

**PURIFICATION OF MONOCLONAL ANTIBODIES
UTILIZING A NOVEL CHROMATOGRAPHY RESIN
OPERATED IN FLOW-THROUGH MODE**

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biochemistry

by

Brooke Czapkowski
April 25, 2013

APPROVED:

James Hamzik, Senior Scientist
R&D Process Solutions
Product Development Chromatography
EMD Millipore
MAJOR ADVISOR

David Adams, PhD
Biology and Biotechnology
WPI Project Advisor

ABSTRACT

Monoclonal antibodies have proven to be an extremely successful class of protein therapeutics. Monoclonal antibodies and other biopharmaceutical products used in humans, are required to meet strict purity standards. Cation-exchange (CIEx) chromatography has become a staple of monoclonal antibody purification platforms. In this project, an experimental chromatography resin specifically designed to be operated in flow-through mode and replace traditional CIEx during monoclonal antibody polishing has been characterized. The results show that this new resin can achieve higher loading capacities and reduced buffer consumption, resulting in improved process economics, while being an effective replacement for currently available CIEx resins that are designed to be operated in bind-and-elute mode.

TABLE OF CONTENTS

Signature Page	1
Abstract	2
Table of Contents	3
Acknowledgements	4
Background	5
Project Purpose	16
Methods	17
Results	21
Discussion	27
Bibliography	29

ACKNOWLEDGEMENTS

I would like to thank Jim Hamzik, who has been an excellent supervisor and mentor to me. Jim's guidance and patience were keys assets to my success during this project. Jim spent many hours, outside of his regular work week, to assure that my project succeeded. I truly appreciate all his help more than words can even describe. I would also like to thank David Beattie, who provided me with the wonderful opportunity to intern at EMD Millipore this summer, which led to this Major Qualifying Project at Millipore. And thank you to Lars Peeck, Joaquin Umana, Kristina Cunningham, Yogesh Mudaliar, Lina Mpanda, Yuanchun Zeng, Neil Soice, and Benjamin Duckless for all of their assistance throughout the duration of my project. Finally, I would like to thank Dave Adams for being an extremely supportive advisor throughout this project.

BACKGROUND

Monoclonal Antibodies

Monoclonal antibodies (mAbs) are generated in the laboratory by using a single clone of a B cell. B cells are the cells within the immune system that produce antibodies. Antibodies, which are also referred to as immunoglobulins (Igs), are proteins which assist the immune system in identifying foreign agents, such as viruses and/or bacteria, within the body.

Immunoglobulin gamma (IgG) is the most abundant type of antibody, and is present in blood and other bodily fluids. Antibodies function by binding to a foreign invader, also known as an antigen, so the antigen is recognized as being foreign and it is triggered to be eliminated from the body. Unlike polyclonal antibodies which bind to a variety of antigens, mAbs can bind very specifically to one target antigen. (Longe, 2010)

The basic molecular structure of most antibodies is “Y” shaped, and consists of variable and constant regions (**Figure 1**). The variable regions vary in sequence and structure, which allows them to develop binding specificities against a wide range of antigens. The constant region is conserved among antibodies, and is responsible for interacting with the effector system and triggering elimination of the antigen from the body. Researchers have taken advantage of mAb’s ability to recognize various targets with high specificity to produce many important therapeutic and diagnostic tools. (Nelson PN, 2000)

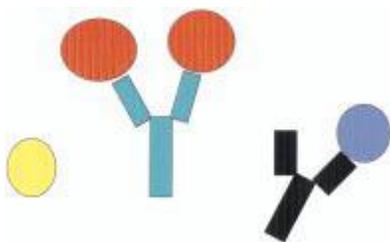


Figure 1: Schematic representation of an antibody molecule highlighting the “Y” shaped structure (Nelson PN, 2000)

Monoclonal Antibodies as Therapeutic Drugs

Monoclonal antibodies are useful within laboratory research and as pharmaceutical therapeutics to treat a wide variety of diseases and illnesses. “By 2009, forty-six different monoclonal antibodies had been licensed for the treatment of a variety of diseases, including cancer, asthma, and rheumatoid arthritis, and for use in transplantation.” (Wood, 2010) Depending on the required therapeutic effect, mAbs can act by blocking or neutralizing a receptor, or they can take an active role by targeting an effector function.

One successful therapeutic mAb, Herceptin, is used to treat a specific form of breast cancer. Herceptin binds to Her-2, a growth receptor found on the surface of breast cancer cells. Her-2 is responsible for sending signals towards the inside of breast cancer cells, requesting the cells to divide and grow. Herceptin slows this process by binding to Her-2 and inhibiting the cell signals, therefore, slowing down the cell division process.

Monoclonal antibodies can also work to fight cancers by enabling the immune system to attack and eliminate the cancerous cells. This is the case for antibodies such as Rituximab, which are used to treat leukemia. These antibodies bind to leukemia cancer cells and then signal the immune system to kill the cells. Monoclonal antibodies are also useful when it comes to suppressing the immune system. Suppressing the immune system can have therapeutic value during allergic reactions and transplantation procedures. (Wood, 2010)

As a result of their proven therapeutic and diagnostic applications, mAbs have gained a huge amount attention and investment from researchers and businesses worldwide.

Forecasted Economic Outlook of Monoclonal Antibodies

Monoclonal antibodies are becoming a staple of the pharmaceutical industry. In terms of the growing market for protein-based therapeutics, mAbs are market leaders based on volume sales (Gottschalk, 2009). The ten mAbs currently utilized for cancer therapy collectively generated US\$17 billion in revenue in 2009. Evaluate Pharma estimates that by 2016, products made from monoclonal antibodies will represent 11 of the top 50 selling therapeutic products in the world, 6 of them being the top 10 selling products. (Becker, 2010) By the end of 2011, it had been estimated that monoclonal antibodies alone generated revenue of about US\$48 billion. (Becker, 2010)

It is forecasted that mAbs will play a major role in the future of disease treatment; consequently, there is currently a significant amount of resources being expended on the research and manufacturing capabilities of mAbs. As a result, it is of importance for scientists to ensure mAbs can be safely and economically manufactured, and distributed as therapeutics to patients.

