PRECIPITATION OF ANTIBODIES USING THE NUCLEOTIDE BINDING SITE

A Thesis

Submitted to the Graduate School
of the University of Notre Dame
in Partial Fulfillment of the Requirements
for the Degree of

Master of Science
in Chemical Engineering

by

Pierre Miranda

__________________________
Basar Bilgicer, Director

Graduate Program in Chemical and Biomolecular Engineering
Notre Dame, Indiana
July 2017
Abstract

by

Pierre Miranda

Antibodies have generated a breakthrough within the biotechnology industry over the past decades in both pharmaceutical areas for treatment of immunological, cancer and cardiovascular diseases, as well as endless applications for biomedical research specifically for molecular diagnostics, cellular imaging, among others. However, the production is still maintained at a high cost mainly due to the current purification method used based on the use of packed columns with proteins A / G.

In this way, the present study shows a variant to a method proposed in the scientific literature based on the precipitation of the antibodies through the formation of complexes and their precipitation with ammonium sulfate. The variation relies on the usage of trivalent molecules that have a particular affinity for the unconventional site named as the nucleotide binding site (NBS) on antibodies.
“Nothing in life is to be feared; it is only to be understood. Now is the time to understand more, so that we may fear less.”

Marie Curie
FIGURES

Figure 1.1: Basic scheme showing the structure of IgG\(^8\) .......................................................... 4

Figure 2.1: Diagram of the antibody purification process\(^50\) .................................................. 17

Figure 2.2: Schematic demonstrating the synthesis of the trivalent molecule containing FCA ......................................................................................................................... 22

Figure 2.3: Formations of antibody complexes upon the addition of the trivalent molecule that include bicyclic antibody trimer, a tricyclic antibody hexamer, and a branched polymeric system. .................................................................................. 24

Figure 2.4: Antibody present in the supernatant after precipitation with trivalent ligands and monoclonal antibodies (6\(\mu\)M). ................................................................................. 25

Figure 3.1: The structure of the tetravalent ligand. R represents the aromatic structures. ................................................................................................................................. 26
TABLES

Table 1.1. Relevant Processes involving the action of antibodies. .................6

Table 2.1 Kd for interactions of aromatic structures and the NBS. ..............16

Table 2.2 Compounds used for the synthesis of the trivalent molecules. ....20

Table 2.3 Hydrodynamic radius of the complex determined by DLS. ..........23
ACKNOWLEDGMENTS

I wish to express my sincere thanks and gratitude to Prof. Basar Bilgicer for his unconditional support during the time that I spent working in his laboratory on the subject of my dissertation.

I would also like to thank all of the members in the Bilgicer’s lab for their encouragement and endless help and particularly to Dr. Peter Deak for his kind advice during the development of this particular project and for providing me with the methods and techniques required.
1.1 Antibodies and the immune system

The immune system is a complex and intricate machinery that has the primary function of fighting infections caused by different pathogens including viruses, bacteria, protozoa, and fungi. Also, this system helps establish a balance that maintains an equilibrium with other systems including the nervous, metabolic and endocrine systems in a process called homeostasis\(^1\).

By taking into consideration the responses of the immune system to pathogens, they have been classified into two broad groups that are primarily differentiated by the mechanisms that involve pathogen recognition:

The innate response - It is the first line of defense and consists of the detection of patterns conserved in the pathogens, carried out approximately during the first five days post infection. The mechanism of detection is based on molecular and cellular events, through proteins and phagocytes, respectively\(^2,3\).
The Adaptive Response - This response can take days and even weeks to develop. The recognition of pathogens is performed in the same way through molecular and cellular mechanisms including proteins such as antibodies and T and B lymphocytes. These cells unlike the ones mentioned above have specific recognition sites known as B-cell Receptors (BCR) and T-cell receptors (TCR).

B lymphocytes or B cells are individually responsible for producing proteins called antibodies, which are of keen interest for the present project. These molecules are secreted after there has been a pathogen recognition event through specific sections in the pathogenic structure known as antigens. The primary function is the activation of the cellular and molecular components of the innate system, but not the direct destruction of the pathogen.

