low flow-rate		High flow-rate			
	10%	50%	10%	50%	10%	50%	10%	50%		
Q Sepharose XL	5	5	5	6	176	196	188	215	>130	244
UNO Q-1	7	8	7	8	24	26	26	27	20	-
Poros 50 HQ	3	4	2	2	45	58	47	58	60 - 70	73
Toyopearl QAE 550c	6	9	6	9	33	53	21	47	70±10	68
Separon HemaBio 1000Q	0.5	0.6	0.4	0.5	18	19	18	19	N.A.	25
Q-Cellthru Bigbeads Plus	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	0.1	< 0.1	< 0.1	61	< 1
Q Sepharose HP	7	10	6	10	64	73	63	74	70	78
Toyopearl SuperQ 650s	< 0.1	0.2	< 0.1	0.1	66	83	48	66	130±25	98

From the results presented, it is clear that commercially available resins cover a wide range of properties. Also, it was noted that the dynamic capacity for all anion exchange resins increased at higher pH values with the percent change varying per resin [16]. For cation exchange resins, an inverse trend was observed where the dynamic binding capacity decreased at higher pH values [14].

Nash et al. also completed a resin comparison study where the changes occurring due to different flow rates were examined. Dynamic binding capacities were determined at a set load concentration and 5% breakthrough for varying flow rates [18]. It was concluded that the dynamic capacities decreased with increasing flow rates. Also, via further investigation it was noted that the resolution and resin performance were compromised at higher flow rates. It is important to note that the degree of change in the mentioned factors varied significantly and was dependant on the resin matrix.

In another study, Horvath et al. determined the dynamic capacity of A-Hyper D resin with 50 mM sodium acetate, pH = 4.5 and 50% breakthrough using a constant load concentration [19]. It was concluded that the dynamic capacity decreased only slowly with increasing flow rate. To be specific, a five fold increase in flow rate resulted in less than 12% decrease in dynamic capacity.

Lagerlund et al. also ran different flow rates through ANX Sepharose 4 fast flow [20]. However, instead of looking at dynamic capacities at the various flow rates, they compiled pressure profiles at different flow rates and different solutions such as 1M sodium chloride and 1M acetic acid. Examining such profiles can be helpful for choosing possible solutions and flow rates in industrial settings.

Furthermore, rather than examining several resins, Lagerlund et al. chose one resin and characterized its properties including capacity, titration curves and stability [20]. ANX Sepharose, the resin chosen, is an anion exchange resin developed by Amersham Pharmacia Biotech. The dynamic binding capacity was determined at only one set of pH and conductivity but using different load proteins. A large part of the study was also focused on investigating the long term stability of the resin in various solutions. Long term stability is a critical parameter when considering the resin for industrial applications.

Another critical parameter investigated by various groups is the resin's base matrix with attempts made to correlate differences in performance trends to changes in the base matrix of the resin. P. R. Levison et al. examined both anion and cation exchange resins and categorized the results based on the base matrix of the resins [12]. The base matrices investigated included agarose, cellulose, dextran, and polymeric material.

P.R Levison et al. note the wide range of capacities obtained especially for the polymeric based resins but did not attempt to compose generalized trends when comparing the various base matrices [12]. However, it was noted that the conditions at which the capacities were determined were the same and hence not optimized per resin. Hence, the results and conclusions obtained could be misleading. As Levison and coworkers noted, this methodology is appropriate for mass screening but optimization of conditions is necessary to fine tune the process.

Furthermore, Levison and coworkers recorded the column packing density for each of the resins screened [12]. This parameter is not recorded by many researchers but can be significant for comparing different resins especially when evaluating economical feasibility for commercial applications. Determining the packing density makes it possible to determine capacity in terms of mg of protein per ml of packed resin, a parameter that is essential for completing an accurate cost assessment.

Also, even though a comparison of resin capacity is standard practice, noting changes in performance at different conditions is also important. Some researchers, including Levison et al., noted changes in resolution, an indication of performance, at changing flow rates [21]. The result of such a study is being able to determine which resins' resolution is more sensitive to changes in flow rates. Such information might help in choosing a resin for an industrial process depending on the range of process conditions expected.

Also, with respect to evaluating resolution, J. Horvath et al. varied the load concentration for various resins and recorded resolution obtained using gradient elution [19]. It is important to note that parameters such as column size and flow rate were different for the different resin systems. Examining resolution versus protein load showed that changes in resolution varied for the different resins examined. For example, the resolution obtained for S Hyper D remained unchanged while the resolution of Poros HS/H was reduced by 50% when the mass of protein loaded was increased by six fold. This could be attributed to resin differences or could be the result of different degrees of parameter optimization for the different resins.

Another resolution related experiment performed by Horvath and coworkers involved determining the effects of flow rate on resolution [19]. In these experiments, not only were the initial resolutions obtained different but the changes in resolution with increased flow rate were also resin dependent. Once again, the experimental conditions were varied per resin and hence, result comparison may not lead to accurate conclusions. For future experiments, it would be beneficial to either fully optimize the conditions for each resin or hold conditions constant to allow for more a meaningful comparison.

Along with resolution, several other factors covered in the literature are used to evaluate performance. As mentioned earlier, in a review completed by E. Boschetti, several factors were suggested to examine performance [13].

After having discussed different experiments designed and parameters considered for comparing resins, general resin performance trends expected at changing conditions and theories modeling these trends are discussed next.

2.5 Resin Performance Trends

One of the main papers referenced to explain protein behavior within ion exchange chromatography is that by C. Brooks and S. Cramer [7]. They developed a theoretical background, referred to as the steric mass action (SMA) ion-exchange equilibrium that has been used to explain protein adsorption to ion exchange resins. The SMA model assumes a stoichiometric protein exchange reaction where the nature of

protein and steric hindrance are taken into account in the model equation shown in Equation 1 below [22].

$$q_p = Kc_p \left(\frac{c_s}{\Lambda - ((z_b + \sigma)q_p)} \right)^{-z_b} \quad \text{Equation 1}$$

where q_p is the concentration of adsorbed protein, K is an equilibrium constant, Λ is the resin ion capacity, z_b is characteristic charge, c_s and c_p are salt and protein concentration in the mobile phase respectively and, σ is the steric factor.