Impurity Clearance of Monoclonal Antibodies

Monoclonal antibodies have proven to be an extremely successful class of protein therapeutics, and when used as human therapeutics, they along with other biopharmaceutical products, are required to meet strict regulatory and purity standards. Commercial production of mAbs is divided into two major steps, each is comprised of smaller unit operations to complete the process. First, animal cells expressing an antibody are grown in a stirred bioreactor containing a nutrient rich liquid medium. Once the antibody reaches a target titer in the medium, it needs to be separated from the cells and harvested from the bioreactor. The aforementioned is commonly referred to as upstream processing. The antibody is then purified by separating the

target molecule from various soluble impurities, generally by means of filtration and chromatography, and this is referred to as downstream processing. **Table 1** displays the major impurities requiring removal validation, and some of the characterization/quantification techniques used during downstream processing.

Impurity/Characterization	Detection Method
Host Cell Proteins (HCP)	Chinese Hamster Ovary (CHO) Host Cell Proteins ELISA
Leached Protein-A	Protein-A ELISA
Aggregates	Size Exclusion High Performance Liquid Chromatography
DNA	QPCR, PicoGreen Assay
Protein Concentration	Ultra Violet (UV) Absorbance
Viruses	QPCR, Infectivity Assay

Table 1: During the downstream process, various bio-analytical tests are performed to determine the purity and characterization of the monoclonal antibodies during the manufacturing process.

Importance of Aggregate and Protein A Clearance from Monoclonal Antibodies

Two related impurities which must be sufficiently removed from monoclonal antibodies during downstream processing are aggregates and leached Protein-A. These must be removed from the mAbs because of their immunogenicity (Farhner, 2001), and can be sufficiently cleared by employing cation-exchange chromatography (Gottschalk, 2009).

Most mAb preparations start out with some level of aggregates, typically 1-15%, and efforts need to be made to reduce the level in the final product per FDA guidance (FDA, 2013). Typical target levels for the final product are less than 0.5%, as protein aggregates are known to cause negative immune responses within patients treated with antibodies which contain high levels of protein aggregates. As a result of there being many different factors involved in immunogenicity, it has been difficult for researchers to pinpoint the exact linkage between

aggregates and immunogenicity. Recently, a few hypotheses have been proposed to explain the alteration of immunogenicity of protein aggregates, including their: “(1) enhanced immunogenicity by oxidation-induced structural change; (2) enhanced immunogenicity by formation of repetitive structures in oxidation-derived aggregates; and (3) reduced immunogenicity by alteration or even loss of immunogenic epitopes in protein aggregates after different treatments” (Wang, 2012). However, it is understood that because the aggregates have similar sequences as the monomer they can also cause the patient to have an immune response towards the therapeutic monomer thus causing neutralizing antibodies to decrease the efficacy of the monomer overtime (De Groot, 2007).

Protein-A, a bacterial cell wall protein, is immobilized onto a solid media, and is often used as the first step of purification of mAbs as an affinity chromatography capture step. Because of its high specificity for antibodies, the Protein-A chromatography step results in >95% purity of the target molecule in one step. However, during this process, some of the Protein-A comes off of the column and into the antibody sample. This “leached” Protein-A can be potentially immunogenic, and can have other physiological consequences, therefore, it is also required to be removed during downstream processing of the monoclonal antibodies. (Farhner, 2001)

Much research has been conducted on how to most effectively produce and manufacture an extremely pure, high yield of mAbs. After years of types of research and investments into facilities, column chromatography has become the preferred platform for maintaining high yields of product and to remove impurities from during downstream processing (Gottschalk, 2009).

Column Chromatography

In the biotechnology industry, column chromatography is the most utilized technique to separate impurities from their target molecules. Chromatography columns are cylindrical in shape, and can be constructed from glass, stainless steel, or acrylic. There are multiple different types of column chromatography currently utilized, some of which include: affinity, anion/cation exchange, mixed-mode, gel filtration (size exclusion), hydrophobic interaction, and reverse phase. Chromatography columns are most often packed with resin beads, also referred to as “media.” The media can capture the impurities and allow the target molecules to flow-through (flow-through mode), or can capture the target molecule and differentially bind the impurities (bind-and-elute mode). **Figure 2** shows a chromatography schematic to visualize flow of the target molecules through resin beads in a chromatography column. (Fetterolf, 2009)

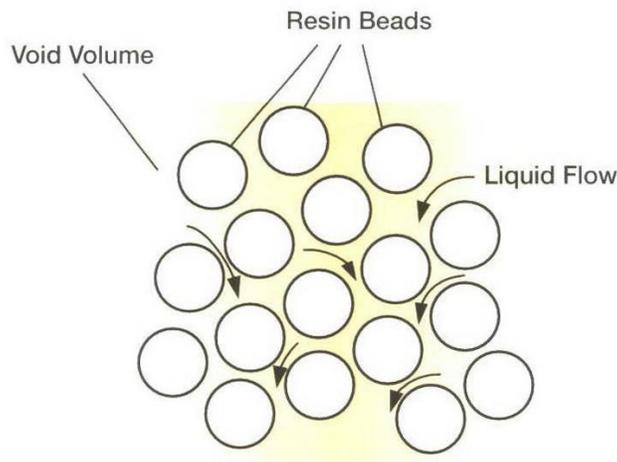


Figure 2: Diagram of the flow in a packed chromatography column. (Fetterolf, 2009)

Column chromatography, like most other laboratory procedures, is defined by a step-by-step procedure. The order of the process run in bind-and-elute mode is: (1) Equilibration, (2) Loading, (3) Washing, (4) Elution, (5) Strip and Cleaning-in-place (CIP). In equilibration, a

buffer solution rinsed through the column for the resin and the entire system itself to be at the desired pH, conductivity, and temperature. Once the column is equilibrated, the product is ready to be loaded into the column. In “bind-and-elute” chromatography, the target molecules bind to the resin, and the impurities either flow through the column or are retained more strongly than the target molecule and remain on the column after the product is eluted. The goal of bind-and-elute is to have virtually none of the target molecules in the “flow through” or remaining on the column after elution.

