Genentech Senior Design Project:  
Purification of a Ranibizumab-like Protein

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Bachelor of Science in Chemical Engineering and the Honors Program

by

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entitled

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Purification of a Ranibizumab-like Protein

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BACHELOR OF SCIENCE IN CHEMICAL ENGINEERING

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Abstract

This project was completed by a Senior Design team of the Department of Chemical and Materials Engineering at the University of Nevada, Reno. The goal of this project was to design a process to isolate and purify a monoclonal antibody similar to Ranibizumab, or “Lucentis”, which is used in the treatment of wet age-related macular degeneration of the eyes, a leading cause of blindness. A focus on the chromatographical purification of Lucentis is provided, and both base and alternate cases for a complete process are presented. A discussion of relevant engineering economics and considerations for scale up of such a process are also included.
Table of Contents

Abstract ............................................................................................................................ i
Table of Contents .......................................................................................................... ii
List of Tables .................................................................................................................. iii
List of Figures ................................................................................................................ iv
Introduction ................................................................................................................... 1
Timeline ......................................................................................................................... 2
Theory ............................................................................................................................. 5
Literature Review .......................................................................................................... 6
Base Case ....................................................................................................................... 7
  1. Ion Exchange Chromatography (IEX) ................................................................... 8
  2. Principles of Operation ......................................................................................... 8
  3. Effects on Design ................................................................................................. 10
  4. Hydrophilic Interaction Chromatography (HIC) .................................................. 11
Alternate Case ............................................................................................................. 11
  1. Multimodal Chromatography ............................................................................. 11
  2. Monoclonal Antibody Purification ..................................................................... 13
  3. Monolithic Chromatography ............................................................................. 13
Modeling ....................................................................................................................... 16
  1. Velocity Modeling ............................................................................................... 16
  2. Flow and Pressure Drop Modeling .................................................................... 17
  3. Adsorption Modeling ......................................................................................... 18
Laboratory Scale Equipment Specifications .............................................................. 19
Industrial Scale Equipment Specifications ................................................................. 22
Experimental Procedure ............................................................................................ 25
Experimental Results ................................................................................................ 28
Conclusion ................................................................................................................... 29
Bibliography ............................................................................................................... 31
Appendix A .................................................................................................................. 33
Appendix B .................................................................................................................. 35
List of Tables

Table 1: Ion Exchange Differences .................................................................9
Table 2: Design Parameters for Monolithic Chromatography .........................16
Table 3: List of Laboratory Materials and Prices ..............................................20
Table 4: List of Industrial Materials and Prices .................................................23
Table 5: Scale-Up Parameters and Estimated Cost ............................................25
List of Figures

Figure 1: Gantt Chart for the Chromatography team of the Genentech Project ..............3
Figure 2: Base Case for Purification Process .................................................................8
Figure 3: Alternate Case for Purification of a Lucentis-like Protein .........................12
Figure 4: CIMmultus™ SO3-8000 Advanced Composite Column for Monolithic Chromatography ..........................................................15
Figure 5: Linear flow rate plot of POROS 50 HS resin .............................................20
Figure 6: Linear flow rate plot for Capto Q ImpRes resin .......................................21
Figure 7: Specifications for the GE BPG 300/500 Glass Chromatography Column ....22
Figure 8: Cation chromatography column and pump setup .....................................26
Figure 9: BCA Assay Curve .........................................................................................27
Figure 10: Results of Gel Electrophoresis ..................................................................28
Figure 11: Results of the BCA Assay .........................................................................29
Figure 12: BCA Assay Results Curve .......................................................................30
**Introduction**

Ranibizumab, more commonly known by the trade name “Lucentis,” is a drug developed by Genentech in San Francisco, California to treat wet age-related macular degeneration, one of the leading causes of legal blindness. Lucentis is a monoclonal antibody fragment produced in *Escherichia coli* using industry-standard recombinant DNA technology. This means Lucentis has been isolated in and is produced by a cloned strain of bacterial cells, which all come from a parent cell. Now, Lucentis is targeted to bind to vascular endothelial growth factor A (VEGF-A) which prohibits VEGF-A binding to its proper receptors. By stopping VEGF-A from binding to its receptors, Lucentis is able to reduce the progression of wet age-related macular degeneration.

A Senior Design team of the Department of Chemical and Materials Engineering at the University of Nevada, Reno were given the task to analyze and design the process of antibody fragment separation for a protein “similar to” Lucentis. The students studied processes involving homogenizers, chromatography, membrane separation, and centrifugation. These processes will be combined in a way that will most efficiently purify the target antibody on a commercial scale. The desired final product will be an aqueous solution of the pure antibody in solution.

The objective of this design project is to purify a solution of “Lucentis-like” protein provided by Genentech to a 99% purity. Lucentis is known to have a molecular weight of approximately 48 kDa. Lucentis’ light chains and heavy chains have molecular weights of approximately 23 kDa and 25 kDa respectively. The challenge is to remove to undesired waste proteins from the “Lucentis like” to produce a useable product.

The overall goal of this project is to optimize purity and step yield for each step in
the process and for each student to have a full understanding of the mechanism designed and how this goal is achieved. The students have conducted an economic analysis on their process and provided operational and raw material costs for the mechanism proposed.

In order to achieve the most efficient mechanism, and to understand each process step as thoroughly as possible, the process was divided into three main sections: bioreactor, tangential flow filtration, and chromatography, with a group of 4-6 students working on each section. This paper focuses on the chromatography aspect of the process and investigates the multiple types of chromatography used in both the base case and alternative case presented with a focus on Ion exchange (IEX) chromatography.

