Protein A Chromatography in Monoclonal Antibody Purification

by

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Abstract

The bio-pharmaceutical industry began over 30 years ago with the production of human insulin and has shown incredible growth ever since. With forecasted annual worldwide sales of over $450B in 2025 for biopharmaceuticals, they are expected to be at least 25% of the entire pharmaceutical market. Therapeutics based on monoclonal antibodies (mAbs) make up roughly a third of all biopharmaceutical sales with indications from asthma, to cancer, to Parkinson's disease. The recent approval of the first biosimilar mAb products in the US and Europe has exposed up to 20 of the top grossing biologic products to competition for the first time, while 75% of the US market is expected to lose patent exclusivity by 2020. With the increased competition from biosimilars, the costs associated with producing mAb based therapeutics will be a constraint on maintaining market share going forward.

The majority of the total manufacturing costs for mAbs resides in the downstream processing where Protein A chromatography is the predominantly employed technology for the primary capture step. With Protein A's high unit cost of up to $15,000 per liter and susceptibility to deamidification when exposed to high pH cleaning and sanitization chemicals, it is no surprise that many mAb manufacturers are considering alternatives.

The objective of this work is to review the production process of mAb therapeutics, with a specific focus on the advantages, disadvantages, and alternatives to Protein A affinity chromatography as the primary capture step in downstream processing.

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List of Abbreviations

AEX	anion exchange chromatography
ANVISA	Brazilian Health Authority
ApA	artificial protein A
ATPE	aqueous two-phase extraction
ATPS	aqueous two-phase system
BET	Brunauer-Emmet-Teller isotherm
BHK	baby hamster kidney
CCF	cell culture fluid
CDR	complimentary determining region
CEX	cation exchange chromatography
cGMP	current Good Manufacturing Practices
C_{Hx}	constant domains in an antibody (x = 1, 2, 3, 4, etc.)
CHO	Chinese hamster ovary
CHOP	Chinese hamster ovary proteins
CIP	clean-in-place
cMEM	complete mammalian cell culture medium
CMOs	contract manufacturing organizations
COGS	cost of goods sold
COS	cell lines derived from monkey kidney
CV	column volume(s)
DBC	dynamic binding capacity
DF	diafiltration

EMA	European Medicines Agency
E. coli	*Escherichia coli* bacteria
Fab	antigen binding fragment
Fc	fragment crystallizable
FDA	Food and Drug Administration
HCP	host cell proteins
HEPA	high-efficiency particulate air
HIC	hydrophobic interaction chromatography
HMW	high molecular weight
HT/THP	chromatography on hydroxyapatite
IEX	ion exchange chromatography
Ig	immunoglobulin
IgA	immunoglobulin of class A
IgD	immunoglobulin of class D
IgE	immunoglobulin of class E
IgG	immunoglobulin of class G
IgM	immunoglobulin of class M
IMAC	immobilized metal ion affinity chromatography
mAb	monoclonal Antibody
MMC	multimodal or mixed mode chromatography
NS0	cell line derived from nonsecreting murine myeloma
PAM	protein A mimetic
PEG	polyethylene glycol

RPLC	reversed-phase liquid chromatography
SIP	steam-in-place
SEC	size exclusion chromatography
SEM	scanning electron microscopy
SMB	simulated moving bed
Sp2/0	cell line derived from nonsecreting murine myeloma
SPA	*Staphylococcal* protein A
SPR	surface plasma resonance
TCFF	tangential cross flow filtration
UF	ultrafiltration
V_L	light chain variable domain
V_H	heavy chain variable domain
WFI	water for injection

Chapter 1 - Introduction

The biopharmaceutical industry is currently undergoing a significant challenge with the first monoclonal antibody (mAb) biosimilar (interchangeable biomolecule) approvals by health authorities in the United States and Europe. This competition is applying new cost pressures for commercially successful products with the benefit of reduced cost to patients at the expense of lost market share for current manufacturers. In order to minimize the financial impact, biopharmaceutical manufacturers will either need to ensure a steady pipeline of new products through research and development to offset the lost revenue from legacy products, or commit to competing with biosimilar manufacturers on the average selling price. It is unlikely that current mAb producers will relinquish multiple billions of dollars per year in sales on a single product without some level of engagement in the price competition.

The greatest opportunity for reducing production costs is in the downstream purification processes that can contribute up to 80% of the total cost of goods and where chromatography is heavily employed in multiple separation sub-processes. Protein A chromatography is almost exclusively utilized as the primary capture step in purification and is the most expensive resin type with a cost of up to $15,000 per liter, making it the ideal candidate for operational improvements or replacement with a less expensive alternative. Operational improvements to Protein A resin will likely be an interim step in cost reduction efforts due to the high resin cost.

The path to identifying cost effective and chemically stable alternative affinity ligands is paved with the understanding of the 3-D structure of immunoglobulins (or mAbs) and how they bind to Protein A ligands. This knowledge has already produced many commercially available ligand alternatives to Protein A at a reduced price per liter and many more with the potential for future commercial viability.

Monoclonal Antibodies (mAbs)

Antibodies or immunoglobulins are proteins made up of two identical large polypeptide subunits (heavy chains) consisting of ~430 amino acids and two identical small polypeptide subunits (light chains) consisting of ~214 amino acids (Shuler et al., 2017). The heavy and light chains are linked by disulfide bonds resulting in a flexible "Y" shaped molecule with a constant or non-variable end identified as the fragment crystallizable (Fc) region that binds to Fc receptors in cell walls and variable ends identified as fragment antigen-binding (Fab) region that binds specifically to antigens such as proteins on the surface of virus or bacteria cells. The light and heavy chains each consist of one variable domain (V_L or V_H), while each light chain has one constant domain (C_L) and the heavy chains each have three or four constant domains (C_H1, C_H2, C_H3, and C_H4) (Sommerfeld and Strube, 2005). Each variable domain includes three complimentary determining regions (CDRs) that determine the specific antibody binding. In human blood, there are five major classes of antibodies based on differences in the sequence of amino acids in the Fc region: IgA, IgD, IgE, IgG, and IgM. A simple diagram of the most abundant human antibody, immunoglobulin G (IgG), is shown in Figure 1-1.

An animal's immune system produces antibodies in B lymphocytes when a foreign substance or antigen is detected. The complex formed by the antibody and antigen consists of hydrogen bonding, van der Waals forces, hydrophobic interactions, and electrostatic forces. It is also known as the animal's immune response. Monoclonal antibodies (mAbs), are engineered antibodies which bind to a single antigen or region on an antigen called an epitope. This specificity is what makes mAbs highly valuable in the biopharmaceutical industry. While mAbs were originally produced using recombinant DNA technology with bacteria (*E. coli*), hybridoma

technology in murine myeloma cells (Mumab or 100% mouse protein) was developed to ensure they were quickly cleared in the body due to immunogenic response (Rodgers and Chou, 2016).

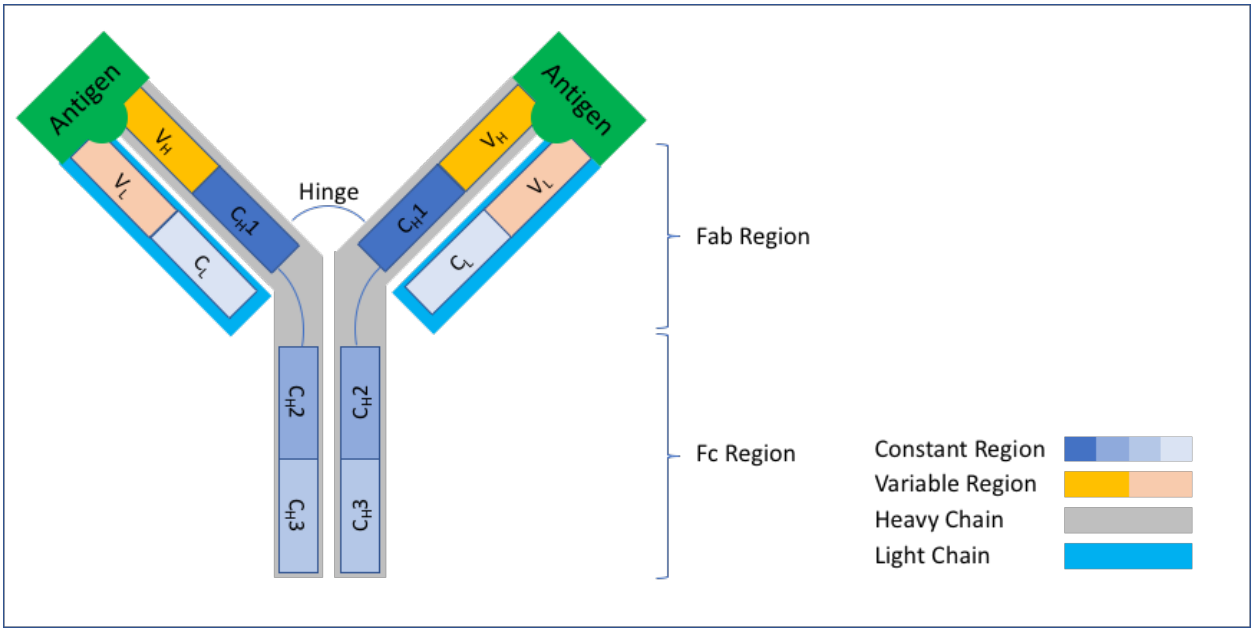


Figure 1-1 Structural diagram of Immunoglobulin G (IgG)

Simplified diagram identifying the two identical heavy and two identical light chains or subunits, constant regions (C_L, C_{H1}, C_{H2}, C_{H3}), variable regions (and V_L, V_H,) as well as the "Y" shaped hinge. Not shown are the complimentary determining regions (CDRs) within each variable region, which determine the specific binding to the antigen. Modified from Sommerfeld and Strube, 2005.

Additional technological advances in modifications to the CDRs within the mAb variable region, allowed for the development of Chimeric mAbs, which were approximately 65% human protein and 35% mouse protein. Phage display techniques increased the human protein content to approximately 95% with the intent of minimizing the immunogenic response (Gombotz and Shire, 2010). Chinese hamster ovary (CHO) cells are the most common type of cells to produce humanized mAbs which will be discussed in this work, but others exist, including: nonsecreting murine myeloma (NS0 and Sp2/0) mouse cells, monkey kidney (COS) cells, and baby hamster kidney (BHK) cells. (Costa et al., 2010).

Demand for Therapeutic mAbs

There have been a significant number of mAb based therapeutics to receive FDA approval since the first mAb therapeutic, Orthoclone OKT3, in 1986 for the treatment of acute kidney transplant rejection (Shukla and Thommes, 2010). In the last ten years, there have been 54 approvals with nearly half approved in the last two years alone. The increased number is illustrated in Figure 1-2 compared to all FDA approvals through 2017, while a complete list of approvals is listed in Appendix A (U.S. Food and Drug Administration, 2018). The worldwide demand is driven by an increasing world population in addition to emerging markets, such as China, Russia, and Brazil all increasing their standard of living (Ecker et al., 2015).

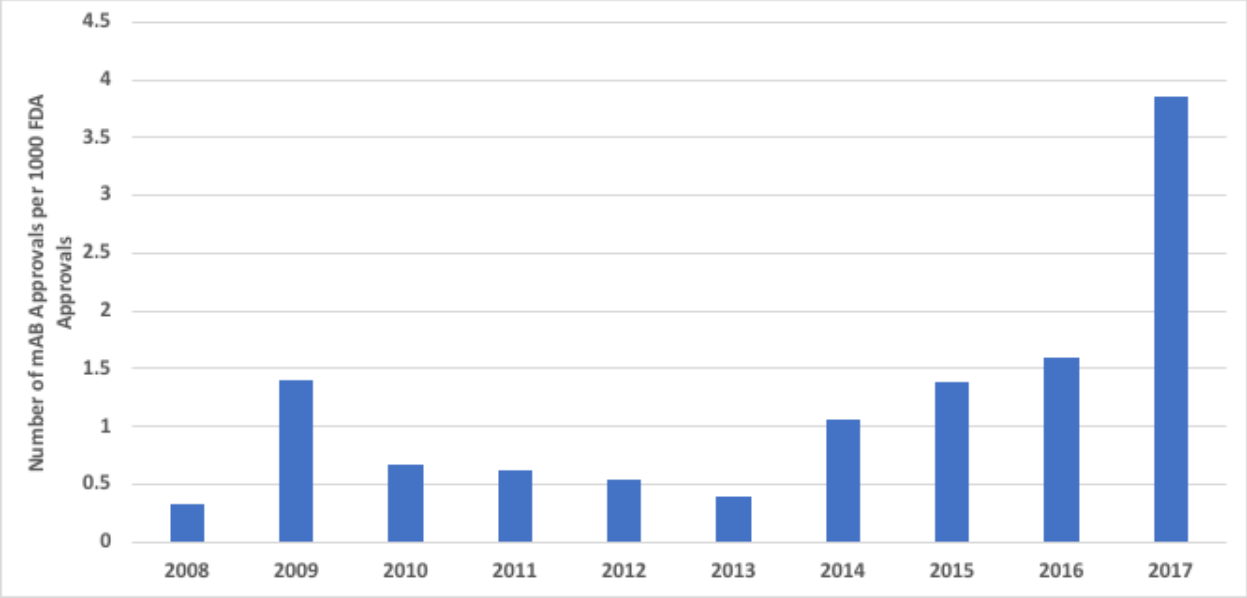


Figure 1-2 FDA approvals of mAbs per Thousand Approvals by Year: 2008-2017

FDA approvals with significant increase in 2017. (U.S. Food and Drug Administration, 2018)

The introduction of biosimilars (molecules interchangeable with an already approved product) to the market has the potential to increase competition as well as worldwide access to mAb therapeutics. Abbot/AbbVie's blockbuster Humira, with ~$10B in peak annual sales

(King, 2013) now faces competition for the first time with the approvals of the biosimilars: Amjevita, Inflectra, and Cyltezo (U.S. Food and Drug Administration, 2018). Further pressures will be exerted on established products as more biosimilars are approved. In 2017, Kent et al. forecasted that biosimilars have the opportunity to achieve a market value of $80B by 2020. Table 1-1 includes those first to feel the effects of this new market pressure (U.S. Food and Drug Administration, 2018, King, 2013).