One of the main factors to be considered during any column chromatography procedure is flow rate. The higher the flow rate, the less probable the target molecules will bind to the resin due to decreased contact time, also referred to as “residence time.” During the wash phase, which is preparation for the elution phase, the same buffer solution used during the equilibration step is flowed through the column to rinse away any loose impurities which may still be present in the column. The elution step is when the target molecules are released from the resin and are collected to form the final product pool. In ion exchange chromatography, this step is achieved by either increasing the salt concentration, or causing a shift in pH of the column by eluting the column with a buffer with a higher salt concentration or pH than the previously used buffer. In terms of the strip and CIP, an extreme elution buffer, with a high salt concentration is used to remove any leftover target molecules still bound to the resin via ionic interactions, and the CIP is typically performed with a caustic wash such as 0.5 N NaOH. The caustic wash can also function as a column sanitization. This is an important step because resin is expensive and columns are often re-used multiple times to improve process economics; therefore, it is pertinent to make sure any remaining material is removed before the next cycle of materials. (Fetterolf, 2009)

Despite the numerous types of current column chromatography methods and platform purification processes, bioprocess developers are always looking for new technologies to improve the robustness and economics of mAb production.

Traditional 3-Step Chromatography Process

The effective purification of mAbs can require three or more sequential chromatography steps. A commonly reported chromatography series includes: Protein-A affinity chromatography, cation-exchange chromatography, and anion-exchange chromatography (Farhner, 2001).

Protein-A Affinity Chromatography uses porous beads with immobilized Protein-A. As discussed previously, Protein-A is a bacterial cell wall protein which specifically binds to antibodies, allowing impurities to flow through. An example of a resin that is used for Protein-A chromatography is ProSep® Ultra Plus media (EMD Millipore). The importance of this Protein-A step in a mAb purification is it concentrates the product, and removes cell proteins, DNA, and media components. This step alone achieves >95% purity, however, it does not remove product related aggregates, and it adds a new, process related impurity, leached Protein-A, into the feed.

Cation-exchange uses beads with negatively charged sites that bind molecules that are positively charged. An example of such beads is Fractogel® SO3 media. **Figure 3** shows a SEM of negatively charged Fractogel® beads (Staby, Jacobsen, Hansen, Bruus, & Jensen, 2006). This step is used to remove impurities such as aggregates and Leached Protein-A. Typically, cation-exchange chromatography is operated in bind-and-elute mode, and requires increased conductivity to elute the target molecule. The resulting high conductivity product pool requires dilution back to low conductivity or the use of salt tolerant chromatography for the next step, anion-exchange chromatography, to be effective. This MQP project is based on exploring the

utility of a novel cation-exchange resin with a proprietary surface modification that can be operated in flow-through mode without an increase in conductivity.

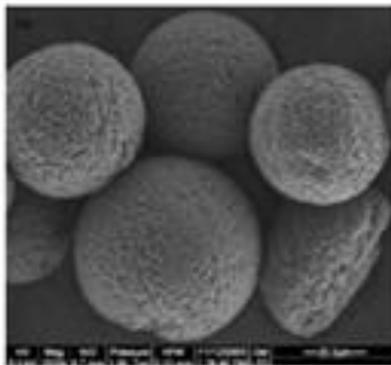


Figure 3: Scanning electron microscope photograph of the surface of Fractogel[®] bead. (Staby, Jacobsen, Hansen, Bruus, & Jensen, 2006)

Anion-exchange is often the last chromatography step of the three-step process; this step uses a flow-through method. Anion-exchange uses positively charged sites to bind negatively charged solutes. Anion-exchange uses porous beads or membranes with positively charged sites such as Fractogel[®] TMAE media and ChromaSorb[™] membrane adsorber (EMD Millipore). Fractogel[®] TMAE media are anion-exchange beads with positively charged sites that bind molecules that are negatively charged, and ChromaSorb[™] membrane adsorber is a salt-tolerant anion exchange membrane absorber. In this flow-through method, the feed is flowed through the resin, the impurities bind to the resin, and the purified product flows through the resin. This step removes DNA, viruses, and residual host cell protein to produce the final product.

Traditional Bind-and-Elute Cation-Exchange Chromatography

As discussed previously, in cation-exchange chromatography, the chromatography media used in the chromatography column has negatively charged ligands on the surface of the resin.

These negatively charged ligands capture the positively charged target molecules and allows for the negatively charged impurities to flow through the column. Once the negatively charged impurities flow through the column and the positively charged target molecules are captured on the negatively charged resin, a buffer with increased sodium chloride is used to create a salt gradient during the elution phase. The positively charged sodium competes with the target molecule for negative sites and on the resin, and the target molecules are dissociated and flow into the elution pool. The resulting pool has a high conductivity, which requires a dilution or diafiltration before moving onto the next step, anion-exchange chromatography, which requires low conductivity. The dilution step can be problematic especially for large facilities that have installed intermediate tanks of a fixed size. Also water-for-injection which is used for the dilution can be very expensive, and the increase in volume adds time and labor to the process. (Zhou, 2007)

In a new flow-through chromatography method that has been designed (discussed below), there is no need for an increased conductivity. Therefore, there is no need for a dilution step between the cation-exchange step and the anion-exchange step.