**Timeline**

This project was completed over a two-semester timeline beginning in the Fall of 2016 and ending the Spring of 2017. The Gantt chart in Figure 1 was created at the beginning of the project and provides an overall timeline for the project with anticipated deadlines and important events. This chart was followed very closely throughout the whole project. The Gantt Chart helped to keep the team on track and organized throughout both semesters and helped to ensure that all due dates were met.
Figure 1: Gantt Chart for the Chromatography team of the Genentech Project.

The first half of this project was dedicated to studying engineering economics and determining a plan of action while the second half of the project was spent designing, analyzing, and testing different models to achieve our results.

The economics learned in the first semester helped students complete a full economic analysis on the completed process design as well as assisted with a full understand of scale-up economics for the process studied. The rest of the semester focused on learning as much about the process as possible before testing began. This included developing design ideas and finalizing a design, gathering necessary materials and equipment, preparing a proposal presentation for the Genentech staff, and visiting the Genentech site in South San Francisco.

The second semester focused on experiment design, process modeling, and testing and was split up into four quarters. The first quarter was spent doing extensive literature
reviews on the types of chromatography used in the base and alternative cases. Through literature reviews and consulting with multiple faculty members, the team decided to focus on chromatography techniques of anion exchange, cation exchange, and hydrophobic interaction. This decision was made on the basis of purifying a Lucentis-like protein; a different protein would require different strategies and techniques for purification. Following important design decisions for the parameters of a chromatography column, the team prepared a list of all materials and procured all necessary materials and equipment. By February 6th, began work on mathematical modelling, examined various scale up designs and an experimental design for the project. A standard operating procedure for experimentation was then created and approved by Dr. Mike Kivistik, the supervisor of the labs worked in. Starting the week of February 13th, experimentation began with the preparation of the first chromatography column.

During the second quarter of the semester, modeling equations and calculations were finalized and were able to provide the team with insight into how the chromatography columns would perform. The team also continued to look into scale up calculations and considerations for the proposed mechanism. During the week of March 13th, the team received new 20 cm x 2.5 cm columns, and began to pack one of them with anion exchange resin. The team’s biggest progress during this quarter was has been obtaining a Masterflex mechanical pump to assist in packing and chromatography experiments. Previously, the columns were gravity-fed, and operated at a tedious rate.

The third and fourth quarters were spent running the protein through the column and testing the protein concentration of the effluent in order to determine the level of purity obtained. This quarter was also spent preparing the final presentation for the Genentech
staff and compiling results with the other two teams, researching bioreactor operation and tangential filter flow.

**Theory**

Chromatography is a powerful tool used to separate the Lucentis-like protein from the lysed, meaning shredded, bacteria that comes from earlier processes. Different types of chromatography can separate molecules suspended in a fluid based on mass, hydrophobicity (how eager the molecule is to bind with water), electric charge, and various other properties. In our case, we used Cation, Anion (both variations of IEX chromatography), and hydrophobic interaction (HIC) chromatography methods.

**Chromatography principles**

Chromatography involves sending some mixture of molecules, traditionally a molecule of interest as well as several impurities all suspended in a solution, through a column filled with a semi-solid resin. A resin can have various internal geometries, but is traditionally a porous matrix of small beads, that can be tightly packed within a tubular column. When the solution flows through the column, some molecules will take longer to pass by nature of some type of interaction, while others may be caught by the resin and unable to leave the column entirely, which means different materials will separate out.

As an example, in Cation exchange chromatography the resin inside the column is positively charged. This means that any negatively charged ions will get caught inside the column while the positive ions will pass through the column unimpeded. How well the negative ions bind to the resin is called ionic capacity and is a measurement of how many
ions can attach the resin per volume. Now, in order to elute the column, meaning release the caught ions, a buffer must be run through the column. The buffer has a strength measured in ions per volume called ionic strength, but it also has a specific pH. The ionic strength of the buffer and its pH can remove the bound proteins and ions from the resin. This can work by either removing the bound things by having a stronger ionic strength than the resin, or by changing the pH of the molecules and changing their charges. Say for instance, the protein we are collecting has a negative charge at pH 6 but becomes neutral at 9. You could remove the protein from the positive resin by changing its pH to 9, thereby stopping the ionic interaction. An Anion exchange chromatography would bind positive proteins to its resin, and a hydrophobic resin would attract lipids, or fats. Using these techniques, it is possible to individually separate the cell waste from the desired proteins.

**Literature Review**

The concept of Ion-exchange chromatography is based on the interaction between charged molecules on the support material and the charged molecules and ions in the mobile phase. In anion-exchange chromatography, negatively charged molecules compete for the positive sites on the support material. These anionic groups are usually tertiary ammonium moieties such as diethyl amino ethyl. In cation-exchange chromatography, positively charged molecules compete for the negative sites on the support material. These cationic groups are often sulfonic acid moieties such as sulfo-propyl or carboxymethyl.