Equation 1 shows that the adsorbed protein concentration is inversely proportional to the conductivity of the mobile phase [22]. In other words, as conductivity increases, the resin dynamic binding capacity decreases. This trend occurs because as conductivity increases, the charge shielding around the protein increases [8]. In ion exchange chromatography, this translates to a decrease in capacity due to decreased electrostatic binding between the protein and the resin. This is referred to as the equilibrium regime.

Q. Shi et al. illustrated this trend by determining capacity as a function of the conductivity of the liquid phase [22]. The data was plotted as $\log k'$ versus $\log (1/c_s)$ where k' is a capacity factor. A linear plot was produced and fit to Equation 2 obtained by manipulating Equation 1 shown previously.

$$\log k' = \log(\beta K \Lambda^{z_b}) + z_b \log \left(\frac{1}{c_s} \right) \quad \text{Equation 2}$$

where β is the column phase ratio. The plot obtained by Q. Shi is shown in Figure 4.

Figure 4. Capacity factor as a function of the conductivity of the mobile phase [22].

However, at Genentech, Inc., it was discovered that two different performance regimes occur, equilibrium and exclusion. In the exclusion regime, an opposite trend was observed where an increase in conductivity resulted in an increase in capacity. In this mechanism, proteins adsorbing to the resin encounter repulsion forces from similarly charged protein molecules adsorbed to the resin. As conductivity increases, the charge shielding around the proteins increases. Also, proteins diffusing into the pores of the resin encounter steric hindrance from proteins already bound to the resin [9].

Along with conductivity, pH is a critical factor in this exclusion mechanism. In the equilibrium regime, it is expected for capacity to increase as pH decreases and proteins become more charged enhancing the electrostatic interactions between the protein molecules and the resin beads [23]. However, in the exclusion mechanism, at higher pH values, capacity increases due to reduced repulsive forces as the protein

molecule become less charged [23]. This shift in behavior with changing pH as well as conductivity is illustrated in Figure 5 [9].

Figure 5. Dynamic binding capacity as a function of solution pH.

To further demonstrate this behavior and shift in trends, protein adsorption can be monitored via confocal microscopy [24]. Confocal microscopy is a tool that allows observing the depth of protein concentration within the resin beads at specific conditions and a specific point in time. Anders Ljunglöf and coworkers recorded confocal microscopy images for monoclonal antibody (Mab) transport through a resin at 5 minutes at different pH values and conductivities. The images obtained are shown in Figure 6 [23]. The extent of penetration illustrated in Figure 6 agrees with the trends discussed earlier and represented in Figure 5.

Figure 6. Confocal images recorded after 5 minutes by A. Ljunglöf at GE Healthcare [23].

2.6 Summary

Looking at previous research and information available in literature, a few weaknesses are noted and are discussed below. Usually, either a pH or conductivity value is chosen and then, the second parameter is optimized. This approach might not offer the complete picture and hence, result in misleading conclusions. Ideally, especially in light of results obtained at Genentech, Inc., it is important to vary both factors to gain full understanding of performance within the desired range. Furthermore, given that results and trends can vary significantly with resin, pH and protein, it would be beneficial to define industrially significant parameters as to optimize commercially viable processes.

Moreover, experiments performed at Genentech, Inc. that examined the exclusion mechanism focused on determining dynamic binding capacities as a function of pH and conductivity. The aim of this project was to further characterize the exclusion mechanism through dynamic binding capacities. This project also further studied the effect of the exclusion mechanism on purity and yields. Two commercially available resins were chosen and their performance (capacities, yields, and purity) was studied as a function of pH and conductivity.

CHAPTER 3 RESEARCH OBJECTIVES

3.1 Research Objectives

In process development and optimization, understanding resin performance in terms of capacity, yields, and purity is essential. Characterizing the exclusion phenomena recently documented in literature over a range of values of pH and conductivity is therefore necessary.

So, the objective of this study was to determine dynamic binding capacities and performance for SP SepharoseTM Fast Flow (SP FF) and SP SepharoseTM XL (SP XL) resins as a function of pH and conductivity within industrially significant conditions. The analysis was completed for two monoclonal antibodies, Mab1 and Mab2.

Conditions under which the exclusion effect occurs were noted. Furthermore, performance was determined at the different conditions by evaluating purity, capacity, and percent yields. Any differences in performance, specifically purity, that occur under the exclusion regime versus the equilibrium regime were noted. The results were also analyzed with respect to the resin and protein properties.

CHAPTER 4

RESEARCH APPROACH

The overall scope of this research was to further characterize the exclusion phenomena previously documented in cation exchange resins at Genentech, Inc. In addition to documenting capacity as a function of pH and conductivity, yields and purity were also addressed.

4.1 Materials and Methods

The cation exchange chromatography runs were performed on an AKTA 100 Explorer system from GE Healthcare. The resins studied, SP Sepharose™ Fast Flow (SP FF) and SP Sepharose™ XL (SP XL) were also obtained from GE Healthcare. For this study, the resins were packed in Omnifit glass columns with 1 cm diameter and 40 cm bed height.

To enable comparison of the results obtained from both resins, Table 3 summarizes the main characteristics for both the SP FF and SP XL resin.

Table 3. Summary of comparison of characteristics of SP FF versus SP XL resin.

	SP Sepharose™ Fast Flow	SP Sepharose™ XL
Base Matrix	6% agarose, 94% water	6% agarose, 94% water
Particle Size	45-165 µm (average 90 µm)	45-165 µm (average 90 µm)
Surface extender	None	Dextran
Ionic Capacity	0.18 - 0.25 µmol/ml	0.18 - 0.26 µmol/ml

The characteristics highlighted include base matrix, particle size, surface extender and ionic capacity. Direct measurement of the resin pore size is not available. However, given that the SP FF has the same base matrix as SP XL but does not have the surface extender, it is expected to have a larger effective pore size in comparison to SP XL.

Two proteins, Mab1 and Mab2, were used to complete the runs. The load material used was representative feedstock for a cation exchange step obtained from the manufacturing facilities at Genentech, Inc. Several feedstocks were used per protein due to availability restrictions. Therefore, the load protein concentrations and impurity levels varied but were documented.