Novel Flow-Through Chromatography

In a new 3-step process designed by scientists at EMD Millipore, a flow through chromatography resin and method has been developed. In order to achieve this novel flow-through process, specially designed beads, with a proprietary ligand capable of retaining aggregates and leached protein-A impurities, are used as the chromatography media. While these impurities bind to the beads, the purified protein product is able to flow through the beads and collect for subsequent purification. In this new process the traditional bind-and-elute cation

exchange step is replaced with a step that achieves several times the resin loading capacity with a resulting low conductivity pool. This results in improved process economics, and circumvents a costly and sometimes facility-constrained dilution step.

PROJECT PURPOSE

This MQP project worked towards characterizing an experimental chromatography resin that is specifically designed to be operated in flow-through mode during mAb polishing. The data indicate that this new resin can be an effective replacement for currently available cation-exchange resins designed to be operated in bind-and-elute mode.

Goal and Scope:

Characterize a novel EMD Millipore chromatography resin, and explore the utility of this new tool in mAb purification.

Objectives:

1. Gain a background understanding of monoclonal antibody purification.
2. Use a Chromatography Workstation to characterize a library of resin prototypes and determine optimal resin design for mAb purification.
3. Explore this new resin's effectiveness in replacing a traditional cation-exchange resin towards a completely flow-through mAb polishing platform.

METHODS AND MATERIALS

General Overview of the Experimental Process

1. Obtain clarified cell culture medium containing mAbA.
2. Generate Protein-A Pool.
3. Screen various prototype flow-through resins.
4. Pack columns with prototype flow-through resins and load them onto an AKTA explorer. Program the AKTA to run method as outlined in Table 2.
5. Run the method to bind the impurities, flow through the purified product, and elute out the impurities at the strip phase while collecting fractions.
6. Analyze collected fractions for aggregate removal using Size Exclusion HPLC.
7. Measure protein concentrations of each fraction using NanoDrop.
8. Perform three step Chromatography purification process using new flow-through resin as the intermediate polishing step and Fractogel® TMAE media and/or ChromaSorb™ adsorber as the final anion-exchange step.
9. Analyze fractions for impurity (Host Cell Protein, Leached Protein-A, DNA, Aggregates) clearance.

Detailed Descriptions of Experimental Processes

A clarified cell culture expressing a test monoclonal antibody (MAbA) was obtained using a proprietary process. The MAbA was partially purified with Protein-A affinity chromatography to produce an intermediate step pool. The protein concentration of pool was measured by a 2000c NanoDrop (Thermo SCIENTIFIC). The NanoDrop software was then used to calculate the protein concentration of the Protein-A pool.

A library of experimental flow-through chromatography matrices were prepared by a proprietary method. The matrices all had various iterations of surface chemistry designed to interact with proteins more or less strongly. The types of matrices which were used included both membranes and resin beads.

All of the flow-through chromatography experiments were performed on an AKTA Explorer (GE Healthcare) using 50 mM Sodium Acetate at pH 5 for the equilibration buffer, 50

mM Sodium Acetate at pH 5 as the loading buffer, using the equilibration buffer for the wash step, using equilibration buffer plus 300 mM NaCl for the strip buffer, 0.5M NaOH as the cleaning buffer, and then a final equilibration wash with the equilibration buffer (**Table 2**). For the resin bead experiments, glass columns with internal diameters of 6.6mm were packed to a bed height of 3cm, which provided total volumes of 1mL of resins and then loaded onto the AKTA Explorer. For the membrane experiments, 3, 4, and 5 layers of membranes were loaded in membrane devices and then loaded onto the AKTA explorer. The feed MAbA Protein-A pool was loaded into a super loop on the AKTA Explorer. The specific method for each experiment was programmed onto the AKTA software (Unicorn). Once a method was begun, the AKTA pumped cell culture through the columns or membranes to bind the impurities, flow through the purified product, and elute out the impurities at the regeneration phase. The end products were collected as 2 mL fractions of purified MAbA into a 96-well plate. The output of the software at the end of each experiment was a detailed chromatogram for each individual experiment.

Process Step	Buffer	Residence Time (min)	Duration (Column Vol.)
Equilibration (EQ)	50 mM Sodium Acetate, pH 5	3	8
Loading	EQ buffer + MAb	3	mL = 100, Load density = 400 mg/mL
Wash	EQ	3	10
Strip	EQ + 300 mM NaCl	3	5
Clean-in-place	0.5 M NaOH	3	5
EQ	EQ	3	5

Table 2: Example of method used for AKTA Explorer cation-exchange chromatography experiments. *NOTE: different runs had varying method conditions.

After these 2mL fractions were collected from the AKTA Explorer, a chromatogram was output by the AKTA software, a sample of such a chromatogram can be viewed in **Figure 4**.

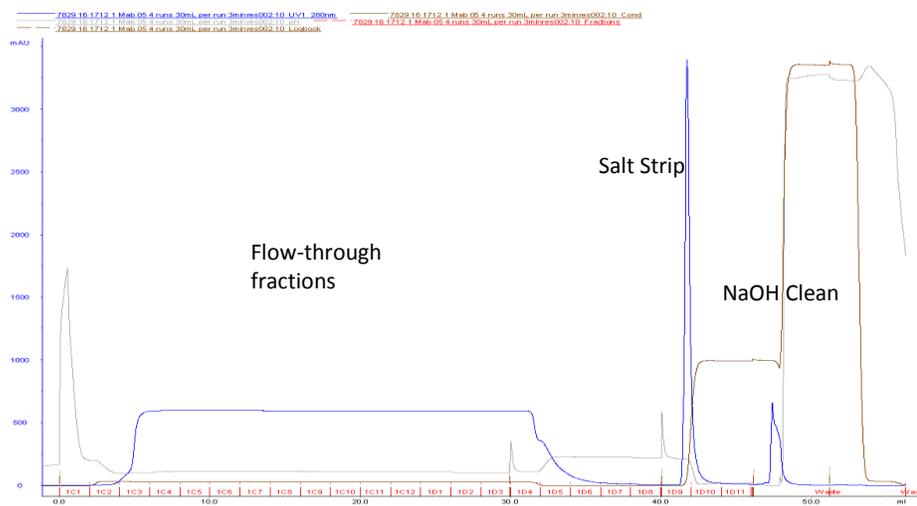


Figure 4: Sample chromatogram run on the AKTA Explorer (GE Healthcare), using the new EMD Millipore flow-through resin.

