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Modified Antibody for Targeted Drug Delivery and Reduced Immunogenicity

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by

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Abstract

Traditional cancer therapies suffer from toxicity, lack of selectivity, and resistance to drugs. In order to increase the dose reaching the tumor tissue, targeted therapeutic approach is being used. Nanoparticles (NPs) can carry larger amount of drugs, however the amount of these NP systems reaching is less than 10%. Most of these NPs get taken up by cells of the immune system, and segregate in organs of the body such as liver, lungs and spleen. Adding a polymer coating such as Poly ethylene glycol reduces the non-specific uptake. Adding a targeting ligand on the NP surface allows specific interaction with the tumor target and intracellular delivery of the payload. However, the protein coating covers the PEG surface. This study undertakes modification of the antibody of interest (Herceptin) with short PEG moieties. It deals with the characterization of the plain and modified Herceptin and immune response of the mouse macrophage cell line RAW 264.7 to NP-antibody/ NP-modified antibody system. Herceptin and modified Herceptin were characterized using Matrix Assisted Laser Desorption/ Ionization (MALDI) and Mass Spectrometry. Two cytokines, TNF-α and IL-1β were measured by Enzyme Linked Immuno Sorbent Assay (ELISA). Fluorescent Microscopy study was undertaken. Systematic study of the applicability of the Bradford Assay to the modified antibody was done. Two standard proteins: Bovine Serum Albumin (BSA) and Bovine Gamma Globulin (BGG) were compared with Herceptin response. Modeling of the Herceptin structure was done using the protein sequence available in the Protein databank.
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List of Abbreviations:

BGG- Bovine Gamma Globulin
BSA- Bovine Serum Albumin
DAPI- 4′,6-diamidino-2-phenylindole
ELISA- Enzyme Linked Immuno Sorbent Assay
EPR- Enhanced Permeation and Retention
FDA- Food and Drug Administration
GM-CSF- Gram Macrophage Colony Stimulating Factor
HER2- Human Epidermal Growth Factor Receptor 2
IL-1β- Interleukin -1β
mAb- Monoclonal Antibody
MALDI- Matrix Assisted Laser Desorption/Ionization
MW- Molecular Weight
NP- Nanoparticle
NHS- N-hydroxysuccinimide
LPS- Lipopolysaccharide
PBMC- Peripheral Blood Mononuclear Cells
PBS- Phosphate Buffer Saline
PDB- Protein Data Bank
PEG- Poly (ethylene glycol)
PLGA- Poly (lactic co-glycolic) acid
RES- Reticulo Endothelial System
RGD- Arg-Gly-Asp
TBTA- Tris[(1-benzyl-1 H-1,2,3-triazol-4-yl)methyl]amine
TFA- Trifluoroacetic acid
TNF-α- Tumor Necrosis Factor-α
Chapter 1: Introduction

1.1 Cancer:

For every eight deaths in the world, one is caused by Cancer.[1] Cancer is believed to be a collection of as much as 100 different diseases, increasing the complexity of the treatment. A list of the common types and the statistics is given at the end of the write up. Underlying causes and risk factors are equally diverse. Within a single cancer type, significant tumor heterogeneity can be found. In a cancer genome, there are between 30 and hundred mutations, which include single point mutations (most frequently observed), insertions and deletions or gene amplification.[2] Even a single substitution in a critical gene in the DNA can cause mutation leading to cancer. Cell division and growth is generally a controlled process, the cells are a dynamic environment where growth and death are a part of their program in a highly regulated manner. Mutations occur throughout the life cycle of an individual, and these mutations go on accumulating over the course of a lifetime. Not all mutations are harmful; however, a single critical mutation can lead the involved cell to turn cancerous. Starting locally during early stages, advancing cancer spreads or metastasizes through the body. Early detection can be of great help, but more advanced stages of cancer are extremely difficult to treat.

The distinguishing features of cancer, according to Hanahan and Weinberg are: insensitivity to anti-growth signals, self-sufficiency in growth signals, evasion of apoptosis; sustained angiogenesis; tissue invasion and metastasis; and limitless replicative potential.[3] There are various defenses in place to avoid these changes from occurring under normal circumstances. Each of these steps in tumorigenesis indicates the breakdown of the regulatory checks. Because the microenvironment changes with each of these changes, a single treatment regime fails to work in cancer treatment.
1.2 Traditional Cancer Therapies:

Cancer can be treated using chemotherapy, radiation and surgery, depending on the type, and the stage at which it is diagnosed. The earliest chemotherapeutic drugs were introduced around the period of World War II i.e. 1940’s which affected and killed rapidly diving cells.[4] The currently used drugs also interfere with the cell replication. Cells which undergo frequent cell division are susceptible to these drugs. However, some classes of healthy cells also divide frequently, like the lining of the digestive tract, the blood cells in the bone marrow, and hair cells. Since there is no way to distinguish between the healthy and cancerous rapidly diving cells for these drugs, chemotherapy has been seen to have severe side effects, including a suppressed immune system and compromised immunity, toxicity, hair loss, loss of appetite, nausea.[5] The immune system cells originate in the bone marrow.[6] The quality of life of these patients is poor.[5] The therapeutic index for these drugs is low, thus limiting the amount of drug that can be safely administered.[7] Some of these drugs are hydrophobic; insoluble in aqueous medium, thus lowering the bioavailability.[8] Resistance also sets in to these drugs on repeated injections, rendering them ineffective.[9, 10] If possible, the frequency of dosing required should be low, which means the drug once injected should stay in circulation and remain available for longer period of time. Hence, in many cases, the therapy is not effective enough to combat the disease due to low availability of the drug. The main objective behind the use of drug delivery systems, especially in cancer therapy, is to selectively destroy the unhealthy cells without the indiscriminate killing of healthy cells in the body.[11] By using different drug delivery methods, the idea is to reduce the cytotoxicity for healthy cells and increase the selectivity and dosage that can be administered to the cancer mass. There are two broad scale approaches to delivering therapeutics to selective targets: the small molecule approach and the nanoparticle approach.
1.3 Newer approaches:

Normally, cells grow because of receipt of external growth signals binding to specific receptors on the cell surface. Similarly, anti-growth signaling molecules also bind to their corresponding receptors on the cell surface. These cell surface receptors, on binding to their corresponding ligands, regulate the inter-cellular machinery. As cancer develops, one of the ways it ensures growth is by overexpressing these receptors, which can receive more growth signals, and continue to proliferate.[3] If this signaling mechanism is interfered with, the cancer growth can be stopped. Other receptors which participate in helping cancer grow can be targeted. Hence, one of the current approaches to cancer therapy is to identify the unique markers or receptors that are lacking or are few in number on normal tissues and are otherwise overexpressed on cancer tissues. Ideally, the target should be exclusive to the cancerous tissue. Hormones, antibodies, antibody fragments, aptamers, growth factors, and other small molecules are seen in reports as targeting agents.[12, 13, 14, 15, 16]

1.3.1 Antibodies:

Antibodies are widely used for cancer therapeutics.[4, 17, 18, 19] Paul Ehrlich had first envisioned the concept of magic bullets that would specifically destroy their corresponding targets. Antibodies have been used to detect analytes since before 1950s.[20] Large scale production of antibodies became possible due to the contribution of Kohler and Milstein in 1975. Since then, antibody production has seen evolution from full mouse antibodies to fully human antibodies. Antibodies are roughly Y shaped heavy proteins of about 150 kDa. There are five subclasses of antibodies: IgA, IgD, IgE, IgG, and IgM. IgG is made up of four polypeptide chains: two heavy and two light.[21] Most of the IgG structure is common to all IgGs, except for the small regions at their tips known as the complementarity determining regions (CDRs). These
are a small sequence of amino acids that confer the specificity associated with antibodies. Primarily ionic (electrostatic) interactions, but also hydrogen bonding and van der Waals forces are the major contributors to the intrinsic affinity between the paratope on the antibody and the epitope on the antigen. There is no covalent binding between antibody and antigen. The association constant (Ka) of the binding between an antibody and its cognate antigen is a measure of the antibody’s affinity. It can range from $10^3$ to $10^{10}$ liters per mole. The higher the intrinsic affinity of the antibody, the lower the concentration of the antigen needed for the available binding sites of the antibody to become saturated (reach equilibrium). Antibodies are targeting agents, though not classed with small molecules.[22] Monoclonal antibodies are a homogeneous population of immunoglobulin that binds to a single epitope. These are produced by a single B-cell from one animal and thus, are immunochemically similar. Antibodies specific to corresponding transmembrane cell surface receptors bind to them, and stop signaling cascades inside the cell. Additionally, their Fc receptors are also flags for recognition by the cells of the immune system. Termed as ‘Antibody dependent cell cytotoxicity’ (ADCC), this adds another feature to their use by recruiting immune cells to fight disease progression.[22] However, resistance to antibodies is also known to develop by various mechanisms, e.g. by change of receptor structure, antigen shedding, and change in signaling cascade.[23] These therapies are usually given in combination with chemotherapy. Advantages of antibodies include: Since they have been in use for decades now, properties of antibodies including pharmacokinetic and systemic interactions have been extensively studied. Their large size prevents loss due to kidney filtration, and they are unlikely to be degraded by proteolytic enzymes. Overall, they have a higher circulation time as compared to smaller peptide molecules. Their size does become a limitation due to poor tissue penetration for such large molecules. Other issues include batch to
batch variation; scale up is limited, sensitivity to high temperatures, and limited storage life.[20] A list of commercial antibodies is given at the end of the chapter. Antibody fragments are also being used as targeting moieties.[13] They have higher tissue penetration, though lower binding affinity.