Table 1-1 Name Brand mAb Therapeutics and their FDA Approved Biosimilars

Brand Name	Company	Indication	Peak Annual Sales[1]	Biosimilar(s)	Biosimilar Company
Humira	Abbot/AbbVie	Immunology	$10B	Amjevita	Amgen
				Inflectra	Celltrion
				Cyltezo	Boehringer Ingelheim
Remicade	Janssen Biotech	Immunology	$7B	Ixifi	Pfizer
				Renflexis	Samsung Bioepsis
Avastin	Genentech	Oncology	$6B	Mvasi	Amgen
Herceptin	Genentech	Oncology	$6B	Ogivri	Mylan

Introduction of biosimilars and their potential on four high value commercial mAbs. (U.S. Food and Drug Administration, 2018. [1]King, 2013)

Indications for Therapeutic mAbs

The high specificity in antibody-antigen binding has resulted in an ever-increasing number of indications treated by mAb therapeutics, including biologics, such as asthma, dermatitis, hemophilia, macular degeneration, and multiple sclerosis. These are outside the more traditional immunology, diabetes, and oncology, which make up approximately $110B annually, and over half the revenue of all biologics combined. These underrepresented or non-represented

indications are only expected to increase as 25% of the current biologic pipeline is addressing indications which are not met by a biologic (Kent et al., 2017). The breakdown of the data in Figure 1-2 by indication are included in Figure 1-3, showing the significant increase in Immunology, Oncology, Neuroscience and others in 2017 (nearly 90% increase from 2016). This is likely due to mAbs frequently having a quick path from molecule development to a clinical trial (Ecker et al., 2015).

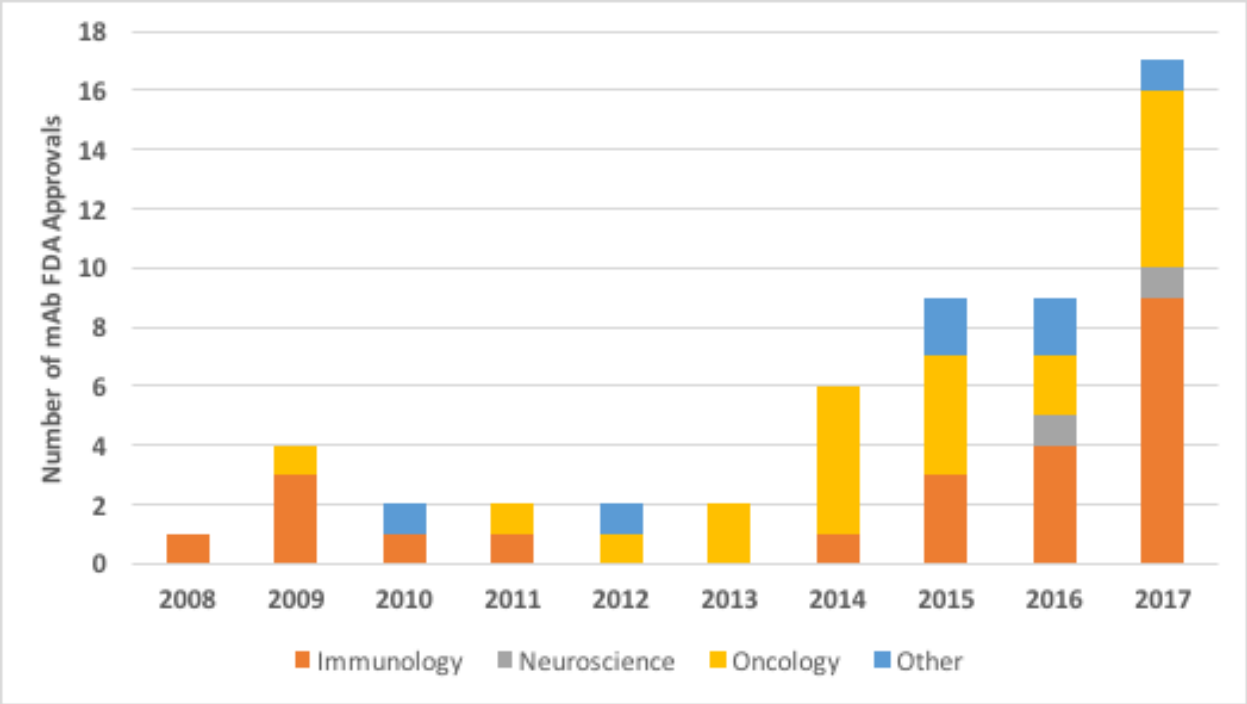


Figure 1-3 mAb FDA Approvals by Indication by Year: 2008-2017

Breakdown of new FDA mAb approvals by indications. Significant increases in all areas with particular growth in immunology. (U.S. Food and Drug Administration, 2018)

Chapter 2 - Overall mAb Production Process

Unlike traditional pharmaceuticals that are produced through traditional chemical reactions, mAbs are produced by living cells. The production is however required to meet current Good Manufacturing Practices (cGMP) as regulated by health authorities such as the U.S. Food and Drug Administration (FDA), European Medicines Agency (EMA), Brazilian Health Authority (ANVISA) and others. These requirements are constantly evolving (hence, the lower-case c) and cover Buildings, Equipment, Process Controls, Laboratory Controls, and Record keeping, among other areas. These are the minimum requirements for a production facility to meet and all producers should far exceed them through policies and procedures. The equipment and unit operations used in the production of mAbs are strictly controlled with high-efficiency particulate air (HEPA) filtration, clean-in-place (CIP), steam-in-place (SIP), Water for Injection (WFI), Process Air, etc. to avoid contamination of the product as it will be administered to patients intravenously.

Large and small-scale production of mAb therapeutics are broken down into two major processing areas: Upstream Processing and Downstream Processing. Upstream processing consists of two sub-processes (1.1 Inoculum and 1.2 Cell Culture) and Downstream processing consists of six sub-processes (2.1 Primary Recovery, 2.2 Primary Capture, 2.3 Viral Inactivation, 2.4 Polishing, 2.5 Viral Filtration, and 2.6 Ultra Filtration / Diafiltration). The order of the downstream sub-processes is often dependent on the specific mAb produced, but Figure 2-1 provides a depiction of a generic mAb production process flow. Certain products may neglect downstream processing steps or repeat them as defined during the mAb development. A summary of the upstream and downstream process steps is provided in Figure 2-1 and described in more detail below.

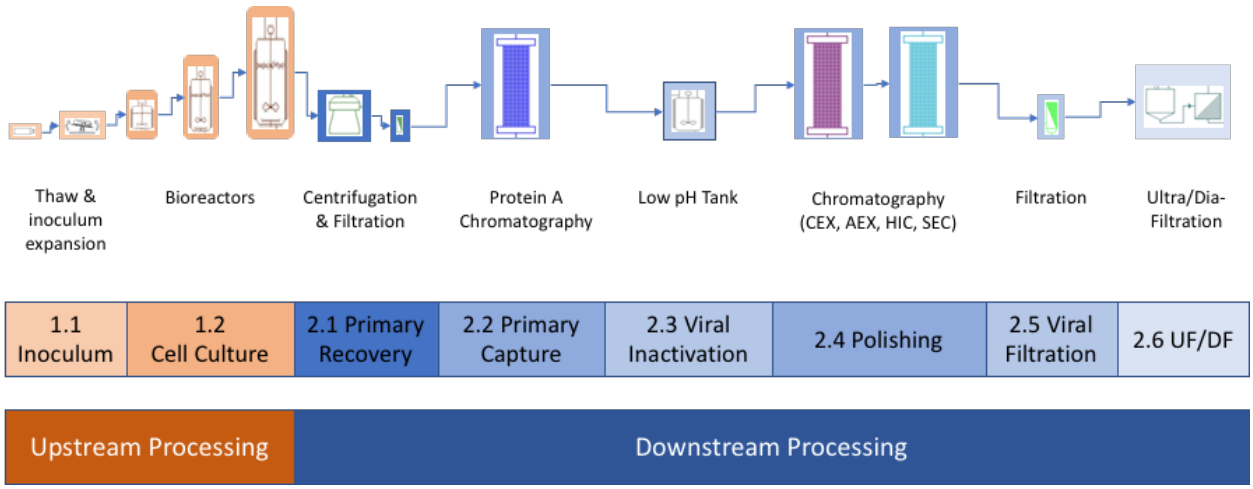


Figure 2-1 Generic mAb Production Process Flow

All mAb production consists of Upstream and Downstream processing steps. These downstream processing steps are an example of a generic process and varies for the specific mAb. Modified from Nfor et al., 2008, Shukla and Thommes, 2010, Shukla et al., 2017, Sommerfeld and Strube, 2005, and Steinebach et al., 2016.

Upstream Processing

Inoculum

Once the desired mAb is engineered in the lab, the cells are stored in a freezer between minus 20°C and minus 80°C to minimize degradation. When required for production of a commercial or clinical product, the cells are thawed in a controlled manner, combined with sterile filtered growth media, and allowed to multiply and expand under controlled conditions. The cells are successively transferred from a smaller volume container to a larger volume container (e.g. 225 ml flask → 2.2 L bottle → 100 L bag → 200 L bag).

Cell Culture and mAb Production

Similar to the inoculum expansion, the cell culture also includes continued cell growth, but in larger and larger fixed bioreactors (up to 12,000 L). Each transfer between bioreactors requires additional cell culture media and supplements to further the cell's growth. Upon completion of this step, in addition to the mAb product, the final bioreactor contains the

remaining cell culture fluid (CCF), which consists of the following: Host cells, host cell proteins (HCP) or Chinese hamster ovary proteins (CHOP), amino acids, vitamins, cell debris, viruses, DNA, endotoxins, and high molecular weight (HMW) aggregates. These components will be separated from the mAb in the downstream processing.

Downstream Processing

Primary Recovery

Cells and cell debris are removed from the liquid stream using centrifugation and filtration. The remaining product stream maintains nearly equal volume to that of the bioreactor as the supernatant of the centrifuge.

Primary Capture (Chromatography)

A chromatography column is used to bind the product mAb to the functional group of the resin. The entire volume of the stream from the primary recovery step passes through the column with the majority of the contaminants passing through the column and discarded in the waste stream. The column is regenerated, which releases the bound mAb, and is fed to a 0.2 μm filter before future processing. Protein A is the most common type of resin used in the primary capture step and will be discussed in more detail in Chapter 4 - (Low et al., 2007).

Viral Inactivation

A low pH incubation step is often included post Primary Capture chromatography as the pH of the mAb stream leaving the column is acidic and can be easily reduced below pH 3.8 with the addition of phosphoric acid for a specific amount of time (approximately 30 minutes) to allow any viruses to be inactivated (Shukla et al., 2017). Prior to feeding the next step, the stream pH is raised to 7 and then passed through another 0.2 μm filter.

Polishing (Chromatography)

Polishing is the second process step that relies on chromatography. This often encompasses a combination of chromatography steps, including: Cation Exchange (CEX), Anion Exchange (AEX), Hydrophobic Interaction Chromatography (HIC), Size Exclusion Chromatography (SEC), and Immobilized Metal Affinity Chromatography (IMAC).

Table 2-1 provides a summary of the type of chromatography by separation (Giacometti and Josic, 2013). Some of these will be discussed in depth in subsequent chapters.

Table 2-1 Methods of Liquid Chromatography

Type of Separation	Type of Chromatography
Size and Shape	Size-Exclusion Chromatography (SEC)
Net Charge	Ion-Exchange Chromatography (IEX)
Hydrophobicity	Hydrophobic-Interaction Chromatography (HIC) Reversed-Phase Liquid Chromatography (RPLC)
Biological Function	Affinity Chromatography (AC)
Metal Binding	Immobilized Metal Ion Affinity Chromatography (IMAC)
Other	Chromatography on Hydroxyapatite (HT/THP)

Chromatography methods based on type of separation required. Modified from Giacometti and Josic, 2013.

Viral Filtration

This is an additional filtration step where the product stream is passed through a 0.2 μm filter to remove any remaining viruses.

Ultrafiltration / Diafiltration

This process step is the final filtration step, which employs tangential cross flow filtration (TCFF) that allows the smaller remaining contaminants to pass through the filter membrane and

be discarded as waste, while retaining the mAb product in the concentrate stream. The periodic addition of clean buffer solution allows for the washing of impurities prior to a final pass through another 0.2 μm filter.

Application of Downstream Processing on Commercial mAbs

In 2005, Sommerfeld and Strube analyzed the downstream production processes for six mAbs therapeutics (Herceptin, Rituxan, MabCampath, Synagis, Remicade, and Simulect) and compared the order of the processes to a generic process flow. Their results are aligned to Figure 2-1 and are presented in Table 2-2 (excluding MabCampath, as it has been withdrawn from the market). All but one of the products rely on Protein A as the primary capture step and the generic order of the first three downstream processing steps. Each product utilizes multiple polishing steps.

Table 2-2 Order of Generic mAb Downstream Production Processes for different mAbs

Process Step	Herceptin	Rituxan	Synagis	Remicade	Simulect
2.1 Primary Recovery	1	1	1	1	1
2.2 Primary Capture	2	2	2[1]	2	2
2.3 Viral Inactivation	3	3	4	3	3
2.4 Polishing	4, 5, 6	4, 5	3, 6, 8	4, 6, 7	4, 5
2.5 Viral Filtration		6	5, 7	5	6
2.6 UF/DF	7	7	9	8	7

The number under each product indicates the order in the process. [1]All products employ Protein A as the primary capture step except Synagis, which requires Anion Exchange. Data from Sommerfeld and Strube, 2005 aligned to downstream processing steps in Figure 2-1.

There have been significant improvements in the upstream processes over the last decade resulting in higher titers (> 5 g/L) and a process as described above should recover at least 70%

of the mAb with over 95% purity (Chon and Zarbis-Papastoitsic, 2011). This increase in titer has challenged the downstream processing steps as the production costs have shifted to downstream, which can now be anywhere from 50% to 80% of the total cost of production (Roque et al., 2004). Protein A is the most commonly implemented chromatography process in the primary capture step and is a major contributor to the significant cost of total production with unit costs between $8,000 and $15,000 per liter (Menegatti et al., 2012). As demonstrated by a recent survey of biopharmaceutical companies, over half were considering an alternative to Protein A as the primary capture step for new products and half of contract manufacturing organizations (CMOs) had already implemented an alternative (Langer, 2016). This work will focus on the important aspects of Protein A chromatography including its benefits, limitations and alternatives.

Chapter 3 - Chromatography Fundamentals

Chromatography is an adsorption process, where mass is transferred between a mobile and stationary phase. In liquid chromatography, the fluid flow is the mobile phase and the resin particles are the stationary phase where the adsorption occurs. The purification and polishing of mAbs rely on cylindrical packed columns, containing varying functional groups in the stationary phase (resin) depending on the type of chromatography, to ensure a large adsorption area per unit volume. An example of a chromatography column is presented in Figure 3-1, identifying the major components of the column as well as potential flow paths (tortious and channeling). Preparation of the resin is very important to allow the particles to settle in a tightly packed bed. Incorrect preparation can result in channeling flow paths that will reduce contact time with the resin, greatly decreasing performance.