As for the proteins, at a pH lower than the isoelectric point of both proteins, Mab2 is more highly charged than Mab1. To aid in understanding and comparing the behavior of the 2 proteins at each of the pH values, the protein charge is summarized in Table 4.

Table 4. Net Charge of Mab1 and Mab2 as a function of pH.

pH	Net Charge	
	Mab1	Mab2
4	100	108
5	46	58
6	22	36

To determine protein concentration in the load material as well as the various phases from the chromatography runs, a Shimadzu UV-1601 UV/Visible spectrophotometer was used. The absorbance was determined at 280 nm and Beer's law, shown in Equation 3, was used to determine the concentration of a solution. In Equation 3, c is the protein concentration, b is the path length, and ϵ is the molar absorptivity.

$$Absorbance = c * b * \epsilon$$

Equation 3

As for the chromatography runs, the buffers used and the length of the various phases were kept constant throughout the study except for the load, equilibration buffer, and wash 1 buffer conditions. The various phases and buffers used are outlined in Table 5. The load, equilibration buffer, and wash 1 buffer were adjusted to match the pH and conductivities tested. Also, the linear flow rate used was 200 cm/hr which translates to a 12-minute residence time for the column dimensions used.

Table 5. Chromatography run conditions.

Phase	pH	Conductivity (mS/cm)	Phase Length in Column Volumes
Equilibration	Same as Load	Same as Load	5
Load	Per experimental matrix Table 6	Per experimental matrix Table 6	(10% BT)
Wash 1	Same as Load	Same as Load	5
Wash 2	7.1	0.8	5
Wash 3	5.5	0.4	5
Elution	5.5	3 – 26	10 CV Linear Gradient
Sanitization	13.2	100	5
Storage	13.0	24	3

4.2 Experimental Design

The initial set of pH and conductivities that were investigated are outlined in Table 6. This matrix was completed for both resins, SP FF and SP XL, and two proteins, Mab1 and Mab2. These conditions were chosen to cover a range of industrially significant conditions.

Table 6. Proposed experimental matrix.

Conductivity (mS/cm)	pH		
	4	5	6
2	✓	✓	✓
4	✓	✓	✓
6	✓	✓	✓
8	✓	✓	✓
10	✓	✓	✓
15	✓	✓	✓

In the process of completing the proposed experimental matrix, some modifications were made to the matrix shown in Table 6. First, if at a specific pH and conductivity a dynamic binding capacity ≤ 10 mg/ml is obtained, then a run at a higher conductivity and the same pH was not performed. Also, if applicable, a run at a conductivity of 1 mS/cm was added in order to better determine the maximum dynamic binding capacity and better characterize the exclusion phenomena.

The pH and conductivity of the load material, equilibration buffer, and wash 1 buffer were adjusted before each chromatography run. The pH of the load was adjusted with either acetic acid or 1.5M Tris base which were used to lower or increase pH respectively. Also, if the desired conductivity was lower than that of the load obtained,

the load was diluted with pure water to attain the desired conductivity. To increase the conductivity, 5M sodium chloride was used for the load, the equilibration buffer, and the wash 1 buffer. No adjustments were made to the pH of the equilibration buffer or the wash 1 buffer since they were made to match the desired pH.

4.3 Analysis and Calculations

The elution pool from each chromatography run was collected and its concentration was determined and used to calculate a percent yield. The protein concentration in the load flow through as well as the three washes, also collected, were determined and used to complete a mass balance for the system. Protein concentrations were determined using the UV/Vis spectrophotometer at 280 nm.

Equations 4 and 5 were used to calculate yield and complete the mass balance respectively where $V_{initial}$ and V_{end} are the volume at the start and end of the load phase respectively.

$$\text{Yield (\%)} = \frac{\text{Elution Pool Volume (ml)} * \text{Elution Pool Concentration} \left(\frac{\text{mg}}{\text{ml}} \right)}{(V_{end} - V_{initial}) \text{ (ml)} * \text{Load Concentration} \left(\frac{\text{mg}}{\text{ml}} \right)} * 100 \quad \text{Equation 4}$$

$$\text{Mass Balance (\%)} = \frac{\text{Total Mab (All Phases Collected) (mg)}}{(V_{end} - V_{initial}) \text{ (ml)} * \text{Load Concentration} \left(\frac{\text{mg}}{\text{ml}} \right)} * 100 \quad \text{Equation 5}$$

The dynamic binding capacities (DBC) at 10%BT and 1%BT were also determined for each of the conditions by utilizing Equation 6 where volume loaded is the

volume of protein loaded onto the column before the 10% or 1% breakthrough is reached, whichever appropriate. The dynamic capacities were then plotted as a function of conductivity and pH.

$$\text{DBC} \left(\frac{\text{mg}}{\text{ml resin}} \right) = \frac{\text{Volume Loaded (ml)} * \text{Load Concentration} \left(\frac{\text{mg}}{\text{ml}} \right)}{\text{Column Volume (ml resin)}} \quad \text{Equation 6}$$

The volume loaded was determined using the logbook generated by the AKTA Explorer 100 and it accounts for the hold up volume. The hold up volume includes both the system hold up and the column volume. The system hold up was determined via passing a protein solution through the system and observing the point at which an increase in absorbance on the chromatogram was obtained. The column volume was determined using the column dimensions and is 31.4 ml for this study. The load concentration was determined using the UV/Vis spectrophotometer.

To evaluate performance, purity was determined by submitting samples for the ELISA assays which were run by the analytical operations department at Genentech, Inc. The assays were used to determine the clearance of Chinese Hamster Ovary Protein, CHOP, as well as Leached Protein A. For CHOP, several serial dilutions were submitted in order to obtain several readings within the detectable range. As for the L. Protein A assay, the samples were diluted to target a specific Mab protein concentration. The lower detectable limit for both assays is 2 ng of impurity per mg of Mab, which is 2 ppm.

Also, the percent variability in the dynamic binding capacities obtained was determined. In experiments previously conducted at Genentech, Inc., it has been determined that results from similar cation exchange chromatography experiments were

reproducible with 5% variation. In order to confirm the reproducibility, 10% of the runs were repeated to establish a percent error in the values recorded. Also, some of the runs were repeated using a different column as to account for any differences due to column packing. Trends observed were studied with respect to the resin and protein properties.

