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COUNTERCURRENT STAGED DIAFILTRATION FOR
MONOCLONAL ANTIBODY FORMULATION

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ABSTRACT

There is significant interest in moving current biomanufacturing operations from traditional batch to continuous processes due to the potential for increased productivity, enhanced product quality, and greater flexibility. Considerable progress has been made in the development of continuous perfusion bioreactors and continuous chromatographic processes. However, the ultrafiltration / diafiltration step, which is currently used for concentration and final formulation of essentially all biotherapeutics, is currently an inherently batch operation. The objective of this project was to examine the feasibility and performance characteristics of a countercurrent staged diafiltration system for continuous protein formulation.

Experimental studies were performed using Pall Cadence™ Inline Concentrators, which are specifically designed for continuous single-pass operation with high conversion (ratio of permeate to feed flow rate greater than 50%). Serum IgG was used as a model protein, with vitamin B₁₂ as a model impurity.

Data obtained in a 2-stage system with 90% conversion in each stage provided 98.8% removal of vitamin B₁₂ with essentially 100% recovery of the IgG. Impurity removal was increased to 99.7% by increasing the conversion to 95%. The experimental results were in excellent agreement with model calculations based on the countercurrent staged configuration. 99.99% impurity removal could be achieved with 4 countercurrent stages, while using 40% less buffer due to the internal recycling of the buffer between the stages. These results provide the first demonstration that one can successfully use countercurrent staged diafiltration with single-pass tangential flow filtration modules for the continuous formulation of high value biological products.
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Chapter 1

Introduction to Monoclonal Antibodies

1.1 Structure and Function

In general, antibodies are “Y” shaped proteins (Figure 1) that are generated by the immune system and can bind to specific molecules called antigens [1]. Antigens can be simple or complex molecules and may contain multiple sites, called epitopes, where an antibody can bind [2]. These antigens can be bacteria (or a molecule secreted by a bacteria), viruses, or cancer cells that need to be targeted and eliminated from the body.

![Figure 1. Structure of an antibody [3].](image)

The two arms of the “Y” shaped antibody are identical, and they recognize and bind to specific epitopes [4]. They are known collectively as the Fragment antigen binding (Fab) region. Differences in the amino acid sequences in the Fab region among antibodies give rise to the epitope specificity of each antibody. While the trunk of the “Y” does not possess antigen-binding capability, it confers the antibody with effector functions that activate natural killer cells, the
complement pathway, and phagocytosis that help destroy the bacteria or virus [2]. Since this fragment of the antibody crystallizes easily, it is known as the Fragment crystallizable (Fc) region. The amino acid sequence of the Fc region is relatively constant among antibodies.

It is important to distinguish between the two types of antibodies: monoclonal and polyclonal. Monoclonal antibodies (mAbs) are produced by a single B cell clone and bind to a single epitope on an antigen. Polyclonal antibodies (pAbs) are mixtures of monoclonal antibodies produced by multiple B cell clones and can bind to multiple epitopes [5].

1.2 Therapeutic Applications

Since the early 1980’s, there has been significant interest in developing commercial therapeutic antibodies to treat diseases such as cancer, asthma, and arthritis [6]. mAbs are particularly attractive as biotherapeutics due to the very high epitope specificity, which reduces the probability of cross reactivity [5] and minimizes adverse side effects. While mAbs are produced by a single B cell clone in vivo, they are produced commercially using recombinant DNA technology. An expression vector containing the gene for the antibody is transfected into a host cell which then expresses and secretes the antibody. Chinese Hamster Ovary (CHO) cells are the most widely used expression system for the production of recombinant mAbs since these cells can provide the desired glycosylation and disulfide linkages [7].

The first commercial mAb, Orthoclone OKT3, was approved in 1986 to prevent rejection during human kidney transplants. From 1986 to 2014, forty-seven therapeutic mAbs have received approval in the U.S. or Europe to treat a wide range of diseases including leukemia (Rituxan), lung cancer (Avastin), and rheumatoid arthritis (Humira) [6]. With sales increasing
90% over just 5 years from $39 billion in 2008 to $75 billion in 2013, the market for therapeutic mAbs is the most rapidly growing sector in the pharmaceutical industry [6].

1.3 Manufacturing Process

Given the growing importance of mAbs for healthcare, substantial effort has been devoted to increase the efficiency, reduce the cost, and improve the sustainability of the current manufacturing process. The following generic process describes the typical sequence of unit operations used to manufacture mAbs in industry.

![Figure 2. Generic process for manufacturing monoclonal antibodies [8].](image-url)
The generic process shown in Figure 2 can be divided into two main parts: upstream processing (USP) and downstream processing (DSP). In USP, the main objective is to produce the antibody from cell culture. Since mAbs are manufactured on a large scale in industry, multiple fermenter trains are frequently used. Centrifugation and filtration steps accomplish primary recovery of the product by removing cells and cell debris from the culture medium which contains the secreted antibody. Although centrifugation is followed by microfiltration on the generic process in Figure 2, microfiltration has now been replaced by a depth filter in most companies. Similarly, the ultrafiltration step before the Protein A chromatography has largely been eliminated in current process designs, with the Protein A chromatography used directly on the clarified cell culture fluid.

In DSP, the main objectives are purification and formulation. Chromatography and membrane processes play a critical role in achieving these objectives [9]. Protein A affinity chromatography captures the desired antibody from the complex media, removing most of the host cell protein (HCP) contaminants. Additional polishing chromatography steps, such as ion exchange and hydrophobic interaction chromatography, remove leached protein A and any remaining contaminants such as DNA and endotoxin. Virus filtration provides clearance of endogenous retroviruses and adventitious viruses. The final ultrafiltration (UF) and diafiltration (DF) process concentrates the mAb and places the product in the desired formulation buffer to provide the antibody with the desired stability needed for a long shelf-life.

Recent efforts to improve the current mAb manufacturing process have focused on both USP and DSP. In USP, advances in cell line engineering, media development, and bioprocess optimization have led to more than a 100-fold increase in the product titer from 50 mg/L in 1986 to 5–20 g/L today [9,10]. Moreover, the idea of switching from predominantly batch to
continuous manufacturing is gaining momentum in the biopharmaceutical industry. Continuous processes have the following advantages over their batch counterparts [11]:

- smaller equipment size and lower equipment costs,
- no need for intermediate storage tanks between unit operations (as shown in Figure 2),
- increase in productivity since all equipment is being used simultaneously at any point in time,
- greater product quality due to steady-state operation.

Perfusion bioreactors, in which there is continuous addition of nutrients and continuous removal of products, can generate higher cell densities and require roughly ten times less reactor volume than fed-batch bioreactors [12]. Apart from increased productivity, shorter residence times of the product inside the bioreactor reduce the likelihood of protein denaturation and aggregation, thereby improving product quality [9].

One consequence of the steady increase in product titer is the need to increase the capacity and/or efficiency of the DSP equipment to avoid a “bottleneck” in the overall manufacturing process. For example, if the product concentration in the clarified culture medium is doubled due to improvements in cell culture, the volume or efficiency of the affinity chromatography column must double. The large improvements in USP have created significant strain on the capacity and efficiency of the purification steps in DSP.

