Antibody Conjugated Nanoparticles for Targeting Metastatic Triple Negative Breast Cancer

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Vidhi Devendra Khanna

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Jayanth Panyam

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Dedication

This thesis is dedicated to my wonderful, loving and supportive parents – Devendra Khanna and Aarti Khanna.
Abstract

Early detection and the availability of new treatments have improved the survival rates of patients presenting with local or regional breast cancer to as high as 99% and 85%, respectively. On the contrary, patients with metastatic disease have a dismal 5-year survival rate of 17%.\(^1\) Thus, there is an urgent need for treatment strategies directed towards metastasis.

Our lab has developed antibodies (Clone 6 and AM6) capable of recognizing tumor cells that have undergone epithelial-to-mesenchymal transition (EMT), a key step in the generation of circulating tumor cells and metastasis. The goal of the current study was to determine whether we use these antibodies as targeting ligands for directing anticancer drug-loaded polymeric nanoparticles to metastatic triple negative breast cancer cells as a novel therapeutic option.

Polymeric PLGA nanoparticles loaded with paclitaxel, a chemotherapeutic agent, were functionalized with the antibodies using thiol-maleimide chemistry. We optimized the conjugation reaction in order to achieve maximal cell uptake of nanoparticles without compromising antibody binding. \textit{In vitro} studies were carried out in an MDA-MB-231 derivative cell line with enhanced lung metastatic potential as well as a melanoma metastatic cell line M12. Clone 6 nanoparticles and AM6 nanoparticles showed significant improvement in cellular uptake as well as retention. A competition experiment confirmed target-mediated uptake of nanoparticles. Cytotoxicity studies showed improved cell kill using Clone 6 nanoparticles and AM6 nanoparticles. Based on these promising \textit{in vitro} results, we are currently carrying out \textit{in vivo} studies in mice. The development of a targeted drug delivery system for the treatment of metastatic triple negative breast cancer can significantly enhance the survival rate for patients who often times have a life-expectancy of less than one year.\(^2\)
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1. Introduction

Breast Cancer is the most common cause of malignancy in females, representing an astounding 29% of all diagnosed cases of cancer in the US.\textsuperscript{3} The diversity in molecular markers of breast cancer has made it difficult for physicians to recommend a uniform treatment methodology for all patients. However, the availability of these molecular markers in breast cancer can be a powerful tool for the development of more personalized therapies.

The recent classification schemes of breast cancer classification have been focused on genotype profiling. Perou et al\textsuperscript{4} characterized gene expression patterns of primary tumors from 42 patients along with 3 normal breast tissue samples. They found that breast epithelial tissues could be categorized into four sub-types – normal breast tissue, estrogen receptor (ER) positive (expressed a genetic and phenotypic profile closely related to breast luminal cells), ER negative (basal-like) and HER2 (also known as ERBB2+) expressing. The HER2 expressing tissues were also ER negative and thus were considered as a sub-category of a broader ER negative class.\textsuperscript{4} Sorlie et al\textsuperscript{5} refined this classification using a larger dataset and found that the ER positive group could also be further sub-divided into at least two sub-groups – Luminal A and Luminal B (and potentially even Luminal C).

\textbf{Figure 1: Classification of breast cancer based on genotypic profiling and molecular markers}
The luminal sub-type (ER positive) is the most common type of breast cancer and is most likely to be grade 1. It is associated with the highest survival rates due to its significant response to hormonal therapy and the lowest rate of TP53 mutations. Sortirou et al have noted differences in the survival rates between the different luminal sub-types. The HER2 positive variety of breast cancers is more likely to have mutations in the tumor suppressor gene TP53 (71%) and are also more often grade 3 tumors. Trastuzumab, a targeted antibody for the HER2 receptor has significantly improved outcome of patients presenting with HER2 positive breast cancer. Basal-like breast cancers, have a similar rate of TP53 mutations (82%) and are strongly associated with grade 3. These types of tumors are also termed as triple negative breast cancer (TNBC) due to the absence of ER, HER2 and PR (progesterone receptor). Recently, a third type of ER negative tumors have been identified that resemble the basal-like subtype closely, but have lower expression of the tight junction proteins – claudins. TNBCs are unfortunately not responsive to Trastuzumab due to absence of HER2 expression. Currently, the only options available to TNBC patients are conventional therapies resulting in significantly lower survival rates (figure 2).