CHAPTER 5 EXPERIMENTAL RESULTS

5.1 Completed Experimental Matrix

Following the proposed experimental matrix and the criteria set forth in the experimental design, the runs completed are summarized in Table 7. This matrix was complete for SP FF and SP XL using both proteins, Mab1 and Mab2.

Table 7. The conductivity and pH values tested on SP FF and SP XL using two proteins, Mab1 and Mab2.

Conductivity (mS/cm)	pH		
	4	5	6
1		✓	✓
2	✓	✓	✓
4	✓	✓	✓
6	✓	✓	✓
8	✓	✓	✓
10	✓	✓	✓
15	✓	✓	

For Mab1, the load material used had an initial concentration of 6.2 – 7.1 mg/ml with 1000-2000 ppm CHOP and less than 3 ppm of Leached Protein A. For Mab2, the load material used had an initial concentration of 5.1 – 8.9 mg/ml with 4000-7000 ppm CHOP and 2-7 ppm of Leached Protein A.

The variation in impurity levels within the load material was present due to the fact that several material lots were used. It was not possible to use one material lot per

protein due to material availability. However, for each resin, per protein, per pH, material from the same lot was used in most cases to enable the comparison of purity results across runs.

Another factor not addressed in the scope of this study is the effect of varying load concentration on the obtained results. However, some information on this parameter was obtained from the replicate runs performed where different feedstocks with varying load concentrations were used. In the most extreme case, the load concentration was 8.2 and 5.1 mg/ml for the original and replicate run respectively. Given that no significant differences were obtained in the dynamic binding capacities for both runs, it seems that, within the range investigated, varying the load concentration does not significantly affect the dynamic binding capacities obtained

5.2 Result Variability

Using the experimental matrix outlined in Table 7, a total of 76 runs were completed. In addition, 7 runs, i.e., 10% of the original runs, were repeated in order to establish a percent variation in the dynamic binding capacities obtained. It was shown that the chromatography runs completed resulted in dynamic binding capacities with less than 5% variation. The results are summarized in Table 8.

Also, within the repeats, two different columns per resin were used to complete the runs. This was to establish that the results obtained were not significantly influenced by column packing related issues.

Table 8. Percent error in the dynamic binding capacities obtained via replicate runs.

Run Conditions				DBC (mg/ml)		Percent Error (%)
Resin	Protein	pH	Conductivity (mS/cm)	Original Run	Replicate Run	
SP XL	Mab1	5	4	124	128	3.3
	Mab2	5	4	127	131	3.1
		5	6	122	119	2.5
SP FF	Mab1	5	4	66	69	3.6
	Mab2	5	4	63	66	4.3
		5	6	63	62	2.1
		5	8	62	61	2.3

5.3 Resin Performance Results

Performance results (percent yields, dynamic binding capacities, and impurity clearance) are presented next.

5.3.1 Mass Balance and Percent Yields

For all the runs completed, percent yield was calculated and mass balance was completed. For the majority of the runs, the mass balance was completed to $100 \pm 5\%$. The cases in which the mass balance was less than 95% corresponded to the presence of a larger absorbance peak in the sanitization phase. The sanitization phase was not collected during this study and hence, was not included in the completed mass balance because the

sodium hydroxide used for the sanitization phase interferes with absorbance of the proteins at 280 nm [25].

Only two extreme cases were encountered using SP FF at pH = 4 and conductivity = 2 mS/cm using both proteins where the mass balances were completed to 64 % and 65 % for Mab1 and Mab2 respectively. Reviewing the chromatograms for both of those runs, it was clear that the elution conditions were not sufficient to elute all of the protein off the column. The rest of the protein was eluted in the sanitization phase and therefore was not included in the mass balance.

Also, 55 of the 76 runs performed had yields over 90%, 8 runs had yields between 75 and 90%, and 11 runs had yields lower than 75%. With regards to the runs with less than 75% yields, those included the 2 cases discussed earlier where the protein was not completely eluted. The rest of the runs were at low binding conditions where a significant fraction of the protein was lost during the washes prior to elution. Furthermore, for 2 of the runs at low binding conditions, all of the protein loaded was eluted during the washes and hence, no elution peak was obtained and percent yield was not calculated.

5.3.2 Dynamic Binding Capacities

For all the runs completed, dynamic binding capacities were determined at 1% BT, 10% BT, as well as based on the elution peak. The dynamic binding capacities were plotted as a function of conductivity. Figures 7, 8, and 9 show the 10% BT dynamic

binding capacities versus conductivity for SP XL at pH 4, 5, and 6 respectively. Each of the plots shows the results for both Mab1 and Mab2.

Figure 7. Dynamic binding capacity at 10% BT in mg/ml as a function of conductivity in mS/cm for SP XL and pH = 4 (Mab1 ■, Mab2 □).

Figure 8. Dynamic binding capacity at 10% BT in mg/ml as a function of conductivity in mS/cm for SP XL and pH = 5 (Mab1 ■, Mab2 □).

Figure 9. Dynamic binding capacity at 10% BT in mg/ml as a function of conductivity in mS/cm for SP XL and pH = 6 (Mab1 ■, Mab2 □).

From Figures 7 - 9, the critical dynamic binding capacities can be determined and are summarized in Table 9 along with percent yields. Note that for both proteins at pH = 6, the critical dynamic binding capacity potentially occurs at a conductivity ≤ 1 mS/cm since the points obtained seem to be within the equilibrium regime. Therefore, in Table 9, the capacities at pH = 6 and conductivity = 1 mS/cm are included even though the critical DBC might be at a lower conductivity.

Also, the trends observed are the same for both Mab1 and Mab 2. However, the plots from Mab 2 seem to be right shifted; that is, a higher ionic strength is needed in order to shield the charge of the protein. This is consistent with Mab2 being more charged than Mab1 at the same pH.

Table 9. The critical dynamic binding capacities in mg/ml at 10% BT, 1% BT, and based on elution for SP XL.