Antibodies are also called immunoglobulins (Ig) because they are a type of globulin that is present in the blood serum but is distinct from albumin. It is important to mention that antibodies are not only found in serum, but also in fluids circumventing tissue. It should be emphasized that they can be considered as free molecules or anchored in the membrane of B lymphocytes (BCR). Generally, all antibodies are in monomeric form except IgA and IgM isotypes, which will be described below because they have the tendency to form dimers and pentamers, respectively.
1.2 Structure of the antibodies

The structure of antibodies is characterized by the presence of four peptide chains linked by disulfide bonds\(^7\). The larger chains are known as heavy chains and are characterized by a molecular weight of about 50 kDa, while the smaller chains called light chains have a molecular weight of approximately 25 kDa\(^8\).

Certain sections of an antibody have been classified as constant and variable regions, as well as antigen binding fragments or Fab and constant or crystallizable fragments or Fc\(^9\).

Antibodies have been classified according to the different types of variations that exist in their chains. For instance, differences in the constant region Fc have allowed the identification of five classes or isotypes designated by the Greek letters \(\alpha\) or IgA, \(\gamma\) or IgG, \(\delta\) or IgD, \(\varepsilon\) or IgE and \(\mu\) or IgM\(^3\). For the case of IgA and IgG, there has been a distinction, and several subclasses have been identified. On the other hand, the variations in the light chain have allowed the classification of each isotype into \(\kappa\) or \(\lambda\)\(^10\).

Structurally speaking, immunoglobulins are formed by repetitive segments of \(\beta\)-sheets spanning 110 amino acids, which are known as the immunoglobulin domains\(^3\). The domain closest to the amino terminal end has been termed as a variable region whereas the remaining domains correspond to the constant regions. As it can be seen in the following figure, the light chains have a single constant domain whereas the heavy chains contain three for the case of IgD, IgG or IgA. However, in the case of IgM and IgE, a constant domain is added which is directed towards the carboxyl terminus. This feature causes the flexible region to disappear for the immunoglobulins above, leading
to an enhanced rigidity of the molecule and a lower capacity for interaction with the antigens\(^1\).

On the other hand, the disparity of the variable regions originates because there are three areas of approximately ten different amino acids denominated hypervariable region\(^7\).

Figure 1.1: Basic scheme showing the structure of IgG\(^8\).
1.3 Function

As detailed in the previous section, the primary function of immunoglobulins is the detection of individual antigens from pathogens. Antigens are complex structures generally of protein origin and larger in comparison to an antibody. Therefore, the recognition process occurs through a particular section of an antigen named as an epitope which is not necessarily repetitive in an antigen structure\(^1\). Depending on the spatial configuration, linear and conformational epitopes can be found\(^{11}\).

The forces involved in the recognition process are primarily weak forces and noncovalent interactions such as hydrophobic interactions, electrostatic forces, van der Waals forces, and hydrogen bridges\(^{12}\). Depending on the type of interaction, monovalent or multivalent interactions may exist with an antigen. For the case of multivalent interactions, since they are cooperative, they will prime the system with stronger interaction than its monovalent counterpart (enhanced avidity)\(^{13}\).