In addition, if a truly end-to-end continuous process for mAb production is to be realized, then continuous modes of operation must be developed for the predominantly batch membrane-based and chromatographic steps in widespread use today. Continuous chromatographic processes, such as periodic countercurrent chromatography (PCC), multicolumn countercurrent solvent gradient purification (MCSGP), and continuous countercurrent tangential
chromatography (CCTC), have already been developed [11]. Recently developed single-pass tangential flow filtration (SPTFF) modules can provide very high conversion (ratio of permeate to feed flow rate), enabling sufficient concentration of the product in a single pass through the module [13]. This has advantages in terms of both equipment cost and product quality over batch ultrafiltration, which requires a feed tank and multiple passes of the protein product through a retentate recirculation pump.

Due to their high conversion, SPTFF modules can also be employed to develop a continuous diafiltration process. In this case, the diafiltration (formulation) buffer would be mixed with the mAb feedstream, with excess fluid removed in the SPTFF module. The use of a buffer flow rate that is 9 times the mAb feed flow rate would result in a 10-fold reduction in the concentration of the original buffer components, with the product then re-concentrated in an SPTFF module. More effective use of the diafiltration buffer can be achieved using countercurrent staging, similar to that employed in the design of classical mixer-settler systems for extraction [14]. This is discussed in more detail in the next Chapter.

Several recent studies in the literature have demonstrated the feasibility of continuous diafiltration processes. Mohanty and Ghosh showed that a tangential flow ultrafiltration membrane cascade in countercurrent configuration can be used to continuously purify the monoclonal antibody Campath-1H from a simulated mammalian cell culture supernatant [15], although the maximum purification factor obtained in this work was less than 12. Lin and Livingston demonstrated the use of a countercurrent nanofiltration membrane cascade for organic solvent exchange [16], although the final product contained less than 77% of the desired solvent. Lin and Livingston also showed how the solvent requirements could be reduced by employing countercurrent staging [16], improving sustainability and lowering raw material costs.
While these studies have helped establish the feasibility of continuous diafiltration processes, they fall well short of the 1,000-fold (or greater) degree of purification / buffer exchange required for the formulation of monoclonal antibody products.

### 1.4 Research Objectives

The overall goal of this research project was to explore the design and application of a continuous countercurrent staged diafiltration process for monoclonal antibody formulation with reduced buffer requirements. The main objectives were to:

- demonstrate the operation of a 2-stage countercurrent diafiltration process,
- study the start-up behavior associated with running a 2-stage countercurrent diafiltration process,
- validate the theoretical equations for the performance of an N-stage countercurrent diafiltration process,
- compare the impurity removal and buffer requirements of the continuous process with the batch process,
- examine the key design variables governing the performance of a countercurrent staged diafiltration process, including the number of stages and the conversion in each stage.

Chapter 2 provides a theoretical treatment of batch and countercurrent staged diafiltration and introduces the mathematical equations that can be used to predict impurity removal in each case. Chapter 3 describes the materials (antibody, impurity, buffer), protein and impurity assays, and experimental apparatus (membrane modules, pumps, etc.) used in the diafiltration experiments.
Chapter 4 presents and analyzes the experimental results. Chapter 5 summarizes the findings of the project, and proposes directions for future work.
Chapter 2

Theoretical Background

Ultrafiltration / diafiltration (UFDF) is a process where the feed is effectively “washed” so that small impurities are removed in the permeate while fresh diafiltration buffer (either water or an appropriate buffer) is added to the feed. Batch diafiltration is most commonly performed with the diafiltration buffer added at the same flow rate as the permeate is removed to maintain a constant retentate volume throughout the process [17]. It is the technique of choice for the final formulation of virtually all mAb products [18]. This chapter describes batch and continuous countercurrent DF in greater detail and goes through the derivation of the mathematical equations describing solute removal based on solution of the appropriate mass balances.

2.1 Batch Diafiltration

A schematic for diafiltration performed in batch mode is shown in Figure 3 below.

Figure 3. Schematic of batch diafiltration.
In batch diafiltration, the feed is pumped through an ultrafiltration module as shown in Figure 3. The molecular weight cut-off (MWCO) of the membrane is smaller than the size of the monoclonal antibody product (~150 kDa) but bigger than the size of small solute impurities; a 30 kDa membrane is most commonly used for antibody processes. Therefore, the product remains in the retentate and is recycled back to the feed tank, and the impurities pass through the membrane into the permeate. To maintain a constant feed volume, \( V_F \), diafiltration buffer is added at the same flow rate as the permeate that leaves the system (\( Q_{DF} = Q_P \)). In antibody formulation, the diafiltration buffer is chosen based on the desired properties for the final formulation, with the initial buffer gradually replaced with formulation buffer over the course of the diafiltration process. Note that two tanks are typically used during batch DF, one for the feed and one for the diafiltration buffer.

The concentration of a solute (e.g. a small impurity) as a function of time during the constant volume diafiltration process can be obtained by solving an overall mass balance on the solute

\[
V_F \frac{dC}{dt} = -Q_P C S_o
\]  

(1)

where \( C \) is the solute concentration in the feed tank at any given time, \( V_F \) is the constant feed volume, \( Q_P \) is the permeate flow rate, and \( S_o \) is the observed sieving coefficient. \( S_o \) is the ratio of the solute concentration in the permeate to the solute concentration in the feed (\( S_o = C_P / C \)); thus, \( S_o = 1 \) for a completely permeable solute. Note that Equation (1) is valid for a solute that is not present in the diafiltration buffer; a term involving \( Q_P C_{DF} \) would be required if the solute were also added to the feed tank during the diafiltration. Separation of variables and integration yields
\[
\int \frac{dC}{C} = -\frac{Q_P S_o}{V_F} \int_0^t dt
\]

\[
\ln \frac{C}{C_o} = -\frac{Q_P S_o}{V_F} t
\]

Noting that \(Q_F = Q_{DF}\) and that \(Q_{DF}\) multiplied by \(t\) is the volume of diafiltration buffer added to the feed tank, \(V_{DF}\), Equation (3) can be rewritten as

\[
\ln \frac{C}{C_o} = -\frac{V_{DF} S_o}{V_F} t
\]

The ratio of \(V_{DF}/V_F\) is defined as the number of diavolumes \(N_D\). Applying this definition to Equation (4) and taking the exponential of both sides yields

\[
\frac{C}{C_o} = \exp(-N_D S_o)
\]

We define the impurity removal \(f\) as

\[
f \equiv 1 - \frac{C}{C_o} = 1 - \exp(-N_D S_o)
\]

and the fold removal \(R\) as

\[
R \equiv \frac{C_o}{C} = \exp(N_D S_o)
\]

Physically, Equations (6) and (7) state that the more we wash the feed with diafiltration buffer (higher the \(N_D\)), the greater the impurity removal. In industry, batch DF is typically performed using 8-10 diavolumes. For a completely permeable solute \((S_o = 1)\), this corresponds to an impurity removal of 99.97% to 99.995%, or equivalently, a 2,980 to 22,000-fold reduction in impurity concentration.

The volume of DF buffer required per unit mass of mAb product is given by

\[
V_{req} = \frac{V_{DF}}{V_F C_F} = \frac{N_D}{C_F}
\]
where \( C_F \) is the concentration of the mAb product in the feed during the diafiltration process. For a 10 diavolume process performed at a mAb concentration of 50 g/L, the buffer requirement is 0.2 L/g mAb or 200 L/kg mAb. If 100 kg of mAb are being processed, then 20,000 L of buffer are required. This is a significant consumption of water and buffer salts. Countercurrent staging has the potential to reduce buffer requirements; an analysis of the performance of a countercurrent staged DF is presented in the next section.