Figure 2 (©National Academy of Sciences): Probability of overall survival depending upon molecular sub-type of breast cancer
Apart from classification based on molecular sub-types, all cancers are also classified into different stages from a clinical stand-point. The localized stage refers to when the primary tumor is localized in the originating organ, the regional stage represents presence of tumor cells in nearby lymph nodes, and the final stage is distant metastasis which signifies spread of tumor cells to organs other than the originating organ. Years of extensive research, improved diagnostic techniques and novel targeted therapies have extended the 5-year survival rates in the early stages of breast cancer to as high as 99% (localized stage) and 85% (regional stage). On the other hand, metastatic breast cancer has a 5-year survival rate of only 17%. Considering that TNBC patients have limited treatment options available to them, metastatic TNBC (M-TNBC) presents an even graver situation.

1.1. The Metastatic Cascade and Epithelial to Mesenchymal Transition (EMT)

In order to understand why metastatic breast cancer has significantly lower survival rates, it is important to first understand the metastatic cascade (figure 4). The structure of epithelial tissues involves a specialized extracellular matrix (ECM) known as the basement membrane (BM) that separates the epithelial and stromal compartments. Carcinomas, such as breast cancer, being epithelial in nature, initially develop on the epithelial side of this BM. The presence of the BM is a significant limiting factor for tumor growth. In order to overcome this barrier, cancer cells undergo a transition known as the Epithelial to Mesenchymal Transition or EMT. The most prominent transcription factors responsible for EMT are Slug, Snail and Twist (figure 3). Cells begin to lose their epithelial phenotype and acquire a more mesenchymal phenotype. The most notable changes include the loss of E-cadherin (protein involved in cell-cell adhesion), loss of cell polarity, increased invasiveness and increased motility. In addition, there is an imbalance in the levels of proteases and protein inhibitors, leading to increased activity of matrix metalloproteinases (MMPs). These proteases contribute to the break-down of the BM and remodeling of the surrounding ECM. Remodeling of the ECM leads to the release of tethered growth factors that further promote proliferation of cancer cells. The
loss of the BM followed by movement of the cells into the stromal compartment forms the first step of the metastatic cascade i.e., invasion.

Once within the stromal compartment, the mesenchymal properties of the tumor cells enable them to move into lymph and blood microvessels. This forms the next step of the metastatic cascade, ‘intravasation’. This step is dictated by a number of molecular mechanisms. One of the major players is transforming growth factor-β (TGF-β), which enhances cell penetration and invasiveness. Additionally, tumor associated macrophages (TAMs) also promote intravasation. Once in the blood and/or lymph vessel, these cells are termed as circulating tumor cells (CTCs). It is unclear how CTCs withstand the harsh circulation environment to reach distant organs where they extravasate (‘extravasation’) to seed micrometastasis. A select few of these micrometastasis are able to colonize the tissue and form macroscopic metastatic tumors.
Figure 4 (Reprinted from Biology of Cancer, published by Garland Science): The steps involved in the metastatic cascade – invasion, intravasation, transport through blood or lymph vessels, extravasation, micrometastasis, colonization.

It is clear that metastasis is a multi-step progression, with each step encompassing distinct molecular mechanisms. In order for the metastatic cascade to proceed, one key rate-limiting step is EMT. EMT forms the basis for the phenotypical changes necessary for a cell from the primary tumor to form a macroscopic metastatic tumor. Recently, EMT has been linked to acquiring not only a mesenchymal phenotype but also stem cell-like properties and drug resistance.  

1.2. EMT, Cancer Stem Cells and Drug Resistance

Anchorage independence, increased motility and invasiveness and enhanced proliferation are a few of the properties acquired by cancer cells as they undergo EMT. In addition to these properties, a study by S. Mani et al13 revealed a significant correlation between metastasis and cancer stem cells. In one of their preliminary experiments, they extraneously induced EMT in normal human mammillary epithelial (HMLE) cells. In sync with EMT induction, they observed an increase in the population of cells with stem
cell like characteristics i.e. CD44$^{\text{High}}$/CD24$^{\text{Low}}$ (figure 5). These characteristics correlated with a small sub-population of cells found in tumors known as cancer stem cells (CSCs) or tumor initiating cells. CSCs are unique in their abilities of seeding tumors at much lower cell numbers and continued self-renewal as compared to fully differentiated tumor cells. In fact, one of the latter experiments by Mani et al tested the tumor seeding capability of EMT induced, CSC-like cells, and found that a 100-fold higher number of normal HMLE cells were required to seed a tumor as compared to EMT-induced HMLE cells.