Some of the biological mechanisms that occur before the interaction/recognition of an antibody with antigens are listed below.
<table>
<thead>
<tr>
<th>Process</th>
<th>Isotype</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement activation</td>
<td>IgE IgG IgA</td>
<td>Activation of the immune’s system molecular machinery for the transmission and execution of signaling events.</td>
</tr>
<tr>
<td>Neutralization</td>
<td>IgE IgG IgA</td>
<td>Reduces and obliterates the function of the pathogen directly.</td>
</tr>
<tr>
<td>Phagocytosis/Opsonization</td>
<td>IgG IgA</td>
<td>Development of signaling events that will lead to the interaction of phagocytic cells with the antigen and posterior destruction.</td>
</tr>
<tr>
<td>Antibody-dependent cell-mediated cytotoxicity (ADCC)</td>
<td>IgG IgE</td>
<td>Similar to opsonization in that it creates a lytic response against a particular cell where specific antibodies have bound.</td>
</tr>
<tr>
<td>Inflammation</td>
<td>IgG IgE</td>
<td>Intracellular signaling cascades generated upon antibody binding and secretion of inflammatory substances such as cytokines.</td>
</tr>
<tr>
<td>Neo and prenatal immunity</td>
<td>IgG IgA</td>
<td>These isotypes have the ability to protect the fetus and protect him/her until birth.</td>
</tr>
</tbody>
</table>
1.4 Monoclonal Antibodies

As it has been observed and elucidated previously, the immune system to defend itself against pathogens generates a great variety of antibodies from B lymphocytes that have mutated to commit and differentiate into plasma cells or antibody producing cells in a process known as somatic hypermutation\textsuperscript{17}. Each of these cells is capable of producing a single type of antibody specific for an antigen or epitope sequence. Through these observations, scientists have realized that mammalian biological systems are capable of generating antibodies specific to certain active molecular regions that produce an immune response (immunogenicity). It is noteworthy that through extensive experimentations, not only the molecules associated with the pathogenic antigens can be detected by the immune system but also other small molecules which can generate an immunogenic reaction on their own. In some circumstances, they need to be conjugated to transport molecules to induce this type of response (hapten)\textsuperscript{18}.

The first methods of antibody production were therefore based on persistent immunizations in laboratory animals such as mice, rabbits, chickens, and goats, in which antibodies lack a unique specificity for an active region. Since the antibodies extracted in this case come from a variety of plasma cells, they were named as polyclonal antibodies\textsuperscript{19}.

Through the significant advances in the field of genetic engineering in recent years, production methods have evolved to the point where animals are not regularly needed (in vitro techniques), and antibodies can now be produced with a single specificity (monoclonal) derived from a single clone plasma cell.
• Hybridomas - The different plasma cells are extracted from the immunizations effectuated in animals, explicitly taken from the spleen. Because primary cells cannot proliferate indefinitely, cells must be immortalized through fusion with myeloma cancer cells. They should then be cultured to exclude non-fused cells. Subsequently, a selection of the clone that produces the best response to the molecule of interest is performed. Once selected, antibodies can be produced in vitro or vivo\textsuperscript{20, 21}.

• Recombinant protein production - This technique is based on the identification of genes characteristic of antibodies in plasma cells or fragments that encode areas of interest, which will be then introduced into a phage vector into other host cells including bacteria, yeasts or mammalian cells\textsuperscript{22, 23}.

Currently, more than thirty monoclonal antibodies have been approved by the US Food and Drug Administration (FDA) which represent approximately half of total sales in the multi-billionaire global pharmaceutical market\textsuperscript{24}.  

1.5 Applications of antibodies in life sciences and medicine

Immunology research over the past fifty years has demonstrated that their incredible specificity allows them to be used for several applications including the treatment of particular types of cancers, immune-related diseases including multiple sclerosis and viral infection, cardiovascular diseases and transplant rejection. Additional applications include diagnostics such as in Western Blot, immune dot blot, and immunohistochemistry; and for purification of individual components (immunoprecipitation).

But the area of greatest focus for monoclonal antibodies is the treatment of cancer. Currently, the most common treatments include surgeries, chemotherapy, radiation therapy, targeted therapy, and immunotherapy. Of these treatments, the first three are the most commonly used. But they are also the ones associated with greater adverse effects on the health of the patients because they are not directed specifically to the cancer cells. The treatments rather affect all the cells with which they come in contact. The most common side effects in patients undergoing these treatments include vomiting, hair loss and chronic fatigue due to a greater cytotoxic effect was seen in the cells that proliferate more rapidly as is the case of the mucous membrane, hair, and gastrointestinal tract, among others.