The isoelectric point (pI) is important in determining what type of ion-exchange chromatography is needed. If the protein is more stable above its pI, then an anion exchanger is used. On the other hand, if the protein is more stable below its pI, then a cation
exchanger is used. Cation-exchange chromatography is most suitable for protein purification due to their stability in an acidic environment.\(^1\)

Other forms of chromatography that will be employed in the alternative case include multimodal and monoclonal chromatography. Multimodal chromatography combines multiple types of chromatography such as ion exchange, hydrophobic interaction, affinity, and size exclusion chromatography to improve the selectivity of certain protein purification. It effectively combines complementary methods and reduces the total number of columns needed in the system. Hydroxyapatite and hydrophobic ion exchange ligands are popular types of mixed-mode media available for use in multimodal chromatography.\(^2\)

Monoclonal chromatography often includes at least one ion exchange chromatography step. This helps to remove impurities such as viral particles, host cell proteins, and residual DNA. Monoclonal chromatography can use both flow through, which removes impurities from a product that flows through the column, and bind-and-elute, which elutes out impurities while the product remains in the column.\(^3\)

**Base Case**

The base case incorporates three types of chromatography, cation ion exchange chromatography, anion ion exchange chromatography, and hydrophobic interaction chromatography. As can be seen in Figure 2 which outlines the process, cation exchange chromatography occurs directly after the protein leaves the centrifuge and is followed by anion exchange chromatography. After the protein leaves the anion chromatography column it passes through a tangential flow filter before entering the hydrophobic
interaction chromatography column.

Figure 2: Base Case for Purification Process.

**Ion Exchange Chromatography**

Ion exchange chromatography (IEX) is a powerful technique for protein purification, and is frequently used in industry because of the high binding capacity of the column and the unlikelihood of denaturing processed proteins. Cation exchange chromatography uses a negatively charged ion exchange resin to remove particles in the liquid mobile phase with a positive charge. Anion exchange chromatography uses a positively charged ion exchange resin to remove particles in the liquid mobile phase with a negative charge.

**Principles of Operation**

Ion Exchange chromatography separates charged molecules in a mobile phase with charged molecules in a stationary resin. Proteins in particular are captured by manipulating the charge of the protein of interest. Proteins consist of many individually positive or negative amino acids, and so can have a positive charge, a negative charge, or no charge, depending on the surrounding pH. The pH at which a molecule has no net charge is called its isoelectric point (pI). By raising the pH, the molecule adopts a negative charge, and by
lowering the pH, the molecule adopts a positive charge. By surrounding the protein in a buffer of the appropriate pH, the protein can be given a charge and forced to bind with an appropriate counter-ion in the IEX resin. Table 1, below, clarifies the circumstances of anion IEX and cation IEX.

Table 1: Ion Exchange Differences

<table>
<thead>
<tr>
<th>Ion Exchange type</th>
<th>Net protein charge</th>
<th>Resin charge</th>
<th>Running pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cation IEX</td>
<td>+</td>
<td>-</td>
<td>Below pI</td>
</tr>
<tr>
<td>Anion IEX</td>
<td>-</td>
<td>+</td>
<td>Above pI</td>
</tr>
</tbody>
</table>

Under normal operation, a protein is run through the ion exchange column in a buffer solution until the protein of interest is entirely captured by binding to the resin, while some contaminants which cannot bind exit in the outlet. Afterwards, the protein and perhaps other bonded contaminants are released from the ion exchange resin. This is normally done by increasing the salt concentration in the column (to a maximum of 1 molar salt), which in turn increases the ionic strength of the solution. The counter-ions supplied by the salt eject the protein of interest as well as other contaminants by binding to the resin in their place. This process is called elution, and if the salt concentration is increased carefully, the protein of interest will separate from all other contaminants, resulting in a purified protein. It should be noted that anion IEX is generally preferable for protein purification due to general trends in protein stability.

Other strategies for protein purification using ion exchange chromatography do
exist, namely a flow-through elution strategy (as opposed to the traditional bind-and-elute method) and chromatofocusing. In the flow through method, the product flows through while impurities and contaminants are instead captured by the IEX resin. However, this method is not as consistent in its results as binding-and-eluting, and does not do much to concentrate the protein of interest, which is also a goal for this project. Chromatofocusing is the process of carefully changing the pH of the IEX column until it approaches the pI of the protein of interest. This will cause the protein of interest to elute as it no longer holds a charge, meaning it will no longer bind to the resin. Although this method provides similar resolution to elution with a changing salt gradient, it is often difficult to implement.

Effects on Design

The material safety data sheet for Lucentis provides an isoelectric point of 7.1. As Lucentis should be similar to the Lucentis-like-protein (LLP) being studied, it can be assumed that the LLP has a similar isoelectric point. Industry standard is to operate IEX at roughly 1 pH above or below the isoelectric point of the protein of interest. As such, a Tris-HCl buffer with a pH of 8.1 was decided upon for anion IEX, and a Sodium Phosphate buffer with a pH of 6.1 was chosen for cation IEX. Both IEX columns will be eluted with a stepwise gradient of NaCl. These choices for buffers and counterions in the salt are appropriate to recommendations in the literature. It is worth noting that an MES buffer would better suit elution at a pH of 6.1 for the cation IEX, but MES is expensive and dangerous.

Additionally, both chromatofocusing and a flow-through elution strategy were decided against for individual reasons. Chromatofocusing was deemed too difficult to
effectively use in the given laboratory setting. A flow-through elution strategy would not really increase protein concentration, and so it was also deemed unsuitable for the given objectives.

*Hydrophilic Interaction Chromatography*

Hydrophilic interaction chromatography, or hydrophobic interaction liquid chromatography (HILIC) is a type of chromatography which is primarily used to separate biomolecules based on polarity. Specifically, by utilizing a polar stationary phase and a partially aqueous eluent, polar substances are able to be separated \(^6\). HILIC has grown significantly more popular since its introduction, and is well-suited to the separation and purification of proteins. Hemstrum and Irgum fully detail hydrophobic interaction liquid chromatography (HILIC) in their paper, including information regarding usage, application, and common issues in troubleshooting operation of HILIC columns. Although the team is primarily focused on IEX at the current moment, it will be necessary to consider implementation of HILIC in a laboratory setting in the near future.