Apart from enhanced tumor inducing capability, EMT has also been observed to confer cells with drug resistance by upregulating the expression of ABC transporters on the surface. A study by Hollier et al showed an increase in the IC50 for paclitaxel on inducing EMT in MCF7 breast cancer cell line. A similar study by Saxena et al on HMLE cells showed an increase in IC50 for doxorubicin in EMT phenotypic cells. All the above evidence suggests that not only is EMT responsible for the development of secondary tumors, but it may also be responsible for the development of more aggressive, resistant tumors, contributing to poor therapeutic outcomes for patients with metastatic cancer.
A slightly different perspective of why metastatic cancers are harder to treat is provided by Dr. Weinberg in his book “The biology of Cancer” — in the case of breast cancer, the primary tumor is in a non-vital organ that can easily be resected from the body. On the other hand, the most common sites for breast cancer metastasis are liver, bone and lung. Metastasis in these vital organs can lead to compromised functionality and irreversible, fatal consequences.

1.3. Therapeutic Options in Breast Cancer

Targeted therapies have shown much promise in breast cancer. Trastuzumab (Herceptin), the therapeutic antibody that has been developed for the treatment of HER2+ patients, improved the overall survival by 37%. HER2 is one of many transmembrane tyrosine kinase receptors responsible for cell growth and survival. In general, this family of receptors is activated by ligand binding, followed by receptor dimerization. In contrast, HER2 is able to acquire a dimerized state without activation by any ligand. Receptor overexpression (often observed in cancer) is also able to cause dimerization, promoting cell proliferation. Binding of trastuzumab to the extracellular domain of HER2 possibly leads to increased destruction of the receptor, inhibition of turnover of the receptor, and/or antibody-dependent cellular cytotoxicity. Considering that the HER2 receptor is overexpressed in almost 20-30% of breast cancer patients, the development of trastuzumab has been a remarkable achievement in the field of breast cancer.

The luminal subtypes of breast cancer also have targeted hormonal therapies available such as Tamoxifen, Anastrozole, Exemestane, and Letrozole. Unfortunately, despite all the progress made with to targeted therapies, this success has been largely limited to patients overexpressing either the ER/PR and/or the HER2 receptor. On the other hand, basal-like breast cancer (ER-/PR-/HER2-) patients are forced to resort to more traditional chemotherapy and radiation options due to the lack of targeted therapies. Interestingly,
Carey et al\textsuperscript{20} studied the response of luminal type, basal like and HER2+/ER- breast cancers to primary chemotherapeutic compounds doxorubicin and cyclophosphamide. Basal-like or TNBC tumors were most responsive (85\%) to chemotherapy. A similar observation was made by Liedtke et al\textsuperscript{21} where, TNBC patients responded to neoadjuvant chemotherapy better than non-TNBC patients. Thus, although primary TNBC has a higher sensitivity to traditional treatments as opposed to other forms of breast cancer\textsuperscript{21}, the metastatic disease is aggressive and resistant to these drugs resulting in significantly lower survival rates.

1.4. Metastatic Triple Negative Breast Cancer (M-TNBC)

On an average, about 11\% of all breast cancer patients present with TNBC.\textsuperscript{2} A study by Onitilo A. et al\textsuperscript{2} looked at the clinicopathological features and survival of breast cancer patients and found that patients presenting with TNBC had the worst overall and disease-free survival rate. They found that TNBC had a significantly higher risk of recurrence post diagnosis and that there was a rapid progression from distant recurrence (metastasis) to death. As high as 34\% of TNBC cases result in distant metastasis.\textsuperscript{2} As a result, patients presenting with M-TNBC have a poor prognosis and a life expectancy of less than one year.\textsuperscript{2}

A major portion of clinical trials being conducted in M-TNBC have been focused on combination therapy. One such trial by Miller et al\textsuperscript{22} tested paclitaxel with bevacizumab (anti-VEGF antibody Avastin\textsuperscript{®}). The combination therapy prolonged the progression-free survival in patients but there was no change in the overall survival. Another study by the same group (Miller et al\textsuperscript{23}) tested combination therapy of capecitabine with bevacizumab, which found no change in progression-free or overall survival. A number of other trials have met similar, significant but insufficient results.\textsuperscript{24-26}

One of the most prominent class of newer drugs being tested for M-TNBC are Poly(ADP-ribose) polymerase (PARP) inhibitors. PARP is an enzyme responsible for
repair of single-stranded breaks in the DNA. PARP inhibitors have a synergistic effect with BRCA1/2 mutations, since BRCA1/2 proteins are responsible for repair of double-stranded breaks. Considering that BRCA1/2 mutations are more common in the basal-like phenotype of breast cancer, PARP inhibitors could be a promising approach for the treatment of M-TNBC. Although several clinical trials with PARP inhibitors have shown promising results, they have also faced issues of toxicity and inadequate end-points. Thus, even though PARP inhibitors have shown potential, there is still no drug available specifically for the treatment of M-TNBC. This follows that newer, targeted drug delivery systems, capable of targeting TNBC in its metastatic form, would fulfil a much needed gap in cancer therapeutics.