1.3.2 Aptamers:

Aptamers are sequences of nucleic acids that bind to specific sequences. Aptamers are produced artificially in an in vitro chemical process called Systematic evolution of ligands by exponential enrichment (SELEX). Aptamers are also highly specific to the ‘apatopes’ they are targeted against. Advantages include the lack of bacterial or viral contamination like antibodies, smaller size ensuring better tissue penetration as against antibodies, thermal insensitivity, non-immunogenicity and scalability. Disadvantages include lack of predictability of PK/PD properties, shorter half-lives, and patent issues (SELEX is a patented process). So far, only one Aptamer has been FDA approved that is Macugen for macular degeneration.[20, 24, 25]

1.3.3 Folate:

Folic Acid is used as a targeting ligand. Folic acid binds to folate receptors on cell surfaces on which they are expressed. Folate receptors are few in numbers on healthy cells and overexpressed on cancer cells. Instances include ovarian cancer, breast, colon prostate, lung cancers, and chronic and acute myelogenous leukemias. Payloads attached to folates are internalized by receptor mediated endocytosis, similar to antibody internalization.[26]

1.3.4 Small molecules:

Small molecules approach refers to molecules designed to interfere in the signaling process which causes cellular dysfunction by binding to their targets bringing about
conformational change in the target. These are usually peptides, and are very selective and are usually tailored to bind only to their specific antigen. Examples would be different kinase inhibitors, siRNA, RGD peptides.[27, 28, 29] Specific examples include Imatinib, a tyrosine kinase inhibitor being used for the treatment of chronic myeloid leukemia (CML) [2] Imatinib, or Gleevec, is approximately 500Da, and binds to the enzyme tyrosine kinase that stops the subsequent signaling cascade that allows the cancer cell to grow.[30] These small molecules can reach their sites of action easily and if are directed towards surface markers, do not need to penetrated into tumor tissues. However, these can diffuse out more easily and can have shorter circulating time.[31, 32] Also, in many cases, multiple signaling pathways are involved in disease progression, and a single molecular target may not be enough.[22] It is reported that a single molecule therapy almost always develops resistance.[2]

1.3.5 Antibody Drug Conjugates:

Another class of therapeutics being studied extensively and also being introduced commercially is antibody-drug conjugates (ADC’s). ADCs are monoclonal antibodies (mAbs) linked to cytotoxic payloads. Drugs and toxins can be directly coupled to antibodies.[4, 6, 33, 34, 35, 36] Key considerations include the ability to bind to the target should remain intact, should have reasonable circulation times in the body, and the payload should be released effectively. A commercial example is Genentech’s T-DM1, which has been released commercially as Kadcyla. DM1 is a potent cytotoxic drug. Trastuzumab is linked to DM1 through a nonreducible
thioether linkage (SMCC), which is only broken by cellular enzymes in the lysosomes.[37, 38, 39] Kadcyla was approved by FDA in 2013 for metastatic breast cancer. However, one of the biggest drawbacks for ADCs is that the number of drugs that can be carried by each molecule is low (Ranging from 3-8).[40] The potency of drugs used is extremely high; hence the stability of such ADCs is a critical issue.

<table>
<thead>
<tr>
<th>Targeting ligands</th>
<th>Target</th>
<th>Example of tumor target</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGD</td>
<td>Cellular adhesion molecules such as ανβ-3 integrin</td>
<td>Vasculature endothelial cells in solid tumors</td>
</tr>
<tr>
<td>NGR</td>
<td>Aminopeptidase N (CD 13)</td>
<td>Vasculature endothelial cells in solid tumors</td>
</tr>
<tr>
<td>Folate</td>
<td>Folate receptor</td>
<td>Cancer cells overexpressing folate receptor</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Transferrin receptor</td>
<td>Cancers that overexpress transferrin receptor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>GM-CSF receptor</td>
<td>Leukaemic blasts</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>Galactosamine receptors on hepatocytes</td>
<td>Hepatoma</td>
</tr>
</tbody>
</table>

Table 1: Some targeting ligands in use [13]

1.4 Differences between Tumor and Healthy tissues:

The difference between the structures of tumor and healthy tissues are prominent. [41, 42, 43] These differences can be used to advantage to design suitably sized nanosystems. Tumor is a fast growing mass of cells, which needs continuous supply of nutrition to sustain the rate of growth. New blood vessels are formed from the pre-existing ones.
to keep up with the growing nutrition demand. Growth factors which promote blood vessel formation such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) help formation of new vessels.[3, 42] Due to the dynamic interactions between the pro-growth and anti-growth factors, there is poor regulation of blood vessel growth, resulting in poorly formed structures.[42] The lining of the newly formed vessels are incompletely formed, hence there are large channels through which Nanoscale materials can flow out into the tumorous tissue, which is not possible in normal tissues. Blood supply around the tumor is chaotic and heterogeneous. The blood velocity surrounding the tumor may be much lower than in normal tissues. [44] One other characteristic is that the lymphatic vessels in tumors are blocked due to the excessive pressure of the growing mass. Material accumulating at the tumor site tends to remain trapped as there is poor outflow from the site. This phenomenon is referred to as the Enhanced Permeation and Retention (EPR) effect, and is the basis of using nanoparticles for drug delivery.[32, 41, 45, 46, 47, 48, 49]

1.5 Nanoparticles

Nanoscale systems carrying drugs show different pharmacokinetic and pharmacodynamic properties than that of the free drug.[10] Nanoparticles can carry higher payloads of chemotherapeutic - drugs, in required doses. They also have lower systemic toxicity than free drug.[50] The rationale is that due to the EPR effect, correctly sized and biocompatible nanoparticle system can accumulate at the tumor site, thus delivering drugs in sufficient quantities, increasing the tumor site accumulation of the drug as compared to the healthy tissues. This is referred to as ‘Passive Targeting’. There is a huge diversity in the materials utilized for creating nanoparticles for drug delivery purposes. From single walled and multiwalled carbon nanotubes (CNT’s) to metallic nanoparticles (iron, gold), inorganic materials like Silica, and a
diverse range of polymeric materials, many approaches are in existence each having their pros and cons. For example, Si NP’s are porous and can hold drugs within these pores.[51, 52] They can be manipulated to optimal size for escape from immune cells, and proper drug release profile. However, their lack of biodegradability is a concern for repeated use. Gold nanoparticle can be modified and drugs can be conjugated to their surface. However, the drugs are exposed to the other tissues and organs along their path, leading to toxicity issues and drug inactivation. Drug encapsulation in polymeric nanoparticles reduces the drug exposure to other organs before it can reach its target, and also carry higher amounts of drugs. Polymeric nanoparticles are quite often biodegradable and their surface properties can be optimized. Examples include poly(vinyl pyrrolidine) (PVP), poly(vinyl alcohol), polyglutamic acid (PGA), poly(malic acid), N-(2 hydroxypropyl)methacrylamide (HPMA), poly(lactic-co-glycolic acid) (PLGA) copolymer, polylactic acid (PLA), polyglycolic acid, poly(alkyl cyanoacrylate), poly(methyl methacrylate)(PMMA), and poly(butyl)cyanoacrylate.[53] Some nanoscale systems are described below:

1.5.1 Polymeric micelles:

Micelles are nanoscale (5-150 nm) colloidal particles. They are made of amphiphilic materials i.e. composed of both hydrophilic and hydrophobic parts. At a critical concentration, individual amphiphilic chains self-assemble to form particular aggregates with the hydrophilic part exposed to the surface, while the hydrophobic part constitutes the inner core. This core can hold hydrophobic drugs, thus making micelles capable of being use as drug delivery vehicles. Individual micellar units are made of di-block or tri-block co-polymers or graft co-polymers. The physical and chemical properties of these micelles depend on the length of the hydrophilic and hydrophobic chains along with their chemical characteristics. The most commonly used
hydrophilic block is PEG, but other materials have also been reported such as poly (N-vinyl-2-pyrrolidone) (PVP), poly (ethylene imine) (PEI), poly (vinyl alcohol) (PVA), etc. For the hydrophobic core, materials reported are propylene oxide, L-lysine, caprolactone, D-L, lactic acid, styrene, aspartic acid, β-benzoyl-L-aspartate, and spermine.[53]

1.5.2 Dendrimers:

Dendrimers are small, branched polymeric macromolecules. They have a large surface areas and a number of functional groups that can be used for drug and targeting ligand attachments. Most commonly reported dendrimers are of Poly (amidoamine) (PAMAM) that are being used in pharmaceutical industries and as imaging and drug delivery agents. Attractive characteristics of dendrimers include controlled sizes and shapes, monodispersity and that the surface functional groups can be designed according to the required application. Drugs can either be attached to the surface or can be encapsulated in the inner cavity. However, some difficulties have been observed in diffusion of encapsulated drugs from the dendrimeric structures.[54]

1.5.3 Liposomes:

Liposomes are nanoscale particles with a lipid layer surrounding an aqueous core.[55] Commercial liposomes containing the drug Doxorubicin were one of the first nanoparticle therapeutics approved, and are being used for the treatment of Kaposi’s sarcoma, ovarian and breast cancer. The first liposomal therapy, Myocet was a non-PEGylated formulation, is used to treat metastatic breast cancer. 190 nm in size, it did not retain the drug very well, losing 90% of the encapsulated drug in 24 hours. It did reduce the dosing frequency to some extent. Doxil is the PEGylated version of liposomes, about 100 nm in size. The circulation half-life is 46 hours, and also has a high drug loading capacity. The stability and ability to retain drug is high. However, prolonged circulation time and repeated dosage has been seen to cause toxicity issues.[56]
1.5.4 Active Targeting Nanoparticles: 

The small molecular targeting ligands mentioned above are being conjugated to these nanoparticle based drug delivery systems for more specificity and better cellular uptake. Active targeting refers to the presence of a targeting ligand such as folate, aptamer, small peptides/molecules, antibodies, etc.[4, 57, 58] The choice of the targeting ligand is based on the type of cancer, the molecules that are overexpressed. Receptor expression should be high, for example, in case of Herceptin therapy, HER-2 expression must be about 10,00,000 receptors per cell to be effective.[13] Internalization of the targeting ligand is also necessary in some cases, while undesired in some. Targeting ligand attached to the payload drug must enter the cell for the encapsulated drug to work. If the binding is too strong, the complex may not get internalized, instead stay stuck on the exterior cell surface. Hence, these considerations must be analyzed and appropriate ligand must be employed. These targeting ligands may not help with directing the payload to the tumor site, but they play a role in the effective internalization of the attached payload.[27, 31, 59] This has been demonstrated by using different targeting ligands such as cell adhesion molecules (CAMs), antibody fragments.

In all of the strategies mentioned above, the critical factor is the tolerance. These systems are not native to the body, and the immune system kicks in when it comes in contact with either the small molecules or the nanoparticle systems.

1.6 Immune system response:

The human body reacts to anything that is not native to the body. The magnitude of this response varies, but it is an important aspect of therapeutics design. Proteins, peptides, antibodies and their fragments have shown immunogenicity when used as therapy. On administration, these proteins are seen to elicit immune response.[60]
Nanomaterial introduced into the body can either suppress the immune system or stimulate it.[50] Stimulation of the immune system can help in certain cases, for example while delivering a vaccine. However, if the stimulation follows a therapeutic dose meant to deliver drugs to diseased region, the purpose of administration is lost, with the addition of complications in the diseased state.