The plate was then placed onto the HPLC (Gilson). The method was run on the HPLC software in order to analyze aggregate removal in each sample. During this method, the liquid handler would insert itself into an individual well of the 96-well plate and take 20 uL of the sample and send it through the GFC (HPLC) column (SEC HPLC TSKsw3000). The output of the software at the end of each experiment was a chromatogram which displayed peaks which referred to the percent of aggregate in the sample vs. the percent of monomers in each fraction, as seen in **Figure 5**.

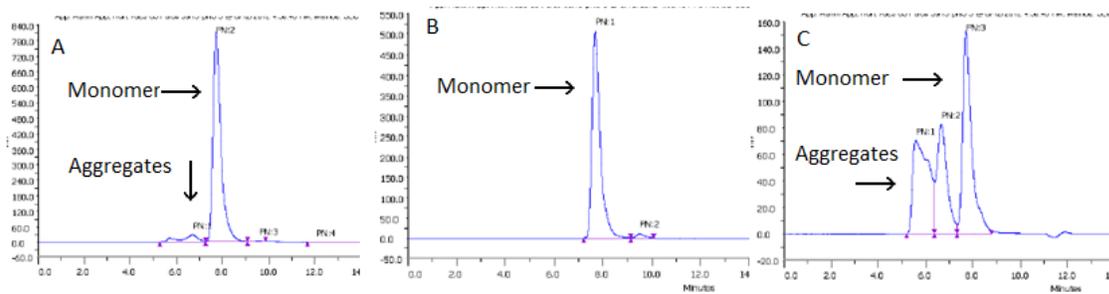


Figure 5: Analysis of Fractions for Aggregates using Size Exclusion HPLC. **A:** Protein-A Pool; **B:** CIEx Flow-Through, **C:** CIEx Strip.

The protein concentrations of each of these samples were measured using a 2000c NanoDrop. 2 uL of each sample was dropped onto the NanoDrop platform using a 10 uL pipette. The NanoDrop software was then used to calculate the protein concentration of each individual fraction.

The samples of purified MAbA with a low aggregate percentage were then pooled together. This pool was then loaded onto the super loop on the AKTA. Fractogel[®] TMAE was loaded into a column and a ChromaSorb[™] membrane was loaded onto the AKTA. In the anion-exchange step of the three-step protein purification chromatography process, the pool was sent through the Fractogel[®] TMAE column and/or the ChromaSorb[™] membrane in order to further purify the samples. These final samples were then assayed using validated protocols. The assays used include:

- Aggregates: Size Exclusion Chromatography (SEC) HPLC
 - See protocol mentioned above; same steps used.
- Protein Concentration: NanoDrop Absorbance
 - See protocol mentioned above; same steps used.
- HCP: CHO Host Cell Proteins 3rd Generation ELISA (specifically used for Chinese Hamster Ovary MAbs)
- Leached Protein A: Protein A ELISA
- DNA: PicoGreen Assay
- Non-Reducing SDS-PAGE gel

RESULTS

We received over 20 various prototypes of resin with varying levels of surface modification, and evaluated them for aggregate removal from a test mAb preparation. The prototypes were loaded onto the AKTA in glass columns with an internal diameter of 6.6mm, a bed height of 3cm and a total volume of 1mL. A mAb solution of 13.5 mg/mL with 2.0 % aggregates was flowed through the resin beads at 3 minute residence time, and various assays were used to analyze the efficiency of the resin beads. **Figure 6** shows four of the prototypes and their ability to separate aggregates. We concluded that prototypes #4 (purple dotted curve) had the best ability to separate monomer from aggregates in flow-through mode so we used this resin for the remainder of the experiments going forward.