*Alternate Cases and Readings*

*Multimodal Chromatography*

Multimodal or ‘Mixed Mode’ chromatography is a type of chromatography, which combines several chromatographic techniques together to improve the selectivity protein purification. By combining various methods, the strengths of different separation techniques are able to be combined into one work-step, which simultaneously reduces the number of columns required and associated costs. Multimodal chromatography is
optimized by varying the parameters relevant to each mode, or type of separation, included in the process. Unfortunately, some parameters may not be unique to a particular mode, and so improving the ability of one mode to purify may simultaneously reduce the ability of another mode to purify.\textsuperscript{10}

All the same, multimodal chromatography is still a very effective purification process, and can be especially useful as a final polishing step in which trace impurities and product variants are removed\textsuperscript{11}. Additionally, multimodal chromatography can be used with both the bind-and-elute as well as the flow-through method of elution. In fact, one of the advantages of multimodal chromatography is the added efficiency of the flow-through method being a generally good choice. For these reasons and more, Multimodal chromatography is included in the alternate case as a semi-final purification step, as shown in figure 3, below.

![Figure 3: Alternate Case for Purification of a Lucentis-like Protein.](image)

The proposed multimodal resin is GE Capto adhere ImpRes, which is a strong anion multimodal exchange resin, designed specifically for the high-resolution polishing of monoclonal antibodies\textsuperscript{12}. An anion resin was chosen to remove contaminants, which may
have passed by the cation IEX preceding the multimodal chromatography. Conveniently, the same Tris-HCl buffer used in the base case can be used for the multimodal chromatography step. Additionally, the adhere ImpRes resin features a high dynamic binding capacity (85 mg/mL), fast mass transfer between the stationary and mobile phases, and high resolution with small column volumes. As a multimodal resin designed for the large-scale purification and polishing of monoclonal antibodies, this seems perfect for the objectives of this project.

**Monoclonal Antibody Purification**

Ranibizumab is an antibody fragment (Fab) from a monoclonal antibody (mAB). As Ranibizumab is the primary “active ingredient” in lucentis, and the team is observing a lucentis-like protein, it can be assumed that purification techniques for a mAB can be applied to the LLP. Most mAB purification processes include an ion exchange chromatography, which reinforces the previously made decision to include IEX in the base process. Moreover, Liu et al. also explain the efficacy of both the flow-through and bind-and-elute elution strategies for purifying mAB’s. However, this is with respect to several contaminants like viral particles, host cell proteins, residual DNA, and more. As none of these contaminants are present in the LLP, the flow-through elution strategy will be discarded as previously discussed.

**Monolithic Chromatography**

Monolithic chromatography is a type of chromatography, which involves a singular silica-based monolith as the stationary medium rather than a columnar packed bed. High
contact between the stationary phase and the mobile phase is provided by the incredibly high porosity and internal surface area of the monolithic column. Additionally, both the size of the through pores of the column and the size of the column’s internal structures can be selected separately, whereas in traditional media, both properties are linked. Other benefits include a relatively high efficiency, with a 10 cm monolithic column having 11,200 theoretical plates.

The proposed industrial monolithic chromatography column is a ‘BIA Separations CIMmultus™ SO3-8000 Advanced Composite Column’, shown in figure 4. The chosen column is a monolithic strong cation exchange column of the largest commercially available size, designed to concentrate the product, remove large impurities, reduce backpressure, and quickly isolate products. As the lucentis-like protein is particularly stable at a slightly acidic pH, a cation IEX column was deemed most appropriate. The same sodium phosphate buffer used in the base case for cation IEX is also used in this alternate case.
Figure 4: CIMmultus™ SO3-8000 Advanced Composite Column for monolithic chromatography. This column is designed for flow-through operation, providing a contrast to other cases.

The design parameters and manufacturer-suggested heuristics for the monolithic column are shown in table 2, below. Note that 40 Column volumes of preparing and cleaning the column is associated with each run of 1000 L (which would be 125 column volumes by itself). Additionally, note that processing the full 1000 L takes just over two hours. As such, it is possible that up to eleven monolithic chromatography columns could be run in series to more drastically purify and concentrate the LLP. This would necessitate a re-ordering of the existing process diagram to make the monolithic chromatography the final purification step, but this may be worthwhile.
Table 2: Design Parameters for Monolithic Chromatography.

<table>
<thead>
<tr>
<th>Design Parameter</th>
<th>Parameter Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column Volume</td>
<td>8 L</td>
</tr>
<tr>
<td>Flowrate</td>
<td>10 L/min</td>
</tr>
<tr>
<td>Pressure</td>
<td>1.4 MPa</td>
</tr>
<tr>
<td>pH</td>
<td>8.1</td>
</tr>
<tr>
<td>Temperature</td>
<td>4 – 40 °C</td>
</tr>
<tr>
<td>Column Volumes per Batch</td>
<td>165</td>
</tr>
<tr>
<td>Time for total operation</td>
<td>2.2 hours</td>
</tr>
</tbody>
</table>

**Modeling**

*Velocity Modeling*

Due to the presence of solid material in the column, the mobile phase can only take up a certain amount of the column volume. The fraction of the column volume occupied by the mobile phase is denoted by $\varepsilon_u$. When modeling flow through a chromatography column, it is important to distinguish between the different types of fluid velocity. Superficial velocity, $u_s$, is the flow per unit area, given in equation 1.

$$u_s = \frac{F}{A}$$  \hspace{1cm} (1)
The superficial velocity is insignificant when modeling flow through a packed column, but can be used to calculate the mobile phase velocity, $u_m$. The mobile phase velocity is given in equation 2.