Run Conditions				Critical Dynamic Binding Capacity (mg/ml)			Percent Yield (%)
Resin	Protein	pH	Conductivity (mS/cm)	10% BT	1% BT	Based on Elution	
SP XL	Mab1	4	10	123	116	125	96
		5	4	124	123	134	102
		6 *	1	164	157	169	102
	Mab2	4	10	110	75	104	90
		5	4	131	116	132	97
		6 *	1	158	151	164	103

Furthermore, in most cases, the capacities at 10% BT and 1% BT were very close indicative of a sharp breakthrough curve. However, at pH = 4 and Mab2, the 10% and 1% BT capacities are significantly different. Also, the capacities obtained based on the elution pool were also close to the 10% BT.

Next, the dynamic binding capacities obtained for SP FF are plotted as a function of conductivity. Figures 10, 11, and 12 show the 10% BT dynamic binding capacities versus conductivity for SP FF at pH 4, 5, and 6 respectively. Each of the plots shows the results for both Mab1 and Mab2.

* The critical dynamic binding capacity at this pH might be at a lower conductivity; no exclusion was observed.

Figure 10. Dynamic binding capacity at 10% BT in mg/ml as a function of conductivity in mS/cm for SP FF and pH = 4 (Mab1 ■, Mab2 □).

Figure 11. Dynamic binding capacity at 10% BT in mg/ml as a function of conductivity in mS/cm for SP FF and pH = 5 (Mab1 ■, Mab2 □).

Figure 12. Dynamic binding capacity at 10% BT in mg/ml as a function of conductivity in mS/cm for SP FF and pH = 6 (Mab1 ■, Mab2 □).

Comparing the results for SP FF to those obtained with SP XL, similar trends are observed. For SP FF, Mab1 and Mab2 exhibit similar trends across the conditions studied with no significant shift observed when comparing the results from both proteins. A summary of the critical dynamic binding capacities observed is shown in Table 10.

Examining the capacity plots, the exclusion regime is observed for both resins with both proteins and at all pH values. However, at pH = 6 with Mab1, a plateau region was reached but exclusion is not fully characterized at the conductivities tested. So, in Table 10, for the critical dynamic capacity with Mab1 at pH = 6, the capacity at conductivity = 1 mS/cm is included since that is within the plateau region observed. However, the actual critical dynamic capacity may occur at a conductivity of less than 1 mS/cm.

Also summarized in Table 10 are the percent yields obtained, all of which are equal to or greater than 90%. This also holds true for the yields obtained with SP XL, which are shown in Table 9.

Table 10. The critical dynamic binding capacities in mg/ml at 10% BT, 1% BT, and based on elution for SP FF.

Run Conditions				Critical Dynamic Binding Capacity (mg/ml)			Percent Yield (%)
Resin	Protein	pH	Conductivity (mS/cm)	10% BT	1% BT	Based on Elution	
SP FF	Mab1	4	8	80	73	78	90
		5	2	73	66	72	94
		6*	1	71	66	73	101
	Mab2	4	8	73	65	74	94
		5	6	63	60	68	94
		6	2	66	61	67	97

Next, the results of resin performance in terms of purity are presented. For all subsequent plots, the dynamic binding capacity at 1% BT will be used since these values would be more industrially applicable.

5.3.3 Impurity Clearance

The results for CHOP clearance are summarized in Figures 13 through 18 below. The figures plot dynamic binding capacity (mg/ml) and CHOP concentration (ng/ml) in

* The critical dynamic binding capacity at this pH might be at a lower conductivity; no exclusion was observed.

the elution pool versus conductivity. Each of the figures summarizes the results at a specific pH and a specific protein for both resins.

From Figures 13-18, it can be seen that for all the conditions, the CHOP concentration, ng/ml, in the elution pool follows the same trend as the dynamic binding capacity. The only exception occurs with SP XL and Mab1 at pH = 6 where at the lower conductivities, CHOP does not follow the dynamic binding capacity trend. Furthermore, the CHOP concentrations in ppm at the critical dynamic binding capacities are summarized in Table 11.

Figure 13. Dynamic binding capacity at 1% BT in mg/ml (SP XL ■, SP FF □) and CHOP concentration in ng/ml (SP XL ♦, SP FF ◇) for Mab 1 at pH = 4 as a function of conductivity in mS/cm.

Figure 14. Dynamic binding capacity at 1% BT in mg/ml (SP XL ■, SP FF □) and CHOP concentration in ng/ml (SP XL ♦, SP FF ◇) for Mab 1 at pH = 5 as a function of conductivity in mS/cm.

Figure 15. Dynamic binding capacity at 1% BT in mg/ml (SP XL ■, SP FF □) and CHOP concentration in ng/ml (SP XL ♦, SP FF ◇) for Mab 1 at pH = 6 as a function of conductivity in mS/cm.

Figure 16. Dynamic binding capacity at 1% BT in mg/ml (SP XL ■, SP FF □) and CHOP concentration in ng/ml (SP XL ♦, SP FF ◇) for Mab 2 at pH = 4 as a function of conductivity in mS/cm.

Figure 17. Dynamic binding capacity at 1% BT in mg/ml (SP XL ■, SP FF □) and CHOP concentration in ng/ml (SP XL ♦, SP FF ◇) for Mab 2 at pH = 5 as a function of conductivity in mS/cm.

Figure 18. Dynamic binding capacity at 1% BT in mg/ml (SP XL ■, SP FF □) and CHOP concentration in ng/ml (SP XL ♦, SP FF ◇) for Mab 2 at pH = 6 as a function of conductivity in mS/cm.

Table 11. CHOP concentrations in ppm in the elution pool at the critical dynamic binding capacities.

Run Conditions				CHOP concentration in the elution pool (ppm)
Resin	Protein	pH	Conductivity (mS/cm)	
SP XL	Mab1	4	10	890
		5	4	330
		6	1	166
	Mab2	4	10	730
		5	4	1310
		6	1	419
SP FF	Mab1	4	8	560
		5	2	190
		6	1	103
	Mab2	4	8	210
		5	6	690
		6	2	1130

Examining the results summarized in Table 11 it can be noted that at pH 6, both resins provide similar CHOP clearance. However, at pH = 4 and 5, SP FF results in better CHOP clearance in comparison to SP XL.

The concentration of Leached Protein A in the elution pool was less than 2 ppm, the lower detectable limit, in almost all cases. The few exceptions were on SP XL, Mab2 where the Leached Protein A levels in the elution pools were 2 – 4 ppm.