### 2.2 Continuous Countercurrent Staged Diafiltration

A schematic for diafiltration performed in a continuous countercurrent N-staged configuration is shown in Figure 4 below.

![Figure 4. Schematic of countercurrent N-staged diafiltration.](image)

The feed, which contains solute at a concentration of \( C_o \), is pumped into Stage 1 at a flow rate of \( Q_F \). At the other end of the process, the DF buffer is pumped into Stage N countercurrent to the feed at a flow rate of \( Q_{DF} = Q_{P1} \) to maintain the same volumetric flow rate at the entrance to each module. In general, the retentate of each stage is pumped to the next stage (moving from left to right), while the permeate is recycled to the previous stage (moving from right to left). For
example, the retentate from Stage 1 is diluted by the permeate recycled from Stage 2, and this forms the feed for Stage 2. The retentate of Stage N is the final diafiltered product.

Countercurrent staged diafiltration is analogous to other staged equilibrium unit operations in chemical engineering, such as liquid-liquid extraction involving a combination of mixer-settlers. In extraction, the DF buffer is analogous to the extraction solvent, the retentate of Stage N to the raffinate, and the permeate of Stage 1 to the extract (note that in this case the impurity is being “extracted”). The “mixers” are simply the T-connectors at which the retentate and permeate streams are combined while the “settlers” are the membrane modules that separate the feed into separate retentate and permeate streams. Therefore, the equations governing solute removal for countercurrent staged diafiltration look very similar to those for extraction.

Again, simple (in this case steady-state) mass balances will be used to evaluate the overall rate of solute removal. The following assumptions will be made:

1. The membrane is completely permeable to the small impurity \( S_0 = 1 \).

2. When the retentate of Stage \( K-1 \) is combined with the permeate of Stage \( K+1 \) to form the feed for Stage \( K \), there is perfect mixing. From this and Assumption 1, it follows that the solute concentration in the retentate leaving Stage \( K \) is equal to that in the permeate leaving Stage \( K \).

3. The conversion, defined as the ratio of the permeate flow rate to the total feed flow rate into each stage, is the same for all stages. Along with the fact that \( Q_{DF} = Q_{P1} \) and \( Q_F = Q_{R,N} \), it follows that \( Q_F = Q_{R1} = Q_{R2} = \ldots = Q_{R,N} \) and that \( Q_{DF} = Q_{P1} = Q_{P2} = \ldots = Q_{P,N} \).

The mass balance on the impurity in Stage \( N \) is simply

\[
Q_{R,N-1}C_{R,N-1} = Q_{R,N}C_{R,N} + Q_{P,N}C_{P,N} \tag{9}
\]
From Assumptions 1 and 2, $C_{P,N} = C_{R,N}$. From Assumption 3, $Q_{R,N-1} = Q_{R,N} = Q_F$ and $Q_{P,N} = Q_{DF}$.

Therefore, Equation (9) can be simplified to

$$Q_F C_{R,N-1} = Q_F C_{R,N} + Q_{DF} C_{R,N}$$

(10)

Dividing throughout by $Q_F$ and factoring out $C_{R,N}$ from the RHS yields

$$C_{R,N-1} = C_{R,N} \left(1 + \frac{Q_{DF}}{Q_F}\right)$$

(11)

We define $\alpha$ as the ratio of the DF buffer flow rate to the feed flow rate

$$\alpha = \frac{Q_{DF}}{Q_F}$$

(12)

Substituting Equation (12) into (11) gives

$$C_{R,N-1} = C_{R,N}(1 + \alpha)$$

(13)

Working backwards from Stage N, the mass balance on the solute in Stage N–1 is

$$Q_{R,N-2} C_{R,N-2} + Q_{P,N} C_{P,N} = Q_{R,N-1} C_{R,N-1} + Q_{P,N-1} C_{P,N-1}$$

(14)

By applying Assumptions 1 to 3, dividing throughout by $Q_F$, and using the definition of $\alpha$ in a similar manner as before, Equation (14) can be simplified to

$$C_{R,N-2} + \alpha C_{R,N} = C_{R,N-1}(1 + \alpha)$$

(15)

Substituting Equation (13) for $C_{R,N-1}$ and rearranging for $C_{R,N-2}$ gives

$$C_{R,N-2} = C_{R,N}(1 + \alpha + \alpha^2)$$

(16)

By studying the forms of Equations (13) and (16), we can see that if we keep working backwards till Stage 1, we will get

$$C_o = C_{R,N}(1 + \alpha + \alpha^2 + \cdots + \alpha^N)$$

(17)

The expression inside the parentheses is a geometric sum which can be rewritten as

$$\frac{C_N}{C_o} = \frac{\alpha - 1}{\alpha^{N+1} - 1}$$

(18)
where $C_{R,N}$ has been replaced with $C_N$ for convenience.

Equation (18) describes how the solute concentration in the diafiltered product depends on the process conditions, i.e., the number of stages $N$ and the ratio of the DF buffer flow rate to the feed flow rate $\alpha$. Note that the equation for countercurrent staged extraction is typically written as [14]

$$\frac{C_N}{C_o} = \frac{E - 1}{E^{N+1} - 1}$$

Equation (19) where $E$ is the extraction ratio, the product of the partition coefficient and extractant flow rate divided by the feed flow rate. The “partition coefficient” in the membrane system is by definition equal to one since the membrane is assumed to be non-selective to the impurity (permeate concentration is equal to the retentate concentration).

For countercurrent staged DF, the solute removal $f$ is given as

$$f \equiv 1 - \frac{C_N}{C_o} = \frac{\alpha^{N+1} - \alpha}{\alpha^{N+1} - 1}$$

and the fold removal $R$ is

$$R \equiv \frac{C_o}{C_N} = \frac{\alpha^{N+1} - 1}{\alpha - 1}$$

Equation (21) predicts that for a fixed solute removal $R$, $\alpha$ decreases with increasing $N$.

Physically, this means that as the number of stages increases, less DF buffer is required. This is analogous to how the volume of extraction solvent required is reduced with increasing number of mixer-settlers. However, for a fixed number of stages, the solute removal increases with $\alpha$. This shows that for a fixed $N$, there is a trade-off between buffer requirement and solute removal.

Explicitly, the buffer requirement per unit mass of mAb product for the continuous process is
where $C_F$ is the concentration of the mAb product in the feed. Equation (22) shows that as $\alpha$ increases, the buffer requirement increases. The membrane conversion defined in Assumption 3 is given by

$$\text{conversion} = \frac{\text{permeate flow rate}}{\text{total feed flow rate}} = \frac{Q_{DF}}{Q_F + Q_{DF}} = \frac{\alpha}{1 + \alpha}$$

In the next section, solute removal and buffer requirement will be compared for batch and continuous DF as a function of the number of stages $N$ and the flow ratio $\alpha$.

### 2.3 Solute Removal and Buffer Requirement

First, we will study the dependence of the fold solute removal $R$ on the flow ratio $\alpha$ and the number of stages $N$. Equation (21) was used to calculate $R$ at different values of $\alpha$ and $N$, and the results are plotted in Figure 5.