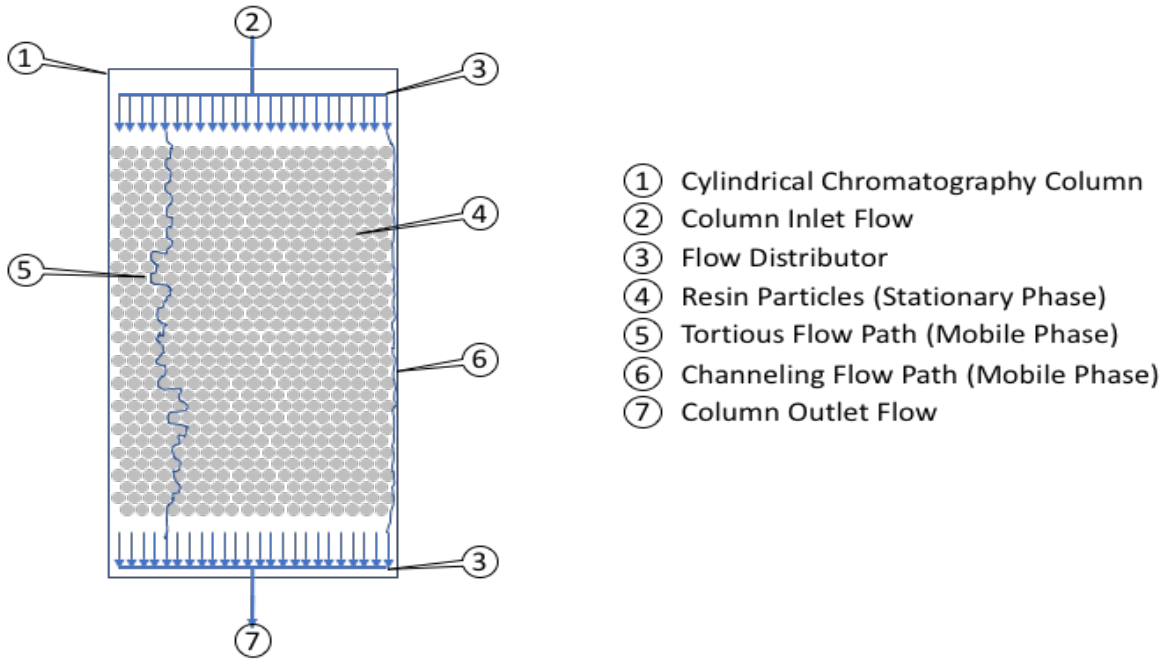


Figure 3-1 Typical packed bed chromatography column

Fluid flow through a cylindrical chromatography column (1) should follow a tortuous path (5) rather than channeling (6) to improve contact time with resin particles (4).

Mass Transfer Mechanisms

The mass transfer between the mobile and stationary phases consist of multiple simultaneous mechanisms occurring within the column, starting with the bulk convective flow and mass transfer through the film diffusion (1) which occurs at the interface between the stationary and mobile phases. This is then followed by the diffusion within the pores of the resin (2), and finally by adsorption kinetics (3) at the functional group on the surface. Since mAbs are large molecules, the diffusion into the solid (resin matrix) is minimal and neglected. Figure 3-2 depicts the mechanisms and processes for a single porous resin bead in a packed chromatography column.

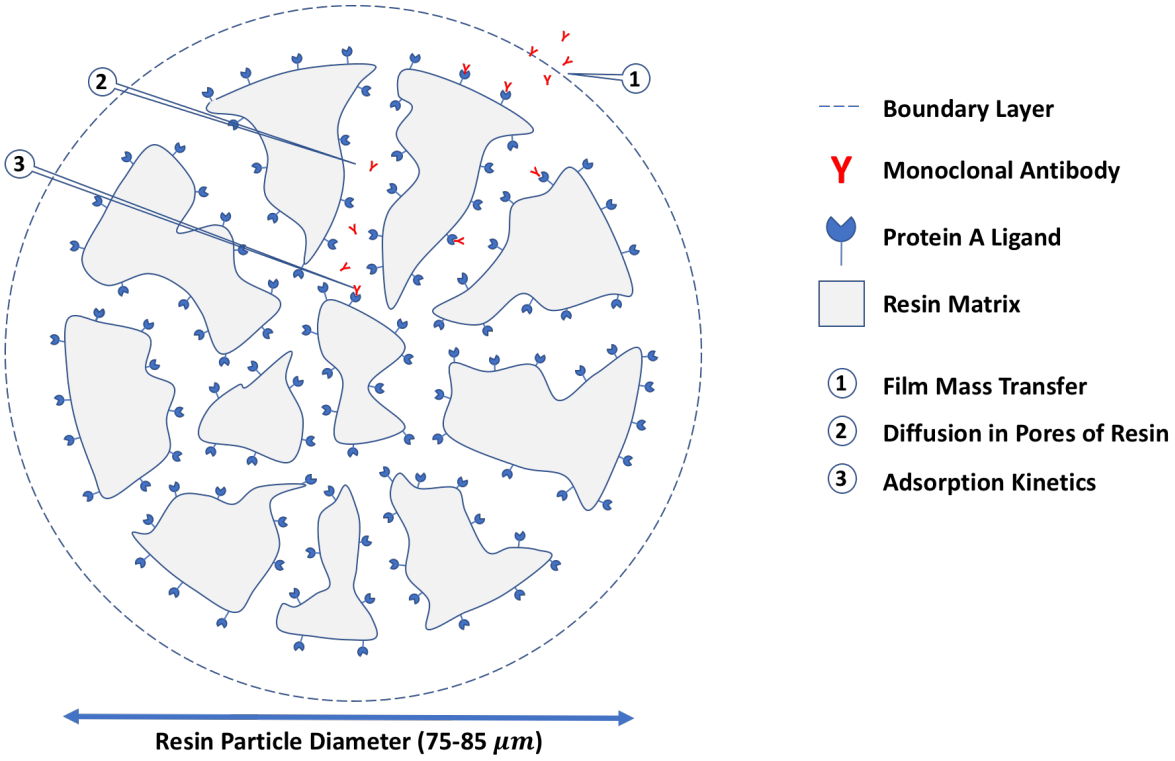


Figure 3-2 Mechanisms of Mass-Transfer with Protein A resin.

Mass transfer begins as the monoclonal antibodies crossing the boundary layer between the resin particle surface and the bulk fluid (1), followed diffusion into the pores (2), and finally with the adsorption kinetics at functional group on the resin (3). Typical resin particle diameter for protein A resins described in this work.

Mode of operation

Chromatography can operate in an on-off (or bind and elute) elution process or flow-through elution process. The difference between the two modes of operation is when the product is eluted. In the On-Off process, the compound to be purified (product) is bound to the functional groups on the resin particles in a column and the majority of the impurities pass through the column and are disposed of as a waste stream. Next, the product is washed from the column with an elution buffer and collected for further processing and the resin particles are regenerated for another cycle. The flow-through elution process allows the product to pass through the column and be collected, while impurities bind to the functional groups of the resin. The flow-through process also completes a regeneration step allowing for another cycle. Resins are normally designed to be used for many cycles to minimize the operating costs. Below is a basic description of the on-off elution process.

Feed

The entire stream from the primary recovery step is fed to the column with a feed buffer (often sodium phosphate and/or sodium chloride at approximately neutral pH for Protein A) where the mAb binds to the functional group, while other contaminants (HCP, DNA, Viruses, Endotoxins, etc.) pass through the column. Once breakthrough of the mAb is detected in the column effluent, the feed flow is stopped.

Wash

To ensure all non-bound contaminants pass through the column, multiple column volumes (CV) of the feed buffer (at neutral pH or slightly basic pH) are fed through the column and discarded as waste. The mAb remains bound to the functional group throughout this step.

Elution

Multiple CV of an elution buffer, such as sodium acetate, are fed through the column to elute the mAb where it is captured for further purification/polishing. The elution buffer differs from the feed buffer either by pH (lower) and/or salt concentration (higher). It is the change in pH and/or salt concentration that causes the bonds between the mAb and the functional group on the resin to begin to repel, which enables the elution. The pH and/or salt concentration in the elution buffer is decreased/increased linearly to elute the mAb in this step. This is then followed with multiple CV of the feed buffer to equilibrate the column.

Regeneration

Clean in Place (CIP) and Sanitization of the column are achieved by multiple CV of dilute sodium hydroxide (<0.5 M), before equilibrating the column with multiple CV of the feed buffer. The column is then ready to repeat the process multiple times. Prior to first use, the regeneration step should be completed. This process has been shown to maintain acceptable mAb yields >90% up to 300 cycles (Low et al., 2007). A depiction of the 4 steps in the On-Off elution scheme described above is presented in Figure 3-3.

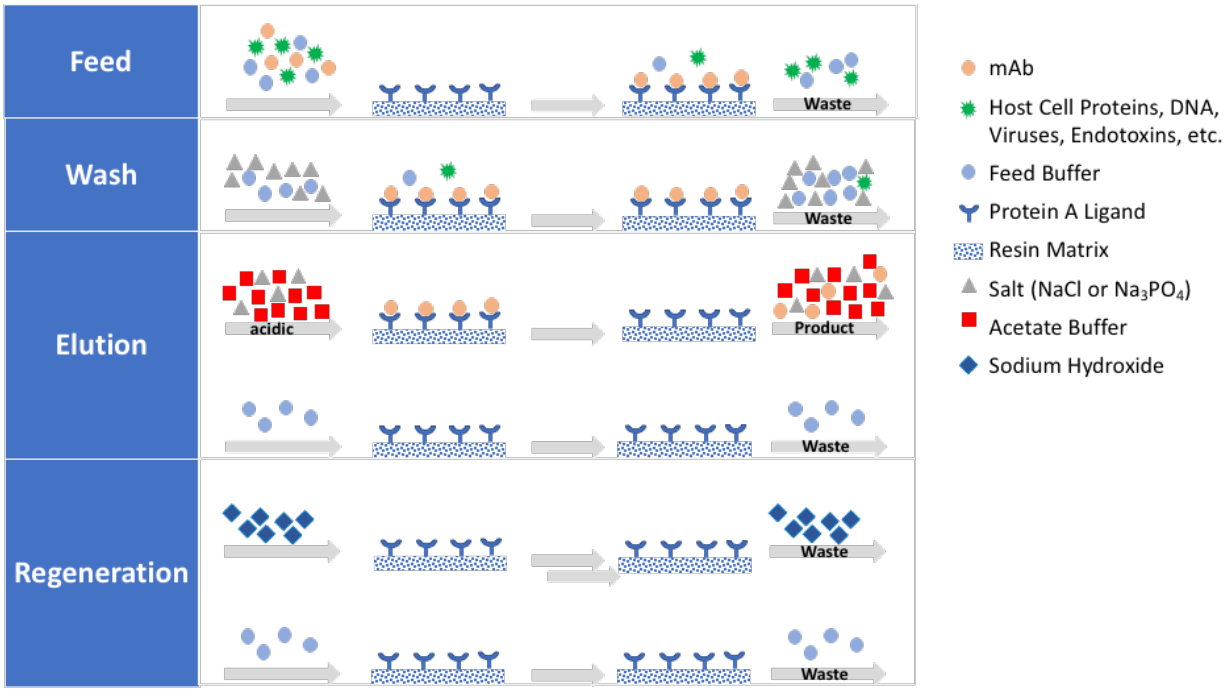


Figure 3-3 On-Off Elution Scheme for Liquid Chromatography (Protein A)

The Feed, Wash, Elution and Regeneration steps are presented for a typical Protein A chromatography column employed as the primary capture step in downstream processing. Modified from Schwarz, 2000.

Theoretical Models

There have been numerous theoretical models developed to describe chromatography from the simplicity of a chemical equilibrium-based model, to the complexity of multicomponent mass transport-based models. This work will focus on adsorption equilibrium and a standard single component transport model, while providing a summary of many others that have been developed.

Adsorption Equilibrium

The resin used in chromatography columns have a theoretical capacity based on the number of available adsorption sites and can be expressed as a reversible equilibrium reaction.

$$c^* + S \overset{K_{eq}}{\leftrightarrow} c^*S \tag{1}$$

Where, c^* is the equilibrium concentration of the mAb, S the concentration of adsorption sites, c^*S is the equilibrium concentration of the complex formed by the mAb and adsorption site, and K_{eq} is the equilibrium constant for the reaction. The equilibrium constant can be expressed in terms of the concentrations as well as reaction rate constants for adsorption and desorption k_a and k_d:

$$K_{eq} = \frac{[c^*S]}{[c^*][S]} = \frac{k_a}{k_d} \tag{2}$$

With the total number of adsorption sites equal to the bound sites and the unbound sites.

$$S_{total} = [S] + [c^*S] \tag{3}$$

Solving equation (2) for $[c^*S]$, substituting into equation (3), and solving for S results in.

$$[S] = \frac{S_{total}}{\left(1 + K_{eq}[c^*]\right)} \tag{4}$$

Finally, substituting equation (4) into equation (2) and renaming $[c^*S]$ as q^* (equilibrium concentration of the analyte in the stationary phase) and $S_{total} = q_{max}$ (maximum concentration of analyte in stationary phase), results in

$$q^* = \frac{q_{max}K_{eq}[c^*]}{\left(1 + K_{eq}[c^*]\right)} \tag{5}$$

Equation (5) is the Langmuir isotherm, which can be used to describe the adsorption of a mAb in the Feed step described above in the On-Off Elution scheme. It relies on three underlying assumptions: (1) the mAb in the mobile phase is strongly attracted to the surface of the stationary phase, (2) there is a fixed number of binding sites in the stationary phase, and (3) only

a monolayer of mAb can attach to the stationary phase. There are other isotherms that also exist for adsorption equilibrium (Freundlich, BET, etc.) depending on the reaction order of the adsorption and desorption (see Figure 3-4) (Harrison et al., 2015).