CHAPTER 6

DISCUSSION

The experimental matrix shown in Table 7 was completed to investigate resin performance as a function of pH and conductivity for SP XL and SP FF resins using 2 proteins, Mab1 and Mab2. Resin performance was assessed based on dynamic binding capacities, yield, and purity.

For each set of conditions, the dynamic binding capacities based on the 10% BT, 1%BT, and elution were calculated. In most cases, the three capacities calculated were very close. This is indicative of a sharp breakthrough curve as well as high yields. The exception was with SP XL at pH = 4 and Mab2 where the 10% and 1% BT capacities are significantly different indicating a shallower breakthrough curve. One explanation could be that compared to the other conditions investigated, more exclusion occurs with SP XL at pH = 4 and hence, the process is more diffusion limited. Therefore, since diffusion is a slow process, it takes longer for the protein to diffuse and bind to the resin, resulting in a shallower breakthrough curve.

The critical dynamic capacities obtained are summarized in Tables 9 and 10. From those values as well as Figures 7 - 12, it is obvious that the dynamic binding capacities obtained with SP XL are significantly higher than those obtained with SP FF. More specifically, the critical dynamic capacities obtained on SP XL were approximately 35 – 50% higher than those obtained on SP FF. However, between SP XL and SP FF, there is a trade-off between capacity and purity and this will be addressed further on.

The results obtained were also used to examine and confirm the occurrence of the exclusion phenomena. By examining Figures 7 - 12, the exclusion effect can be observed for both resins and both Mab1 and Mab 2. For SP XL, the exclusion effect is most pronounced at pH = 4 and 5 with both proteins but is more drastic with Mab2. For SP FF, exclusion is also apparent at pH = 4 and 5 with both proteins. At pH = 6, all of the points obtained for SP XL are within the equilibrium regime and no exclusion is observed with either protein. In comparison, for SP FF at pH = 6, a plateau region was reached and some exclusion was observed.

Within the observed exclusion regime, the data follows the trends documented in earlier studies. That is, within the exclusion regime, as the conductivity increases, the dynamic binding capacity increases until the equilibrium regime is reached after which the dynamic binding capacity decreases with increasing conductivity. Also, within the exclusion regime, an increase in pH results in an increase in dynamic binding capacity. However, an opposite trend occurs in the equilibrium regime.

Also, the behaviors of both Mab1 and Mab2 are similar with the differences being attributed to the difference in charge between Mab1 and Mab2. A summary of the proteins' charge at the different pH values is shown in Table 4. From the results, it can be noted that the differences between Mab1 and Mab2 follow the expected trends given that, at the same pH, Mab2 is more charged than Mab1. For example, at pH = 6 with SP XL, higher conductivity is needed to shield the charge of Mab2 when compared to Mab1. Also, the differences between both proteins are more apparent with SP XL since more exclusion occurs on SP XL versus SP FF.

Next, the purity performance of the resins is assessed and the results are summarized in Figures 13 - 18. From those figures it can be observed that the CHOP concentration, in ng/ml, in the elution pool follows the same trend as the dynamic binding capacity (higher DBC translates to a higher Mab concentration in the elution pool). The only exception occurs with SP XL and Mab1 at pH = 6 where at the lower conductivities, CHOP does not follow the dynamic binding capacity trend.

This purity result seems to be counter intuitive since CHOP is significantly smaller in size than the monoclonal antibodies and hence, would not be expected to be as affected by the exclusion phenomena. If CHOP did experience less exclusion than the proteins, this would mean that impurity levels in the elution pools, in ppm, would be higher at the conditions where the protein experiences more exclusion. Therefore, a trade-off between capacity and purity would be expected.

However, from the results obtained, it is clear that CHOP experiences the same degree of exclusion as the proteins. One explanation could be that the CHOP is associated with the Mab and therefore similar trends are observed for both the Mab and CHOP. Hence, given the above conclusions, it is possible to operate at higher capacities while maintaining the desired purities in terms of CHOP in ppm in the elution pool. This result is extremely important for process development where operating at the critical dynamic capacity would be ideal.

Moreover, comparing the performance of the resins between the exclusion and the equilibrium regimes, no major differences seem to be present. Good yields are obtained under both regimes. Also, the purity obtained follows the same trend regardless of the

regime under which the resin is being operated. The above is true for both resins tested, SP XL and SP FF.

The CHOP concentrations in ppm at the critical dynamic binding capacities are summarized in Table 11. From Table 11 it can be noted that, in general, SP FF provides significantly better CHOP clearance than SP XL. Even though at pH = 6 both resins provide similar CHOP clearance, SP FF achieves significantly better clearance than SP XL at both pH = 4 and 5. Therefore, when comparing the two resins, a trade-off between capacity and purity is observed.

Furthermore, the CHOP clearance obtained for Mab1 and Mab2 with both resins seem comparable, even though, the CHOP concentrations in the loads for Mab1 and Mab2 were different. Given that it is possible to have different CHOP populations in the load material for different products coupled with the assay variation, an additional level of complexity in analyzing and comparing results across various proteins is apparent.

Also, the yields obtained with each of the resins are comparable. In general and especially at conditions of interest, i.e. conditions at which critical dynamic capacities occur, the yields obtained were over 90%. These results are presented in Tables 9 and 10. This indicates that the salt gradient applied within the elution phase was sufficient for adequately eluting both proteins, Mab1 and Mab2.

It is difficult to make many conclusions regarding Leached Protein A clearance because the loads initially contained low Leached Protein A levels. However, the Leached Protein A was cleared in almost all cases. The few exceptions were on SP XL, Mab2 where the Leached Protein A levels in the elution pools were 2 – 4 ppm which is

close to the assay's detection limit. With further investigation and optimization of elution and pooling conditions, it may be possible to increase purity performance while maintaining high yields.

The major characteristics for both resins are summarized in Table 3. Both resins have the same base matrix, made of 6% agarose as well as the same particle size. The major difference is the dextran extenders on the SP XL which are not present on SP FF. Those dextran extenders provide a larger surface area for protein binding and hence, resulting in the significantly higher capacities observed on SP XL. However, also due to the presence of the dextran extenders, the pore size for SP XL is also smaller than that of SP FF. Due to the smaller pore size, the exclusion effect is magnified in SP XL leading to more extreme changes in capacities as a function of conductivity. In contrast, for SP FF at pH values where both regimes are observed, a significant plateau region exists rather than a sharp peak.