![Figure 5. Fold removal as a function of flow ratio and number of stages.](image-url)
As seen in Figure 5, as the ratio of the DF buffer flow rate to the feed flow rate increases, the fold removal of solute increases. In addition, at a fixed flow ratio, the fold removal increases with increasing number of stages. For example, at $\alpha = 19$:

- $R = 380$ for $N = 2$,
- $R = 7,200$ for $N = 3$,
- $R = 138,000$ for $N = 4$.

In Figure 6, the flow ratio that is required in staged DF to produce the same solute removal as in batch DF is plotted as a function of the number of diavolumes used in the batch DF process.

\[ \text{Flow Ratio, } \alpha = \frac{q_{DF}}{q_{Feed}} \]

\[ \text{Number of Diavolumes, } N_D \]

**Figure 6. Flow ratio versus number of diavolumes at equivalent solute removal.**

For a batch DF performed at 8 diavolumes (2,980-fold solute removal), a 3-stage continuous DF requires $\alpha = 14$ for equivalent removal. The flow ratio can be reduced to $\alpha = 7.1$ for $N = 4$. 
Next, we will compare the buffer requirements for the batch and staged DF. Figure 7 shows the buffer requirements for staged DF, performed at a fixed feed mAb concentration of 100 g/L, as a function of solute removal and the number of stages.

The plots demonstrate that for staged DF, the buffer requirement can be reduced significantly by increasing the number of stages. The red line shows the buffer requirement for batch DF performed at a mAb concentration of 60 g/L. Staged DF consumes less buffer than a batch DF process (per gram of mAb product) when the curves lie below the red line. For batch DF performed at $R = 1,000$, the buffer requirement is 0.115 L/g mAb. For staged DF performed at the same fold removal, the buffer requirement is reduced to 0.096 L/g for $N = 3$, 0.053 L/g for $N = 4$ (one-half of the batch value), and 0.037 L/g for $N = 5$ (one-third of the batch value).

\[ \text{Buffer Required, } V_{DF} \text{ (L/g)} \]

\[ \text{Fold-Removal, } R \]

\[ C_F = 100 \text{ g/L} \]

Figure 7. Buffer requirement versus fold removal at $C_F = 100$ g/L.
The buffer requirement for staged DF can be further reduced by increasing the mAb concentration in the feed. Figure 8 shows the buffer requirements for staged DF when the mAb concentration is doubled from 100 g/L to 200 g/L.

By doubling the feed mAb concentration, the buffer requirement decreases by an additional factor of 2. At a fold removal of 1,000, the buffer requirement is 0.048 L/g for N = 3, 0.027 L/g for N = 4, and 0.019 L/g for N = 5.

Figure 8. Buffer requirement versus fold removal at \( C_F = 200 \) g/L.

In summary, the model calculations for staged DF demonstrate the following:

- the fold removal of solute increases with increasing \( \alpha \) and N,
- at fixed solute removal, the buffer requirement decreases with increasing \( C_F \) and N.

The next Chapter describes the materials and methods that were used in the batch and staged DF experiments.
Chapter 3
Materials and Methods

3.1 Materials

Membranes

For batch diafiltration, a regenerated cellulose (Ultracel) tangential flow filtration membrane (Pellicon® 3 cassette, C screen, 88 cm$^2$) with a nominal molecular weight cut-off (MWCO) of 30 kDa was obtained from MilliporeSigma.

For countercurrent staged diafiltration, two Delta regenerated cellulose single-pass tangential flow filtration modules (Cadence™ Inline Concentrator, 650 cm$^2$) with a nominal MWCO of 30 kDa were obtained from Pall.

Model Antibody

Serum immunoglobulin G (IgG) powder (~150 kDa molecular weight) was obtained from SeraCare (Milford, MA) and used as the model antibody in all experiments. Serum IgG is much less expensive and more readily available than commercial monoclonal antibodies.

Model Impurity

Vitamin B$_{12}$ (cyanocobalamin, 1355 Da), obtained from Sigma-Aldrich (St. Louis, MO), was used as the model impurity in all experiments. Vitamin B$_{12}$ was chosen for two reasons: 1)
its small size ensures that it easily permeates the 30 kDa membranes, and 2) its concentration can be measured using UV-Vis spectrophotometry.

Buffer

10 mM acetate buffer solutions of pH 5 were prepared by diluting a concentrated 3M sodium acetate buffer solution (Sigma-Aldrich) with deionized water. To prepare 1 L of 10 mM acetate buffer solution, 3.3 mL of stock solution was diluted with 1,996.7 mL of deionized water. All solutions were pre-filtered through 0.2 μm Supor® 200 filters (Pall) prior to use. The solution conductivity was measured using a 105APlus Conductivity meter (Thermo Orion), and the pH was measured using a 420APlus pH meter (Thermo Orion).

3.2 Assays

Vitamin B\textsubscript{12} Assay

Vitamin B\textsubscript{12} concentrations were determined using a NanoDrop 2000c Spectrophotometer (Thermo Scientific). Before any diafiltration experiments were performed, a calibration curve for B\textsubscript{12} absorbance at 550 nm was constructed using solutions of known concentrations. Another calibration curve was constructed for B\textsubscript{12} absorbance at the absorption wavelength of IgG (280 nm) to correct for B\textsubscript{12} absorbance in the IgG assay. The calibration curves, shown in Figure 9, were highly linear for B\textsubscript{12} concentrations below 100 mg/L. The equations of the best-fit lines were used to calculate B\textsubscript{12} concentration from absorbance (Abs).
IgG Assay

IgG concentrations were determined using a NanoDrop 2000c Spectrophotometer (Thermo Scientific). Before any diafiltration experiments were performed, a calibration curve for IgG absorbance at 280 nm was constructed using solutions of known concentrations. The calibration curve was linear for IgG concentrations below 1 g/L. Another calibration curve was constructed for IgG absorbance at the absorption wavelength of vitamin B₁₂ (550 nm) to correct for IgG absorbance in the B₁₂ assay. The calibration curve was linear even until a high IgG concentration.
concentration of 100 g/L. Both calibration curves are shown in Figure 10. The equations of the best-fit lines were used to calculate IgG concentration from absorbance (Abs).

\[
C_{IgG,280 \text{ nm}} (g/L) = \frac{Abs(au) - 0.010}{1.259}
\]  
(26)

\[
C_{IgG,550 \text{ nm}} (g/L) = \frac{Abs(au) - 0.0004}{0.0008}
\]  
(27)

![Calibration curves for IgG absorbance at 280 nm and 550 nm.](image)

**Figure 10.** Calibration curves for IgG absorbance at 280 nm and 550 nm.

**Procedure**

Each absorbance measurement was carried out with 300 μL of sample in a plastic cuvette (Eppendorf UVette®). The spectrophotometer was blanked with 10 mM acetate buffer solution before samples were analyzed. If the concentration of vitamin B₁₂ or IgG in the sample was above the upper limit of the calibration curve, aliquots of the sample were diluted with acetate buffer in a centrifuge tube to achieve a concentration within the range of the calibration curve. The dilution factor was determined based on the expected concentration of B₁₂ or IgG in the sample.
3.3 Batch Diafiltration