Immunotherapy offers the advantage of a greater specificity towards cancer cells since tumor-specific antigens can be created that are only characteristic of certain types of cancers because of specific mutations that can result in proteins entirely different from those found in healthy cells or an overproduction of growth factor receptors.
Because the antibodies interact with the growth factor receptors, possible interactions are reduced in a manner that tends to stabilize cell proliferation behavior. Also, the detection and subsequent destruction of these aberrant cells can be facilitated by the immune system\textsuperscript{31}.

Molecular conjugations of these antibodies with chemotherapeutic agents or radioisotopes have become another method to eliminate the undesirable effects of systemic toxicity\textsuperscript{32}. 


1.6 Antibody purification

Costly and complex manufacturing processes are required to produce antibodies with particular attention to the purification methods. These methods can be classified into three categories which include physicochemical fractionation, class-specific affinity, and antigen-specific affinity\(^3^3\).

Physicochemical fractionation includes techniques based on the separation by charge, size, and precipitation. Ex: Ammonium sulfate precipitation and size exclusion and ion exchange chromatography\(^3^4\).

Class-specific affinity techniques are based on immobilization with ligands such as proteins and peptides that have a particular affinity for the constant region of immunoglobulins (Fc). Ex: Protein A, G, and A/G\(^3^5\).

Antigen-specific affinity techniques are based on the purification via the recognition of the antigen binding domain. Ex: Protein and peptide antigen immobilization\(^3^6\).

For the case of monoclonal antibodies, the most used purification technique in pharmaceutical companies correspond to the second category and particularly to protein A/G since its interactions with immunoglobulins are particular and they have been well studied\(^2^1\).

It is important to mention that protein A, G, and A/G are genetically engineered bacterial proteins in which sequences that are not relevant to the interaction with the constant region of the immunoglobulins have been removed\(^3^7\). They don’t only have one reactive site but several sites along their structure which allows them to have a greater
probability of interaction with the immunoglobulin even though they are covalently immobilized onto resins, and some of the sites become blocked or hindered\textsuperscript{33}.

The general procedure involved in a purification using these proteins involves four steps. The first one, binding, is typically performed under physiological conditions where the sample containing immunoglobulins and contaminants is passed through a column to allow for a proper interaction between the proteins. The second step, washing, requires the usage of phosphate-buffered saline (PBS) to remove contaminant and nonbound proteins from the column. The third phase, elution, requires an acidic elution buffer to dissociate the bound immunoglobulin from the resin. The fourth step, neutralization or exchange buffer, which requires the identification of the protein of interest with a protein assay and the subsequent neutralization of the buffer or the usage of desalting column or dialysis to remove other small size components and change the buffer into a physiological pH\textsuperscript{37-41}.

Remarkably, the efficiency obtained with protein A/G is high with a range between 80-95%. However, there are several limitations and drawbacks associated with the technique:

- Acidic elution buffer can potentially affect the structure of the protein\textsuperscript{42,43}.
- It cannot differentiate between bivalently active mAbs or mAbs with a damaged or impaired portion of the variable region such as the nucleotide or antigen binding sites (ABS)\textsuperscript{44}.
- There is a limited lifetime of the columns due to hydrolyzation\textsuperscript{45}.
- Histone proteins in host cell DNA can interact with the resin coated with protein A/G\textsuperscript{33}.
Ammonium sulfate precipitation has been widely used for the purification of proteins due to its ability to reduce the solubility as the concentration of ammonium sulfate increases, leading to the precipitation of proteins in decreasing order based on their molecular weight. This particular phenomenon is also known as salting out. However it is important to mention that this process can be opposite for other salts. The parameter that determines this behavior has been experimentally characterized by the Hofmeister series or lyotropic series.\(^{46,47}\)

The observed effect of the change in solubility is associated with the increment in the ionic strength of the solution achieved by decreasing hydrophobic interactions as the solvent surface tension increases without damaging the protein structure.\(^{48,49}\)