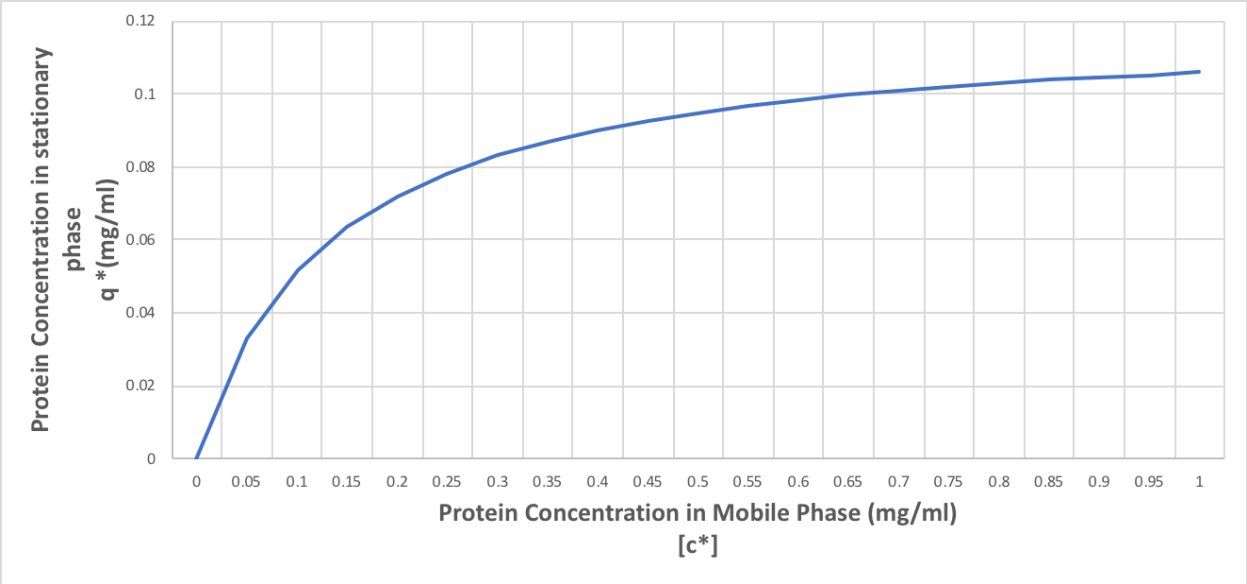


Figure 3-4 Example Langmuir Isotherm

Langmuir Equilibrium adsorption isotherm with $K_{eq} = 7.5$ and $q_{max} = 120\mu g/ml$. Values for K_{eq} and q_{max} from Harrison et al., 2015.

The values of K_{eq} and q_{max} are determined through equilibrium adsorption experiments in lab-scale chromatography columns and the specific resin and mAb and a linearized version of equation (5).

$$\frac{c^*}{q^*} = \frac{1}{q_{max}} c^* + \frac{1}{q_{max} K_{eq}} \tag{6}$$

Plotting the ratio $^{c^*}/_{q^*}$ vs c^* will result in $^{1}/_{q_{max}}$ as the slope, which can be used to easily determine K_{eq}. The equilibrium-based model can also be applied to the elution step by including an additive such as sodium ion in a cation exchanger (discussed in Chapter 5 -) as shown below.

$$c_{Na} + S \overset{K_{eq,Na}}{\longleftrightarrow} c_{Na}S \tag{7}$$

The total number of adsorption sites in Equation (3) now becomes:

$$q_{max} = [S] + [c_{Na}S] + q^* \tag{8}$$

The bound protein isotherm is then

$$q^* = \frac{q_{max} K_{eq} [c^*]}{\left(1 + K_{eq,Na}[c_{Na}] + K_{eq}[c^*]\right)} \tag{9}$$

By adding progressively greater concentrations of sodium in the elution buffer, the adsorption isotherm for the mAb begins to flatten out causing it to elute from the column as shown in Figure 3-5.

The same elution mechanism is experienced with changing pH through the change in the charge of a mAb due to the change in the equilibrium constant. Salt and pH gradients are often used, for mAb elution. Temperature gradients also have a similar effect, but are very rarely applied as increased temperatures have the potential to denature the protein and render the product inactive.

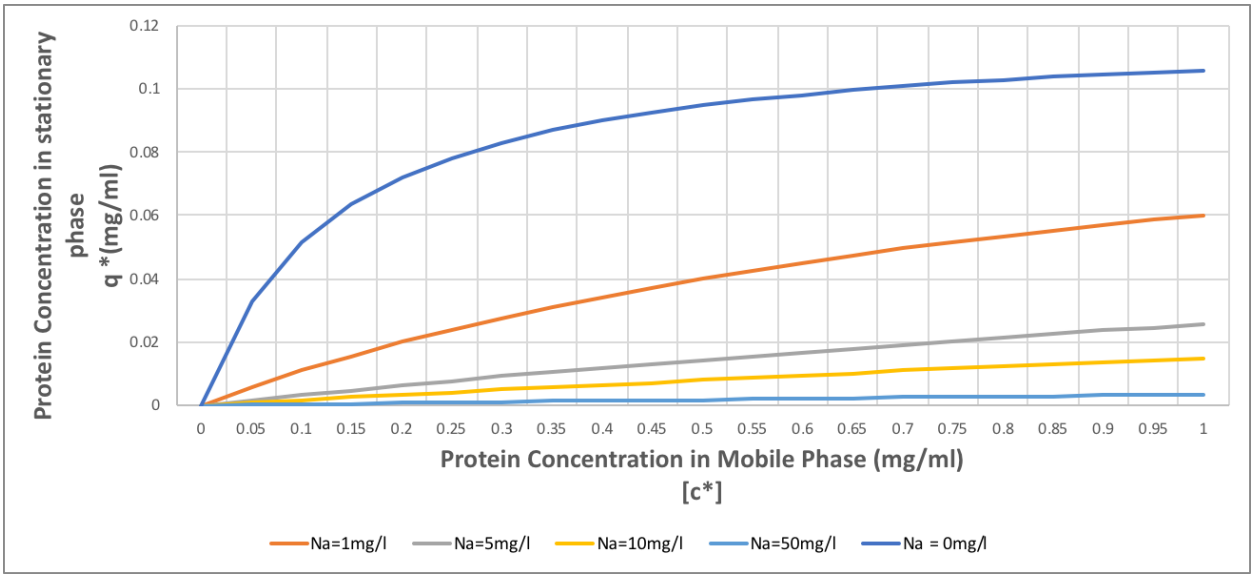


Figure 3-5 Example Elution Step Langmuir Isotherms

Langmuir Equilibrium adsorption isotherms with $K_{eq} = 7.5$, $K_{eq,Na} = 2$, and $q_{max} = 120\mu g/ml$ with varying concentrations of Sodium to reduce the adsorption of the protein. Values for K_{eq}, $K_{eq,Na}$, and q_{max} from Harrison et al., 2015

Chromatography Column Mass Balance

A macroscopic view of a packed bed with radial gradients neglected, results in the generic form of the mass balance of a chromatography column.

$$\frac{\partial c}{\partial t} + \frac{(1-\varepsilon)}{\varepsilon}\frac{\partial q}{\partial t} + \frac{v}{\varepsilon}\frac{\partial c}{\partial z} = D\frac{\partial^2 c}{\partial z^2} \tag{10}$$

Where c is the concentration of the analyte in the mobile phase, q is the adsorbed concentration of the analyte, ε is the bed porosity (ratio of liquid volume to empty column volume), D is the axial dispersion coefficient, and v is the velocity of the mobile phase. In addition to the mass balance of equation (10), an additional equation is required for the adsorption on the stationary phase. The reaction-dispersion model assumes the adsorption and desorption are the rate limiting steps with first order kinetics.

$$\frac{\partial q}{\partial t} = k_a c - k_d q \tag{11}$$

Yang et al. 2015 used surface plasma resonance (SPR) with adsorption experiments to determine the values of k_a and k_d. Without this technique one of the following assumptions is required.

1. The rate of reaction is a linear driving force in the mobile phase, which is applicable when the feed concentration is low as demonstrated in the adsorption isotherm (Grom et al., 2018)

$$q = K_{eq} c^* \ , \quad \frac{\partial q}{\partial t} = k_m \left(c - c^*\right) \tag{12}$$

2. The rate of reaction is a linear driving force in the stationary phase (Ng et al, 2012)

$$q^* = f(c^*) \ , \quad \frac{\partial q}{\partial t} = k_m (q - q^*) \tag{13}$$

These assumptions require an estimate for the axial dispersion coefficient D and lumped mass transfer coefficient k_m. These parameters can be obtained from empirical correlations or lab experiments (Ng et al., 2012, Grom et al, 2018, Sandoval et al, 2012, Pabst et al, 2018). Applying the parameters, equations (10) and (12) or (13) can be solved numerically, while analytical solutions have also been completed by rewriting them in dimensionless form to predict elution or concentration profiles for Protein A chromatography, minimizing the need for costly intermediate scaleup of columns prior to commercial use (Harrison et al., 2015). These models can also be applied to multicomponent mixtures to predict impurity adsorption as well as multiple elution steps. Lienqueo et al., 2012, were able to simulate the elution curves for a mixture of mouse IgGs with varying affinities to Protein A using this type of model. They were

even able to calculate the experimentally observed nonlinearity of the pH gradient (Lienqueo et al., 2012).

Other Models

There are many other models that have been developed to describe chromatography. Xu et al. completed a summary of many models developed to describe fixed-bed adsorption in aqueous systems including those described in this work. A summary of other models not discussed in this work is provided in Table 3-1 (Xu et al., 2013).

Table 3-1 Summary of other models for fixed-bed adsorption aqueous systems

Model	Basis
Bohard-Adams	Lumped kinetic model
Clark	Simplified continuity equation with Freundlich isotherm
Constant Pattern Theory	Wave velocity with self-sharpening wave
Dose-response	Simplified mass transfer
General Rate Model	Reaction kinetic model with mass transfer
Linear Driving Force	Lumped Mass Transfer Coefficient
Steric Mass Action	Lumped kinetic model with multi-components
Thomas	Simplified lumped kinetic model
Wang	Simplified mass transfer
Wave Propagation Theory	Wave velocity/concentration velocity
Wolborska	Lumped kinetic model with constant initial kinetics
Yoon-Nelson	Probability of adsorption and breakthrough

Summary of other models not discussed in this work. Modified from Xu et al., 2013.

Limitations of Chromatography

As discussed previously, chromatography is heavily relied upon in the downstream processing of mAbs, but it has limitations that must be considered in its design and operation.

Channeling of flow through a column can result in significant and costly yield loss in downstream processing and is often a result of issues encountered during the column loading and packing process. The procedures for this process are crucial in ensuring a successful column run. Issues with channeling also limit the maximum diameter of a chromatography column at 2m, which increases equipment costs for multiple columns for large batch sizes (Chon and Zarbis-Papastoitsic, 2011).

Utilization of available capacity within a column is also a limitation due to the typical mode of an On-Off elution scheme in Protein A or Cation Exchange resin where the mAb is fed to the column and stopped when breakthrough is detected before proceeding to the subsequent wash/elute/regen steps. In this mode the functional sites on the resin are not fully loaded and are underutilized. The Dynamic Binding Capacity (DBC in mg mAb to ml resin) is measured in a chromatography column to determine the amount of mAb that can bind to the resin under operating conditions (Pathak and Rathore, 2016).

$$DBC_{xx\%} = \frac{c_0(V_L - V_0)}{V} \tag{14}$$

Where c_0 is the inlet mAb concentration (mg mAb/ml), V_L is the volume (ml) fed through the column to achieve $xx\%$ of the feed concentration in the effluent, V_0 is the column void volume (ml), and V is the empty column volume (ml). It is common to measure $DBC_{10\%}$, but any percentage breakthrough can be measured. With the breakthrough at or near 0% of the feed concentration, there is approximately 50% of the total capacity still available within the column to bind the target mAb that are not utilized under normal operating conditions, which necessitate larger and more costly columns (DiLeo et al., 2017). Another limitation to the utilization of the resin functional groups is steric hindrance from large mAb molecules bound to the resin that

effectively block functional sites from exchanging with additional mAb molecules. This underutilization is depicted in Figure 3-6 for CEX chromatography.

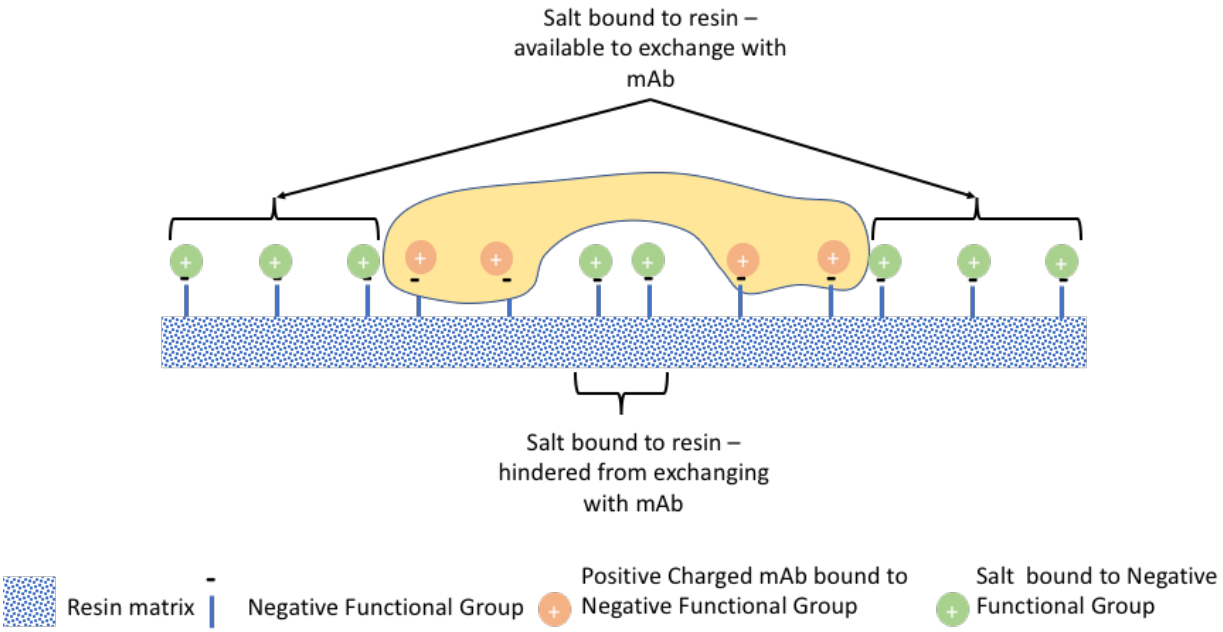


Figure 3-6 Visualization of Steric Hindrance with Cation Exchange

Typical adsorption of a mAb on a cation exchange resin, where the large mAb hiders multiple binding sites from other mAb molecules. Modified from Diedrich et al., 2017.