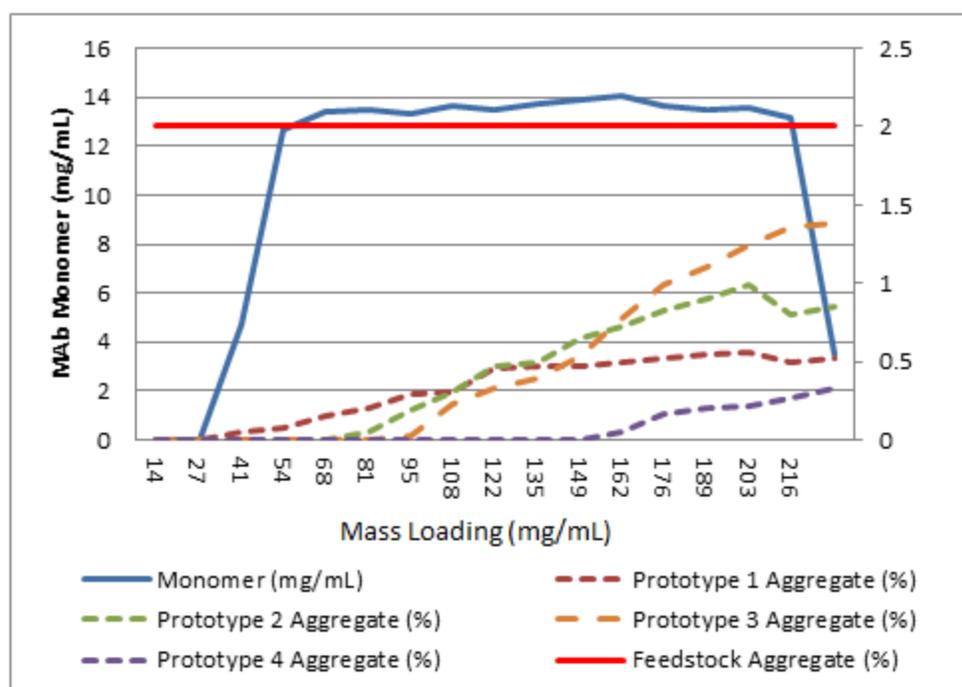


Figure 6 shows four of the prototypes tested and their ability to separate aggregates. Prototype #4 (purple dotted curve) displayed the best ability to separate aggregate from monomer which is determined by the late aggregate break-through (right side) of the aggregates at 150 mg/mL.

To determine the effect of residence time (flow rate) on the ability for resin prototype #4 we tested the resin at 1, 3, and 6 minute residence time (**Figure 7**). For example the shorter the residence time, the less time it takes to exchange a column volume. Since aggregates are large molecules, we were concerned with a dramatic drop off of capacity at short residence time (faster flow rates) because the molecule would have less time to access the internal surface area of the bead.

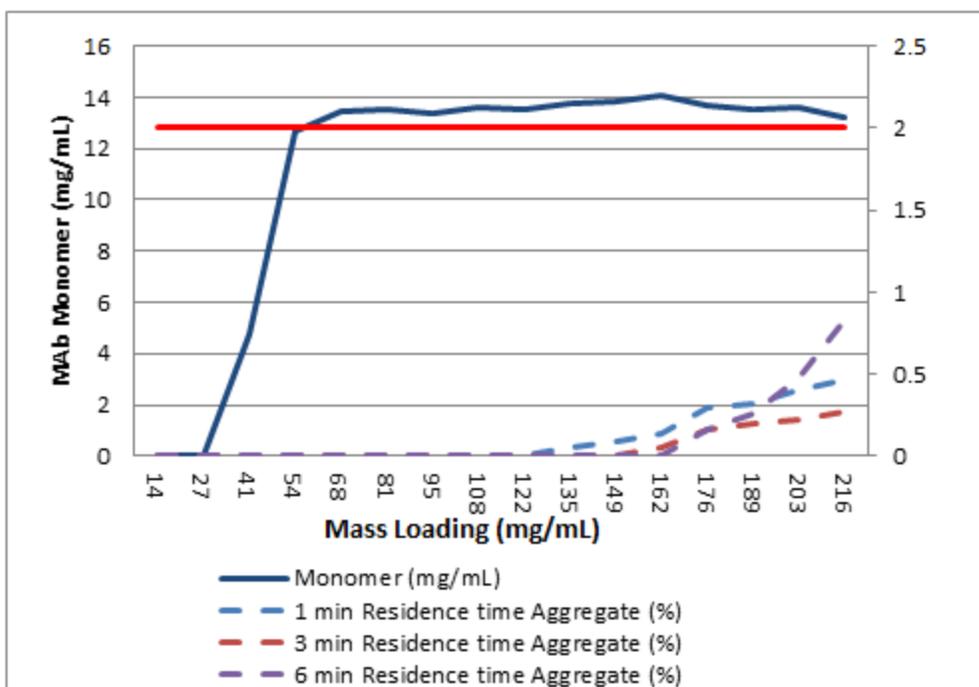


Figure 7 shows the impact of applying the sample at 1, 3, and 6 minute residence times. This shows that there is not a large decrease in dynamic binding capacity for aggregates between 1 and 6 minute residence time. This is important as we begin to assess the industrial applications of this technology because processing time is an important factor that contributes to process economics.

To evaluate the applicability of resin beads as a chromatography medium for the 3-step chromatography purification template, we tested a full purification train and applied the appropriate assays. To begin the experiment we generated a Protein-A pool from a cell culture

containing a monoclonal antibody. The protein A pool had 5.4% aggregates determined by (SEC) HPLC. Next, we adjusted the pH of the protein-A pool to pH 5.0 and performed the new flow-through process for removal of aggregates. Finally, the last step consisted of either ChromaSorb™ membrane adsorber or Fractogel® chromatography resin. The results are shown below on **Table 3**. Also, **Figure 8** shows a non-reducing SDS-PAGE gel for the three step process.

Protein A Pool		Starting 5.4 %	Starting 251 (ppm)	24000 (ppm)
Protein Load Density (mg protein/mL resin)	% Protein Recovery	% Aggregates 6min	[LProtein A] (ppm)	[HCP] (ppm)
16	14	0.0%	0.4	1
32	94	0.0%	1.8	1300
48	94	0.0%	1.9	4400
64	95	0.0%	2.1	9300
80	98	0.5%	2.4	19400
96	100	0.7%	2.5	24800
112	99	1.1%	3.1	25500
New FT Chromatography Pool	>90 %	<0.5%	0.8 (ppm)	11500 (ppm)
ChromaSorb™ membrane adsorber	>90 %	<0.5%	NA	60
Fractogel® media TMAE	>90 %	<0.5%	NA	200

Table 3 shows a three step process of purifying a monoclonal antibody from crude cell culture. This process includes a protein-A step, a novel flow-through process to remove aggregates, and one of two separate anion-exchange flow-through steps.