If left ‘unprotected’, these systems will be kicked out of circulation within a few minutes. The immune system with its various components and cell types is designed to distinguish between the ‘self’ and the ‘non-self’. [61] The neutrophils and macrophages are usually the first line of defense against foreign invaders into the body. Typically, these cells of the immune system have receptors on their surface which recognize pathogenic markers, foreign proteins and material. This first line of defense response is termed as the ‘innate immune response’, and is non-specific, mainly given by the cells of the innate immune system. These cells include neutrophils, eosinophils, basophils, monocytes/macrophages, dendritic cells (DC), and mast cells. Monocytes are the circulating cells in the blood stream, and the spleen and the bone marrow. Macrophages are resident cells in the various tissues in the body. There are various pathways/ mechanisms by which these particles on being introduced into the body get eliminated by these phagocytic cells.[50]

When nanoparticle based delivery systems are introduced into the blood stream, proteins present in the blood stream, such as fibrinogen, albumin, and immunoglobulins and complement proteins, form a coating on them. This process is known as ‘opsonization’. [62, 63, 64] Such a protein coat not only changes the physical size and charge characteristics of the nanoparticle system, it results in ‘marking’ of these nanoparticles as targets for the RES cells. Monocytes/Macrophages recognize these protein coated particles which enhances their clearance
process. These phagocytic cells have receptors on their surface that recognize these opsonins, and get activated. On recognition of interacting bacteria or pathogen, via receptor binding, the phagocytic cells engulf the detected invader, and also secrete molecules called cytokines. Immune cells have corresponding receptors for these cytokines. These cells ingest the interacting particles, and depending on whether the particles are biodegradable or not, their processing is carried out.[65] In case of non-biodegradable material, it is transported and collected in the liver, lungs and spleen. In order for the nanoparticles or any agent for targeted delivery to be useful, the first consideration is for the agent to reach the tumor location. For that to occur, the agents have to remain in circulation for extended periods of time. Some of such systems failed due to the immediate clearance of these particles from the blood stream within a few minutes.[63, 65] These particles get transported to liver, kidneys and spleen, and thus the major dose ends up in these organs causing toxicity.

1.7 Factors affecting the Immune response:

There are a number of parameters that determine the interactions between the NP system and the body environment. Prominent among them are: the material characteristics, surface charge, size, tendency for aggregation , the stability and thickness of surface coatings, etc.[50] Though the parameters are known, the exact criteria and effect relationship is not known.

Size is an important parameter, though there are varying reports on what the optimal size should be. Particles having less than 200 nm in size have a better chance of staying in circulation, but should be greater than 5 nm to avoid renal cut off. It has been observed that hydrophobic material gets taken up by RES cells more prominently.[58]

Surface composition and charge dictate the interactions of the nanosystems with their surrounding in vivo. It has been seen that nanosystems with near neutral or slightly negative zeta
potential are more likely to circulate longer. However, contradictory reports exist. Increasing the surface charge can decrease protein adsorption, but the opposite trend has also been reported. Because of the diversity of materials, parameters, and techniques employed in these reported studies, arriving at a uniform conclusion has been extremely difficult. Each formulation has to be evaluated independently. The mechanisms involved in immune response are not uniform. The complement system which is the cascade that is involved in pathogen/foreign material removal has two pathways: the classical complement pathway, which is activated due to the formation of antibody-antigen complexes; and the alternate pathway which works without the involvement of antibody interactions. Carbon nanotubes (CNTs) and liposomes have been found to activate the classical pathway. But, although both single walled (SWNTs) and double-walled (DWNTs) CNTs activate the classical pathway, only DWNTs have been found to activate the alternate pathway, the reason for which is not clearly known.

1.8 Strategies to increase circulation:

Two strategies can be used of to reduce the immune system response: make materials more biocompatible or add a stealth mechanism to avoid detection. An example of the first instance would be antibodies over the years. The first antibodies that were used were from mouse, which were not compatible. Advances in genetic engineering led to production of chimeric antibody (30%
mouse, 70% human) to humanized antibodies (90% human, 10% mouse) and finally to human antibodies. This has led to a large number of therapeutic antibodies reaching clinical trials and getting approvals to be used as commercial therapeutics.\[6, 17, 19, 22\] Small molecules faced enzymatic degradation and immunogenic issues as well. Since 1970’s, attachment of Polyethylene glycol to small molecules has been in practice to increase the circulation time, and better stability.\[66, 67, 68, 69\] Many such conjugated proteins have been approved for therapy: PEG-asparaginase (Oncaspar), PEG-adenosine deaminase (Adgen), Somavert, a PEGylated growth hormone receptor antagonist, PegIntron (Schering-Plough, USA), form of interferon (IFN)-α2b and Pegasys (Hoffman-La Roche, Inc., USA), form of IFN-α2a, both for the treatment of hepatitis C; Neulasta (Amgen, USA), a granulocyte colony stimulating factor (G-CSF) for the treatment of chemotherapy-induced neutropenia; and Mircera (Hoffman-La Roche, Inc., USA).\[66\] Such modification has led to reduced immunogenicity of proteins. While reduced activity of modified proteins is a drawback, the improved bioavailability has led this class of therapy to be useful.

Nanoparticles are conjugated to PEG to reduce the immune response.\[58, 62, 63, 70, 71, 72, 73\] PEG remains the most commonly used hydrophilic material, though other materials have been studied for the same reason, such as poly (vinyl alcohol) (PVA) poly (N-vinyl-2-pyrrolidone) (PVP), and poly (ethylene imine) (PEI). PEG introduces hydrophilicity which is shown to reduce protein adsorption to the surface. Numerous success stories of PEG conjugation have been reported. For example, it was reported that the half-life of latex NP’s increased dramatically by 40 times from 20 minutes to 13 hours on conjugation to PEG.\[74\] PEG is also attributed to for offering other useful advantages, such as reducing hemolysis. Hemolysis refers to the damage caused to erythrocytes or the red blood cells by NPs. Rupture or damage to the
RBC’s could lead to serious medical conditions such as anemia and acute immune reactions. [50, 75] It is an FDA approved, biocompatible polymer. It reduces nanoparticle aggregation, thus helps maintain the size of the system. PVA, Chitosan, Poloxamer, Poloxamine, Polysorbate are some of the other materials explored, but PEG is the most widely used. Hence, the nanoparticles being used in recent times are made up of two or more blocks, one of them being PEG, or some other polymer with stealth properties, though PEG is the most popular.

An effective drug delivery system, in our particular case for cancer, would be a system that would deliver the payload to the tumor site at optimal doses while reducing the exposure of the healthy tissues to the chemotherapeutic drugs. It would also be compatible with the immune system to a higher degree in order to reduce the non-specific toxicity to the organs of the immune system. The current reports on tumor accumulation of such different systems is less than 10%. [76] Most of the NP systems end up in liver, lungs, kidneys and spleen depending on their characteristics. [58] Nanoparticles conjugated to a targeting ligand are expected to work coherently to confer an advantage over plain nanoparticulate systems. Plain nanoparticles

![Figure 4: %Injected dose taken up by tumor as a function of protein concentration. The increase is not a continued trend, decreasing between 5 and 10% (Top). Uptake in the organs however increases between the two different amounts (Bottom) [77](image)]
without protein conjugation have a PEG coating exposed to the external surfaces that they come in contact with. However, once coated with antibodies, the PEG coating would be covered thereby losing any advantage PEG offers in reducing the non-specific uptake by the cells of RES.[23] In general, presence of the protein coating is reported to increase the RES uptake, however, there is an optimum amount of protein that can keep the uptake low and still bind effectively to the target.[77] In vivo biodistribution and tumor uptake of PLGA-PEG-aptamer complex is shown from paper by Gu, Langer et al.[77] Increasing the aptamer density from 5% to 10% increased the uptake of the complex in liver from 40% to over 60%. Increased uptake was attributed to coverage of PEG surface by the protein and the loss of PEGs shielding effect in the manuscript. Hence, studies must be done on a case to case basis for the optimum amount of protein coating on the surface. Even then, the antibody coating can interact with other blood proteins. Loss of epitope recognition ability and increased opsonization are the likely consequences. We wanted to evaluate the addition of more PEG onto the antibody coating on the NPs and test the immune response of macrophages to such a system. The hypothesis is that at certain optimal concentration of protein coating and extra PEG linkers, we will be able to see a reduced immune response of macrophages to such a system, and give us an idea of the working parameter range for optimizing such a system. The idea is to attach small enough linkers to the antibody so as to add hydrophilicity, at the same time minimize conformational changes to retain its binding ability.

1.9 Mass Spectrometry Study:

Antibodies have a large number of lysine sites. For e.g.: Herceptin has 86 lysines available. Adding linkers will give rise to heterogeneous populations of modified antibodies.[78] Also, there needs to be a study of the efficiency of conjugation and location of the modifications.
Depending on the site of modification, the properties such a system can be different in vivo.[79] Hence, quantification of these modifications becomes important, for repeatability and reproducibility. There are reports of other amino acids apart from lysines getting modified such as threonines, serines and tyrosines.[80, 81, 82, 83] Hence a detailed study on the other possible modifications should be undertaken to assess extension of the usability of lysine chemistry to other antibodies. If any of the above mentioned groups fall in the epitope binding site of the antibody in use, the binding capacity may be compromised. Mass spectrometry studies have been seen in literature for such a purpose. One study did not take into account any other modifications apart from lysine. Another showed a few other possible sites of modification, however, the mass being attached to the antibody was large. Our linkers of interest are small molecules, hence the complexity increases. Detailed literature review is presented in a subsequent chapter relating to mass spectrometry studies.

1.10 Protein Quantification

Protein modification for conjugation to other moieties such as radioactive dyes, nanoparticles, drugs and polyethylene glycol for longer circulation has become fairly common. Thus, protein quantification in such cases is of interest to find out the level of conjugation. Bradford Assay is seen to be used for the purpose.[84, 85] Based on the commonly described procedure in the literature, BSA is used to build a standard curve for proteins, despite of the variability in the types of proteins used. Despite the recommended use of a protein with similar structure, if not the pure protein under investigation, the use of Bovine Gamma Globulin or Immunoglobulin has not been commonly seen in cases where antibodies or antibody fragments have been used for conjugation. Chemical crosslinking moieties are often present for attachment of proteins to polymers; however, discussions on their effect on protein quantification are not
seen in detail. Efforts have also been made to investigate the applicability of Bradford Assay to chemically modified proteins. Although modification of lysine groups has been reported to have no effect on the results of the Bradford Assay, no actual data was reported.[86] Recent reports have been contradictory.[87] While the Bradford Assay was not tested on modified, PEGylated protein, the modification is expected to affect the accuracy of the quantification. PEG interference has also been reported, the range of molecular weight was much higher than our application called for.[88] The reported did not account for attachment of PEG to the protein and blocking lysine sites. Hence, in order to estimate the amount of Herceptin attached to the NPs we studied the effect of modification, if any, on the Bradford assay, and the data is presented in a later chapter.
Chapter 2: Materials and Methods

This study contains an antibody (Herceptin) conjugated to PLGA-PEG nanoparticle system via a linker using click chemistry. The three components, thus, become an antibody as a targeting ligand, the NHS-ester as the linking entity, and the nanoparticles as the payload. Selection of all three components has multiple factors that need to be considered. To link the antibody to the payload, multiple sites can be used, however, maintaining the functionality of the targeting ligand is critical. Multiple sites are available on proteins for modification, with their pros and cons. Selection of the linker is dependent on the functional groups present on the protein and the NP. The length, solubility, and polarity of the linker together determine factors such as aggregation, selectivity, therapeutic index, pharmacokinetics as well as the stability of the conjugate.[33] Bioconjugation techniques need to be assessed to ensure the efficacy of the end product, and the use of bio-compatible, relatively benign processing is required. The background on protein modification methods, click chemistry, and the system used is as follows:

2.1. Protein Modification:

Proteins, with various amino acids in their framework, have different functional groups and side chains that can be used to modify proteins. Polymeric chains can be introduced with one end functionalized to react with proteins and the other end to NPs. Functional groups on proteins that are commonly utilized are:

a. Amines: Lysines and α-amino acids are employed for modification. Lysines are usually surface exposed, and available in large numbers. The ε-primary amine is usually the target. Amines on lysines are good nucleophiles above pH 8.0, reacting easily with a large number of reagents and yield stable bonds.
b. **Thiols**: This group includes Cystines, Cysteines and Methionines. Cysteine has a free thiol group that is more reactive than the amine group, and can react with the same functional groups that react with amines, and also those which are specific to thiols. An additional advantage is that thiols react even at neutral pH. However, due to their high reactivity, they do not exist freely, are often present as disulfides. Methionine contains sulfur in the form of a thioether linkage. These bonds are needed to be broken to make use of thiols. This can affect the structure and stability of the whole protein.

c. **Carboxylic Acids**: Aspartic and Glutamic Acid contain dicarboxylic acid in their side chains, while proteins in general contain carboxyl group at their terminal position. However, the reactivity of this group is low in water. Selective modification becomes challenging. To use carboxylic acid as a modification site, it is first converted to a reactive ester by using carbodiimide, which makes it water soluble. Further, it is reacted with an amine or a hydrazine. Since the number of carboxylic groups are limited, and due to the multiple processing steps involved, this functional group has limited usability.

d. **Other amino acids**: Other amino acids are not used often for modification. Some of them like arginine need harsh conditions for modification that may harm the protein function. Similarly, tryptophans also need extreme conditions. Photooxidation and iodoacetate reactions have been reported for Histidines, but they are not commonly seen to be used.[89]

**2.2 NHS Ester Reactions:**

With the above considerations, modification of primary amines is the mostly commonly employed method of protein modification.[80, 81, 82, 83] The structural integrity of the antibody is critical, and breaking of the disulfide bonds could be detrimental to the purpose of
employment, and hence we focused on lysine modification. Many commercial products have reported the use of lysine modification chemistry, such as Kadcyla (Trastuzumab emtansine, T-DM1), Mylotarg (Gemtuzumab ozogamicin, withdrawn in 2010), etc.[33] However, the resulting heterogeneity in lysine modification is a persistent issue and is duly recognized. Amine reactive agents like N-hydroxysuccinimide (NHS) ester are the most popularly used agents. These are acylating agents, and react with nucleophiles. In the reaction, a stable imide or an amide bond is formed releasing the free ester.[80] NHS esters react with primary or secondary amines like the free N-terminus and ε-amino groups in lysine side chains, have half-lives of hours at near neutral or physiological conditions (pH 7-7.5).[90] However, the ester undergoes hydrolysis as the pH increases; hence the pH we use for our modifications is around 7.2.

2.3 Click Chemistry:
Nature builds a variety of compounds like proteins, DNA, RNA, etc. with unparalleled efficiency. Incorporating ideas of synthetic organic synthesis in making newer materials needs to be promoted so as to increase the selectivity and yield of the current practices. Some of the principles that are sought to be followed such as: mild reaction conditions, a stable linkage, should not exhibit cross-reactivity with other groups, should have low number of side products.[91] Sharpless and others worked on identifying a set of reactions that confirm to the above conditions, under the tag of “click” chemistry. Reactions belonging to the category are extremely specific, mild conditions, high yields and fewer side products that do not need extensive purification steps. A common example is the 1, 3-dipolar cycloaddition of alkynes and azides.[53] Click chemistry has been used in bioconjugation reactions for attaching ligands to NPs.[92, 93] Few classes of reactions that have been identified as fitting into the criteria for click chemistry are:

1. Cycloaddition of unsaturated species: 1, 3-dipolar cycloaddition.

2. Cycloaddition of unsaturated species: [4+2]-cycloaddition (Diels–Alder).


5. Addition to carbon–carbon multiple bonds.[91; 94]

Click chemistry involves formation of Carbon-
heteroatom bonds (which are seen recurring in natural products).[95] The features of click reactions include:

1. High yield and high selectivity reactions, under mild conditions and low temperatures.

2. These relatively harmless reaction conditions are necessary to work with proteins which are temperature sensitive, and whose structure must be preserved to retain their functional integrity.

3. Purification steps are simple, and overall the processing of these bio conjugates has few steps.

The NHS ester linker has an alkyne terminal group that is designed to react with the azide group on the NP. The reaction is catalyzed by copper sulfate. If the catalyst is absent, the cycloaddition is slow and not regioselective. However, it was shown that a small amount of Cu (I) working as a catalyst allows for regioselection in the reaction, and can be performed at room temperature.[96]

2.4 Herceptin as targeting ligand:

Among various targeting ligands discussed in the previous chapter, the use of antibodies is widely seen. We have been working with breast cancer cells for in vitro analysis of this system, and naturally, the type of cells being used influences the choice of the targeting moiety. Using HER-2 expressing cells call for the use of an anti-HER 2 agent. While the use of anti-HER 2 aptamer has been reported, the use of aptamers as yet is limited due to the prohibitive cost and unknown clinical characteristics. On the other hand, the antibody used in this study, Herceptin is an FDA approved monoclonal antibody therapy for metastatic breast cancer. Herceptin (Transtuzumab) is a recombinant antibody that recognizes the extracellular domain of the HER2 receptor in HER2 overexpressed breast cancer. Herceptin as a targeting ligand has many
advantages, apart from being FDA approved. It works via multiple mechanisms of action causing cell death:

1. Herceptin treated cells show G1 cell cycle arrest, leading to reduction in cell multiplication.

2. In clinical trials, Herceptin was shown to reduce tumor size.

3. The HER-2 surface receptor has been seen to undergo proteolytic cleavage, which is a kind of resistance mechanism. Increased levels the shed receptor in the serum is associated with poor prognosis, and decreased response to treatment in patients with advanced breast cancer. Herceptin has been shown to block HER-2 receptor proteolytic cleavage and shedding in vitro.

4. Overexpression of HER-2 in human tumor cells is associated with increased angiogenesis and increased expression of vascular endothelial growth factor (VEGF). Studies showed that treatment of HER-2-overexpressing breast cancers with Herceptin reduced tumor volume and decreased blood vessel density in vivo. Expression of multiple pro-angiogenic factors was reduced, while expression of anti-angiogenic factors was increased in Herceptin-treated tumors relative to control-treated tumors in vivo.

5. Works with chemotherapeutic agents by inhibiting DNA repair.[23, 97, 98, 99, 100, 101, 102, 103]
6. The epitope binding region does not contain a lysine group, which is necessary for avoiding modification and thus impaired binding of the antibody.

2.5 Role of PEG and PLGA:

Biodegradable polymers have been studied and utilized for the purpose of controlled release of drugs since these can be processed or reabsorbed by the body. The removal of these agents would not require surgical intervention. Most commonly employed biodegradable polymers are poly(lactic acid), poly(glycolic acid), and their copolymers, like poly-lactic co-glycolic acid. If the monomer ratios during processing are varied, the formed polymer can release drugs over sustained periods, for months and years.[104] Encapsulating high potency drugs in such biodegradable polymeric pouches decreases the dosing frequency, maintains a constant concentration of drug availability in the body. Cancer treatments can be improved using these new methods of drug delivery.

Anti-fouling materials are of interest in diagnostics, implants and therapeutics.[63] Reducing the interactions with blood components would prevent formation of protein coating on material entering the body, helping it retain its structural and functional integrity. It can also reduce immune cell activation, blood coagulation, and infection. For example, it has been reported that as low as 10ng of protein adsorption on an area of 1 cm² can lead to platelet adhesion, causing failure of implanted device. In case of therapeutics, this is necessary to keep the drug in circulation and reduce the uptake by the RES by reducing the protein adsorption. Some of the materials that have been explored as anti-fouling agents such as polyamides, PEG, polysaccharides have some common properties such as they form hydrogen bonds, thus hydrophilic, and are electrically neutral. The formation of a water layer surrounding the hydrophilic material is believed to be what prevents protein adsorption on the surface. The
effectiveness of the anti-fouling material depends on the surface packing, and molecular weights. Surface packing includes length of polymer chain, i.e. film thickness and density of packing. The surface density of the hydrophilic polymer has an optimum, since too high or too low density has been seen to increase protein adsorption. Individual PEG chains are highly flexible. Water layer forming around individual chains allow the stealth effect of PEG. However, if the packing density is too high, the flexibility and the water cover are lost, leading to loss of the stealth effect. Modeling studies have been performed to assess the parameters affecting the non-fouling behavior.

Molecular weight of the blocks of polymers used plays a role in directing the nanoparticle interactions with blood proteins, as well as the composition. In one study of PLA and PLA-PEG nanoparticles, it was shown that proteins such as apolipoproteins A-IV and E (which are suspected in participating in the immune system recognition response) could stick to PLA nanoparticles, but were absent on the PLA-PEG nanoparticles.[63]

2.6 System used in this study:

Nanoparticles were made using PLGA-PEG blocks with different molecular weights as mentioned in the following chapters. They were made by Zilan Zhou, using nanoprecipitation method, which is beyond the scope of current study. NHS ester linkers were
used for protein modification of different molecular weights and functional groups. A long, 2000 Da linker was purchased from Jenkem, USA, having NHS ester group at one end and azide on the other, the central link being PEG repeating chains. This is a solid, and is water soluble. The 225 Da dPEG NHS ester linker (Propargyl dPEG) was purchased from Quanta BioDesign, OH. The initial and terminal end are the same as the 2000Da linker, the central portion contains a single PEG unit. This linker is water insoluble, and needs to be dissolved in dimethyl sulfoxide (DMSO) prior to its dilution with water. A 330 Da linker, also from Quanta BioDesign, was used for the immune tests to modify Herceptin with a methoxy group terminal, instead of an azide group. For the click chemistry, three catalysts were employed: 0.10 mM Cu (II) SO₄, 0.50 mM sodium ascorbate, 0.50 mM TBTA. TBTA is a chelating agent that has been used in literature. [93] Cu₂SO₄ catalyst solution, TBTA catalyst solution and sodium ascorbate were added in the given order. The samples were incubated for 24 hours at 4°C. The samples were filtered using Amicon filters (300K). The filtrate was used to determine the amount of protein conjugated using Bradford Assay.
Chapter 3: Characterization of Antibody Modification

To confirm the attachment of the linkers and to find the extent of modification and the number of linkers attached to the protein, the pure protein samples and the modified samples were analyzed by MALDI-TOF. MALDI, short for ‘Matrix Associated Laser Desorption/Ionization’ is an

![Figure 9: Confirmation of Herceptin at ~ 147800 Da](image1)

![Figure 10: Close up of Herceptin peak](image2)
ionization technique widely used for the characterization of large polymers and proteins.\[105, 106\] It is referred to as a soft ionization technique, due to the use of a ‘holder’ or the matrix on which the sample to be analyzed is deposited, and laser is used to ionize the matrix. The matrix molecules ionize taking the protein sample along. This is particularly useful since proteins are temperature sensitive and degrade at high temperatures. The other ionization technique seen in literature is Electrospray Ionization (ESI), but MALDI is preferred for large molecules. We were interested in the detection of the whole antibody and the species formed after conjugation. The molecular weight difference between the two gives an idea about the degree of conjugation. Because of the very large size of the protein (148kDa), gauging the extent of modification by looking at the mass shift proved to be a challenging task. Figure 9 shows the spectrum acquired on an ABSciex 4800 MALDI-TOF/TOF Instrument. The peak is for Herceptin, which on further resolution shows a range of about 3000Da. Modification due to the large 2000 Da linker was detected, and on an average 1-3 linkers were detected.
To determine the mass shift caused by a linker of 225Da, the level of conjugation (the number of linkers attached) would need to be high (more than 15 linkers). Even then, getting a distinct peak would have been difficult. Further complications arose when on modifying the antibody with the 225Da linker, the peak disappeared, the reason for which could not be satisfactorily explained. One of the causes of interference was thought to be the presence of large quantity of dimethyl sulfoxide (DMSO) in which the linker needs to be dissolved prior to its dilution. Due to the short length of the 225 Da linker, and with the alkyne group at one end, the linker is insoluble in aqueous solvents, and first needs to be dissolved in organic solvents like DMSO.