The plateau region would be ideal for commercial operation since performance is not very sensitive to changes in one of the critical parameters, specifically conductivity. This would provide a wider acceptable operating range. Therefore, one advantage of SP FF over SP XL is the existence of this plateau region.

It is notable that few papers in literature, if any, investigate performance while varying pH and conductivity. Usually, a set of conditions is chosen without taking into consideration the interactive effects between both factors. Following that approach would have lead to missing the exclusion mechanism and, hence, not being able to fully take advantage of the resin performance and optimize the process.

By examining dynamic binding capacities as a function of both pH and conductivity, it was possible to thoroughly characterize the performance of the resins. Therefore, it was possible to better understand the exclusion regime, the conditions under which it occurs as well as to relate the capacities to the purity performance obtained.

The data obtained from this study are also important for extracting industrially significant information such as pool volumes. This data is essential for process development and ensuring that the process is feasible within the manufacturing facilities available. Furthermore, having a thorough understanding of the behavior of the resin can be utilized in integrating the cation exchange step within the rest of the process. For example, the loading conditions onto the cation exchange column can be chosen not only to obtain the best performance but also decrease the load modifications required prior to loading onto the cation exchange column. All of this is crucial for designing an efficient and robust process to produce the desired product with excellent purity.

CHAPTER 7

CONCLUSIONS

By following the experimental procedure set forth, the performance of SP FF and SP XL resin was characterized as a function of pH and conductivity. The conditions under which the exclusion effect occurs were noted and performance in terms of dynamic binding capacities, yields, and purity was assessed. For purity assessment, CHOP and Leached Protein A clearance was determined.

The exclusion effect was observed for both resins with both proteins. For SP XL, the exclusion effect is most pronounced at pH = 4 and 5 with both proteins. For SP FF, exclusion was also apparent at pH = 4 and 5 with both proteins. Also, the trends obtained within the exclusion regime were as expected where as the conductivity increases, the dynamic binding capacity increases until the equilibrium regime is reached. Furthermore, within the exclusion regime, an increase in pH results in an increase in capacity. Even though at the same pH, Mab2 is more highly charged than Mab1, the trends for both proteins at the same conditions were similar.

Even though the exclusion mechanism has been recently documented in literature, this is the first study to systematically assess and compare the purity obtained during the equilibrium and the exclusion effect.

Moreover, it has been shown that CHOP levels, in ng/ml, in the elution pools follow the dynamic binding capacities except at the lower conductivities for SP XL with Mab1 at pH = 6. This suggests that CHOP experiences exclusion to the same extent as the proteins at all conditions, even though CHOP is significantly smaller in size. This

could be due to the fact that under the conditions tested, the CHOP is associated with the Mab and hence, under both the equilibrium and exclusion regimes the CHOP trends were comparable. The yields obtained were also consistent throughout both regimes. This means that it is possible to take advantage of the higher capacities occurring at the intersection of the equilibrium and the exclusion regime, i.e. the critical dynamic binding capacities, without sacrificing purity in terms of CHOP in ppm in the elution pool.

As for the 2 different resins assessed, significantly higher dynamic binding capacities were obtained with SP XL. For example, with Mab1 at a pH of 5, the critical dynamic binding capacity at 10% BT was 124 and 73 mg/ml for SP XL and SP FF respectively. The higher capacity obtained with SP XL is a result of the dextran extenders present only on SP XL. As for purity, further investigation would be necessary to understand the effect of the dextran extenders on CHOP clearance, if any.

In summary, the results obtained help further characterize the exclusion effect and better understand its impact on purity and yield and hence provide a significant process development tool. This study has reconfirmed the occurrence of the exclusion phenomena in both SP XL and SP FF. It has proven that the major impurity, CHOP, experiences the exclusion effect to the same extent as the Mab proteins which could be due to the CHOP being associated with the Mab. Hence, it is possible to take advantage of the capacity with running at the critical dynamic capacity without sacrificing purity performance. However, when comparing the two resins investigated, a trade-off between capacity and purity exists where higher capacities are obtained with SP XL while SP FF achieves higher CHOP clearance.

CHAPTER 8

FUTURE STUDY

This study has further characterized the exclusion regime and investigated dynamic capacities, yield and purity as a function of pH and conductivity. However, several areas can be further investigated to enhance our understanding of this phenomenon.

Even though a protein mass balance was completed for the monoclonal antibodies, no mass balance was completed for the impurities. Completing a mass balance for the impurities, specifically CHOP, would be important for understanding where the majority of the CHOP is being cleared, e.g. in the washes and sanitization.

Also, collecting the elution peak in fractions and analyzing the profile of the various impurities across the elution peak can aid in understanding the protein and impurity elution profiles. It would be possible to assess the resolution between the protein and the impurities across the elution peak for both resins. Furthermore, with the availability of such details, it would be possible to optimize the pooling conditions to maintain a minimum yield requirement while reducing the impurity levels in the elution pool.

Several parameters not investigated in this study should be addressed in future studies. Evaluating the effect of varying the load concentration on the dynamic binding capacities and purity is a parameter that should be further explored. This would be useful to predict the effect of upstream process changes that result in load material with a significantly different concentration.

Another factor is the effect of residence time on capacity especially in conditions that fall within the exclusion regime. In a study performed in collaboration between Genentech, Inc. and GE Healthcare, the dynamic binding capacity and equilibrium capacity were determined as a function of conductivity [23]. The results are shown in Figure 19 [13]. From Figure 19, it can be observed that within the equilibrium regime the dynamic capacities and equilibrium capacities are very comparable. However, within the exclusion regime, it is possible to achieve significantly higher capacities by operating at longer residence times. Therefore, it would be valuable to investigate the highest obtainable dynamic binding capacities at industrially applicable residence times.

Figure 19. A comparison between the equilibrium and dynamic capacity as a function of conductivity for SP XL at pH = 5 [23].

Also, it would be worthwhile narrowing the range based on the results obtained and further optimizing performance by changing parameters such as elution and pooling conditions.

Once the additional data is obtained, a better decision can be made to choose the desired resin, pH and conductivity per product. It would then be important to test the sensitivity in performance with respect to the various parameters and set a suitable operating range accordingly. At that point further understanding of other factors, such as resin reuse, is necessary.