Apparatus

The setup for the batch diafiltration experiments is shown in Figure 11.
Masterflex L/S 16 (1/8” ID) platinum-cured silicone tubing (Cole-Parmer) was used as the connecting tubing due its smooth surface and low protein binding levels. A Masterflex L/S peristaltic pump (Cole-Parmer) controlled the feed flow rate into the membrane module. The feed pump was fitted with larger ID tubing (1/4”) to reduce the shear stress imparted to the IgG in the feed stream. A Rabbit-Plus peristaltic pump (Rainin Instrument) controlled the flow rate at which the DF buffer was added to the feed reservoir. Before an experiment was performed, both pumps were calibrated by timed volume collection using a digital balance (Mettler Toledo). A metering valve (Swagelok) was used to control the retentate flow rate. The permeate flux was determined by recording the mass of the permeate collection vessel over time. While the permeate was open to atmosphere, the feed and retentate pressures were monitored using stainless steel pressure gauges (Ashcroft) to determine the transmembrane pressure, which is given by

$$ TMP = \frac{P_{\text{feed}} + P_{\text{retentate}}}{2} - P_{\text{permeate}} $$  \hspace{1cm} (28)

**Sample Collection**

300 μL samples were collected from the feed vessel using a pipette (VWR). The sample collection times usually corresponded to times at which an integer or half-integer number of diavolumes was processed. Before performing an experiment, a sample from the feed solution was collected to measure the initial concentrations of IgG and B12 via UV-Vis spectrophotometry. This was done to verify that the measured concentrations were close to the prepared concentrations.
Membrane Pre-Conditioning, Cleaning, and Storage

The following pre-conditioning, cleaning, and storage steps were carried out before and after an experiment was performed.

1. The system was flushed with 2 L of deionized water at a high feed flow rate of 44 mL/min.
2. The system was washed with deionized water for 1 hour at the operating feed flow rate with recirculation of the retentate and permeate to the feed reservoir.
3. The system was washed with buffer for 30 minutes at the operating feed flow rate with recirculation of the retentate and permeate to the feed reservoir.
4. The experiment was performed with the IgG / B₁₂ feed at the operating feed flow rate.
5. After performing the experiment, the system was washed with buffer for a few minutes.
6. The system was washed with 0.5 N sodium hydroxide solution for 40 minutes at a high feed flow rate of 44 mL/min with recirculation of the retentate and permeate to the feed reservoir.
7. The system was flushed with 2 L of deionized water at a high feed flow rate of 44 mL/min.
8. The system was washed with deionized water for 1 hour at the operating feed flow rate with recirculation of the retentate and permeate to the feed reservoir.
9. The membrane cassette was removed from the holder and stored in 0.1 N sodium hydroxide solution in the refrigerator.
3.4 Continuous Countercurrent Staged Diafiltration

Apparatus

Since two single-pass tangential flow filtration (SPTFF) modules were available from Pall, continuous 1-stage and countercurrent 2-stage diafiltration experiments were performed. The setup for both experiments is shown in Figures 12 and 13.

Figure 12. Setup for continuous 1-stage diafiltration experiment.
Masterflex L/S 16 (1/8” ID) platinum-cured silicone tubing (Cole-Parmer) was used as the connecting tubing due to its smooth surface and low protein binding levels. Masterflex L/S peristaltic pumps (Cole-Parmer) controlled the feed and DF buffer flow rates. For the 2-stage DF system, the retentate of Stage 1 was connected to the feed pump to match its flow rate to the feed flow rate. The drive of the feed pump was mounted with two heads (as shown in the bottom diagram).
panel of Figure 13) to accommodate both the feed stream and the retentate stream from Stage 1. The feed pump was fitted with larger ID tubing (1/4”) to reduce the shear stress imparted to the IgG. Before an experiment was performed, both pumps were calibrated by timed volume collection using a digital balance (OHAUS). A metering valve (Swagelok) was used to control the flow rate of the retentate from Stage 2 (diafiltered product). The flow rate for this product stream was determined by recording the mass of the product collection vessel over time. While the permeate of Stage 1 was open to atmosphere, the pressures of the other streams were monitored using stainless steel pressure gauges (Ashcroft) to determine the transmembrane pressure for each stage. Polypropylene Y-connectors (Cole-Parmer) were used to mix the various inlet streams.

**Sample Collection**

Roughly 500 μL samples were collected from the product stream in centrifuge tubes at predefined time intervals. Before performing an experiment, a sample from the feed solution was collected to measure the initial concentrations of IgG and B_{12} via UV-Vis spectrophotometry. This was done to verify that the measured concentrations were close to the prepared concentrations.

**Membrane Pre-Conditioning, Cleaning, and Storage**

The following pre-conditioning, cleaning, and storage steps were carried out before and after an experiment.

1. Each module was flushed with 2 L of deionized water at a feed pressure of 20-40 psig.
2. After all components were connected, the system was flushed with buffer for 30 minutes at the operating flow rate for each stream.

3. The feed line was immersed in the IgG / B₁₂ feed container and the experiment was performed.

4. After performing the experiment, the system was flushed with buffer for 15 minutes.

5. Each module was flushed with 2 L of 0.25 N sodium hydroxide solution at a high feed flow rate. If the retentate flow rate was lower than normal (likely caused by deposition of protein at retentate exit), the module was back-flushed (cleaning solution pumped into permeate side instead of feed side) until the retentate flow returned to normal.

6. After cleaning, each module was capped and stored at room temperature with the sodium hydroxide solution inside the module.

The next Chapter will discuss the batch and continuous DF experiments that were performed and analyze the results that were obtained.
Chapter 4

Experimental Results and Analysis

4.1 Batch Diafiltration Experiments

In order to validate the key experimental procedures, conventional batch DF experiments were performed using the Pellicon® 3 Ultracel membrane cassette using the experimental setup described in the previous Chapter (Figure 11). Data were obtained at several IgG concentrations using the experimental conditions given in Table 1.

Table 1. Experimental conditions for batch DF experiments.

<table>
<thead>
<tr>
<th>IgG conc. (g/L)</th>
<th>Initial B₁₂ conc. (g/L)</th>
<th>Feed volume (mL)</th>
<th>Feed flux (L/m²/h)</th>
<th>Permeate flux (L/m²/h)</th>
<th>TMP (psi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>100</td>
<td>300</td>
<td>40.2</td>
<td>4</td>
</tr>
<tr>
<td>20</td>
<td>5</td>
<td>80</td>
<td>300</td>
<td>34.1</td>
<td>4</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>50</td>
<td>300</td>
<td>19.1</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 1 shows that as the IgG concentration in the feed increases, the permeate flux decreases. This trend can be attributed to the increase in feed viscosity at high IgG concentrations, which leads to concentration polarization at the membrane surface. From Chapter 2.1, the B₁₂ concentration as a function of the number of diavolumes N_D is given by

\[
\ln \frac{C}{C_o} = -N_D S_o
\]  

(29)

where Equation (29) is the result of taking the natural log of Equation (5). A plot of \( \ln \frac{C}{C_o} \) versus \( N_D \) should have a slope of unity since \( S_o = 1 \) for a small solute like B₁₂. A semi-log plot of the experimental data is shown in Figure 14.
The black line has a slope exactly equal to 1, representing the theoretical values of the normalized vitamin B$_{12}$ concentration assuming S$_o$ = 1. The results obtained with pure vitamin B$_{12}$ (no IgG in the feed) were in good agreement with theory. The small deviation between the data and theory after 6 diavolumes is likely due to the large relative error in the measured B$_{12}$ concentration at the very dilute B$_{12}$ concentrations near the end of the experiment where the measured concentrations approach the lower limit of the calibration curve (10 mg/L). However, when IgG was present at 20 g/L, the data showed significant discrepancies after 5 diavolumes and this became even more pronounced when the IgG concentration was increased to 100 g/L. The values of the B$_{12}$ concentration were uniformly higher than the model calculations at large N$_D$ at high IgG concentrations, suggesting that the B$_{12}$ clearance through the membrane decreased with increasing IgG concentration.