Previous studies on the purification of monoclonal antibodies have been done using multivalent scaffold with specific peptides to target the antigen binding site and precipitate the complex formed using ammonium sulfate.\(^{50}\) The results have demonstrated that the technique was successful in purifying mAbs of the same isotype showing >95% efficiency.\(^{51}\)

Notice that the introduction of a scaffold has been necessary to increase the molecular weight of the complex because there are other protein impurities found in cell lysates that would have reduced the purification efficiency, requiring the application of other purification techniques to eliminate the contaminants.
1.7 The nucleotide binding site

The nucleotide binding site (NBS) is a region or binding pocket between the heavy and the light chains of the variable fragment (Fab domain) that was discovered due to its increased affinity for ATP (adenine)\textsuperscript{52}. From this elucidation, Dr. Bilgicer’s research group decided to further characterize this unconventional site by using all available crystalline structures for the different immunoglobulin isotypes. The results found demonstrated a highly conserved region in more than 260 structures analyzed characterized by the presence of two tyrosine residues on the light chain and one tyrosine and one tryptophan or phenylalanine residue on the heavy chain\textsuperscript{53}.

Computational simulations were also used for screening and finding analogs that were potential candidates for noncovalent interactions with this binding pocket. The best analogs found were aromatic ring molecules such as Tryptamine and Indole-3-Butyric Acid (IBA) which demonstrate a $K_d = 1 - 8\mu M$, making this interaction very attractive for potential applications for detection and purification\textsuperscript{7}.
2.1 Overview

Recent studies performed in Dr. Bilgicer’s research group have led to the identification of other aromatic ring cyclic compounds that can be used to generate non-covalent interactions such as the ones seen in the case of IBA, 2-Naphthaleneacetic acid and FCA\textsuperscript{56,59}. These molecules were selected as candidates to create a precipitation system similar to the one outlined in the previous section because they have low-affinity interactions with binding constants in the range of micromolar, but when they are combined in a multivalent system, their interactions will contribute to a much stronger and stable interaction (avidity).
### TABLE 2.1

$K_d$ FOR INTERACTIONS OF AROMATIC STRUCTURES AND THE NBS

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Kd (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBA</td>
<td>2-8</td>
</tr>
<tr>
<td>FCA</td>
<td>1.6</td>
</tr>
<tr>
<td>2-Naphthaleneacetic acid</td>
<td>1.8-3.7</td>
</tr>
</tbody>
</table>

The advantage and versatility of this technique compared to that used with multivalent molecules specific for the antigen binding site is that this technique will not only separate antibodies of monoclonal origin but to all antibodies (polyclonal). This is because the nucleotide binding site is conserved in all immunoglobulins as it has been described.

As described in the section on monoclonal antibodies, there are several sources from which they can be extracted. Each of the sources contains several contaminants which include proteins other than antibodies, fragmented or denatured antibodies and residual host cell DNA\textsuperscript{52}.

One way in which it can be guaranteed that the pollutants are extracted and that has been experimented and reported in the scientific literature is through the application of the three steps that are detailed in figure 2.1.
Figure 2.1: Diagram of the antibody purification process\textsuperscript{60}. 
The first step refers to the elimination of all contaminants having a higher molecular weight than the antibodies (150 kDa). For this, an exact amount of ammonium sulfate must be added so that all high molecular weight components precipitate in solution, while free monoclonal antibodies remain in it. All concentrations of ammonium sulfate that allow this difference or condition form the precipitation window.

The second step of this process is the addition of the trivalent molecules so that the trivalent complexes are generated, which will precipitate from the solution leaving aside all the low molecular weight contaminants.

The third step simply refers to the different techniques that can be used to remove the trivalent molecules from the complexes formed, thus releasing the antibodies. These methods include the use of membranes or filters that allow the filtration of trivalent molecules.