However, presence of DMSO hinders the ionization of the protein sample, and hence the amount must be restricted. Exhaustive purification of the modified Herceptin was carried out by using centrifugal filter (MWCO ~ 50,000 Da) Even then, the peak could not be seen, either for the modified Herceptin or corresponding to the MW of plain Herceptin.
Figure 10 shows a close up of the distribution of the peak. As can be seen, the width of the peak is large (close to 3000Da). Detection of modification therefore required either a rather large mass shift using a large MW linker or a very high degree of modification (large number of small linkers attached)

After a number of unsuccessful attempts to acquire the confirmation for the protein modifications by 225 Da and the 334 Da linkers, a set of samples was sent to Shimadzu. The

![MALDI spectra](image)

Figure 13: MALDI for detection of linkers

The 225 Da and 334 Da spectra were inconclusive (Top) Polydispersity of the 2000Da linker can be seen from the distribution of the MWs (Bottom)
samples were analyzed using the Axima MegaTOF, where all of the linker conjugations were successfully confirmed, along with quantification of the conjugation. An average number of linkers attached were determined.

For the 2000Da linker, two sets with linker to antibody ratios of 200:1 and 1000:1 were tested. The Shimadzu results agree with the initial ones from the Proteomics Lab, East Campus. An additional entity was also detected with about 18 linkers. As predicted, the degree of conjugation heavily depends on the molar ratio used, though to arrive at a correlation, a large number of samples will be needed to gather data. The number of linkers attached is also dependent on the length. The 225 Da linker has a length of 7 A, while the 334 Da linker has a length of 11.7 A. The length of the 2000 Da linker is 20 nm, which in comparison to the size of the antibody (10-15nm) is quite considerable. Hence, on an average, the number of long linkers is low. The need for better instrumentation is clearly noticeable for such studies, especially taking into account the large size of the protein. As seen earlier, a protein of the size of 150kDa constitutes nearly the end limit of detection for some instruments. Instruments used for small protein analysis cannot be used satisfactorily for proteins in the class of IgG. In case where such linkers are used for protein modification, crosslinking of protein must be taken into account. However, the size of the cross linked protein then increases to 300kDa, which would be impossible to detect. Hence, there is a crying need for better instrumentation for such cases. This particular machine, Axima MegaTOF, however, is, as one can imagine, expensive.

<table>
<thead>
<tr>
<th>MW of Linker</th>
<th>Molar Excess</th>
<th>Mass of Modified Protein</th>
<th>Mass of Pure Protein</th>
<th>Mass Difference</th>
<th>Average Linkers</th>
<th>% Lysine coverage</th>
</tr>
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<tbody>
<tr>
<td>225 Da</td>
<td>200</td>
<td>158,108</td>
<td>148,568</td>
<td>9540</td>
<td>42.4</td>
<td>47.11</td>
</tr>
<tr>
<td>334 Da</td>
<td>200</td>
<td>154,974</td>
<td>148,568</td>
<td>6406</td>
<td>19.5</td>
<td>21.67</td>
</tr>
</tbody>
</table>
Table 2: Linker Conjugation Data

<table>
<thead>
<tr>
<th>Linker Size</th>
<th>Linker to Antibody Ratio</th>
<th>m/z 1000</th>
<th>m/z 156,175</th>
<th>m/z 148,568</th>
<th>Intensity Ratio</th>
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<tr>
<td>2000 Da</td>
<td>1000:1</td>
<td>150,911</td>
<td>148,568</td>
<td>2343</td>
<td>1.5</td>
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<tr>
<td>2000 Da</td>
<td>200:1</td>
<td>156,175</td>
<td>148,568</td>
<td>7607</td>
<td>3.11</td>
</tr>
</tbody>
</table>

Figure 14: Shimadzu Data for Modified Herceptin
Herceptin modified with (top left) 225 Da linker and (top right) 334 Da linker; linker to antibody ratio of 1000:1; (bottom left) modified with 2000 Da linker; linker to antibody ratio 200:1 and (bottom right) 1000:1
Chapter 4: Protein Quantification using Bradford Assay

4.1 Introduction:

Protein modification for conjugation to other moieties such as radioactive dyes, nanoparticles, drugs and polyethylene glycol for longer circulation has become fairly common. Thus, protein quantification in such cases is of interest to find out the level of conjugation. Bradford Assay is seen to be used for the purpose.[84, 85] Based on the commonly described procedure in the literature, Bovine Serum Albumin (BSA) is used to build a standard curve for proteins, despite of the variability in the types of proteins used. Despite the recommended use of a protein with similar structure, if not the pure protein under investigation, the use of Bovine Gamma Globulin (BGG) or Immunoglobulin (IgG) has not been commonly seen in cases where antibodies or antibody fragments have been used for conjugation. Chemical crosslinking moieties are often present for attachment of proteins to polymers; however, discussions on their effect on protein quantification are not seen in detail.

Efforts have also been made to investigate the applicability of Bradford Assay to chemically modified proteins. Although modification of lysine groups has been reported to have no effect on the results of the Bradford Assay, no actual data was reported.[86] Recent reports have been contradictory.[87] While the Bradford Assay was not tested on modified, PEGylated protein, the modification is expected to affect the accuracy of the quantification. PEG interference has also been reported, the range of molecular weight was much higher than our application called for.[88] The reported did not account for attachment of PEG to the protein and blocking lysine sites. While working on quantifying the monoclonal antibody, Herceptin, conjugated to nanoparticles which our lab studies, lack of data and contradictory reports of the applicability of Bradford Assay for such estimation led to this study. This investigation was
undertaken mainly to assess the applicability of Bradford assay for estimating degree of protein conjugation when the protein has been modified. Interferences from other materials, if any, have been reported.

4.2 Theoretical Background:

The Bradford Assay is a colorimetric assay, and is widely used for protein quantification.[86, 107] The binding of the Bradford reagent, the main component of which is the Coomassie Blue G250 dye, to the protein present in the solution being measured forms a stable complex which is blue in color. This blue complex is measured at 595 nm. The dye exists in green and red forms in the unbound state. It forms covalent and ionic bonds with the different amino acids of the protein, arginine and lysine being the main amino acids interacting. Histidines, tryptophans, tyrosines and phenylalanine are also reported to interact.[107] It is important to keep in mind that the response of the assay depends heavily on the amino acid composition of the standard and measured proteins. It is recommended that the standard curve for protein measurement should be build using the protein under investigation. If that is not feasible, the two should be as close to each other as possible in terms of the composition. BSA that is widely used is about 66,000 Da. That is almost 1/3rd the MW of Herceptin. The amino acid composition also varies proportionately, and is bound to distinctly affect the accuracy of the assay. Hence, it becomes vital to assess the quality of the standard. Also, the free dye shows absorbance at 450 nm. Using the ratio of absorbances at 595/450 brings much more accuracy and eliminates the variability introduced by different amounts of dye added.[108]

4.3 Materials and Method:

Herceptin (Transtuzumab) was received as a generous gift from Genentech. The vial (440 mg) was reconstituted with sterile water and stored in small aliquots. For removing the
constituents present in packaged sample (L-histidine HCl, L-histidine, α-trehalose dihydrate, and Polysorbate 20), the antibody was buffer exchanged using Amicon filters (50,000MWCO).

NHS-PEG-alkyne linker was purchased from Quanta BioDesign (Powell, OH), which uses the primary amine of preferentially lysine as a binding site. Coomassie Blue reagent from Pierce Biotechnology was used. BGG was purchased from Thermo Scientific. Standard curves for both BSA and BGG were built as per protocol received from Thermo Scientific. Herceptin and BGG were modified with NHS ester linker (a ratio of 1:200) The NHS ester was dissolved in DMSO and diluted to required concentration with PBS (pH 7.2)

4.4 Results and Discussion:
Linearization was carried out to reduce the effect of different dye amounts, by also measuring the absorbance of the samples at 450nm, and the ratio of absorbances (595/450) was plotted and used for analysis.[108] The range of protein concentrations according to the standard protocol for Bradford Assay was from 0-25 ug/ml. We attempted to increase the range, but as can be seen, non-linearity is introduced at higher concentrations. Different linkers and reagents involved in the modification reaction were also tested to determine any detectable interference. The PEG linkers, as well as free NHS did not show any detectable response above the baseline. On modifying Herceptin with the linker, the deviation of the initial concentration reading of Herceptin in presence of the linker from the standard curve is remarkable, and remains high for the two concentrations (65 ug/ml and 72ug/ml) which leads us to add a caveat to the applicability of the Bradford assay for modified protein in the linear range only (0-25 ug/ml) for reasonable accuracy. The deviation is almost negligible for Herceptin at lower concentration (20 ug/ml). This deviation is prominent as a result of the non-linearity of standard curve at higher concentrations. We were interested in determining the applicability of the Bradford assay for the PEGylated protein over an extended period of time, to see if there was a change in the readings.
As can be seen, there is a decreased response that can be attributed to the conjugation of NHS ester to the lysine residues. The increase in the measured absorbance at 48 hours, however, remains to be accounted for. In the linear range, the deviation from the expected varies from -26% to +4%. Similar change over time can be seen for modified BGG at higher concentrations. Thus, application of the Bradford Assay should be assessed on a case to case basis, and possible deviation due to modified lysine residues should be taken into account while applying the Bradford Assay for modified proteins. The range of applicability should be taken into consideration. Interferences from the linker and the by-products formed should be ascertained. For the purpose of our application, modified Herceptin with attached PEG
linkers can be quantified using the Bradford assay as there is no overlap of absorbance wavelengths for any of the linkers used. There is some variation over time, and the quantification shows deviation of about 25% from the expected values. This variation can be due to the modification of lysine sites on the protein. Even then, using Herceptin to build the standard curve gives much more accurate results as compared to using BSA as the standard protein which severely overestimates the amount of protein present. If protein is not available, BGG should be used.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration</th>
<th>A595</th>
<th>A450</th>
<th>A595/A450</th>
</tr>
</thead>
<tbody>
<tr>
<td>225 Da linker</td>
<td>2mg/ml</td>
<td>0.297</td>
<td>0.349</td>
<td>0.851</td>
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<tr>
<td>333 Da linker</td>
<td>2mg/ml</td>
<td>0.285</td>
<td>0.321</td>
<td>0.888</td>
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<tr>
<td>2kDa linker</td>
<td>2mg/ml</td>
<td>0.286</td>
<td>0.332</td>
<td>0.861</td>
</tr>
<tr>
<td>DMSO</td>
<td>2mg/ml</td>
<td>0.284</td>
<td>0.33</td>
<td>0.861</td>
</tr>
<tr>
<td>Lysine</td>
<td>3mg/ml</td>
<td>0.629</td>
<td>0.209</td>
<td>3.009</td>
</tr>
<tr>
<td>NHS</td>
<td>2mg/ml</td>
<td>0.316</td>
<td>0.357</td>
<td>0.885</td>
</tr>
</tbody>
</table>