Figure 14. Normalized B$_{12}$ concentration versus number of diavolumes.
The most likely explanation for the decrease in B_{12} clearance at high IgG concentrations is the presence of binding interactions between B_{12} and IgG, which prevents the passage of bound B_{12} through the membrane. Shao and Zydney demonstrated that reversible equilibrium binding between D-tryptophan (small impurity) and bovine serum albumin (protein product) significantly reduced impurity clearance during diafiltration [19]. Lowering the protein concentration encourages the dissociation reaction, which explains why the deviations for the 20 g/L IgG experiment were lower than those for the 100 g/L IgG experiment. Prentice et al. reported specific evidence for the binding of IgG proteins to the hydroxo form (OH-cobalamin) of vitamin B_{12}, which is formed from the non-binding cyano form (CN-cobalamin) in the presence of light [20]. In these experiments, the feed was prepared using CN-cobalamin. Therefore, photo-degradation of CN-cobalamin to OH-cobalamin caused B_{12} to bind to IgG, which led to reduced clearance.

To test this hypothesis, an experiment was conducted in the dark by shutting off the lights and covering the feed container with aluminum foil. The goal was to see whether reduced exposure to light could prevent the formation of OH-cobalamin and eliminate binding between B_{12} and IgG. A control experiment was performed at the same conditions but in ambient lighting. The experimental conditions are given in Table 2, with the results plotted in Figure 15.

**Table 2. Experimental conditions for batch DF experiments in light and dark conditions.**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>IgG conc. (g/L)</th>
<th>Initial B_{12} conc. (mg/L)</th>
<th>Feed volume (mL)</th>
<th>Feed flux (L/m²/h)</th>
<th>Permeate flux (L/m²/h)</th>
<th>TMP (psi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>50</td>
<td>80</td>
<td>60</td>
<td>300</td>
<td>30.9</td>
<td>6</td>
</tr>
<tr>
<td>Dark</td>
<td>50</td>
<td>80</td>
<td>60</td>
<td>300</td>
<td>28.9</td>
<td>5</td>
</tr>
</tbody>
</table>
In the standard experiment, the normalized $B_{12}$ concentration deviated significantly from the theoretical prediction. In contrast, the data obtained in the dark were in good agreement with theory, with the deviation at larger $N_D$ reflecting the uncertainties in the measured $B_{12}$ concentration at the very low concentrations seen near the end of the diafiltration.

In summary, batch DF experiments showed that vitamin $B_{12}$ clearance is in good agreement with model calculations when the binding between the OH-cobalamin form of $B_{12}$ and IgG is minimized. This can be done by operating at a low IgG concentration in the feed or by reducing the exposure of the apparatus to light, which drives the formation of OH-cobalamin.

Figure 15. Vitamin $B_{12}$ clearance in dark and standard lighting.
4.2 Continuous 1-Stage Diafiltration Experiment

Initial experiments were conducted using a continuous 1-stage DF experiment to validate the experimental protocol for the continuous system. The feed and operating conditions are shown in Figure 16.

The IgG and $B_{12}$ concentrations in the feed were 100 g/L and 5 g/L, respectively. $\alpha$, the ratio of the DF buffer flow rate (45 mL/min) to the feed flow rate (5 mL/min), was set at 9. This resulted in a 10x dilution of the feed when it was mixed with the DF buffer right before entering the Pall Cadence™ SPTFF module. Since $S_0$ should be equal to 1 for a small solute like $B_{12}$, the $B_{12}$ concentration in the retentate and permeate should be the same (0.5 g/L). A metering valve was used to manually set the retentate flow rate at 5 mL/min. Therefore, the conversion, which is the ratio of the permeate flow rate (45 mL/min) to the total feed flow rate (50 mL/min, or 46 LMH), was 90%. This should give a final IgG concentration exactly equal to that in the initial feed (before dilution with the DF buffer).

![Figure 16. Experimental conditions for continuous 1-stage DF.](image_url)
Figures 17 and 18 show the conversion and normalized $B_{12}$ concentration in the retentate over the course of the experiment. The measured conversion data was approximately 90% throughout the experimental run, demonstrating stable operation of the SPTFF module. The normalized $B_{12}$ concentration in the retentate was constant and consistent with the expected value of 0.1 (10x dilution).
4.3 Pall Module Performance Optimization

Before proceeding to countercurrent 2-stage DF experiments, the performance of the Pall Cadence™ SPTFF module was studied at different operating conditions to identify the range of conditions suitable for continuous operation without fouling. The setup was similar to that depicted in Figure 16, but without any DF buffer stream. The performance was studied as a function of

- feed IgG concentration,  
- feed flux,  
- conversion / volumetric concentration factor (VCF),  
- transmembrane pressure (TMP).

Figures 19 and 20 show the permeate flux and conversion as a function of TMP.

![Figure 19. Permeate flux versus TMP.](image1)

![Figure 20. Conversion versus TMP.](image2)

The flux in the absence of any IgG in the feed (deionized water only) showed a linear dependence on the TMP, consistent with a constant value of the membrane permeability. The
addition of IgG caused a reduction in the filtrate flux due to the effects of concentration polarization, with the retained IgG providing an additional resistance to filtration and / or a reduction in the effective pressure driving force due to the osmotic pressure of the protein solution. For 5 g/L IgG at a feed flux of 46 LMH, the permeate flux was only slightly lower than the DI water flux at the same TMP until the permeate flux approached a value essentially equal to the feed flux (corresponding to more than 95% conversion). At a feed flux of 92 LMH (100 mL/min), the permeate flux appeared to approach a pressure-independent region after 10 psi, with the maximum permeate flux being approximately 90 LMH. At the higher IgG concentration of 10 g/L and a feed flux of 46 LMH, the pressure-independent flux was attained at a lower TMP of 5 psi, with the maximum permeate flux of 42 LMH. This behavior is seen more easily in the plot of the conversion in Figure 20. A conversion of 96% (25-fold concentration) could only be achieved using the 5 g/L feed concentration. Increasing the feed concentration to 10 g/L gave a maximum conversion of only 94% due to the greater effects of concentration polarization.

To further study the relationship between feed flux and conversion, experiments were conducted at a constant TMP of 25 psi. The data are plotted in Figure 21. For all the feed IgG concentrations studied, the conversion decreased with increasing feed flux. In addition, at a given feed flux, the conversion increased with decreasing feed IgG concentration. A more concentrated feed will have a higher exit IgG concentration after ultrafiltration, leading to lower permeate flux and lower conversion.
All of the conversion data shown in Figures 20 and 21 were obtained from direct measurements of the permeate flux. Figure 22 shows a comparison of the measured IgG concentration factor (CF), evaluated from the IgG concentrations in the feed and final retentate as determined via the UV absorbance at 280 nm, and the volumetric concentration factor (VCF), determined directly from the measured flow rates. The black line, which has a slope of unity, represents the expected behavior assuming no loss of IgG. The data obtained at 5 g/L, show good agreement with the model, particularly at low VCF. However, at 10 g/L IgG in the feed and a feed flux of 92 LMH, the data points deviated significantly from linearity with the CF values being much lower than the VCF. Similar behavior was observed for the experiment performed at 18 g/L IgG and 46 LMH. This loss of IgG was due to membrane fouling, which caused protein to be retained within the module.