1.5. Nanoparticles for Targeted Anticancer Drug Delivery

Nanomedicine has gained significant importance in cancer therapy over the past few years. The primary advantage of nanoparticles is their size range (1 to 500nm) due to which they are internalized into cells better than larger sized delivery systems such as microparticles. This is essential for chemotherapeutic drugs such as paclitaxel that act intracellularly. The surface properties of nanoparticles can be modified to be hydrophobic or hydrophilic depending upon the requirement. Further, a drug loaded in nanoparticles remains protected for longer and nanoparticles can be formulated to act as a controlled release reservoir for the drug. Nanoparticles have also been engineered for targeted drug delivery to avoid toxicity and improve efficacy. Nanoparticles also have a unique passive targeting feature in case of cancer therapy.

In normal tissues, the blood vessels are lined by tightly packed endothelial cells that are highly selective in terms of what moves out of the lumen into the interstitial fluid. As opposed to this, in case of tumors, the vasculature is leaky and pores between endothelial cells are abnormally large. This is due to rapid and incomplete development of the tumor vasculature. Additionally, the tumor vasculature has a missing layer of pericytes surrounding the vessels. This uniquely leaky vasculature of the tumor allows
nanoparticles to preferentially “leak” out of the blood vessels and move into the tumor interstitial fluid. This would not be possible in case of other organs due to the tight endothelial junctions, liver being the exception.

Under normal conditions, fluid draining from the blood vessels into tissue is cleared via the lymphatic system. However, tumors lack an efficient lymphatic system, as a result of which, there is elevated interstitial fluid pressure (IFP). Thus, the nanoparticles move into the tumor relatively easily and are unable to move out. This phenomenon of preferential uptake of nanoparticles followed by prolonged retention in the tumor is termed as the “Enhanced Permeability and Retention” effect or the EPR effect.

Although the EPR effect has been of the main driving forces for the development of cancer nanotherapeutics, it is important to recognize that the EPR effect is not a universal phenomenon. Not all tumor vessels are leaky to the same extent, not all pores are of uniform size, and thus, not all tumors exhibit the EPR effect.
An extension of the EPR effect is active targeting. Actively targeted drug delivery involves the use of specific ligands that are able to recognize antigens or proteins that are overexpressed by the tumor. The ligands used for active targeting generally involve proteins, the most popular being antibodies and their fragments. In vivo stability, availability of different forms of antibodies (scFV, Fab fragment, dimers, whole antibody), and the availability of a myriad of techniques for antibody engineering is what makes them highly amenable for use in targeted drug delivery. Active targeting enables nanoparticles to “actively” bind to the cell surface and undergo receptor mediated internalization. Not only does active targeting provide the carrier with additional means for endocytosis, but also a target to “hold on” to in the tumor, resulting in improved retention. Recent reports have pointed out that the main advantage of actively targeted nanoparticles is the improved internalization of nanoparticles, however the overall biodistribution remains unchanged. Targeted drug delivery (active or passive) has the potential to reduce the toxicity associated with the drug and improve its overall efficacy.

1.6. Targeted Drug Delivery for M-TNBC

As discussed earlier, patients presenting with M-TNBC are faced with limited treatment options that include traditional chemotherapeutic agents such as paclitaxel, doxorubicin, and docetaxel. These drugs are associated with severe side-effects due to their non-specific toxicity, and development of drug resistance is not uncommon. In fact, drug resistance is one of the major causes for treatment failure in case of M-TNBC. Multiple agents have been tried to overcome this problem including capecitabine, gemcitabine, platinum based therapies, but with limited success. Targeted therapies that have been tried for M-TNBC include EGFR targeted antibodies (bevacizumab) and PARP inhibitors, again with limited success. Aside from these, a number of groups have attempted to develop targeted drug delivery systems for the treatment of M-TNBC. Kutty et al formulated doxorubicin-loaded micelles using Cetuximab (EGFR targeted antibody) as the targeting ligand. They observed a significant improvement in
cytotoxicity towards EGFR overexpressing cell lines. Another recent study by Song et al\textsuperscript{42} described the development of CAR T cells directed towards the folate receptor - \(\alpha\) in TNBC, with some success. Most clinical trials for M-TNBC at this point are focused on combination therapies, with a few antibody-drug conjugates in the pipeline.\textsuperscript{43}

We hypothesized that a delivery system targeting metastatic lesions will improve the efficacy of an existing chemotherapeutic drug, thus providing a viable therapeutic option for M-TNBC patients. Since nanoparticles are highly suitable for use as a targeted drug delivery platform, we decided to formulate paclitaxel loaded targeted antibody-conjugated nanoparticles for the treatment of metastatic breast cancer. Considering that EMT is a critical, rate-limiting step for the development of metastasis, our lab developed an antibody capable of binding to tumor cells with EMT phenotype. This antibody was selected as the targeting ligand for the formulation of targeted nanoparticles.