Table 3: Absorbance ratios for different reagents
Chapter 5: Immune Response

5.1 Introduction:

Immune response can be broadly categorized into innate and adaptive immune response, though both kinds interact frequently and also use some common machinery. Innate immunity includes multiple barriers, physical and chemical in nature. Elements of the innate immune system namely, the complement proteins, cytokines, and acute phase proteins, neutrophils, monocytes, macrophages, provide non-specific and immediate defense response in the host against a foreign agent.

Adaptive immune system is more specific in nature to specific antigens. This is a slower process, but adaptive response is a learned response to antigens, and stronger in nature. Activated macrophages release inter-cellular signaling molecules called cytokines. Introduction of a system not inherent to the human body stimulates the cells of the immune system, primarily macrophages and dendritic cells. On stimulation, these cells have been reported to produce pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8, IL-10, IL-18 tumor necrosis factor

![Figure 20: Some TNF-α and IL-1β results found in literature](image-url)

(Left) In vivo IL-1β levels measured on injection of various types of carbon nanotubes with Platinum.[110] (Right) TNFα level in an in vitro assay to measure the response of PBMC.[59]
alpha (TNF-α).[109] Production of these leads to increased migration of cells of the immune system in the area surrounding the infected area, thus mounting a stronger attack on the invading nanoparticle system.

IL-1α,β, TNF-α,β and perhaps IL-6 are regarded as broad-spectrum inflammatory mediators.[110] Almost all cells of different types are affected by IL-1β and TNF, although the receptors to which they bind are distinct. Their activities are quite similar, and although in vitro assays do not show any significant difference, their in vivo activities are different in subtle ways. IL-1β and TNF cause fever, inflammation, fibroplasia, and angiogenesis. Some of the effects IL-1β and TNF have in common could be because they induce similar cytokines and receptors such as IL 2-receptors, IL 6, colony stimulating factors and acute phase proteins. In some cases, IL-1β and TNF are released together for enhanced response. The study of these cytokines shows that there are some common as well as unique signal transduction pathways for IL-1β and TNF. Similar studies have been done for looking at the cytokine response to antibody infusions, PLGA nanoparticles and platinum carrying carbon nanotubes.[111]

5.2 Enzyme Linked Immuno Sorbent Assay (ELISA):

In vitro assay to assess the response of RAW 267.4 cells, a mouse macrophage cell line was used. There are different formats of ELISA; however the basic principle is the specificity of interaction between antigens and corresponding antibodies. Usually, one of the members of the pair, i.e. either the antibody or the antigen is immobilized or coated onto a surface, the complementary antibody or antigen is detected. Amplification and visualization of the antibody-antigen pair is enabled by using reagents conjugated to enzymes. The enzyme may be linked to a detection antibody. A specific substrate that interacts with the enzyme and shows a color change
proportional to the quantity is used to quantify the immune response. Standard curves are used to quantify the results.[112]

5.3 Procedure:

Multiple formulations were used for this test. NPs of different molecular weight formulations were used. The functional group that reacts with Herceptin-linker conjugate, the azide group, on the nanoparticle surface was controlled and varied for the different samples by varying the ratio of polymers during the nanoparticle formation. Hence, the amount of Herceptin conjugate to the NP was controlled. Samples with 1%, 10%, 50% and 100% Herceptin were tested. Based on the Bradford Assays, the concentration of Herceptin was roughly 50% of the concentration of the NP.

RAW 264.7 macrophage cells were seeded in a 96 well plate. After allowing them sufficient time for attachment, the different samples were dosed at a dilution of 10:1 with culture medium. After an overnight exposure, the supernatant was collected and the cells were lysed by a lysing solution (Triton-X solution). The supernatant was used to test for TNF-α, while the lysate was used to test for IL-1β. Capture Antibodies for TNF-α and IL-1β were coated onto a 96 well plate. After coating, to avoid any other protein coating the residual surface, a blocking agent was used, namely Bovine Serum Albumin (BSA). After washing with PBS and Tween, the test solutions were added to the respective capture antibody coated plates. After two hours, the test solutions were washed away. Primary antibodies for TNF-α and IL-1β were added. After an hour, the primary was washed, and a tag attached secondary was added. After half an hour, the secondary was washed, and the enzyme was added. The coloring substrate was added and the plates were read using a plate reader. Antigen concentration is proportional to the enzyme substrate reaction color development.
5.4 Results:

Dose Response Curve:

Samples: PEG-PLGA NPs – 70K PLGA, 5K PEG, Vehicle: PBS, Concentration: x = 1mg/ml

Response for the NPs was found to be dose dependent for TNF-α, increasing with some variation for IL-1β. Response for IL-1β is based on cell lysate and can be prone to losses. The dose response curve was used to determine a safe concentration which would ensure production of detectable concentration of the test cytokines. 10 x concentrations were chosen. As can be seen, we expected the response to Herceptin to be baseline, while the one for the NP to increase with the dose. However, this curve does not reflect on what the combination would work like.

Following this test, multiple formulations were tested. The results are as follows:
Run: 1 PLGA-PEG NPs - 30K PLGA, 5K PEG, Vehicle - PBS

The first run did not yield any conclusive results especially for IL-1β. In the next run, Herceptin concentration was also changed along with the concentration of NP.
Run 2: PLGA-PEG NPs - 50K PLGA, 5K PEG Vehicle - PBS

![Graph showing TNF-α ELISA results for Run 2](image)

![Graph showing IL-1β ELISA results for Run 2](image)

Figure 24: ELISA results for Run 2

Run 3: PLGA-PEG NPs - 15K PLGA, 5K PEG Vehicle - PBS
Figure 25: ELISA results for Run 3
5.5 Analysis:

Run 2 gave the most consistent trends. The immune response increased with conjugation of NP with Herceptin, but reduced on modifying Herceptin. Also, a higher protein cover might actually be detrimental. Independent binding studies have shown that about 20% Herceptin is enough to ensure strong binding to surface receptors. Higher coverage might indicate multi-layer adsorption which may impair the epitope binding ability of the antibody. Hence, a lower protein cover with modification might be an ideal formulation. However, the high TNF levels of the Vehicle control, which should ideally be at a minimum, indicate that the assessment of TNF levels may be faulty. In case of Run 3, LPS should have hit much higher levels. However, the vehicle and LPS levels are much closer than reason allows for. One possibility could be external contamination in the Vehicle. The other possibility discussed was that the cell-line itself could suffer a mycoplasma contamination. Thirdly, batch to batch variations in sample type and quality could also be adding to the variability.

5.6 Fluorescent Microscopy:

Visualization study using fluorescent microscopy was undertaken subsequently. The process may not be quantitative, but since ELISA did not yield repeatable results, this was a more direct approach. Nanoparticles were prepared with Coumarin-6 dye (responsible for the green color). DAPI was used to stain the nuclei.
Formulation: 15K PLGA-5K PEG, 100% Herceptin coverage

Concentration used: 10mg/ml diluted to 1 part in 10 of culture medium

Incubation time: 45 minute, followed by Washing 3 times with PBS

Another set of pictures were taken at a lower concentration treatment. Concentration used: 5mg/ml diluted to 1 part in 10 of culture medium

Incubation time: 45 minute, followed by Washing 3 times with PBS
5.7 Analysis:

The preliminary microscopy study shows the reduced uptake of the NP-Modified Herceptin samples as against the NP and the NP-Herceptin samples. The visibly apparent difference among the different figures is encouraging. Reduced green fluorescence associated
with the nanoparticles is seen for the modified Herceptin conjugated NP. However, more quantitative studies such as flow cytometric analysis would be needed for substantial verification.
of the hypothesis. This is a more direct test to study the interactions between the macrophage cells and the conjugates. Other cells lines, especially human cell lines can also be tested in the future.

Figure 31: NP-modified Herceptin treated cells
Chapter 6: Mass Spectrometry and Modeling

6.1 Need for Mass Spectrometry studies:

The NHS ester reaction is used to modify Herceptin. When it was initially introduced about 30 years ago, the reaction was said to be exclusive to the N-terminus or the e-primary amine of the lysine residue on proteins. However, a growing number of mass spectrometry studies in the recent times has shown to encounter other amino acids being modified by the NHS ester. One study found modifications on tyrosine and serine side chains on some of the studied peptides. [113] Similar results have been seen in studies by independent groups.[82, 83,114]

Using the same chemistry for modifying antibodies for commercial products needs a quality control study on the heterogeneity of the number and distribution of linkage sites.[115] A few papers have studied this area due to the large complexity in the data analysis for a protein of such size.[116,117]

Herein lays the complexity. Herceptin has close to 90 lysine sites. Hence any of those and depending on the amount of linker present, the degree of conjugation can be a variable. Adding to that, serines, tyrosines and threonines as possible sites of modification, the sheer magnitude of heterogeneity is extremely large. Quantification and assessment of this heterogeneity is necessary for quality control. Such a study is also important to determine the criteria for applying this system of modification to other targeting ligands. Binding of the targeting ligands to the
cognate antigen must remain a point to remember and must be retained reasonably for a successful targeted drug delivering moiety. Mass spectrometry is a widely used and preferred method of analysis for proteins. Mass spectrometric studies have been traditionally done to elucidate structure of proteins and other information using cross linking agents. The sites of modifications can be found using MS/MS studies. However, many challenges are encountered in the process.