While not a permanent loss of capacity, this steric hindrance requires additional resin in the chromatography column (or multiple columns) to ensure adequate recovery. Using resin for multiple cycles (feed → regeneration) results in a cumulative fouling process where contaminants build up on the resin surface blocking or inactivating functional groups that ultimately decrease the binding capacity and increase pressure drop across the column (Lintern et al., 2016). The operation under multiple cycles can also damage the underlying structure of the resin causing portions to leach into the product stream as fines.

Despite the limitations associated with chromatography, it is still used in multiple steps of the downstream processing for all commercial mAb production through the adoption of sensible design and operation.

Chapter 4 - Protein A Chromatography

Advantages of Protein A

Of all the chromatography methods employed in the downstream processing of mAbs, Protein A is the most common method used in the primary capture step (Low et al., 2007). It is an affinity chromatography process, which depends on the highly specific interactions between the Fc region of the mAb and the Protein A ligand. Protein A, or *Staphylococcal* Protein A (SPA), is a cell wall associated polypeptide chain comprised of three distinct regions; S, or signal sequence that is cleaved during secretion, five homologous Ig-binding domains (E, D, A, B, and C) and the anchoring region to the cell wall (XM) (Hober et al, 2007, Kruljec and Bratkovic, 2017, Schwarz, 2000) as depicted in Figure 4-1.

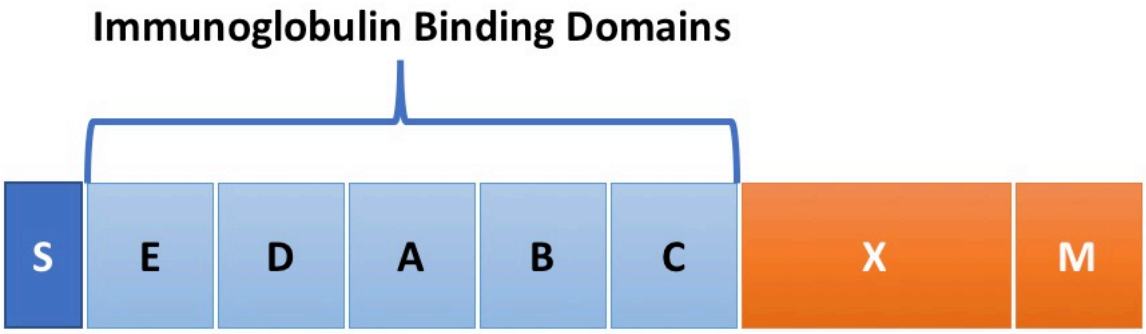


Figure 4-1 Staphylococcal Protein A (SPA)

Polypeptide chain comprised of three regions: (S) signal sequence, (E, D, A, B, and C) homologous Ig binding domains, and (XM) cell wall anchoring region. Modified from Hober et al., 2017.

The SPA is immobilized to a porous resin bead (X M domains), or stationary phase in the packed chromatography column and is operated in an on-off elution scheme with the process steps depicted in Figure 3-3 (Hage, 1999, Pflaunmiller et al., 2013). Protein A is predominantly used

as the primary capture step in mAb purification due to the specificity of the mAb to ligand binding, while allowing contaminants still present in the feed stream to pass through the column, resulting in multi-log clearance of contaminants (Xenopoulos, 2015). Volume reductions >70% and yield on the order of 95-99% are achieved with Protein A (Steinebach et al., 2016). An additional benefit of eliminating a virus inactivation step immediately following primary capture by Protein A has been achieved with at least one mAb due to the high rejection of contaminants (Zhang et al., 2014).

Limitations of Protein A

The cost of Protein A resin is by far the greatest impetus to identify an alternative in mAb purification and with the introduction of biosimilars, this will only increase due to increasing competition. As mentioned previously, with downstream processing making up to 80% of total cost of goods sold (COGS) and Protein A resin costing up to $15,000 per liter or 35% of material costs, the cost pressures are obvious (Roque et al., 2004, Mehta et al., 2007, Menegatti et al., 2012).

Resin life is another significant limitation of Protein A resin (related to its high cost) due to the expected use through multiple regeneration cycles. Unfortunately, the ligands on Protein A resin are susceptible to deamidification when exposed to the high pH cleaning and sanitization chemicals required to comply with cGMP. The deamidification is caused by covalent modifications to asparagine residues in the B domain in the presence of hydroxide radicals and can result in ligand leaching as well as ligand inactivation (Hober et al., 2007). This degradation of the ligand has the single greatest impact on resin performance (Jiang et al., 2009). Lintern et al., in 2016 determined that commercially available resins can lose up to 40% of their initial

DBC after only 100 cycles. In addition to deamidification, resin life can be reduced due to a number of other reasons: hydrodynamic stress, mechanical compression, ligand occlusions, and resin fouling (Nweke et al., 2018). Low pH elution conditions can also lead to aggregation of proteins over time and increase the problem of fouling. Figure 4-2 provides a depiction of the resin lifecycle of a typical Protein A chromatography column.

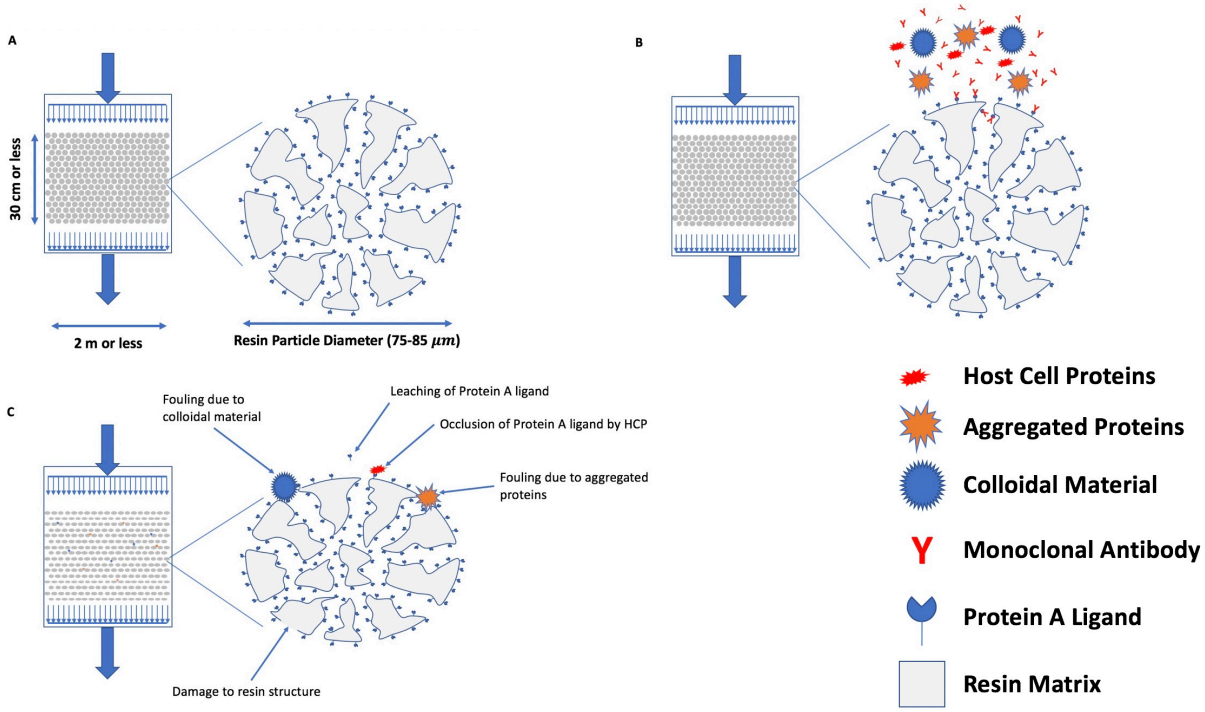


Figure 4-2 Overview of the processes that can affect resin life during bioprocessing.

A. Depiction of a typical protein A column. B. Operating conditions of the column during loading. C. Resulting damage due to repeated cycles indicating resin fouling and ligand leakage. Modified from Nweke et al., 2018.

While the affinity of Protein A ligands for mAbs is a major reason for employing this as the primary capture step, it can also bind with impurities such as DNA and HCP that will elute with the mAb and require removal in additional chromatography steps (Liu et al., 2010).

Improvements Available Using Protein A

Additives

The introduction of certain additives to the regeneration buffer have improved the lifetime performance of Protein A resin. Some manufacturers have proposed the addition of anti-chaotropic salts such as sodium chloride to improve the surface hydrophobicity and improve ligand stability (by disrupting hydrogen bonding), while others have suggested the use of sodium sulfate instead of sodium chloride to reduce the corrosion of stainless-steel processing equipment (chromatography columns, piping, valves, etc.) and increase resin life (Wang et al., 2013). Still others have explored alternative anti-chaotropic salts that are more kosmotropic such as sodium citrate that improves DBC by 10% after 100 cycles compared to sodium sulfate (Yang et al., 2015).

The addition of polyethylene glycol has been proposed as an option to improve resin life by reducing resin fouling and degradation in alkaline conditions through the creation of steric hindrance for the Protein A ligand (Weinberg et al., 2018). Weinberg's experiments resulted in overall increase in resin lifetime, but reduced DBC overall, making the adoption of this strategy unlikely due to the unfavorable economics of lower productivity. Other additives such as sucrose and xylitol can also improve resin stability and increase resin life (Jiang et al., 2009).

The problem of Protein A leaching with each cycle can be reduced through additives in the CCF, such as EDTA, to inhibit proteinases (Liu et al., 2010).

Operational Changes to Protein A Chromatography

Since Protein A capture is most often operated as a near zero breakthrough, there is nearly half the total capacity available for binding with mAbs. Changing to a simulated moving bed (SMB) process, where two or more columns or packed beds are used in series allows the first

bed to operate to a much greater mass loading. Figure 4-3 illustrates the operation in both a single and two-column configuration. The area A represents the mass loading for a single bed operation, while the area A+B is the mass load on the first column in a two-column operation. The remaining area C is the mass loading on the second column in the two-column operation. When the DBCx% is measured at the outlet for column 1, the wash step will wash contaminants from column 1 to column 2, then column 1 will be eluted and regenerated as column 2 continues to operate at a reduced flowrate.

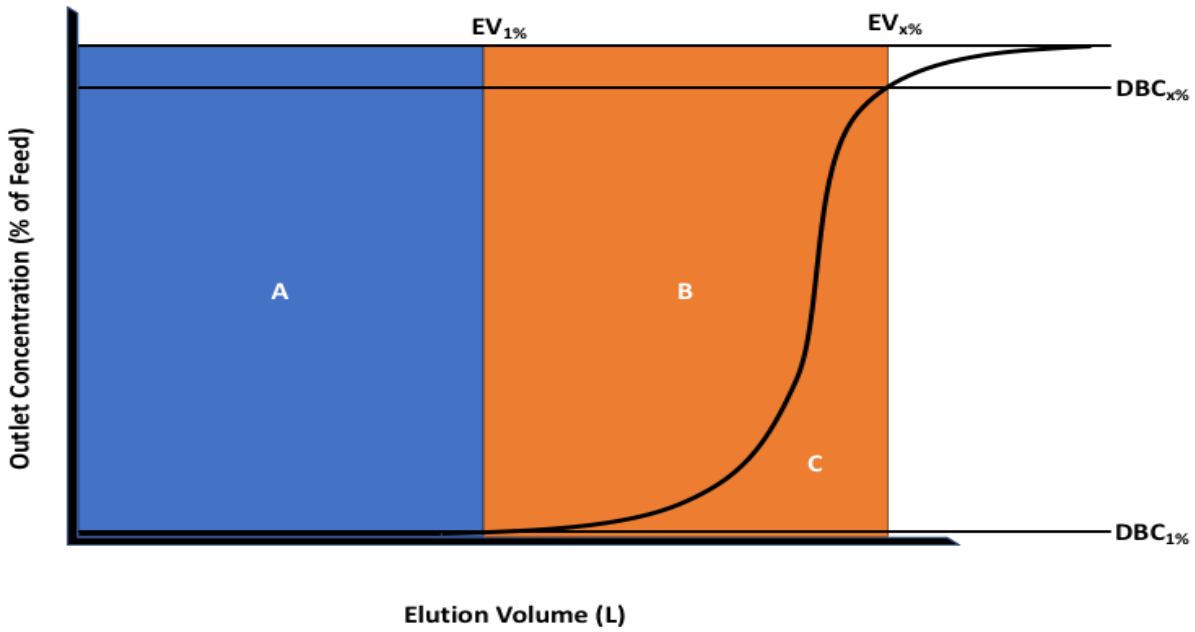


Figure 4-3 Simulated Moving Bed

Breakthrough curve for operation with both a single and two bed operation. EV1% corresponds to DBC1%. Modified from Angarita et al., 2015.

The operating parameters for the SMB process are determined from lab scale adsorption equilibrium and breakthrough experiments. This mode of operation was able to produce significant productivity increases (>30%), reduced buffer consumption (up to 40%), and decreased resin cost (up to 28%) compared to a single Protein A column (Angarita et al., 2015).

A novel approach to bioseparations has recently been applied for mAbs from CHO CCF by relying on a cGMP-compliant magnetic "rotor-stator" high-gradient magnetic separator with functionalized magnetic particles (Ebeler et al., 2018). By pumping CCF with suspended magnetic particles (cross-linked agarose with magnetite inclusions functionalized with a Protein A ligand) up through perforated stainless-steel discs surrounded by electromagnets, the magnetic particles bind to the protein A (see Figure 4-4), which bind to the disc in an On-Off Elution scheme similar to that of the Protein A column depicted in Figure 3-3.