\[ u_m = \frac{u_s}{\varepsilon_u} \]  

(2)

Solving first for the superficial velocity using equation 1, and then plugging that into equation 2 gives the mobile phase velocity. The diameter of the column and volumetric flow rate are given in the heuristics. The fraction of the column volume occupied by the mobile phase is assumed to be 0.25. The calculations for the velocity are shown below.

\[ A = \pi r^2 = \pi (15)^2 = 706.86cm^2 \]

\[ F = 220cm/hr \times 706.86cm^2 = 155,508.84cm^3/hr \]

\[ u_{\|} = \frac{155,508cm^3/hr}{706.86cm^2} = \frac{220.0cm/hr}{0.25} = 880cm/hr \]

\[ 880cm/hr = 14.67cm/min \]

**Flow and Pressure Drop Modeling**

Flow in a chromatography column is nearly always laminar. When flow is laminar it can be modeled using Darcy’s law. Darcy’s law equation is given in equation 3 where $B$ is the specific permeability of the particles in the column and $\eta$ is the dynamic viscosity.\(^1\)

\[ u_{\|} = -\frac{B}{\eta \varepsilon_u} \frac{dp}{dz} \]  

(3)

In liquid chromatography, the pressure drop, $dp/dz$, is constant along the length of the column. The Darcy equation can then be simplified to the form shown in equation 4.
\[ \Delta p = \frac{\epsilon \eta L u_m}{B} \]  

(4)

For a packed column, the specific permeability, \( B \), can be found using the Carman-Kozeny equation given in equation 5.\(^1\)

\[ B = \frac{d^2}{180 \psi^2} \frac{\epsilon_a^3}{(1-\epsilon_a)} \]  

(5)

The specific shape factor, \( \psi \), is 1.7 for porous non-spherical particles. The diameter of the particles, \( d_p \), is assumed to be 0.005cm. The dynamic viscosity is assumed to be 0.00012Ns/cm\(^2\) and the length of the column is given in the heuristics. The calculations for the pressure drop along the column are given below.

\[ B = \frac{(0.005)^2}{180 \times 1.7^2} \times \frac{0.25^3}{(1 - 0.25)^2} = 1.34 \times 10^{-9} \]

\[ \Delta p = \frac{(0.25)(0.00012)(20)}{1.34 \times 10^{-9}} = 448 \text{Pa} \]

\[ 108 \text{Pa} = 0.004 \text{atm} \]

**Adsorption Modeling**

The binding of ions to a charged resin can be modeled by the Langmuir isotherm as shown in equation 6\(^2\).

\[ q_m = (q_i)_m \times \frac{K_{i}c_i}{1 + \Sigma K_j c_j} \]  

(6)

Where \((q_i)_m\) and all of the \( K_i \)'s are experimentally determined constants, \( q_i \) is the amount of solute adsorbed, and \( c_i \) is the concentration of solute in solution. Equation 7 can be used to model the concentration and adsorption of a solute in ion exchange chromatography\(^2\).

\[ K_{AB} = \frac{(\frac{\beta}{\gamma})^{n-1} y_A(1-x_A)^n}{x_A(1-y_A)^n} \]  

(7)
Where A is the ion being exchanged with the ion bonded to the resin, which is B, C and Q are the total equivalent concentrations in the liquid and ion exchanger phases, respectively, and \( x_A \) and \( y_A \) are the equivalent fractions of A in the liquid and ion exchanger phases, respectively. Once \( q_i \) is determined from equation 6, equation 7 can then be used to predict \( x_A \) once the number of equivalents of the solute is determined.

**Laboratory Scale Equipment Specifications**

The equipment utilized for this experiment consists of the following:

- Two 2.5 cm x 20 cm Econo-Column Chromatography Columns
- POROS 50 HS Resin
- Capto Q ImpRes Resin

The specified columns were chosen to obtain a larger elution flowrate, which would enable the possibility for further experiment trials. However, a larger column results in a larger volume necessary to reach a bed height of 15 cm. Below is the following volume calculation:

**Inner column diameter, \( D = 2.5cm; \)**

**Bed height, \( h = 15cm; \)**

**Finding Volume (\( V_1 \)):**

\[
V_1 = \frac{\pi h D^2}{4} = \frac{(2.5^2)(15 cm)\pi}{4} = 73.6 \text{ mL}
\]

Thus, a total resin volume of 73.6 mL is necessary for each column. Therefore, the team needs a minimum of 150 mL of each resin to complete a full packing, as well as having a
reservoir in case the packings needs to be redone. In the table below, the costs for all materials are listed.

**Table 3: List of Laboratory Materials and Prices**³⁴.

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Amount</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 cm x 20 cm Econo-Column</td>
<td>2 Columns</td>
<td>$84</td>
</tr>
<tr>
<td>POROS 50 HS Resin</td>
<td>150 mL</td>
<td>$400</td>
</tr>
<tr>
<td>Capto Q ImpRes Resin</td>
<td>150 mL</td>
<td>$564</td>
</tr>
<tr>
<td><strong>Total Price</strong></td>
<td></td>
<td><strong>$1048</strong></td>
</tr>
</tbody>
</table>

To determine the theoretical flow rates of the ordered columns, the following figures were utilized:

![Figure 5: Linear flow rate plot of POROS 50 HS resin (provided by manufacturer).](image)

Figure 5: Linear flow rate plot of POROS 50 HS resin (provided by manufacturer).⁷
Using Figures 5 and 6, the following calculations were performed to calculate the theoretical flow rates of the columns.