Figure 21. Conversion versus feed flux at a constant TMP of 25 psi.
In summary, the ultrafiltration experiments performed with the Pall Cadence™ SPTFF module showed that

- conversion can be increased by decreasing the feed flux and IgG concentration,
- maximum conversion of 96% (25-fold VCF) was achieved at a feed flux of 46 LMH (50 mL/min) and 5 g/L IgG,
- high feed fluxes and IgG concentrations resulted in membrane fouling and loss of protein.

Figure 22. Concentration factor via UV absorbance versus volumetric concentration factor.
4.4 Countercurrent 2-Stage Diafiltration Experiments

In the 2-stage DF experiments, two Pall Cadence™ SPTFF modules were connected in series in a countercurrent configuration. Several experiments were conducted at different feed fluxes, IgG concentrations, and conversions. This section describes the operating conditions, experimental results, comparison with model predictions, and the buffer requirement for the countercurrent 2-stage diafiltration.

4.4.1 Constant Feed Flux and Conversion Experiments

Initial experiments were performed at a constant feed flux of 46 LMH (50 mL/min) using a conversion of 90%, corresponding to a permeate to feed flow ratio of $\alpha = 9$ as shown in Figure 23. Data were obtained with different IgG concentrations in the feed, with the steady-state results summarized in Table 3.

![Diagram](image)

**Figure 23. Operating conditions for constant feed flux and conversion experiments.**
Table 3. Steady-state data for constant feed flux and conversion experiments.

<table>
<thead>
<tr>
<th>C_{IgG,feed} (g/L)</th>
<th>C_{B12,feed} (g/L)</th>
<th>C_{IgG,exit} (g/L)</th>
<th>C_{B12,exit} (g/L)</th>
<th>TMP_{stage1} (psi)</th>
<th>TMP_{stage2} (psi)</th>
<th>B_{12} removal (%)</th>
<th>Buffer req. (L/g IgG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.9</td>
<td>0</td>
<td>0.026</td>
<td>4.5</td>
<td>3</td>
<td>98.7</td>
<td>-</td>
</tr>
<tr>
<td>100</td>
<td>1.2</td>
<td>105</td>
<td>0.019</td>
<td>12.5</td>
<td>8</td>
<td>98.4</td>
<td>0.09</td>
</tr>
<tr>
<td>50</td>
<td>2.2</td>
<td>46</td>
<td>0.022</td>
<td>5.5</td>
<td>5.5</td>
<td>98.9</td>
<td>0.18</td>
</tr>
</tbody>
</table>

*Note: C_{IgG,exit} and C_{B12,exit} are the concentrations in the diafiltered product stream.*

The first experiment with 1.9 g/L B_{12} and no IgG in the feed was performed to study B_{12} clearance in the absence of any binding interactions with IgG. The other two experiments were conducted in darkness with the feed container covered with aluminum foil to minimize binding interactions. The first experiment with no IgG in the feed had the lowest TMP, while the experiment with 100 g/L IgG had the largest TMP. This was to be expected since the presence of IgG results in a more viscous solution. However, the buffer requirement is lowest for the 100 g/L experiment (0.09 L/g IgG) since buffer requirement (= α/C_{F}) is inversely proportional to the feed IgG concentration. The exit IgG concentrations for the second and third experiment were close to the feed concentrations, suggesting that there was almost complete recovery of protein in the diafiltered product. The B_{12} removal was calculated using

\[ f = 1 - \frac{C_{B12,exit}}{C_{B12,feed}} \]

(30)

The predicted B_{12} removal is given by Equation 20, which is reproduced below.

\[ f = \frac{\alpha^{N+1} - \alpha}{\alpha^{N+1} - 1} = \frac{9^{2+1} - 9}{9^{2+1} - 1} = 98.9\% \]

Since α = 9 and N = 2, the predicted B_{12} removal was equal to 98.9% for all three experiments, which is in good agreement with the measured values. The small discrepancies between experimental and theoretical B_{12} removal for the first two experiments may have been caused by
errors in measuring the concentration of dilute B₁₂ solutions, or it might be due to errors in the flow rates, particularly for the retentate flow rate from Stage 1 which was not directly measured in these experiments. Therefore, an improvement in the experimental setup would be to add an inline flowmeter to measure the actual flow rate.

### 4.4.2 Transient Experiments

The experiments described here examined the transient behavior of the exit IgG and B₁₂ concentrations from start-up to steady-state operation.

**Transient Experiment 1**

The operating conditions for Transient Experiment 1 are shown in Figure 24.

![Figure 24. Operating conditions for Transient Experiment 1.](image)
The feed flux of 46 LMH (50 mL/min) and the conversion of 90% are the same as those used in the experiments described previously in section 4.4.1. The exit B_{12} and IgG concentrations are plotted as a function of time in Figure 25.

![Figure 25. Exit B_{12} and IgG concentrations versus time for Transient Experiment 1.](image)

The initial breakthrough in concentration occurred approximately 5 minutes after start-up of the run (denoted as the time at which the IgG was initially introduced in the feed). The hold-up volume in the system was determined to be approximately 42 mL: 7 mL per module and 28 mL for tubing and gauges used in the 2-stage system. The residence time in the system can be estimated by dividing the hold-up volume by the flow rate, which varies between 5 and 50 mL/min given the large conversion in the module. Using the flow rate of 5 mL/min, which is present in most of the tubing, gives a residence time of just more than 8 minutes, which is consistent with the results in Figure 25. The IgG and B_{12} concentrations increased rapidly after the initial breakthrough and then began to plateau after around 20 minutes of operation. The gradual increase in concentrations suggests that there was considerable mixing within the
system. The concentrations of both species reached steady-state at around 20 minutes. The steady-state IgG concentration was 44.8 g/L, which is very close to the feed concentration of 45 g/L. The exit B₁₂ concentration was 0.024 g/L, giving a removal of 98.8% in good agreement with the predicted value of 98.9%.

**Transient Experiment 2**

A subsequent experiment was performed in which the flow rates were halved while the IgG concentration in the feed was doubled from 45 to 90 g/L. It is important to note that this gives the same throughput of IgG since a feed with twice the concentration was being fed at half the rate. The conversion in each stage was set to 90%. The operating conditions are shown in Figure 26.

![Figure 26. Operating conditions for Transient Experiment 2.](image)

The exit B₁₂ and IgG concentrations are plotted as a function of time in Figure 27. For this experiment, the initial breakthrough in concentration occurred after 10 minutes, which is consistent with the lower feed flow rate. However, the IgG concentration reached steady-state at
20 minutes, which was faster than the time of 30 minutes that it took for the B\textsubscript{12} concentration to reach steady-state. This was in contrast to the behavior observed in Transient Experiment 1 where the concentrations of both species reached steady-state at approximately the same time. The origin of this behavior is unclear and warrants further investigation.

At steady-state, the exit IgG concentration was 92.2 g/L, which is close to the feed concentration of 90 g/L. The exit B\textsubscript{12} concentration was 0.021 g/L, giving an impurity removal of 98.9% exactly equal to the model prediction.