The determination of the precipitation window and more accurate of the ideal ammonium sulfate concentration to be used is the central element of this purification technique. Since the nature of the contaminants is unknown, it’s hard to experiment with the samples extracted from monoclonal antibodies directly. Therefore, an experiment that simulates these conditions is applied. It consists in the determination of the precipitation window through the use of trivalent complexes formed from the antibodies and the trivalent molecules, which simulate the action of high molecular weight contaminants.
Considering this information, the objectives proposed for the project are:

- Synthesize multivalent molecules that can form a complex with two commercially available monoclonal antibodies used for cancer treatment (Rituximab and Trastuzumab). Details of the organic synthesis schemes are presented in the following section. But in general Reversed Phase High-Pressure Liquid Chromatography (RP-HPLC) was used for the purification of the compounds. Additionally, all the collected material was then characterized using a microTOF mass spectrometer.

- The verification of the complex formation was achieved via the measurement of the molecular diameter using a DLS device (Dynamic Light Scattering).

- Utilization of trivalent molecules to purify the mAbs incubated with ascites fluid, and Chinese Hamster Ovary Cells (CHO) conditioned media. Ascites fluid corresponds to the peritoneal fluid that is present during the extraction of monoclonal antibodies in animals. On the other hand, CHO conditioned media refers to the media that is characteristics of monoclonal antibodies extracted from in vitro production methods.

- Determination of the efficacy of the purified monoclonal antibodies by using them to target the receptor Her2/neu for Trastuzumab on the SKOV3 cell line and CD20 for Rituximab on IM9 cell line.

- Double-stranded DNA (dsDNA) and residual host cell protein quantifications for samples obtained from CHO conditioned media and the ascites solution.

- Evaluation of the removal of contaminants by using the technique known as SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) where the samples of purified and non-purified components were run together.

- Comparison of the results of the methodology on the method based on AMS precipitation and the NBS, as well as protein A/G.

If all of the objectives are favorable, an alternative methodology will be proposed that can serve to purify antibodies.
### 2.2 Materials

<table>
<thead>
<tr>
<th>Component-Description</th>
<th>Provider</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMF (N,N dymethylformamide) &gt;99.8%</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>HBTU ((2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate)</td>
<td>Novabiochem</td>
</tr>
<tr>
<td>DIEA (N,N-Diisopropylethylamine)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Amido-\textit{dPEG}_6-acid</td>
<td>Quanta Biodesign</td>
</tr>
<tr>
<td>Ethylenediamine</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>TSAT (Tris-succinimidyl aminotriacetate)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>ACN (Acetonitrile)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>FCA – 96%</td>
<td>Alfa Aesar</td>
</tr>
<tr>
<td>Rituximab</td>
<td>Genentech</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td>Roche</td>
</tr>
<tr>
<td>Ammonium Sulfate</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>PBS (Phosphate Buffered Saline)</td>
<td>Sigma Aldrich</td>
</tr>
</tbody>
</table>
2.3 Synthesis of trivalent molecule

The reaction scheme is detailed in the following figure. It is important to mention that during all reactions the solvent used was dimethylformamide (DMF).

The precursors for the reaction was FCA, to which coupling agent HBTU ((2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and the base DIEA (N, N-Diisopropylethylamine) were incorporated in a molar ratio of 1:1 and 1:50, respectively.

After five minutes of reaction for the activation, Amino-dPEG₆ acid was added with a 1:2 stoichiometric ratio. The reaction conditions for this addition were 18 hours of reaction at room temperature with continuous stirring.

Subsequently, the solvent of the solution was evaporated using a high vacuum evaporative system (roto evaporator) and the lyophilization process of the sample.

Next, the first product was separated through reversed-phase high-pressure liquid chromatography (HPLC) model Agilent 1200 series and a Zorbax C18 semi-preparative column (9.4 mm x 250 mm). The volumetric flow selected for the system was 4 ml/min and the selected solvents that allowed the separation were ACN and water. The addition of ACN was done through gradual increments of 4% ACN per minute, and effluent monitoring was performed through the 220 nm wavelength characteristic of the amide bond formed. The product was characterized using a Bruker micrOTOF II mass spectrometer where the mass expected for this section was confirmed.