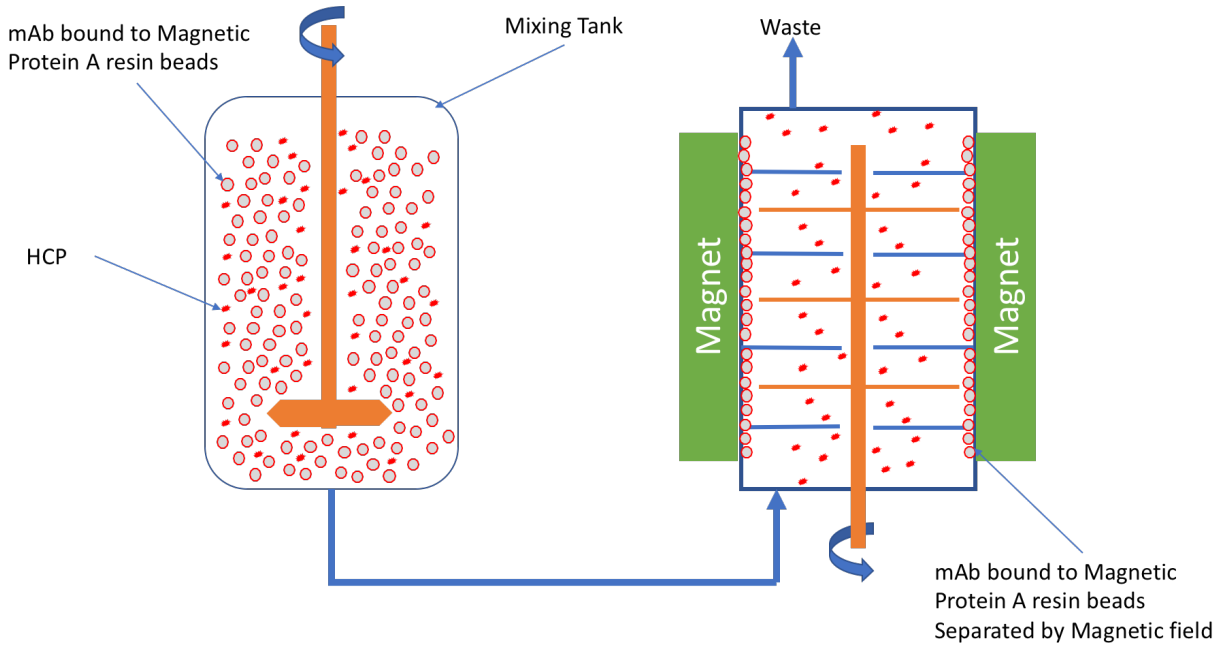


Figure 4-4 cGMP-compliant High-Gradient Magnetic Separator

Process flow diagram of "rotor-stator" high-gradient magnetic separator for mAb purification. The mAb is first bound to to magnetic Protein A resin beads in a stirred tank, then fed to the "rotor-stator" magnetic separator. The magnetic resin is bound to the magnets, while the HCP and other impurities pass through and are discarded as waste. Modified from Ebeler et al., 2018.

This process was able to achieve yields of nearly 90% and purities greater than 95%, while maintaining a productivity 3x greater than that of the comparable Protein A resin column. While the researchers proposed this as a single step separator, the reduction for HCP was less than that

of a typical Protein A column, which would require additional polishing steps to prove commercially viable. Future studies on the cost of ownership for this newly applied technology will be necessary if it is to be a potential replacement for a traditional Protein A column.

Chapter 5 - Alternatives to Protein A

In 2016, Langer completed a survey of biopharmaceutical companies to determine their interest in replacing Protein A for existing and future products. The results were not surprising, with over half of the respondents indicating that they were considering replacing protein A for new products and nearly 20% were even considering alternatives for existing products. This was a continued trend over the previous years. They also learned that half of all contract manufacturing organizations (CMOs) surveyed had already replaced Protein A with an alternative (Langer, 2016). Considering the cost associated with validating downstream processing steps and licensing a product, this supports the need for new alternatives.

Cation Exchange

Fekete et al. (2015), have claimed that, "cation exchange is the gold standard for separating charge sensitive antibodies" due to possessing greater capacity and improved stability while exposed to acidic and alkaline conditions when compared to Protein A. Cation Exchange (CEX) chromatography is based on the exchange of positively charged ions in the mobile phase (protein) with those on the stationary phase (resin particles). Since proteins often exhibit a net positive charge at pH 8 and below (i.e. isoelectric point), this allows them to bind to the negative functional groups on the resin particles, with an On-Off Elution scheme similar to that described previously for Protein A (Giacometti and Josic, 2013). Follman and Fahrner (2004), determined that through factorial screening experiments, that they could successfully achieve equivalent performance to the Genentech standard mAb purification process of Protein A → Cation Exchange → Anion Exchange with two different processes relying on cation exchange as the primary capture step and neglecting Protein A altogether. Their work is compared with CEX as

the primary capture step followed by either anion exchange (AEX), Multimodal Chromatography (MMC, described later in this work), and Hydrophobic Interactive Chromatography (HIC) in Table 5-1.

Table 5-1 Comparison of Processes with Protein A and non-Protein A Primary Capture

Primary Capture	Polish Step 1	Polish Step 2	Overall Yield
Protein A (97%)	CEX (89%)	AEX (98%)	85%
CEX (96%)	AEX (100%)	HIC (89%)	85%
CEX (96%)	MMC (92%)	AEX (100%)	88%

Overall yield for three step purification processes. Protein A → Cation Exchange (CEX) → Anion Exchange (AEX), CEX → AEX → Hydrophobic Interaction Chromatography (HIC), and CEX → Multimodal Chromatography (MMC) → AEX. Data from Follman and Fahrner, 2004.

With comparable performance, higher capacities, no ligand leakage, and improved stability, CEX is a very viable option as the primary capture step in mAb purification (Follman and Fahrner, 2004). When considering that Protein A resins are up to 30x more expensive than CEX resins, the viability of replacing Protein A is even greater. The results are promising and could indicate new mAb products or biosimilars, will employ CEX as the primary capture step. One highly successful mAb therapeutic (Humira) is already produced with CEX as the primary capture step (Kaleas et al., 2014).

Membrane Chromatography

Membrane Chromatography is the ideal replacement technology for column chromatography in mAb purification due to the low cost to produce, potential to mechanically filter while simultaneously binding the mAb at much higher flowrates (due to not being diffusion

limited), and require much smaller unit operations (Orr et al., 2013). The pharmaceutical industry is also moving to adopt single use technologies (eliminates CIP and sterilization), which also make Membrane Chromatography an attractive option as it could eliminate the need for chemical cleaning and sanitization (these steps would be performed by the manufacturer of the membrane prior to shipment). There has been a significant effort spent on replacing the downstream polishing steps with membranes. Giovannoni et al. 2009, developed a membrane-based system to purify mAb that relies on both an AEX membrane followed by a CEX membrane after an initial capture step with traditional Protein A chromatography. While this did not replace protein A as the primary capture step, it achieved a yield of 82% and purity of 99.9%. Others have also shown that replacing traditional AEX column chromatography in mAb purification with an AEX membrane can result in equal performance while also providing a viral clearance step (Gjoka et al., 2017). There has been at least one application of Membrane Chromatography in a lateral flow configuration employed as the primary capture for a mAb from CHO using a HIC membrane to achieve both high purity (97%) and high yield (97%) (Ghosh and Wang, 2006). The more traditional tangential flow membrane separation has been demonstrated by Dutta et al. 2015, with Protein A resin introduction in a countercurrent tangential flow setup in comparison to traditional Protein A resin in a packed column for two different mAbs from CHO CCF with very promising results (see Table 5-2). The system was operated in an On-Off Elution scheme similar to traditional chromatography, where the yield, rejection of high molecular weight (HMW) aggregates, and rejection of host cell proteins (HCP) were equivalent or better than that of Protein A chromatography.

Table 5-2 Membrane and Column Based Primary Capture Performance

Protein	Membrane			Column		
	Yield	HMW	HCP	Yield	HMW	HCP
Mab1	90.4%	8.6%	179ppm	>90%	11%	604ppm
Mab2	92.8%	3.9%	2195ppm	~100%	2.1%	2787ppm

Alternative purification processes utilizing membrane chromatography compared to traditional Protein A chromatography. Equivalent or better performance was achieved with countercurrent membrane filtration. Data from Dutta et al., 2015.

Based on the limited application of Membrane Chromatography as the primary capture step, it will require additional research before its widespread adoption. It does however remain a promising future technology especially as a single use system.

Aqueous Two-Phase Extraction (ATPE)

Aqueous two-phase extraction has been applied to the separation of mAbs for the primary capture step followed by downstream chromatography columns (Rosa et al., 2009, Rosa et al., 2011, Asenjo and Andrews, 2012, Dhadge et al., 2014, de Barros et al., 2016). The most commonly applied aqueous two-phase systems (ATPS) for extraction are polyethylene glycol (PEG) and dextran or PEG and potassium phosphate (Harrison et al., 2015). This process separates the protein in the top (less polar PEG phase), while impurities are separated into the bottom (more polar phase). It is a mild process that does not denature the target protein, making it a viable alternative to Protein A (Asenjo and Andrews, 2012). In a direct comparison to Protein A chromatography, Rosa et al. (2011), were able to achieve comparable yield (95%) and purity (>95%) when purifying a mAb with a titer of 2.5g/l from CCF. They were also able to compare the annual operating costs to show that there is a 39% cost savings when operating an ATPE system over the traditional Protein A initial capture step (Rosa et al., 2011). The use of

magnetic particles enabled Dhadge et al. (2014), to achieve purity of up to 98% for mAb purification with ATPE. The results of several applications of this technology are presented in Table 5-3

Table 5-3 Aqueous Two-Phase Extraction Primary Capture Performance

Protein	Yield	Purity
mAb1 (E. coli. in PEG/Sulfate)	91%	73%
mAb2 (CHO in PEG/dextran)	95%	93%
mAb3 (CHO in PEG/dextran with magnetic particles)	92%	98%

Yield and purity achieved through multiple aqueous two-phase extraction processes. Data for mAb 1 from Asenjo and Andrews, 2012, mAb2 from Rosa et al., 2009, and mAb3 from Dhadge et al., 2014.

Considering the annual operating cost advantages identified by Rosa et al. (2011) as well as the purity achieved by others, ATPE could be a very attractive alternative to Protein A.

Alternative Affinity Ligands

The prospect of being able to engineer a ligand with high stability, high specificity, low toxicity, and economical production process is the ultimate goal of artificial or synthetic ligand development. These alternative binding sites, often referred to as alternative scaffolds can possess similar or better properties to Protein A ligands with a much smaller and less complex structure while produced using combinatorial libraries and phase display technology (Lofblom et al., 2010). Figure 5-1 provides the different affinity ligands discussed in this section moving from high complexity, cost, and lower stability of Protein A to ligands of lower complexity, cost and improved stability.

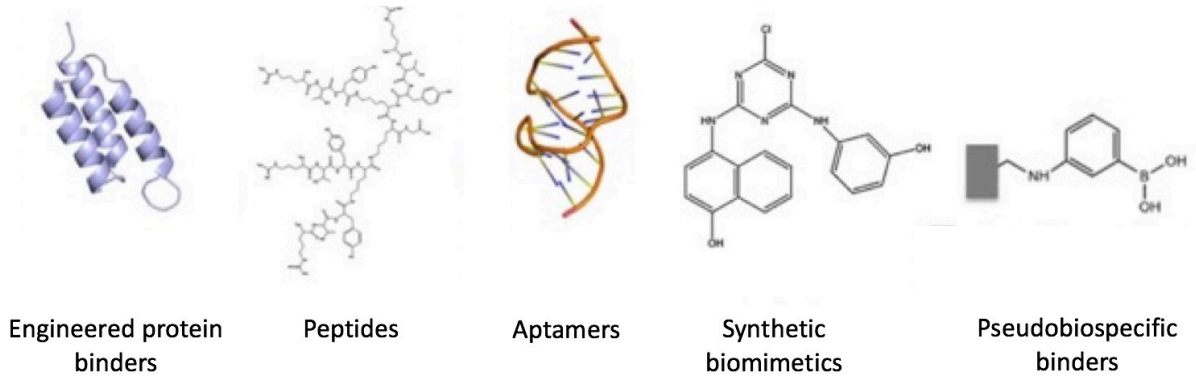


Figure 5-1 Alternative Affinity Ligands for bioseparations

Alternative Affinity ligands discussed in this work ranging from engineered protein binders (affibodies), peptides (cyclic or branched), aptamers (nucleic acids), synthetic biomimetics (triazine), and pseudobiospecific binders (multimodal chromatography). Moving from left to right above decreases molecular complexity (and cost), while increasing stability. Modified from Dias and Roque, 2017.

Affibodies

The most commercially successful alternative ligands are the engineered protein A variants. The engineered variants of the B domain (also known as the Z domain) are the most common alternative scaffolds or affibodies and achieve high yields with high stability (Kruljec and Bratkovic, 2017). These affibody libraries are created through combinatorial engineering principles at the binding surface by randomizing 13 positions within helices one and two of the three-helix bundles in the Z domain (Nygren, 2008, Wallberg et al., 2010, and Stahl et al., 2017). Nygren has reported that the first use of affibodies in affinity chromatography was to bind to Taq DNA polymerase and human apolipoprotein A-1, which resulted in high resistance to repeated pulses of 0.5M sodium hydroxide (Nygren, 2008). Hober and others developed experiments focusing on the necessity of asparagine residues (side chains interacting with the peptide chain of the Protein A ligand) as they are susceptible to deamidification under alkaline conditions. By creating specific mutations at the Ig binding domains and testing them in a chromatography

process with CIP, the impact of each change was measured using surface plasmon resonance to determine changes in affinity while binding studies confirmed the stability of the ligand to alkaline conditions. (Hober et al., 2007). Others have shown that affibody-based chromatography can be successfully used to recover eight different HER2-binding variants produced in *E. coli* CCF with both high recovery and specificity (Wallberg et al., 2010). The use of affibodies has been successfully applied commercially in engineered Protein A resins. In fact, several commercially available engineered Protein A resins from GE Healthcare are listed in Table 5-4 (MabSelect SuRe, MabSelect SuRe LX, and MabSelect SuRe pcc), which are all Z-domain affibodies and have demonstrated alkaline resistance (Pabst et al., 2018). Table 5-4 provides a summary of these alkaline stabilized Protein A resins indicating their $DBC_{10\%}$, CIP pH range, and modified binding domain region.

Table 5-4 Commercially Available Engineered Protein A Resins (Alkaline Stabilized)

Name	Manufacturer	$DBC_{10\%}$ (mg IgG/ml resin)	CIP pH range	Modified Binding Domain (# of repeat units)
MabSelect SuRe	GE Healthcare	35	2-13.7	B (4)
MabSelect SuRe LX	GE Healthcare	60	2-13.7	B (4)
MabSelect SuRe pcc	GE Healthcare	60	2-13.7	B (4)

GE Healthcare engineered protein binding ligands with Dynamic Binding Capacity, CIP pH range and the number of repeating units modified at the B domain. Modified from Pabst et al., 2018.

Increasing the number of repeating units at each binding domain adds additional binding sites and improves capacity while simultaneously increasing stability. Nweke et al, 2018, demonstrated that MabSelect SuRe retained 90% of its original capacity when exposed to alkaline conditions higher than normal for extended periods of time. These resins and their

competitors are currently commonly employed as the primary capture step for mAb production. This modified affinity ligand provides greater resistance to alkaline conditions with comparable affinity and selectivity to Protein A, but does so at similar cost of approximately $12,000 per liter (Stanton, 2017).