Summary of Transient Experiments

A summary of the main results obtained from the transient experiments is presented in Table 4. Data obtained from two other countercurrent 2-stage DF experiments performed at 80% and 95% conversion are also included.
Table 4. Summary of results from countercurrent 2-stage diafiltration experiments.

<table>
<thead>
<tr>
<th>C_{IgG,feed} (g/L)</th>
<th>Conversion (%)</th>
<th>Impurity Removal (%)</th>
<th>Number of Stages for 99.99% Removal</th>
<th>Buffer req. (L/g IgG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>128</td>
<td>80</td>
<td>96.2</td>
<td>6.2</td>
<td>0.03</td>
</tr>
<tr>
<td>45</td>
<td>90</td>
<td>98.8</td>
<td>4.2</td>
<td>0.20</td>
</tr>
<tr>
<td>90</td>
<td>90</td>
<td>98.9</td>
<td>4.2</td>
<td>0.10</td>
</tr>
<tr>
<td>90</td>
<td>95</td>
<td>99.7</td>
<td>3.2</td>
<td>0.21</td>
</tr>
</tbody>
</table>

Note: 8 diavolume batch DF performed at 60 g/L IgG requires 0.13 L buffer/g IgG.

The following trends can be identified from the results:

- impurity removal increases with increasing conversion,
- buffer requirement decreases with decreasing conversion and increasing IgG concentration.

The main takeaway is that at a fixed number of stages, there is a trade-off between impurity removal and buffer requirement. However, both a sufficiently high level of impurity removal and a lower buffer requirement than a traditional batch DF can be achieved under certain conditions.

The model calculations (Figure 8, page 19) show that an impurity removal of 99.99% (or 10,000 fold) can be achieved for a 3-stage DF performed at a mAb concentration of 200 g/L.

Important, the buffer requirement at these conditions is 0.11 L/g mAb, which is lower than the 0.13 L/g mAb required for an 8 diavolume batch DF performed at a mAb concentration of 60 g/L.

The data in Table 4 is plotted with the model calculations in Figure 28. The two data points in the middle correspond to the two 90% conversion (α = 9) experiments. The left-most and right-most data points correspond to 80% conversion (α = 4) and 95% conversion (α = 19), respectively. The experimental data are in good agreement with the model calculations for N = 2.
Figure 28. Fold-removal versus flow ratio from data and model calculations.
Chapter 5

Conclusions

5.1 Summary of Findings

There is considerable interest in the biopharmaceutical industry to transition from traditionally batch to continuous processes for the manufacture of high value biological products. This interest is motivated by the potential for increased productivity, enhanced product quality, and greater flexibility in manufacturing. While significant progress has been made in the development of continuous perfusion bioreactors and continuous chromatographic processes, the ultrafiltration / diafiltration step, which is currently used for concentration and final formulation of essentially all biotherapeutics, is currently an inherently batch operation. The data presented in this research project provide the first demonstration that countercurrent staged diafiltration with single-pass tangential flow filtration modules can be used for the continuous formulation of monoclonal antibody products.

Experiments were performed with Pall Cadence™ Inline Concentrators with 30 kDa molecular weight cut-off membranes, serum IgG as the model antibody, and vitamin B12 as the model impurity. The apparatus was successfully developed to perform countercurrent 2-stage diafiltration experiments.

Model calculations showed that impurity removal can be increased by increasing the ratio of the diafiltration buffer flow rate to the feed flow rate and the number of stages. At a fixed impurity removal, the buffer requirement can be reduced by increasing the mAb concentration in
the feed and the number of stages. A 10,000-fold impurity removal can be achieved for a
countercurrent 3-stage diafiltration performed at a mAb concentration of 200 g/L. The buffer
requirement at these conditions is 0.11 L/g mAb, which is lower than the 0.13 L/g mAb required
for a traditional 8 diavolume batch diafiltration performed at a mAb concentration of 60 g/L.

Experimental data were in good agreement with model calculations. Data obtained in a 2-
stage system with 90% conversion in each stage provided 98.8% removal of vitamin B\textsubscript{12} with
essentially 100% recovery of the IgG. Impurity removal was increased to 99.7% by increasing
the conversion to 95%.

5.2 Suggestions for Future Work

The following suggestions could be promising directions for future work on
countercurrent staged diafiltration:

- perform experiments with 3 or more stages,
- demonstrate operation over longer process times (e.g. 4 hours) with product recovery steps,
- perform experiments with commercial monoclonal antibodies,
- use formulation buffer as the diafiltration buffer and monitor buffer exchange (through pH
  measurements, for example).
BIBLIOGRAPHY


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EDUCATION

Aug 2013 – May 2017
B.S. Chemical Engineering
Schreyer Honors College
Minor in Chemistry
The Pennsylvania State University, University Park, PA

RESEARCH EXPERIENCE

June 2016 – present
Research Assistant, Prof. Andrew Zydney’s Lab
Department of Chemical Engineering, Penn State

- Conducting honors thesis research on utilizing ultrafiltration / diafiltration (UFDF) for monoclonal antibody formulation
- Developing a continuous multistage countercurrent diafiltration process with reduced water usage as compared to existing batch diafiltration processes
- Analyzing the performance of two single-pass tangential flow filtration modules in countercurrent operation

Aug 2014 – May 2016
Research Assistant, Prof. Robert Rioux’s Lab
Department of Chemical Engineering, Penn State

- Developed a continuous packed bed flow reactor for the one-pot synthesis of cyclic carbonates from olefins
- Coupled the orthogonal reactions of methyltrioxorhenium-catalyzed epoxidation and aluminum-catalyzed carboxylation via an in-line liquid-liquid membrane separator
- Optimized process conditions such as temperature, catalyst loading, and residence time

PUBLICATIONS


PROFESSIONAL SOCIETIES

2015 – present  Tau Beta Pi, Engineering Honors Society
2015 – present  Omega Chi Epsilon, Chemical Engineering Honors Society
2013 – present  American Institute of Chemical Engineers
HONORS AND AWARDS

Nov 2016 3rd Place, AIChE National Student Paper Competition
Summer 2016 Summer Research Fellowship, Dept. of Chemical Engineering, Penn State
Apr 2016 1st Place, AIChE Mid-Atlantic Regional Student Paper Competition
Feb 2016 Evan Pugh Scholar Senior Award, Penn State
Dec 2014 3rd Place, Civic Engagement Public Speaking Contest, Penn State
Nov 2013 Best Design Team Award, Engineering Design 100, Penn State

TEACHING

Spring 2017 Instructional Aide, CHE 497C, Penn State
Fall 2016 Teaching Intern, CHE 210, Penn State

LEADERSHIP

Nov 2014 – May 2016 Research Officer, AIChE Student Chapter, Penn State

- Planned faculty-student events and promoted undergraduate research
- Organized 4 CENTER Talks with Prof. Darrell Velegol for over 100 students
- Mentored 5 chemical engineering students to find research positions
- Presented at First-Year Seminars to encourage underclassmen to get involved with undergraduate research

Oct 2013 – Dec 2013 Team Leader, Alcoa Sustainability Project, Engineering Design, Penn State

- Designed a prototype for the ‘Aluminator’, a light reflecting panel that harnesses sunlight and reduces the need for conventional electrical lighting in classrooms
- Won the Best Design Team award
- Led team to the Design Showcase and presented the prototype in front of Alcoa judges