A 1-D gel electrophoresis was carried out at the Proteomics Lab (East Campus). The primary purpose was to attempt to confirm if the 225Da linker was getting successfully conjugated to Herceptin, and hence see a mass shift due to the conjugation, since no peak could be detected on MALDI. The modified sample (in lane 3) shows a considerable shift in comparison to the unmodified heavy and light chains (lane 2). The lanes 6, 7 and 8 show the chains modified with the 2000Da linker. Polydispersity of the product is evident, a natural consequence of the polydisperse nature of the linker. An attempt was made by the lab to run a MS² study on the 225Da linker modified chains. The protein was digested using trypsin and the data was analyzed by MASCOT. 6 lysine sites of modification were found. However, the
antibody to linker ratio was low (1:50). Independent Mass spectrometric studies were proposed to be carried out to investigate the location of modifications. Thanks to Shimadzu’s results, a significant extent of modification (about 43 short linkers) could be seen to be attached.

6.2 Method:

Pure and modified protein were analyzed after digestion with trypsin by LC-MS. Along with the above samples, a blank was analyzed to identify the background. Modified Protein was Herceptin modified by the 225 Da NHS ester linker. Digestion buffer was prepared by adding 20 ul of 8 M urea, 0.4M Ammonium bicarbonate (pH 7.5-8.5) to the protein in water. 5 ul of 45 mM DTT was added to reduce the disulfide bonds, incubating the mixture at 50°C for 15 min to reduce the protein. On cooling to room temperature, 5ul of 100 mM Iodoacetamide was added and left in the dark at room temperature for 15 min. The digestion buffer was diluted to 2 M urea and 0.1 M Ammonium bicarbonate by adding water (35ul). 20 ug of Promega sequence grade modified trypsin was dissolved in 200 ul of 50 mM Acetic acid. 5 ul of trypsin (0.1 ug/ul) was added and the mixture was incubated at 37°C overnight. The digestion was stopped by acidifying the sample with TFA (10 ul of 3 % TFA solution). The data obtained from the experimental digestion was compared with a theoretical digest or in silico digest obtained from the Protein Prospector tool (University of California, San Francisco, http://prospector.ucsf.edu). Both mass values and m/z charge values were matched, with different percentage of matches being found for both. Finally, to identify the possible modification sites, the expected mass addition of 110.038 Da (or 220.076 Da for two linkers) was subtracted from the peak list obtained from the experiments. This data was compared to the in silico digest to spot the sites of modification, bearing in mind that multiple such sites (about 40) should exist according to the intact antibody
analysis. However, only 4 such sites were identified. Because of the discrepancies, further MS$^2$ analysis was not carried out.

6.3 Results:

In section 3A two heavy chain proteins were detected, unnamed protein product and Chain B, X-ray structure of the antigen-binding domains from three variants of humanized anti-p185-her2 antibody 4d5 and comparison with molecular modeling. In the first protein 6 sites of lysine modification with dPEG spacer were identified and in the second 3 sites were identified. In section 3B one light chain protein was identified; Chain A, X-ray structure of the antigen-binding domains from three variants of humanized anti-p185-her2 antibody 4d5 and comparison with molecular modeling, with 3 sites of lysine modification with the dPEG spacer. [Analysis by Proteomics Lab, East Campus, University of Cincinnati]

Section 3A:
Sequence Coverage: 40%

Matched peptides shown in Bold Red and Bold Green if K is modified with spacer
EVQLVESGGG LVQPGGSLRL SCAASGFNIK DTYIHWRQA PGKGLEWVAR
IYPTNGYTRY ADSVKGRFTI SADTSKNTAY LQMNSLRAED TAVYYCSR
GDGFYAMDYW GQGTLVTVSS ASTKGPSVFP LAPSSKSTSG GTAALGCLVK
DYFPEPVTVS WNSGALTSGV HTFPAVLQSS GLYSLSVVVT VPSSSLGTQT
YICNVTNKPS NTKVDKKVEP KSC

Section 3B
Sequence Coverage: 44%
Matched peptides shown in Bold Red and Bold Green if K is modified with spacer:

DIQMTQSPSS LSASVGDRVVT ITCRASQDVN TAVAWYQQKP GKAPKLLIYS
ASFLESGVPS RFSGRSSTGTD FTLTISSLQP EDFATYYCQQ HYTTTPPTFGQ
GTKVEIKRTV AAPSVFIFPP SDEQLKS GTA S V V C L NN FY P REAKVQWKV
DNALQSGNSQ ESVTEQDSDK D STYLSSTLT LSKADYEHKV YACEVTHQG
LSSPVTKSFN RGEC

Search Parameters:

Type of search: MS/MS Ion Search
Enzyme: Trypsin
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Deamidated (NQ), Oxidation (M), Delta: H (6) C (6) O (2) (K)
Mass values: Monoisotopic
Protein Mass: Unrestricted
Peptide Mass Tolerance: ± 1 Da
Fragment Mass Tolerance: ± 1 Da
Max Missed Cleavages: 4
Instrument type: ESI-TRAP

Using Protein Prospector, the corresponding search showed:

DIQMTQSPSSLASVGDRVVTITCRASQDVNTAVAWYQQKPGBKAPKLIYSAFLYSGVPSRFSGS
RSGTDFTLTISSLQPEDFATYYCQQHYTTPTFGQGTKVEIKRTVAAAPSVFIFPPSDEQLKS GTAV
VCLLNFYPREAKVQWKVDNALQSGNSQESVT EQDSDKSTYLSSTLTLSKADYEHKV YACE
VTHQGLSSPVTKSFN RGEC
EVQLVESGGGLVQPGSRLSCAASGFLIKDYTIHWVRQAPG KGLEWVARIDTNGYTRADS V
KGRFTISADTSKNTAYLQMNSLRAEDTAYYCSRWGGDGFWYAMDYWQGTVLTVSSASTKGP
For the search parameters:

Database: User Protein

Considered modifications: Deamidated (Q), Oxidation (M)

Digest Used: Trypsin

Max. # Missed Cleavages: 4

Constant Modification: Carbamidomethyl (C)

Minimum Digest Fragment Mass: 400

Maximum Digest Fragment Mass: 4000

Minimum Digest Fragment Length: 5

6.4 Analysis:

There are a few points of consideration if one is to dig further into the causes of discrepancies and solutions to aid such a study. Herceptin is a 148kDa protein, containing some 1320 amino acids. Some of the previous studies digging into the specificities of the NHS ester reaction were carried out on smaller, linear peptides sequences of roughly 15 amino acids. Such constructs do not need digestion protocol, and simplifies the data range. The possible species formed as a result of the modifications are limited; hence the presence of a distinct species in sufficient amount would allow its indisputable detection. Even in case of monoclonal antibodies,
though the sequence is quite homogeneous, there is an inherent heterogeneity in the final product since the processing includes cell cultures and purification steps. During the processing, many different modifications can be added which would impact the process of characterization. Many modifications have been reported in mAbs, including mutations, C-terminal lysine processing, glycation, oxidation, deamidation, pyroglutamic acid formation, isomerization, disulfide shuffling, peptide bond cleavage, etc. For resolving information specific to structural features of the protein in question, fragmentation of larger proteins into smaller peptides is necessary. The protein is digested into small peptides by a protein cutting protease, followed by LC/MS, or an LC/MS/MS analysis. Proteolytic digestion, combined with LC/MS/MS analysis, provides high structural resolution, however, it needs a large sample, is time-consuming, and labor-intensive. There are limitations to the fragments that can be analyzed/detected. Characterization of fragments greater than 4000Da is difficult, so is that of 2-3 amino acids, due to poor retention.

Getting the data is one part of the process, the challenging one is the data analysis. Using the peak data did not help automatically confirm the protein match using Protein Prospector. The initial knowledge of the sequence made it possible to directly compare the theoretical digest for Herceptin to the peaks found experimentally. Matching the fragment ions to the theoretically calculated ion m/z for each peptide was a labor intensive task.

A minimum of five peptide masses is necessary for matching the protein and 15% of the protein sequence needs to be covered for an unambiguous identification. For pure protein, 12 peptides were matched to the in silico digest, though the sequence match was only about 13%. Now, to take this further, on modification with the linker, the number of distinct species being formed rises exponentially taking the heterogeneity in the original antibody and the number of lysine residues that can be possibly modified. Add to that the other three members that can
possibly change i.e. serine, tyrosine and threonine, the resulting collection is such a diverse spread, that the intensity of resulting peaks may be extremely low, making it hard to identify modifications with confidence. One way to address this issue is to start out with a gel digestion, using separated heavy and light chains which might simplify the data analysis. Also, employing purification assays that will selectively concentrate only the fragments which have the linker present on them will simplify the process. Such a process has been employed to identify small proteins attached to an antibody.[116] The process employed immunoassays to separate and concentrate only the modified fragments, and hence simplified the detection.

6.5 Herceptin 3D Model and Mapping:

Since a number of lysine residues are available on an antibody, heterogeneity in conjugation is a common issue. Other reports have shown some other residues being modified as a result of the NHS ester reaction such as serine, tyrosine and arginine.[82, 83] Assessing this heterogeneity was one of the aims of this study. Studying the efficiency of conjugation and mapping the sites of modification. The general structure of an antibody consists of 4 polypeptide chains, 2 identical light and heavy chains respectively. An antibody can also be subdivided into the Fab and Fc regions. Herceptin is an engineered antibody with a Fab region from a mouse antibody and Fc fragment from human.[118] The Protein Databank (www.rcsb.org) has the structures for both the Fab fragment and Fc region of Herceptin (Entries 1N8Z and 3D6G).[119] The structure 1N8Z consists of 3 unique chains, two of which are of use in reconstructing the model.
These are Herceptin light and heavy chains, and the sequence for the tyrosine kinase receptor which works as the complexing ligand. Similarly, the sequence of the Fc region of Herceptin is available. To build the model, the first step was to duplicate the existing chains and align them with a structurally similar molecule to obtain its co-ordinates. Using Superpose, the two light and two heavy chains and the Fc region was aligned with the IgG structure 1HZH to obtain a set of symmetrical co-ordinates.[16] The PDB files were merged using a freeware called PDBeditor. The merged file was imported into SPDB Viewer.[120] The
SPDBViewer has fitting options, and were used to obtain the 3D structure of Herceptin. MS/MS data was obtained for Herceptin modified with the 225 Da linker. The sites of modification were mapped on the structure.
Chapter 7: Future Work

7.1 Optimization, in vivo response:

A number of parameters govern such nanoparticulate system and the biological responses. In vitro, the homogeneity in samples, lower batch to batch variation, and testing a range of parameters is necessary to establish repeatable results and protocols. In vivo, the conditions and hence the response may be completely different. In case of this system, size of the nanoparticles, surface functionalization and the role of azide and alkyne functional groups used on the cells, purity of processing, need to be separately checked and controlled. Working concentration range must be explored, or the effect of concentration of the samples and the resultant response data must be generated on a larger scale. A range of pre-clinical studies must be done to establish the stability of the system. Importantly, the range of modifications needs to be tested, which was not addressed in this work. Serum protein adsorption and such in vitro tests are needed to confirm the hypothesis. The binding ability of the antibody must be retained and hence a quality control check must be established.

Even for the immune response test, flow cytometric analysis or perhaps quantitative study using image processing software must be done. Using human monocytes for the study would be the next step.