For the POROS 50 HS resin, the following would be the resulting flow rate:

Area of the Column: 4.906 cm$^2$

Pressure at Pump: 1 bar

Thus, the linear flow rate is about: $v = 400$ cm/hr = 6.667 cm/min from Figure 5.

$$F_{\text{lin}} = A_1 \times v = (4.906 \text{ cm}^2) \cdot (6.667 \frac{\text{cm}}{\text{min}}) = 32.71 \frac{\text{cm}^3}{\text{min}}$$

For the Capto Q ImpRes resin, the following would be the resulting flow rate:

Area of the Column: 4.906 cm$^2$

Pressure at Pump: 1 bar

Thus, the linear flow rate is about: $v = 100$ cm/hr = 1.667 cm/min from Figure 6.
Ultimately, these new columns will yield flow rates of 32.71 mL/min and 8.18 mL/min for each respective resin. These flow rates are completely viable for the laboratory scale experiment.

**Industrial Scale Equipment Specifications**

The scaled-up process utilizes the same resins listed previously, however an industry scale column is necessary, as well as a much larger supply of each resin. Below are the specifications for an industrial column that could be utilized.

**GE BPG 300/500 Glass Chromatography Column**

- Ideal for process development and biopharmaceutical manufacture
- Column Diameter: 29.6 cm
- Cross-Sectional Area: 688 cm$^2$
- Bed Height Range: 0 cm to 26 cm
- Max Operating Pressure: 4 bar

Figure 7: Specifications for the GE BPG 300/500 Glass Chromatography Column.$^3$
The chosen column requires a large volume to reach a bed height of 15 cm and the necessary volume calculations below:

*Column Diameter, \( D = 29.6 \text{ cm} \)*

*Bed Height, \( h = 15 \text{ cm} \)*

\[
V = \frac{\pi hD^2}{4} = \frac{(29.6^2 \text{ cm}^2)(15 \text{ cm})\pi}{4} = 10322 \text{ mL}
\]

Thus, a volume of 10.322 L is necessary for each resin. The table below provides the total costs for the whole system.

**Table 4: List of Industrial Materials and Prices\(^{3,4,5}\).**

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Amount</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>GE BPG 300/500 Glass Chromatography Column</td>
<td>1 Columns</td>
<td>$5000</td>
</tr>
<tr>
<td>POROS 50 HS Resin</td>
<td>11 Liters</td>
<td>$26000</td>
</tr>
<tr>
<td>Capto Q ImpRes Resin</td>
<td>11 Liters</td>
<td>$21000</td>
</tr>
<tr>
<td>Total Price</td>
<td></td>
<td>$52,000</td>
</tr>
</tbody>
</table>
To determine the theoretical flow rates of the industrial column, the Figures 5 and 6 are utilized.

For the POROS 50 HS resin, the following would be the resulting flow rate:

Area of the Column: 688 cm$^2$
Pressure at Pump: 2 bar

Thus, the linear flow rate is about: $v = 800$ cm/hr from Figure 5.

$$F = A \times v = (688 \text{ cm}^2)(800 \frac{\text{cm}}{\text{hr}}) = 5.50 \times 10^5 \frac{\text{cm}^3}{\text{hr}} = 550.4 \frac{\text{L}}{\text{hr}}$$

For the Capto Q ImpRes resin, the following would be the resulting flow rate:

Area of the Column: 688 cm$^2$
Pressure at Pump: 3 bar

Thus, the linear flow rate is about: $v = 280$ cm/hr from Figure 6.

$$F = A \times v = (688 \text{ cm}^2)(280 \frac{\text{cm}}{\text{hr}}) = 1.926 \times 10^5 \frac{\text{cm}^3}{\text{hr}} = 192.64 \frac{\text{L}}{\text{hr}}$$

Therefore, the expected flow rates for each respective resin is 550.4 L/hr and 192.64 L/hr for the industrial column, which is completely reasonable for such a large-scale process.
Table 5: Scale-Up Parameters and Estimated Cost.

<table>
<thead>
<tr>
<th>Equipment or Material</th>
<th>Quantity</th>
<th>Unit Price</th>
<th>Cost (year basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatography Columns</td>
<td>9 Columns</td>
<td>$29,731.00</td>
<td>$267,579</td>
</tr>
<tr>
<td>Capto Q ImPres</td>
<td>488 L</td>
<td>$3063 per 1L</td>
<td>$1,494,744</td>
</tr>
<tr>
<td>POROS HS 50 resin</td>
<td>310 L</td>
<td>$2480 per 1L</td>
<td>$768,800</td>
</tr>
<tr>
<td>DI water</td>
<td>12,825 L/day</td>
<td>$21.50 per 1000 gallons</td>
<td>$26,587</td>
</tr>
<tr>
<td>Sodium Phosphate (buffer)</td>
<td>4,988 L/day</td>
<td>$1.1 per kg</td>
<td>$16,449</td>
</tr>
<tr>
<td>Tris (buffer)</td>
<td>7,838 L/day</td>
<td>$1.33 per kg</td>
<td>$31,191</td>
</tr>
<tr>
<td>NaCl</td>
<td>312.29 kg/day</td>
<td>$200 per metric ton</td>
<td>$22,797</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>$2,628,147</td>
</tr>
</tbody>
</table>