After the desired product had been collected, the solvent was evaporated and lyophilized. The product was dissolved in DMF. HBTU and DIEA were added again for
activation and ethylenediamine was then added in a molar ratio of 1:100 for one hour under the same reaction conditions mentioned above. Likewise, the same steps were taken to purify and characterize the desired product.

DIEA and TSAT were finally added in a molar ratio of 1:50 and 1:6, respectively. The conditions of the reaction were the same as for all previous steps, the reaction time of about 18 hours at room temperature. After evaporation of the solvent and purification, the desired product was characterized which had an exact mass of 1898.97 for FCA.

Figure 2.2: Schematic demonstrating the synthesis of the molecule containing FCA.
2.4 Precipitation and complex formation

A stock solution of the FCA molecule was prepared in PBS, including one for the mAbs Rituximab and Trastuzumab and one in water for ammonium sulfate. First, the solution of mAbs was mixed with the solution of the trivalent molecule, and it was incubated for four hours to allow for a better interaction and complex formation. After this time, the ammonium sulfate solution was added and incubated at 4°C overnight before centrifugation at 15000 rpm for a better separation and aggregation of the precipitates.

The complex formation which is specific to the interaction NBS-ligand was confirmed by using a DLS device which showed an increment in the hydrodynamic radius for all the trivalent molecules as it can be visualized on the following table.

**TABLE 2.3.**

HYDRODYNAMIC RADIUS OF THE COMPLEX DETERMINED BY DLS

<table>
<thead>
<tr>
<th>Components</th>
<th>Effective Diameter (nm)</th>
<th>Components</th>
<th>Effective Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trastuzumab</td>
<td>10.59±0.189</td>
<td>Rituximab</td>
<td>10.82±0.161</td>
</tr>
<tr>
<td>Trastuzumab+FCA</td>
<td>14.94±0.273</td>
<td>Rituximab+FCA</td>
<td>14.66±0.39</td>
</tr>
</tbody>
</table>
The exact structures that are being formed cannot be predicted just by using DLS but based on thermodynamic favorability; it will include different types of interactions to form the cyclic structures.\textsuperscript{51}

![Diagram](image)

Figure 2.3: Formations of antibody complexes upon the addition of the trivalent molecule that include bicyclic antibody trimer, a tricyclic antibody hexamer, and a branched polymeric system.

The supernatant was collected and analyzed via RP-HPLC with an analytical column, the percentage of remaining antibody in the solution was calculated considering the elution peak of monoclonal antibody.
Figure 2.4: Antibody present in the supernatant after precipitation with trivalent ligands and monoclonal antibodies (6μM).
CHAPTER 3:

CONCLUSION AND FUTURE WORK

As it can be observed, the solubility of mAbs decreases which is essential for the selectivity of this technique. Moreover, a window where complexes precipitate and monomeric antibodies remain in solution at a particular concentration of AMS is the most important aspect. Unfortunately for the trivalent molecule, this window was very narrow. Therefore the subsequent experiments proposed were not performed, and a tetravalent system was introduced instead.

Figure 3.1: The structure of the tetravalent ligand. R represents the aromatic structures.
Other experiments that have not been performed yet include the comparison between the precipitations with the tetravalent molecule to determine the most efficient system and the best concentrations of the components to use. With that information, the next experiment would be to incubate the mAbs with ascites fluid, and CHO conditioned media and purify them using the protocol depicted in figure 2.1.

Next, an analysis of the effectiveness of the purification would be performed by running an SDS-Page of the purified and non-purified samples. The RP-HPLC would also be used to elucidate and visualize the components in the samples collected at each step involved in this purification technique. Additionally, a quantification of the residual dsDNA and the remaining CHO host cell proteins would be executed using the commercially available kits.

Finally, the purified mAbs would be tested to evaluate if they still retain their binding capacities to CD20 and Her2/Neu receptors overexpressed in certain types of cancers.
BIBLIOGRAPHY