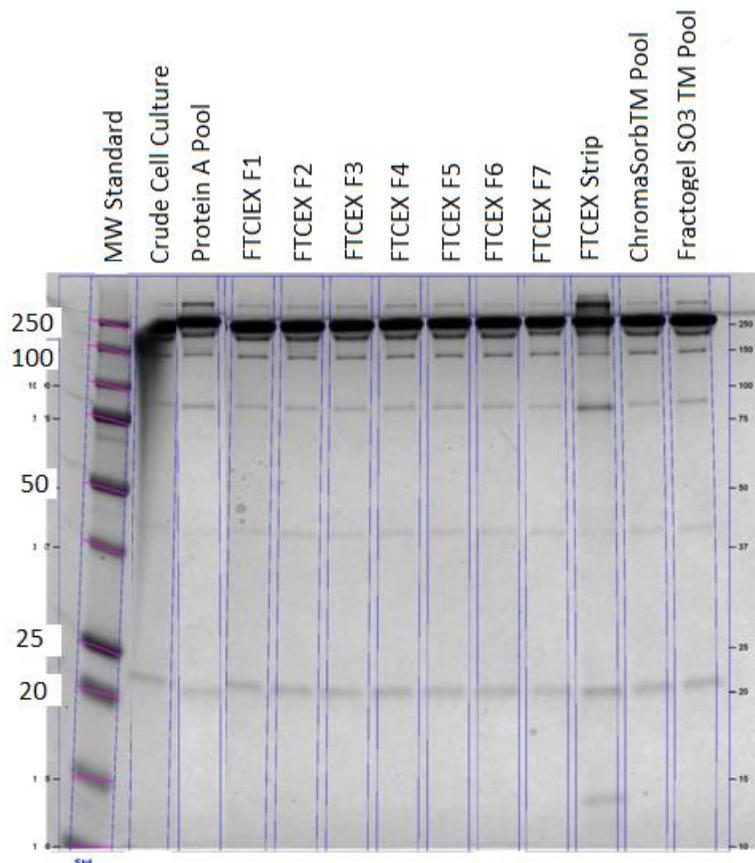


Figure 8 is a photograph of a non-reducing SDS-PAGE gel for the three step process, including fractions of the CIEX flow-through resin. It can be seen from the gel that the crude cell culture (lane-2) contains a wide range of protein species at a dilute concentration which is observed from streak of stain and also contains monoclonal antibody (apparent MW between 100 and 250 kDa). The protein-A pool (lane 3) shows a much cleaner and concentrated protein, but includes a significant amount of aggregates shown by the stain above 250 kDa. The FTCEX fractions show a significant removal of aggregated species. Whereas the salt strip shows that the aggregates are significantly concentrated. The light bands at approximately 25 and 50 kDa in all the samples are likely fragments or light and heavy chain of the monoclonal antibody.

It is important to understand the utility and benefit from a flow-through purification unit operation compared to a traditional bind-and-elute unit operation. We have observed that the ability to load our prototype resin #4 to about 150 mg/mL with 2% aggregate present. This is advantage over traditional bind-and-elute where you are typically limited to around 50 mg/mL loading. However, it is important to make sure that the resulting product is similar between the

two methods if the FT-operation is truly a functional replacement. We compared Fractogel[®] SO3 media performed in bind-and-elute mode verses our FTCEX resin prototype #4 performed in flow-through mode. We loaded the Fractogel[®] SO3 media to 50 mg/mL and prototype #4 to 150 mg/mL. The feed was a protein A pool and had an initial aggregate concentration of 3.4%.

Figures 9A and 9B show the bind-and-elute for Fractogel[®] SO3 media and FTCEX prototype #4, respectively.

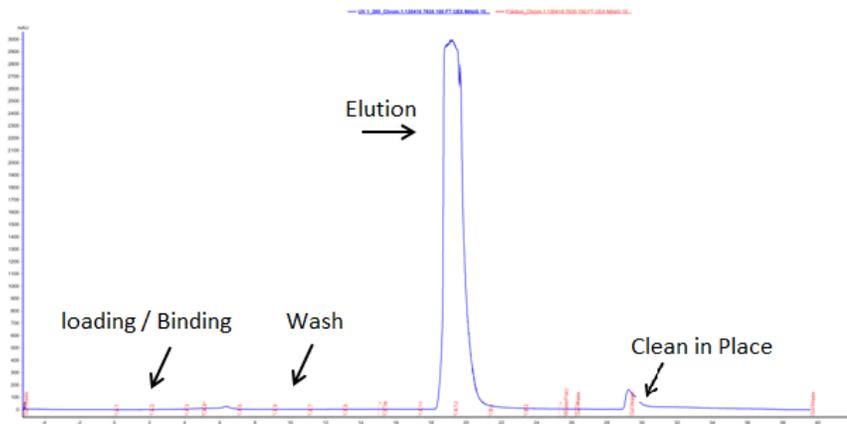


Figure 9A (above) Fractogel[®] SO3 media performed in bind-and-elute mode.

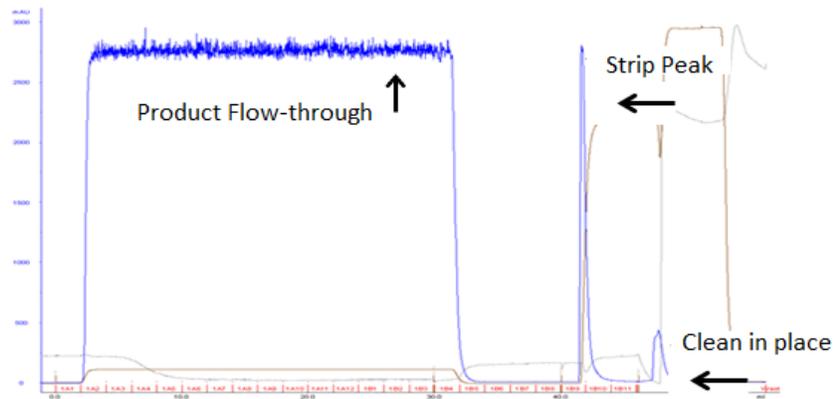


Figure 9B (above) FTCEX prototype #4 performed in flow-through mode.