Peptides

Peptides have been investigated as an alternative to Protein A with the distinct advantages of being less expensive to manufacture, withstanding the harsh conditions of CIP chemicals, and the minimizing ligand leakage through a design process relying on knowledge of the three-dimensional structure of the target mAb, combinatorial peptide libraries and high throughput screening (Kruljec and Bratkovic, 2017). An early example of a peptide ligand for binding with IgG is TG19318 ((Arg-Thy-Tyr)$_4$-Lys$_2$-Lys-Gly), or Protein A Mimetic (PAM). By mimicking the interaction between Protein A and the Fc region of IgG, PAM has successfully been used to bind with other immunoglobulins (IgM, IgA and IgE) (Roque et al., 2004, Hober et al., 2007, and Lund et al., 2012).

Menegatti et al., 2012, investigated several much simpler linear hexapeptide ligands to bind with the Fc region of IgG and determined that HWRGWV (His-Try-Arg-Gly-Trp-Val) was a viable alternative to traditional Protein A resins with significant resistance to NaOH. After 200 cycles employing 0.1M NaOH during regeneration, the yield only decreased from 91% to 85% and the purity remained over 93% (Menegatti et al., 2012). Others have confirmed that HWRGWV can provide performance greater than that of Protein A in similar experiments (Najafian et al., 2017). It has also been marketed commercially as Kaptive-GY for antibody purification. Looking for even simpler peptide ligands, Wei et al. (2015), developed small peptide ligands with fewer than five residues resulting in high selectivity, simple elution and no

immunogenic responses by simulating the molecular dynamics and then synthesizing them chemically. These were then tested with complete mammalian cell culture medium (cMEM) and CHO CCF to test the recovery of IgG and compare to Protein A. One of these small ligands, DWHW (Asp-Trp-His-Trp), achieved equal or better yield and comparable IgG purity to Protein A (see Table 5-5), notably without Arg or Lys residues in the binding domain.

Table 5-5 Peptide Ligand vs Protein A Performance

Ligand	cMem		CHO	
	Yield	**Purity**	**Yield**	**HMW**
Protein A	79.6%	98.1%	85.4%	98.1%
DWHW	87.0%	95.6%	84.6%	95.2%

Comparison of engineered peptide ligand DWHW with Protein A to purify protein from mammalian cell culture medium (cMEM) and CHO. Protein A elution occurred at pH 3, while the modified peptide ligand DWHW was eluted at pH 9. Modified from Wei et al., 2015.

Another small peptide ligand, DAAG was developed by others with comparable $DBC_{10\%}$, recovery, and purity to that of MabSelect SuRe (Lund et al., 2012). As Wei et al., 2015, discovered during their research, there were no known small peptide affinity ligand chromatography resins on the market, indicating additional research is required in this area to provide commercial alternatives to Protein A.

Aptamers

Aptamers are short single-stranded non-protein nucleic acids (DNA or RNA) that fold into complex structures with high affinity and specificity to targeted proteins (Perret and Boschetti, 2018). Non-protein nucleic acids that are used as RNA aptamers with natural ribonucleotides are often unstable when exposed to alkaline conditions. They are also synthesized with combinatorial chemistry using nucleic acid libraries with a specific process:

systematic evolution of ligands by exponential enrichment or SELEX (Zhao et al., 2012). Aptamers are small in comparison to protein-based ligands and cost effective to synthesize based on the SELEX process and are easily modified chemically to improve stability when exposed to the alkaline conditions of the regeneration cycle (Smuc et al., 2013). Inomata et al., 2018, isolated aptamer Apt131 as an alternative to Protein A for the purification of IgG, mAbs, and Fc-fusion proteins with complete stability in 1M NaOH and mild elution conditions that also minimized mAb aggregation on the resin. The results indicated their Apt131 was an acceptable alternative to Protein A.

Aptamers are a new area of investigation for affinity chromatography and while there are no current commercial applications, it is expected to be a busy field of research due to the potential benefits.

Synthetic Biomimetics

Fully synthetic ligands have been developed with the increased knowledge of the 3-dimensional structures of IgG or mAb and how they bind to known affinity ligands such as protein A. Combining this knowledge with solid phase combinatorial synthesis and high throughput screening has led to the development of several synthetic non-peptide ligands. Using these techniques, Teng et al., 1999, were able to narrow down to a single candidate, artificial Protein A (ApA) or ligand 22/8 by relying on triazine chemistry. Ligand 22/8 was demonstrated to purify IgG >99% with relatively mild elution conditions between pH 3.4 and 4.7. Based on these findings, triazine has been used to synthesize many biomimetic ligands (Hober et al., 2007). Ligand 22/8 is very stable when exposed to 1M NaOH for over 140 hours (Roque et al., 2004). Building upon the work to develop ligand 22/8, led to the development of ligand 8/7. Roque et al., 2005 studied the binding of protein L (from *Pepto streptococcus magnus*) to IgG

and developed a triazine based artificial protein L ligand 8/7. There is also a commercially available resin relying on a synthetic ligand (KappaSelect by GE Healthcare) (Kruljec and Bratkovic, 2017).

Pseudobiospecific Binders

There are several classes of pseudobiospecific affinity chromatography resins, including Immobilized Metal Affinity Chromatography (IMAC), thiophilic adsorption chromatography, chromatography on hydroxyapatite, and multimodal chromatography (MMC). This section will focus on MMC as it has been applied as the primary capture step for mAb purification. Additional information can be found with the following references (Hage, 1999, Schwarz, 2000, Giacometti and Josic, 2013, Pflaunmiller et al., 2013, and Kruljec and Bratkovic, 2017)

Multimodal or mixed-mode chromatography relies on two or more modes of binding between the mobile and stationary phases. This is typically through a combination of ionic and hydrophobic or affinity interactions. By employing the multiple modes, the resin has greater resolution than CEX or hydrophobic interaction chromatography (HIC) alone as proteins have both charged and hydrophobic sites. Given the multiple modes, the resin is able to operate at a pH much closer to neutral and require much less salt than normally required with traditional HIC. Near pH 7, the majority of the binding is due to HIC, with little cation exchange due to the salt concentration required (Zhang and Liu, 2016). Work by Joucla et al., 2013, focused specifically on the primary capture step using commercially available MMC resin to capture a mAb produced from CHO cells with yields >90% and purity >95%, while reducing HCP 160-fold. Maria et al. (2015), were able to develop a process for a mAb produced via CHO with two subsequent MMC columns followed by an AEX column that achieved a yield of nearly 90% and final mAb purity of 99.9%. A summary of their results is presented in Table 5-6.

Table 5-6 MMC mAb Purification Process Yields

Primary Capture	Polish Step 1	Polish Step 2	Overall Yield
MMC[1](94%)	MMC (94%)	AEX (99%)	87%
MMC[2](92%)	n/a	n/a	n/a

Alternative Purification processes based on Multimodal Chromatography (MMC). [1]HEA HyperCel MMC by Pall and [2]Capto MMC by GE. [1]Data from Joucla et al., 2013. [2]Data from Maria et al., 2015.

These results also support MMC as another viable option as the primary capture step for mAb purification with commercially available resins especially considering that Protein A resins are priced approximately 10x higher.

This section covered many alternative ligands and though others do exist, those covered here were considered to have the most potential as alternatives to Protein A. A summary of the ligands discussed here are provided in Figures 5-2 through 5-6. Even with the promising Protein A alternatives identified here and elsewhere, their adoption for existing commercial products will be an uphill battle as they were already approved for markets by local health authorities (FDA, EMA, ANVISA, etc.). Changes to the primary capture process would require costly re-filing with these agencies prior to adoption of a Protein A alternative.

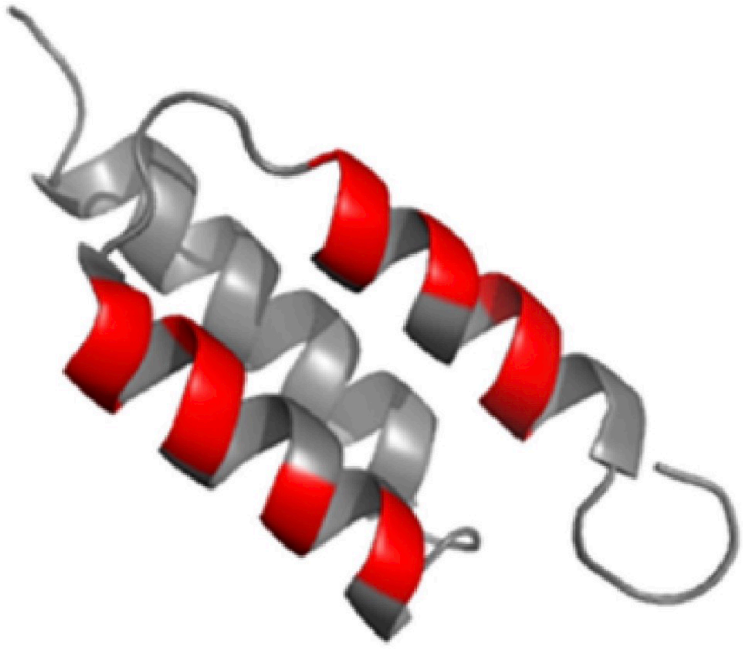
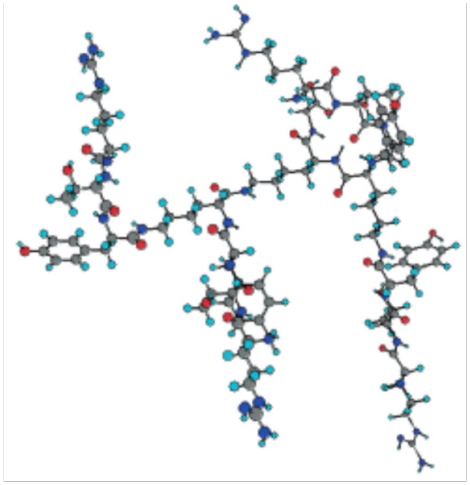
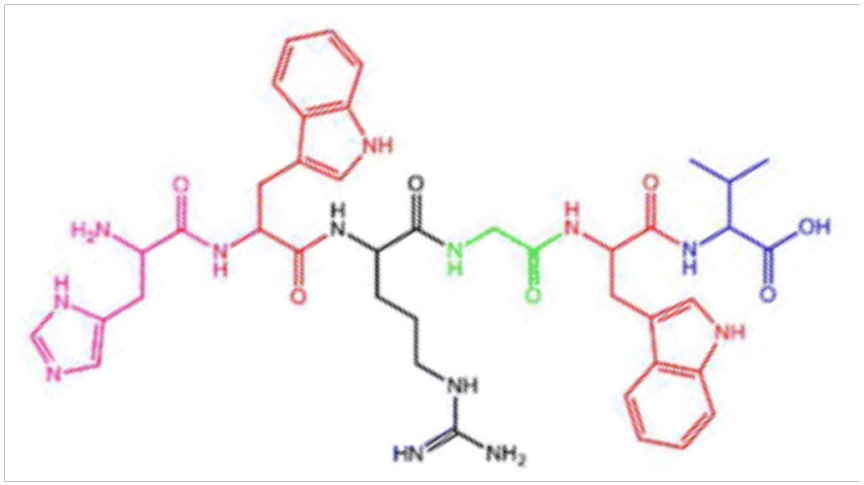


Figure 5-2 Alternative Affinity Ligand: Affibody

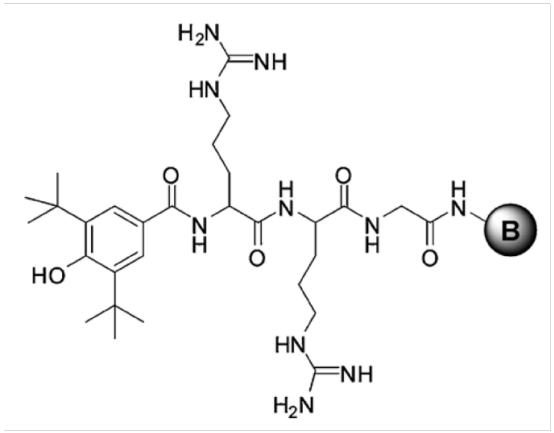
Affibodies are engineered variants of the B domain (also known as Z-domain) in Protein A with improved alkaline stability for the purification of mAbs. Modified from Kruljec and Bratkovic, 2017.