7.2 PEG constraints:

Although PEG has been referred to as non-immunogenic, recent reports have described anti-PEG antibodies in the blood after injecting PEGylated material. Particularly the work on “Accelerated Blood Clearance” has described that repeated injections of PEGylated material causes the body to remove these systems out of circulation pretty quickly. Adaptive immune response to PEG has been observed.[121] Mouse studies revealed that on administering multiple
injections of an enzyme-PEG conjugate (β-glucuronidase-PEG), antibodies were produced for the protein (IgG) and for PEG (IgM).[71, 122, 123] Alternatives to PEG for in vivo applications will become a critical field of study. Several other materials are being tested, and will probably need vigorous testing in the future as better alternatives to PEG. Polyamides and Polysaccharides are being explored. Biomimetic or biomaterials are being studied for the same. Natural proteins such as lysines, arginines, glutamine or aspartic acid are being considered. Another class called poly-zwitterionic materials are also promising candidates. Examples include 2-methacryloyloxyethyl phosphorylcholine (MPC), sulfo betaine, methacrylate (SBMA), and carboxybetaine methacrylate (CBMA) and polyampholytes.[63]

7.3 Heterogeneity in tumor mass:

HER2 directed nanoparticles may prove effective against cells overexpressing HER2. However, cancer masses are normally not homogenous. Cancers have diverse types of cells like epithelial cells, fibroblasts, blood and lymphatic vessels forming cells, immune system cells and mesenchymal cells that may vary from tumor to tumor.[124] Hence, there are limitations to the efficacy of these systems. Applicability to other cancers needs to be ascertained.
References:


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<thead>
<tr>
<th>Cancer Type</th>
<th>Estimated New Cases</th>
<th>Estimated Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bladder</td>
<td>74,690</td>
<td>15,580</td>
</tr>
<tr>
<td>Breast (Female – Male)</td>
<td>232,670 – 2,360</td>
<td>40,000 – 430</td>
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<tr>
<td>Colon and Rectal (Combined)</td>
<td>136,830</td>
<td>50,310</td>
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<tr>
<td>Endometrial</td>
<td>52,630</td>
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<tr>
<td>Kidney (Renal Cell and Renal Pelvis) Cancer</td>
<td>63,920</td>
<td>13,860</td>
</tr>
<tr>
<td>Leukemia (All Types)</td>
<td>52,380</td>
<td>24,090</td>
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<td>Lung (Including Bronchus)</td>
<td>224,210</td>
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<td>Melanoma</td>
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<td>Pancreatic</td>
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<td>Prostate</td>
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<td>Thyroid</td>
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Table 4: Common Cancer types and Occurrence.
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<tr>
<th>International non-proprietary name</th>
<th>Trade name</th>
<th>Type</th>
<th>Indication first approved</th>
<th>First EU (US) approval year</th>
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<tr>
<td>Muromonab-CD3</td>
<td>Orthoclone Okt3</td>
<td>Anti-CD3; Murine IgG2a Fab</td>
<td>Reversal of kidney transplant rejection</td>
<td>1986* (1986#)</td>
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<tr>
<td>Abciximab</td>
<td>Reopro</td>
<td>Anti-GPIIb/IIa; Chimeric IgG1 Fab</td>
<td>Prevention of blood clots in angioplasty</td>
<td>1995* (1994)</td>
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<td>Rituximab</td>
<td>MabThera, Rituxan</td>
<td>Anti-CD20; Chimeric IgG1</td>
<td>Non-Hodgkin's lymphoma</td>
<td>1998 (1997)</td>
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<td>Daclizumab</td>
<td>Zenapax</td>
<td>Anti-IL2R; Humanized IgG1</td>
<td>Prevention of kidney transplant rejection</td>
<td>1999 (1997); #</td>
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<td>Palivizumab</td>
<td>Synagis</td>
<td>Anti-RSV; Humanized IgG1</td>
<td>Prevention of respiratory syncytial virus infection</td>
<td>1999 (1998)</td>
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<td>Infliximab</td>
<td>Remicade</td>
<td>Anti-TNF; Chimeric IgG1</td>
<td>Crohn disease</td>
<td>1999 (1998)</td>
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<td>Trastuzumab</td>
<td>Herceptin</td>
<td>Anti-HER2; Humanized IgG1</td>
<td>Breast cancer</td>
<td>2000 (1998)</td>
</tr>
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<td>Gemtuzumab ozogamicin</td>
<td>Mylotarg</td>
<td>Anti-CD33; Humanized IgG4</td>
<td>Acute myeloid leukemia</td>
<td>NA (2000#)</td>
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<td>Alemtuzumab</td>
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<td>Anti-CD52; Humanized IgG1</td>
<td>Chronic myeloid leukemia</td>
<td>2001 (2001)</td>
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<td>Adalimumab</td>
<td>Humira</td>
<td>Anti-TNF; Human IgG1</td>
<td>Rheumatoid arthritis</td>
<td>2003 (2002)</td>
</tr>
<tr>
<td>Efalizumab</td>
<td>Raptiva</td>
<td>Anti-CD11a; Humanized IgG1</td>
<td>Psoriasis</td>
<td>2004 (2003); #</td>
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<tr>
<td>Cetuximab</td>
<td>Erbitux</td>
<td>Anti-EGFR; Chimeric IgG1</td>
<td>Colorectal cancer</td>
<td>2004 (2004)</td>
</tr>
<tr>
<td>Omalizumab</td>
<td>Xolair</td>
<td>Anti-IgE; Humanized IgG1</td>
<td>Asthma</td>
<td>2005 (2003)</td>
</tr>
<tr>
<td>Drug Name</td>
<td>Trade Name</td>
<td>Therapeutic Target</td>
<td>Indication</td>
<td>Year/Status</td>
</tr>
<tr>
<td>--------------------</td>
<td>----------------</td>
<td>---------------------------------------------</td>
<td>-------------------------------------</td>
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</tr>
<tr>
<td>Ranibizumab</td>
<td>Lucentis</td>
<td>Anti-VEGF; Humanized IgG1 Fab</td>
<td>Macular degeneration</td>
<td>2007/2006</td>
</tr>
<tr>
<td>Certolizumab pegol</td>
<td>Cimzia</td>
<td>Anti-TNF; Humanized Fab, pegylated</td>
<td>Crohn disease</td>
<td>2009/2008</td>
</tr>
<tr>
<td>Golimumab</td>
<td>Simponi</td>
<td>Anti-TNF; Human IgG1</td>
<td>Crohn disease</td>
<td>2009/2009</td>
</tr>
<tr>
<td>Canakinumab</td>
<td>Ilaris</td>
<td>Anti-IL1b; Human IgG1</td>
<td>Rheumatoid arthritis, ankylosing spondylitis</td>
<td>2009/2009</td>
</tr>
<tr>
<td>Catumaxomab</td>
<td>Removab</td>
<td>Anti-EPCAM/CD3; Rat/mouse bispecific mAb</td>
<td>Malignant ascites</td>
<td>2009/NA</td>
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<tr>
<td>Ustekinumab</td>
<td>Stelara</td>
<td>Anti-IL12/23; Human IgG1</td>
<td>Psoriasis</td>
<td>2009/2009</td>
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<tr>
<td>Tocilizumab</td>
<td>RoActemra, Actemra</td>
<td>Anti-IL6R; Humanized IgG1</td>
<td>Rheumatoid arthritis</td>
<td>2009/2010</td>
</tr>
<tr>
<td>Ofatumumab</td>
<td>Arzerra</td>
<td>Anti-CD20; Human IgG1</td>
<td>Chronic lymphocytic leukemia</td>
<td>2010/2009</td>
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<tr>
<td>Denosumab</td>
<td>Prolia</td>
<td>Anti-RANK-L; Human IgG2</td>
<td>Bone Loss</td>
<td>2010/2010</td>
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<tr>
<td>Belimumab</td>
<td>Benlysta</td>
<td>Anti-BLyS; Human IgG1</td>
<td>Systemic lupus erythematosus</td>
<td>2011/2011</td>
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<tr>
<td>Ipilimumab</td>
<td>Yervoy</td>
<td>Anti-CTLA-4; Human IgG1</td>
<td>Metastatic melanoma</td>
<td>2011/2011</td>
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<tr>
<td>Brentuximab vedotin</td>
<td>Adcetris</td>
<td>Anti-CD30; Chimeric IgG1; immunoconjugate</td>
<td>Hodgkin lymphoma</td>
<td>2012/2011</td>
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<tr>
<td>Pertuzumab</td>
<td>Perjeta</td>
<td>Anti-HER2; humanized IgG1</td>
<td>Breast Cancer</td>
<td>2013/2012</td>
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<tr>
<td>Raxibacumab (Pending)</td>
<td></td>
<td>Anti-B. anthrasis PA; Human IgG1</td>
<td>Anthrax infection</td>
<td>NA/2012</td>
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<tr>
<td>Commercial Antibodies</td>
<td>Status</td>
<td>Approved Mabs</td>
<td></td>
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<tr>
<td><strong>Ado-Trastuzumab emtansine</strong></td>
<td>Kadcyla</td>
<td>Anti-HER2; humanized IgG1; immunoconjugate</td>
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<td><strong>Vedolizumab</strong></td>
<td>(Pending)</td>
<td>Anti-alpha4beta7 integrin; humanized IgG1</td>
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<td><strong>Ramucirumab</strong></td>
<td>(Pending)</td>
<td>Anti-VEGFR2; Human IgG1</td>
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<tr>
<td><strong>Obinutuzumab</strong></td>
<td>Gazyva</td>
<td>Anti-CD20; Humanized IgG1; Glycoengineered</td>
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<tr>
<td><strong>Siltuximab</strong></td>
<td>(Pending)</td>
<td>Anti-IL-6; Chimeric IgG1</td>
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<tr>
<td><strong>Secukinumab</strong></td>
<td>(Pending)</td>
<td>Anti-IL-17a; Human IgG1</td>
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<tr>
<td><strong>Nivolumab</strong></td>
<td>(Pending)</td>
<td>Anti-PD1; Human IgG4</td>
<td></td>
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<tr>
<td><strong>Lambrolizumab</strong></td>
<td>(Pending)</td>
<td>Anti-PD1; Humanized IgG4</td>
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<tr>
<td><strong>Dinutuximab</strong></td>
<td>(Pending)</td>
<td>Anti-GD2; Chimeric IgG1</td>
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</tr>
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</table>

Table 5: Commercial Antibodies and their status

*Country-specific approval; approved under concertation procedure; voluntarily withdrawn from market. BLyS, B lymphocyte stimulator; C5, complement 5; CD, cluster of differentiation; CTLA-4, cytotoxic T lymphocyte antigen 4; EGFR, epidermal growth factor receptor; EPCAM, epithelial cell adhesion molecule; GP, glycoprotein; IL, interleukin; NA, not approved; PA, protective antigen; RANK-L, receptor activator of NFkB ligand; RSV, respiratory syncytial virus; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor. Source: The Antibody Society website (http://www.antibodysociety.org/news/approved_mabs.php)