Three Step Process	Step	Recovery (%)	Aggregate (%)	Leached Protein A (ppm)	CHOP (ppm)
Step 1	Protein A Pool	97	3.4	56	600
Step 2	Fractogel [®] SO3 media	84	0.0	LOQ	232
Step 3	ChromaSorb [™] Membrane Adsorber	99	0.0	LOQ	LOQ

*LOQ = below the limit of quantification

Table 4A: Three step process with traditional bind-and-elute CEX as intermediate step.

Three Step Process	Step	Recovery (%)	Aggregate (%)	Leached Protein A (ppm)	CHOP (ppm)
Step 1	Protein A Pool	97	3.4	56	600
Step 2	FTCIEX Pool Prototype 4	87	0.2	0.8	250
Step 3	ChromaSorb [™] Membrane Adsorber	96	0.2	NA	2

Table 4B: Three step process with FT CEX as intermediate step.

DISCUSSION

This project successfully characterized a library of resin prototypes to help determine an optimal resin design. These novel resins appear to be an effective replacement for traditional cation-exchange resins. The over-arching goal towards a completely flow-through monoclonal antibody polishing platform is now closer to becoming a reality.

The data from the results section strongly suggests that the most efficient resin beads used during the cation-exchange process was Resin prototype 4. As seen in Figure 6, Prototype #4 displayed the best ability to separate aggregate from monomer, as evidenced by the late aggregate break-through (purple dotted line) of the aggregates at 150 mg/mL.

As seen from figure 7 the resins are relatively insensitive to flow rate from 1-6 minute residence time. However, there is about 40 mg/mL difference in break through capacity between 1 and 6 minute residence time. This can be explained by the decreased contact time at 1 minute, which likely does not allow the aggregate to diffuse as far into the resin as the longer residence times. However, there is still significant capacity for aggregates at 1 minute residence time, and this is likely the most aggressive the flow rate would be challenged.

Results for the three step process, Table 3, produced a final pool with less than 0.5% aggregates, no detectable leached Protein-A, and low levels of HCP, while retaining high monoclonal antibody recovery. This is encouraging because this was considered an exceptionally challenging feed due to the unusual high levels of HCP in the load material. The increase in impurity and removal of aggregates was confirmed by SDS-PAGE.

It was also important to understand the performance in relation to a more traditional 3-step process that uses bind-and-elute cation exchange chromatography as an intermediate

polishing step. Our new method was loaded to 3X the capacity for traditional CIEX, 150 mg/mL compared to 50 mg/mL. The difference is due to the fact that traditional CIEX needs to bind both the aggregate and the monomer and additional loading would cause loss in yield due to breakthrough of product. The results were very promising for the new method which resulted in similar product yield and product quality. The advantage of the new method is primarily due to the higher loading which requires less resin to process the same mass of monoclonal antibody. This also results in less consumption of buffer due to smaller required column volumes of resin. These process intensifications (reduced materials and labor) could produce substantial cost savings and more attractive process economics.

An interesting find is that these resins displayed ideal performance under the conditions of pH 5 (data not shown) with a residence time of 1-6 minutes. As studies are continued, these conditions will be more closely observed for more of the resins that will be characterized and categorized. Additionally, another experiment to use in the future would be to use high-throughput screening on each resin to determine the optimal pH and salt concentration in order to remove the maximum amount of aggregates while recovering 80-90% of the monomer which is generally considered acceptable. Additional future experiments might include measuring the affinity of the purified mAb for its target to make sure it remains unaltered.

With these experiments, a successful method of unique flow-through chromatography as an efficient way of removing aggregates and other impurities, yielding a purified monoclonal antibody concentrate, has been developed. These findings are important in the quest towards determining the optimal conditions and matrix structure for potential commercial implementation. The next step towards achieving this goal is to add depth to the applications knowledge base of this promising technology.

BIBLIOGRAPHY

- Becker, M. (2010, July 12). Monoclonal Antibody Companies Command Premiums. *Seeking Alpha*.
- De Groot, A. S. (2007). Immunogenicity of Protein Therapeutics. *Trends in Immunology*, 482-490.
- Farhner, R. e. (2001). Industrial Purification of Pharmaceutical Antibodies: Development, Operation, and Validation of Chromatography Processes. 301-327.
- FDA, US. (2013). *Guidances (Drugs)*. U.S. FDA.
- Fetterolf, D. M. (2009). Column Chromatography. *Journal of Validation Technology*, 43.
- Gottschalk, U. (2009). *Process Scale Purification of Antibodies*. Hoboken: John Wiley & Sons, Inc.
- Longe, E. J. (2010). Monoclonal Antibodies. In M. Johnson, *The Gale Encyclopedia of Cancer* (p. 971). Detroit: Gale.
- Nelson PN, R. G. (2000). Demystified... Monoclonal antibodies. *Molecular Pathology*, 111-17.
- Staby, A., Jacobsen, J. H., Hansen, R. G., Bruus, U. K., & Jensen, I. H. (2006). Comparison of chromatographic ion-exchange resins: V. Strong and weak cation-exchange resins. *Journal of Chromatography A*, 168-179.
- Wang, W. e. (2012). Immunogenicity of Protein Aggregates--concerns and realities. *International Journal of Pharmaceutics*, 1-11.
- Wood, P. (2010, November). Monoclonal Antibody Therapy. *Biological Sciences Review*, p. 30.
- Zhou, J. X. (2007). pH-conductivity hybrid gradient cation-exchange chromatography for process-scale monoclonal antibody purification . *Journal of Chromatography A*, 69-80.