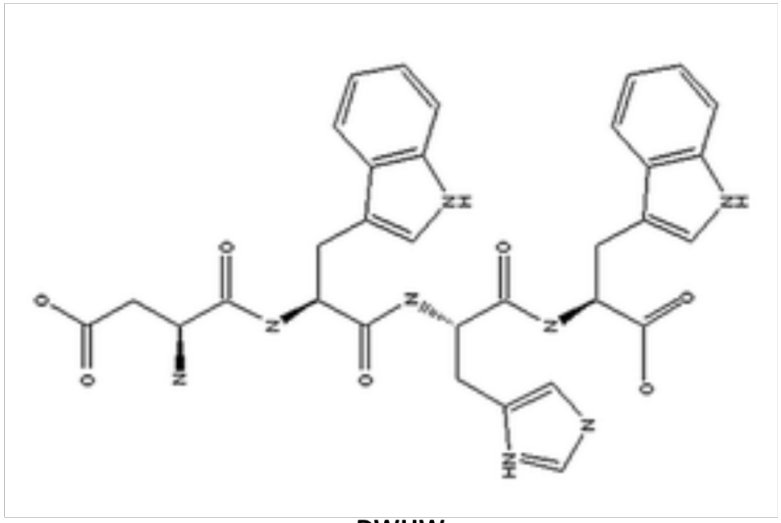
PAM-TG19318



HWRGWV



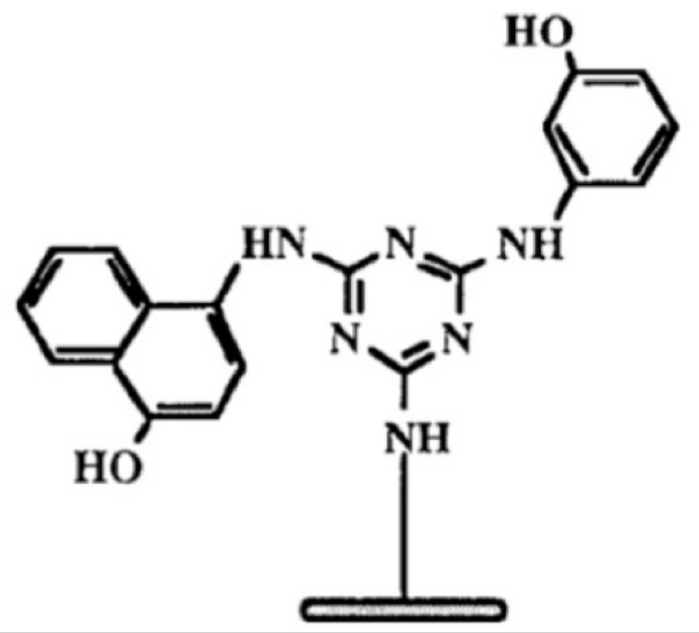
DAAG

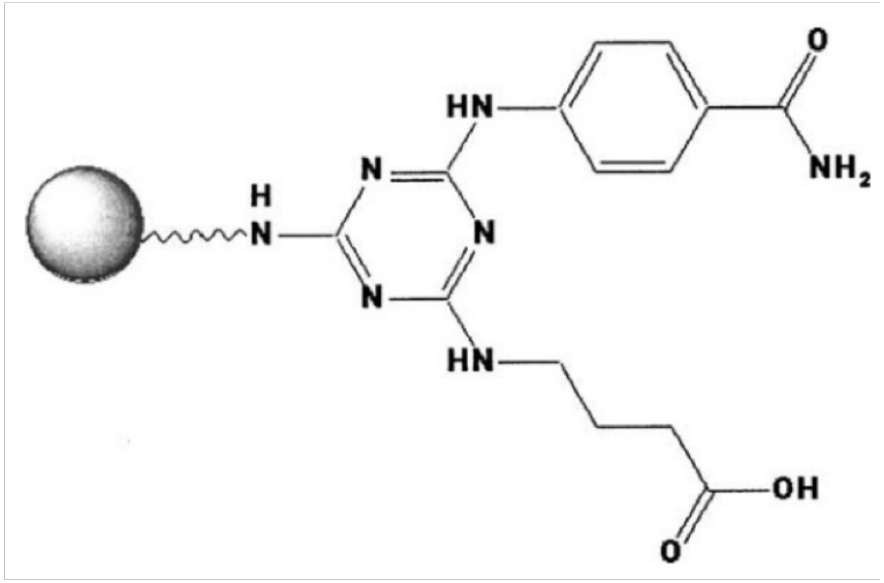


DWHW

Figure 5-3 Alternative Affinity Ligands: Peptides

Several example peptide Protein A mimetic ligands used to purify mAbs with improved alkaline stability. PAM-TG19318 modified from Roque et al., 2004, HWRGWV modified from Shen et al., 2015, DAAG modified from Lund et al., 2012, and DWHW modified from Wei et al., 2015.





Ligand 22/8 **Ligand 8/7**

Figure 5-4 Alternative Affinity Ligands: Synthetic Biomimetics

Two examples of synthetic biomimetic ligands with improved alkaline stability used for mAb purification. Ligand 22/8 modified from Teng et al., 1999 and Ligand 8/7 modified from Roque et al., 2005

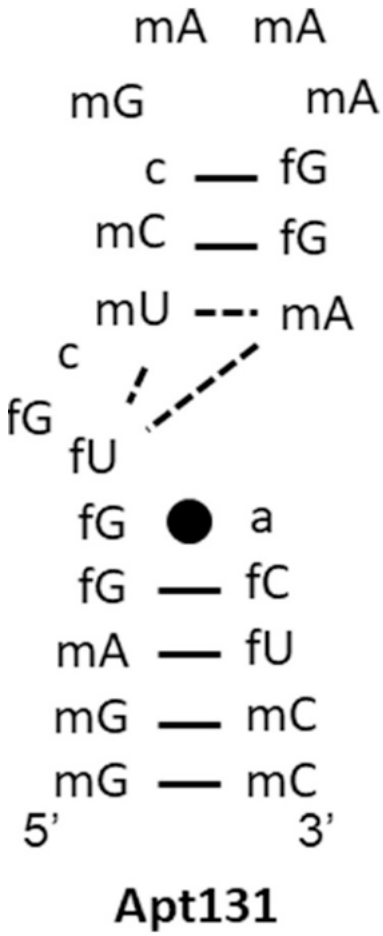
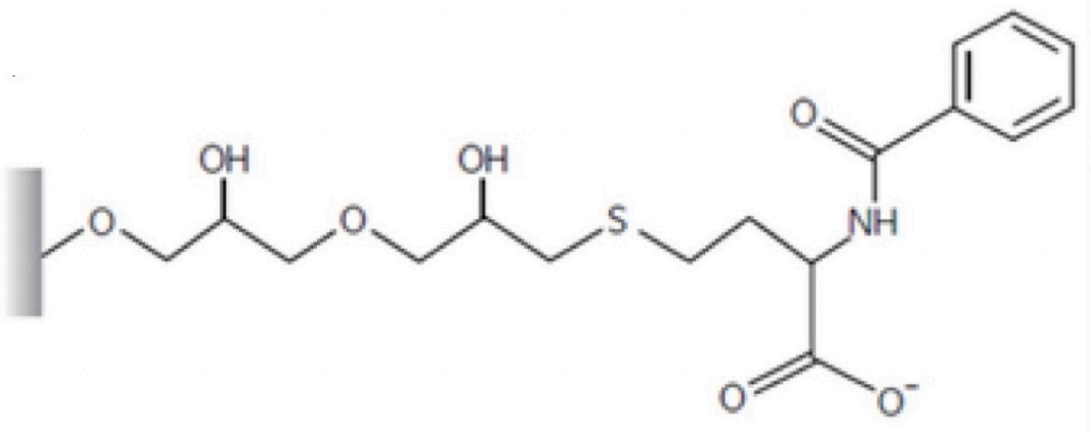


Figure 5-5 Alternative Affinity Ligand: Aptamer

Example aptamer based ligand chemically modified to improve alkaline stability for mAb purification. Modified from Inomata et al., 2018.



Capto MMC

Figure 5-6 Alternative Affinity Ligand: Pseudobiospecific

Example multimodal chromatography ligand for mAb purification with both hydrophobic interaction and cation exchange sites. Modified from Zhang and Liu, 2016

Chapter 6 - Conclusions

Monoclonal antibody (mAb) based therapeutics continue to be a significant portion of the overall pharmaceutical industry and given the recent increase in FDA approvals over the last decade and significant annual increase in 2017 alone, the reliance on mAbs in this sector shows no signs of decreasing in value to patients. Approvals of the first biosimilars in the U.S. and Europe have opened up many of the top biologics to significant competition for the first time and the majority of the US market is expected to lose patent protection in the next few years. This increased competition is expected to help provide additional options and reduced costs for patients. Considering that the downstream processing continues to account for the vast majority of the total cost of goods for current mAbs and Protein A resin is the most commonly employed primary capture step with a cost up to $15,000 per liter, it is not surprising that alternatives to Protein A are being actively considered by many mAb therapeutic manufacturers.

Operational changes offer opportunities to improve existing operations with Protein A or Z-domain affibodies through increased productivity and decreased resin and chemical cost. This has already been achieved by some through changing the chromatography process to a simulated moving bed (SMB). Attempts at implementing chemical additives to improve resin life have been promising but with much less benefit when compared to SMB. With these operational changes, the resin cost is still the major factor in influencing the cost of the primary capture step, which supports the need for additional alternatives.

The development of Z-domain affibodies has greatly improved the resin life, which is a major limitation of the Protein A ligand. Through engineering, resin manufacturers have increased alkaline resistance to cleaning and sanitization chemicals required to comply with cGMP and have successfully been adopted as a replacement for traditional Protein A resin by

many manufacturers as the primary capture step. While this has improved the overall resin life, it has however been achieved at a price equivalent to that of Protein A.

Currently available cost-effective alternatives to Protein A or Z-domain affibodies, such as CEX or MMC offer significantly less cost with greater capacity for mAbs. Unfortunately, only one, CEX which is less that 5% of the cost of Protein A resin, is known to be used in the production of one commercial mAb (Humira). Other alternatives such as membrane chromatography or ATPE are attractive, but have not gained significant adoption in commercial mAb production. As the understanding of the 3-D structure of immunoglobulins or mAbs and how they bind to Protein A has increased over the years, it has provided great insight into future alternatives at potentially much lower cost. It was this understanding that led to the development of Z-domain affibodies and has led to other affinity ligands such as; peptides, aptamers, fully synthetic, and pseudobiospecific binders. While many of these new ligands are not yet commercially available, their reduced complexity is expected to translate to significantly less expensive production costs with the potential of much greater stability and selectivity in the future.

Regardless of the alternatives to Protein A available, now or in the future, there will be resistance to changing existing processes for licensed products due to the significant cost and effort required to perform re-validation activities and complete multiple health authority approvals. Perhaps the introduction of biosimilars will exert additional cost pressures on manufacturers of commercial products and motivate them to move away from Protein A to remain competitive in the new environment. The greatest opportunity to move away from Protein A will be with the introduction of new products prior to validation and health authority approval.

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Appendix A - Recent mAb FDA Approvals

Table A-1 mAbs by Year of FDA approval: 2008-2017

Drug Name	Active Ingredients	Company	Year	Indication	Note
CIMZIA	CERTOLIZUMAB PEGOL	UCB INC	2008	Immunology	Fab
ARZERRA	OFATUMUMAB	GLAXO GRP LTD	2009	Oncology	
ILARIS	CANAKINUMAB	NOVARTIS PHARMS	2009	Immunology	
SIMPONI	GOLIMUMAB	CENTOCOR ORTHO BIOTECH INC	2009	Immunology	
STELARA	USTEKINUMAB	CENTOCOR ORTHO BIOTECH INC	2009	Immunology	
ACTEMRA	TOCILIZUMAB	GENENTECH	2010	Immunology	
PROLIA	DENOSUMAB	AMGEN	2010	Other	
ADCETRIS	BRENTUXIMAB VEDOTIN	SEATTLE GENETICS	2011	Oncology	ADC
YERVOY	IPILIMUMAB	BRISTOL MYERS SQUIBB	2011	Immunology	
PERJETA	PERTUZUMAB	GENENTECH	2012	Oncology	
RAXIBACUMAB	RAXIBACUMAB	HUMAN GENOME SCIENCES INC.	2012	Other	
GAZYVA	OBINUTUZUMAB	GENENTECH	2013	Oncology	
KADCYLA	ADO-TRASTUZUMAB EMTANSINE	GENENTECH	2013	Oncology	ADC
BLINCYTO	BLINATUMOMAB	AMGEN	2014	Oncology	
CYRAMZA	RAMUCIRUMAB	ELI LILLY AND CO	2014	Oncology	
ENTYVIO	VEDOLIZUMAB	TAKEDA PHARMS USA	2014	Immunology	
KEYTRUDA	PEMBROLIZUMAB	MERCK SHARP DOHME	2014	Oncology	
OPDIVO	NIVOLUMAB	BRISTOL MYERS SQUIBB	2014	Oncology	
SYLVANT	SILTUXIMAB	JANSSEN BIOTECH	2014	Oncology	
COSENTYX	SECUKINUMAB	NOVARTIS PHARMS CORP	2015	Immunology	
DARZALEX	DARATUMUMAB	JANSSEN BIOTECH	2015	Oncology	
EMPLICITI	ELOTUZUMAB	BRISTOL MYERS SQUIBB	2015	Oncology	
NUCALA	MEPOLIZUMAB	GLAXOSMITHKLINE LLC	2015	Immunology	
PORTRAZZA	NECITUMUMAB	ELI LILLY CO	2015	Oncology	
PRALUENT	ALIROCUMAB	SANOFI AVENTIS	2015	Other	
PRAXBIND	IDARUCIZUMAB	BOEHRINGER INGELHEIM	2015	Immunology	Fab
REPATHA	EVOLOCUMAB	AMGEN INC	2015	Other	
UNITUXIN	DINUTUXIMAB	UNITED THERAP	2015	Oncology	
AMJEVITA	ADALIMUMAB-ATTO	AMGEN INC	2016	Immunology	Biosimilar (Humira)
ANTHIM	OBILTOXAXIMAB	ELUSYS THERAPEUTICS INC	2016	Other	
CINQAIR	RESLIZUMAB	TEVA RESPIRATORY LLC	2016	Immunology	
INFLECTRA	INFLIXIMAB-DYYB	CELLTRION INC	2016	Immunology	Biosimilar (Humira)
LARTRUVO	OLARATUMAB	ELI LILLY AND CO	2016	Oncology	
TALTZ	IXEKIZUMAB	ELI LILLY AND CO	2016	Immunology	
TECENTRIQ	ATEZOLIZUMAB	GENENTECH INC	2016	Oncology	
ZINBRYTA	DACLIZUMAB	BIOGEN	2016	Neuroscience	
ZINPLAVA	BEZLOTOXUMAB	MERCK SHARP DOHME	2016	Other	
BAVENCIO	AVELUMAB	EMD SERONO INC	2017	Oncology	
BENLYSTA	BELIMUMAB	GLAXOSMITHKLINE LLC	2017	Immunology	
BESPONSA	INOTUZUMAB OZOGAMICIN	WYETH PHARMS INC	2017	Oncology	
CYLTEZO	ADALIMUMAB-ADBM	BOEHRINGER INGELHEIM	2017	Immunology	Biosimilar (Humira)
DUPIXENT	DUPILUMAB	REGENERON PHARMACEUTICALS	2017	Immunology	
FASENRA	BENRALIZUMAB	ASTRAZENECA AB	2017	Immunology	
HEMLIBRA	EMICIZUMAB	GENENTECH INC	2017	Other	
IMFINZI	DURVALUMAB	ASTRAZENECA UK LTD	2017	Oncology	
IXIFI	INFLIXIMAB-QBTX	PFIZER INC	2017	Immunology	Biosimilar (Remicade)
KEVZARA	SARILUMAB	SANOFI SYNTHELABO	2017	Immunology	
MVASI	BEVACIZUMAB-AWWB	AMGEN INC	2017	Oncology	Biosimilar (Avastin)
MYLOTARG	GEMTUZUMAB OZOGAMICIN	WYETH PHARMS INC	2017	Oncology	
OCREVUS	OCRELIZUMAB	GENENTECH INC	2017	Neuroscience	
OGIVRI	TRASTUZUMAB-DKST	MYLAN GMBH	2017	Oncology	Biosimilar (Herceptin)
RENFLEXIS	INFLIXIMAB-ABDA	SAMSUNG BIOEPSIS CO LTD	2017	Immunology	Biosimilar (Remicade)
SILIQ	BRODALUMAB	VALEANT LUXEMBOURG	2017	Immunology	
TREMFYA	GUSELKUMAB	JANSSEN BIOTECH	2017	Immunology	

Source: U.S. Food and Drug Administration, 2018