Experimental Procedure

Due to time, budget, and supply constraints, only the cation exchange chromatography column was utilized and only one run was conducted. Using the Masterflex pump to speed up the elution process, protein was added to the 2.5 cm diameter chromatography column filled with POROS 50 HS strong cation resin which had a bed height of about 11 cm. Figure 8 shows the setup of the chromatography column and pump.
The buffer used was a 50mM sodium phosphate buffer at a pH of 6.1. To elute the protein, buffers of 0.4, 0.6, 0.8, and 1 M of Sodium chloride concentrations in sodium phosphate buffer were gradually added. Seventy-two three milliliter samples were collected of the effluent. Six samples were randomly chosen to run gel electrophoresis on in order to determine if the process worked and the protein was purified. A BCA assay was also run on the 72 samples in order to determine the protein concentration. Figure 9 shows the curve for the BCA Assay which helped determine the protein concentration.
The following steps were taken in order to conduct the BCA Assay using the spectrophotometer:

1. Set the absorbance to the reference 562 nm wavelength

2. Subtract the blank absorbance at 562 nm from the sample and standard absorbances to find the Net A (net absorbance).

3. Plot the Standards Net A to create the BCA Standard Curve (figure to the left)

4. Utilize the calculated Net A for each unknown sample on the curve to find the concentration.

5. Example: Net A = 1, thus the concentration is 750 \( \mu \text{g/mL} \)
**Experimental Results**

*Gel Electrophoresis*

Out of the six samples analyzed in the gel electrophoresis, only two samples showed results. The two samples that showed protein were the Lucentis-like protein in buffer, and the first sample of the effluent. The results of the gel electrophoresis can be seen in Figure 10. The right sample refers to the Lucentis-like protein in buffer and the left is the first elution sample.

![Figure 10: Results of Gel Electrophoresis](image)

From the ladder shown in figure 10 the samples correlate to 180 kDa. From these results, it can be assumed that the protein conglomerated and passed directly through the column without being purified.

**BCA Assay Results**

The BCA Assay results showed that the initial “Lucentis like” protein in buffer and first elution sample were the only samples reading anything. All of the other samples gave a zero reading. Figures 11 and 12 show the results of the BCA Assay. The results agree with the findings of the gel electrophoresis and will be discussed shortly.

![Figure 11: Results of the BCA Assay](image)
Discussion of Results and Conclusions

There are a few possibilities to consider regarding the results from the cation exchange chromatography run. The first possibility, which is believed to be the most accurate, is that the protein has become denatured and thus no longer exhibits characteristics of a protein. This would explain the results obtained from the gel electrophoresis and BCA Assay. The protein is 11 years old, so this is a believable assumption. After consulting with the bioreactor and tangential flow filtration groups, it was revealed that their results correlated to denatured protein as well, which would further support this belief. However, last year the the chemical engineering seniors were able to
obtain results from the same protein and it is unlikely, but not impossible, for the protein to denature in the past year after being fine for 10 years.

Another possibility to consider is the amount of protein loaded into the column. With a column diameter of 2.5 cm and a bed height of 11 cm, the bed volume is about 54 cm$^3$. Only about 5 milliliters of protein were loaded into the column. It is possible that the protein was not denatured and instead the concentration of the protein was so minute it was unable to show up on the gel electrophoresis and BCA Assay.

Lastly, there could have been an error with the operation of the gel electrophoresis. Before loading the samples into the gel electrophoresis, they need to be heated up which will break them down and allow the gel electrophoresis to run them. The samples were only heated to 50 degrees celsius prior to being loaded into the gel electrophoresis, which was possibly not hot enough to allow them to be broken down sufficiently.

For next years chemical engineering seniors, it is advised to test the protein prior to beginning experimentation in order to determine it is not denatured. It is also advised to begin experimentation in the first semester. Time constraints were a huge issue and lead to the team only being able to conduct one trial on one time of chromatography. It would have been wonderful if the team had the ability to run multiple types of chromatography, or at the very least both the cation and anion in order to compare results.
Bibliography


9. ScienceLab.com: Chemicals and Laboratory Equipment. Sodium Chloride MSDS.
10. ScienceLab.com: Chemicals and Laboratory Equipment. Sodium Phosphate MSDS.
11. ScienceLab.com: Chemicals and Laboratory Equipment. Hydrochloric Acid MSDS.
12. ScienceLab.com: Chemicals and Laboratory Equipment. Sodium Hydroxide MSDS.
13. Santa Cruz Biotechnology, Inc. Tris Base MSDS.
Appendix A

Chromatography Heuristics
<table>
<thead>
<tr>
<th><strong>Resin Height</strong></th>
<th>Conventional resin height is approximately 15-20 cm.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diameter</strong></td>
<td>Approximately 2 meters - an increase in diameter of columns will directly correlate with an increase the resin volume.</td>
</tr>
<tr>
<td><strong>Number of Theoretical Plates</strong></td>
<td>Approximately 1,400 to 10,000 theoretical plates. The number of theoretical plates depends on the resin (many small beads).</td>
</tr>
<tr>
<td><strong>HETP</strong></td>
<td>Should be the same as the diameter of the individual resin bead, approximately 50 μm. This is common for almost all columns.</td>
</tr>
<tr>
<td><strong>Pressure Drop</strong></td>
<td>A consistent column height and a larger diameter will prevent an increase in the pressure drop and degradation of the resin.</td>
</tr>
<tr>
<td><strong>Number of Columns</strong></td>
<td>To minimize maintenance costs, smaller columns are run in parallel. Future designs may investigate having fewer larger columns.</td>
</tr>
<tr>
<td><strong>Column Material</strong></td>
<td>Stainless steel is preferred, but acrylic is cheaper and can handle the same amount of pressure and design specs. However, acrylic is harder to sterilize.</td>
</tr>
</tbody>
</table>
Appendix B

Standard Operating Procedure:

Cation and Anion IEX
Standard Operating Procedure: Cation and Anion IEX

*Safety*

<table>
<thead>
<tr>
<th>Safety Equipment</th>
<th>Hazard</th>
<th>Consequences</th>
<th>First Aid/Solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Safety glasses/goggles</td>
<td>Chemical splash back</td>
<td>Eye irritant</td>
<td>Flush eyes for 15 minutes</td>
</tr>
<tr>
<td>Protective Clothing/Nitrile Gloves</td>
<td>Chemical contact or splash back</td>
<td>Skin irritant</td>
<td>Wash with soap and water</td>
</tr>
<tr>
<td>Waste Vessel</td>
<td>Chemical spills/Sewage contamination</td>
<td>Slipping, chemical contact/splash back</td>
<td>Disposal into vessel and wipe up any spills</td>
</tr>
<tr>
<td>Labels</td>
<td>Utilization of incorrect chemical</td>
<td>Unwanted reactions</td>
<td>Label all unidentifiable material</td>
</tr>
</tbody>
</table>

*Refer to the chemical specific MSDS document listed in the References to further review hazards and first aid.*

Table A1: Outline of safety equipment, hazards, consequences, and first aid/solutions for the Cation and Anion IEX process.
**Materials:**

<table>
<thead>
<tr>
<th>40 cm Glass Column</th>
<th>100 mL Beaker (5)</th>
<th>POROS CEX Resin</th>
<th>Stirrer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stand w/ Rubber clamps</td>
<td>Scoopula</td>
<td>DEAE Resin</td>
<td>Pipettes (4)</td>
</tr>
<tr>
<td>Test tubes (10)</td>
<td>Tris Base</td>
<td>Hydrochloric Acid</td>
<td>Sodium Phosphate</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>Graduated Cylinder</td>
<td>pH Strips</td>
<td>Sodium Hydroxide</td>
</tr>
</tbody>
</table>

*Table A2: List of materials needed for the Cation and Anion IEX*

**Procedure:**

**250 mL Tris Buffer Preparation for Anion IEX**

1. Transfer 210 mL of deionized water into a beaker and add the appropriate amount of Tris buffer to the water.

2. Mix the solution until the solution is clear and transparent.

3. Take a pH reading and adjust the pH by adding drops of 10mM HCl or 10mM of NaOH to reach a pH reading of 8 to accommodate for the sample’s isoelectric point.

4. Add additional deionized water to reach a volume of 250 mL, and take a pH reading
to make sure it remains at the desired point. Adjust the pH if it has changed.

5. Label the buffer in order to prevent confusion when utilizing the buffer.

250 mL Sodium Phosphate Preparation for Cation IEX

1. Transfer 210 mL of deionized water into a beaker and add the appropriate amount of the Sodium Phosphate buffer to the water.

2. Mix the solution until the solution is clear and transparent.

3. Take a pH reading and adjust the pH by adding drops of 10mM HCl or 10mM of NaOH to reach a pH reading of 6 to accommodate for the sample’s isoelectric point.

4. Add additional deionized water to reach a volume of 250 mL, and take a pH reading to make sure it remains at the desired point. Adjust the pH if it has changed.

5. Label the buffer in order to prevent confusion when utilizing the buffer.

Chromatography Column Operation

1. Attach 40 cm glass column on rubber clamps and fasten the clamps. Make sure the valve is in the closed position.

2. Mix 85 cm$^3$ of the appropriate resin with water in a beaker. Stir the resin to form a slurry. Utilize a POROS CEX resin for cation exchange, and a Diethylaminoethyl (DEAE) Resin for anion exchange.

3. Allow the resin to settle and then decant the excess liquid from the slurry. Add deionized water to the slurry, and repeat the decanting process until the excess liquid is clear colored. Once this point is reached, leave the resin suspended in water to prevent it
from drying.

4. Stir the resin once again to achieve a mobile slurry, and pour into the column. Additional deionized water is to be added to the resin to allow more resin into the column in order to reach a resin height of 20 cm.

5. Once the resin has settled, open the release valve and drain the water into a beaker until the excess liquid level is at the level of the resin. This is to prevent the resin from drying up.

6. Wash the resin with a buffer solution in 25 cm$^3$ portions. Add the first 5 cm$^3$ using a pipette by running the buffer solution down the side of the column to avoid disturbing the resin layer, and carefully pour the remaining buffer solution down the side of the column. If the resin gets disturbed, allow the resin to settle and gently tap the sides of the column in order to keep the top resin layer even.

7. Drain the buffer solution into the beaker until the liquid level reaches the top layer of the resin. Continue washing the resin with two to three 25cm$^3$ buffer portions in order to prepare the column for the loading of the sample.

8. Load the 5 cm$^3$ Lucentis-like sample by running it gently down the side of the column with a pipette.

9. Elute the sample by draining the liquid into a beaker until the liquid level reaches the top layer of the resin.

10. Add 5 cm$^3$ of the buffer solution by running it down the side of column with a pipette. Elute the buffer until the liquid level reaches the top layer of the resin. This is to ensure
that the loaded sample has traveled down the resin before adding a large portion of buffer solution.

11. Add a 50 cm$^3$ portion of buffer solution by running down the first 5 cm$^3$ down the side of the column with a pipette, and pouring the rest down the side of the column. Be careful to not disturb the top layer of the resin.

12. Line up a rack of 10 test tubes and elute 5 cm$^3$ samples into the test tubes, until all the buffer has been eluted down the column.

13. These samples are then tested by electrophoresis to determine whether the refined Lucentis-like sample had been successfully eluted into some of the test tubes.