Furthermore, the exclusion phenomenon has only been investigated within cation exchange chromatography resins. However, the theory behind the phenomena is expected to hold for anion exchange resins as well. Therefore, a similar study similar should be completed for anion exchange chromatography resins such as Q SepharoseTM Fast Flow, also manufactured by GE Healthcare.

REFERENCES

1. Genentech, Inc. (2004) *The Science of Biotechnology* [Online] Available at <http://www.gene.com/gene/research/biotechnology> (accessed 22 September 2005).
2. California Research Bureau (1996) *An Overview of Biotechnology and Bioindustry* [Online] Available at http://www.library.ca.gov/CRB/96/07/BIOT_CH1.html (accessed 22 September 2005).
3. P. Todd, S.R Rudge, D.P. Petrides and R.G. Harrison, Bioseparations Science and Engineering (Oxford University Press).
4. Wikipedia, the free encyclopedia (2005) *Chromatography* [Online] Available at <http://en.wikipedia.org/wiki/Chromatography> (accessed 22 September 2005).
5. Amersham Biosciences (2005) *The typical ion exchange experiment* [Online] Available at http://www.chromatography.amershambiosciences.com/APTRIX/upp00919.nsf/Content/LabSep_EduC%7ELC_tech%7EIEEX%7EIEXBasic%7EIEXEper?OpenDocument&hometitle=LabSep (accessed 23 July 2006).
6. M.A. Fernandez, W.S. Laughinghouse, G. Carta, "Characterization of protein adsorption by composite silica-polyacrylamide gel anion exchangers II. Mass transfer in packed columns and predictability of breakthrough behavior," *J. Chromatogr. A*, **746**, pp. 185-198 (1996).
7. C. A. Brooks and S.M. Cramer, "Steric Mass-Action Ion Exchange: Displacement Profiles and Induced Salt Gradients," *AIChE J.*, **38**, pp. 1969-1978 (1992).
8. J-C Janson and L. Ryden, Protein Purification: Principles, High-Resolution Methods, and Applications, 2nd ed. (Wiley-VCH).
9. C. Harinarayan, J. Mueller, A. Ljunglöf, R. Fahrner and R. van Reis, "Development of Novel Ion Exchange Process Conditions in Antibody Purification," Presentation at Antibody Production and Downstream Processing, IBC Conference, San Diego, USA (2002).
10. J. S. Fritz, "Review Early milestones in the development of ion-exchange chromatography: a personal account," *J. Chromatogr. A*, **1039**, pp. 3-12 (2004).
11. H. Small, T.S. Stevens and W.C. Bauman, "Novel Ion Exchange Chromatographic Method Using Conductimetric Detection," *Anal. Chem.*, **47**, pp. 1801-1809 (1975).

12. P.R. Levison, C. Mumford, M. Streater, A. Brandt-Nielsen, N.D. Pathiramna, S.E.J. Badger, "*Performance Comparison of Low-Pressure Ion-Exchange Chromatography Media for Protein Separation*," J. Chromatogr. A, **760**, pp. 151-158 (1997).
13. E. Boschetti, "*Advanced sorbents for preparative separation purposes*," J. Chromatogr., **658**, pp. 207-236 (1994).
14. A. Staby, M.B. Sand, R.G. Hansen, J.H. Jacobsen, L.A. Andersen, M. Gerstenberg, U.K. Bruus and I.H. Jensen, "*Comparison of chromatographic ion-exchange resins III. Strong cation-exchange resins*," J. Chromatogr. A, **1034**, pp. 85-97 (2004).
15. A. Staby and I.H. Jensen, "*Comparison of chromatographic ion-exchange resins II. More strong anion-exchange resins*," J. Chromatogr. A, **908**, pp. 149-161 (2001).
16. A. Staby, I.H. Jensen and I. Møllerup, "*Comparison of chromatographic ion-exchange resins I. Strong Anion-exchange resins*," J. Chromatogr. A, **897**, pp. 99-111 (2000).
17. A. Staby, M.B. Sand, R.G. Hansen, J.H. Jacobsen, L.A. Andersen, M. Gerstenberg, U.K. Bruus and I.H. Jensen, "*Comparison of chromatographic ion-exchange resins IV. Strong and weak cation-exchange resins and heparin resins*," J. Chromatogr. A, **1069**, pp. 65-77 (2005).
18. D.C. Nash and H.A. Chase, "*Comparison of diffusion and diffusion-convection matrices for use in ion-exchange separations of proteins*," J. Chromatogr. A, **807**, pp. 185-207 (1998).
19. J. Horvath, E. Boschetti, L. Guerrier and N. Cooke, "*High Performance Protein Separations with Novel Strong Ion-Exchangers*," J. Chromatogr., **679**, pp. 10-22 (1994).
20. I. Lagerlund, E. Larsson, J. Gustavsson, J. Färenmark and A. Heijbel, "*Characterization of ANX Sepharose 4 Fast Flow media*," J. Chromatogr. A, **796**, pp. 129- 140 (1998).
21. P.R. Levison, R.M.H. Jones, D.W. Toome, S.E. Badger, M. Streater and N.D. Pathirana, "*Influence of flow-rate on the chromatographic performance of agarose- and cellulose-based anion-exchange media*," J. Chromatogr. A, **734**, pp. 137-143 (1996).

22. Q. Shi, Y. Zhou and Y. Sun, "*Influence of pH and Ionic Strength on the Mass-Action Model Parameters Around the Isoelectric Point of Protein*," *Biotechnol. Progr.*, **21**, pp. 516-523 (2005).
23. R. van Reis, Downstream Keynote: Charge Exclusion Phenomena In Separation Media, Abstracts of Papers, Best of Biochemical Technology Division of ACS, July 18, 2005 (2005).
24. A. Ljunglöf and J. Thömmes, "*Visualising intraparticle protein transport in porous adsorbents by confocal microscopy*," *J. Chromatogr. A*, **813**, pp. 387-395 (1998).
25. The Hebrew University of Jerusalem (2002) *UV Absorbance (280 nm) – Protein Determination* [Online] Available at <http://www.ls.huji.ac.il/~purification/Protocols/OD280.html> (accessed March 24 2006).