1.7. Antibody Development\textsuperscript{44}

An scFV-based phage display method was used for the development of a humanized IgG antibody that binds specifically to EMT phenotypic cells. This method utilizes large, diverse libraries of bacteriophage (bacterial viruses) displaying antibody fragments on their surface for \textit{in vitro} high-throughput screening against the desired peptide/antigen. Sequential enrichment of antibody fragments that display specificity for the given target eventually leads to a manageable number of candidates that can be reformatted into fully human IgGs using modern molecular biology techniques.

Since in our case the antigen was unknown, we used a bio-panning procedure with paired isogenic mammary epithelial cell lines (HMLE – control mammary epithelial cells, and HMLE-Twist1, an EMT phenotypic cell line) to display the antigen in its native form. A mixed population of the two cell lines was exposed to the phage display library and flow cytometry was used to selectively sort HMLE-Twist1 target cells and collect the binders. Repeated iterations of this procedure successively narrowed down the phage library to
finally yield an scFv with selective affinity to EMT cells. This scFv was subsequently formatted into a fully human IgG, now termed as Clone 6.

Target deconvolution revealed HSPG2/Perlecan Domain 1 as the EMT cell surface antigen bound by Clone 6. Perlecan is a heavily sulfated proteoglycan ubiquitously found in most vascularized tissues.\textsuperscript{45} It is part of a larger group of proteoglycans known as Heparan Sulfate Proteoglycans (HSPGs). It is a critical proteoglycan in the tumor stroma, tethering a number of essential growth factors such as the basic Fibroblast Growth Factor (bFGF). During EMT, there is an increase in the enzyme heparanase which results in cleavage of sulfated glycosaminoglycans. Perlecan is thus cleaved of its sulfated residues causing the release of growth factors promoting metastasis. Perlecan has also been observed to be more closely associated with tumor cells once released from the basement membrane. The drastic increase in perlecan concentration observed in case of melanoma metastasis agrees with the hypothesis that perlecan is a good marker for metastasis.\textsuperscript{46}

Our initial studies have shown that Clone 6 can be used as a diagnostic agent for the detection of mesenchymal CTCs. In order to establish the specificity to a metastatic breast cancer cell line, we tested binding to LM2 cells (an MDA-MB-231 derivative that is derived from ex vivo expanded spontaneous lung metastases) and found the Kd value
to be between 125-275 nM (figure 7A). Due to previous studies showing Perlecan being overexpressed in melanoma metastasis\textsuperscript{46}, we also tested Clone 6 binding to M12 (melanoma brain metastasis cell line developed from patient derived xenograft brain metastasis) and obtained similar results (figure 7B). We have also been successful in improving the affinity of this antibody by using mutagenic PCR to randomize key amino acid residues most responsible for antigen binding. This led to the development of a second antibody with a Kd value of 10 nM, termed as AM6.

1.8. Engineering Antibody-Conjugated Nanoparticles

Poly(DL-lactide-co-glycolide) (PLGA), an FDA approved polymer, has been one of the most commonly employed polymers in controlled release formulations and tissue engineering applications due its safety, acceptable toxicity profile, biocompatibility and biodegradability\textsuperscript{47}. PLGA is degraded \textit{in vivo} by hydrolysis into biologically compatible lactic acid and glycolic acid, both of which are eliminated from the body via the tricarboxylic acid (TCA) cycle. Drug release from PLGA matrix takes place initially by simple diffusion, followed by a combination of drug diffusion and polymer degradation. The rate of drug release from PLGA can be customized by varying the lactide to glycolide ratio and the molecular weight of the polymer.\textsuperscript{48} Additionally, PLGA nanoparticles have been observed to escape endo-lysosomal degradation via a pH dependent charge reversal, prolonging their half-life within the cell.\textsuperscript{49} Based on these desirable properties, we decided to formulate our nanoparticles using PLGA.

Despite its several advantages, PLGA still faces the most common pharmacokinetic problem observed with nanoparticles – rapid clearance by the mononuclear phagocytic system (MPS).\textsuperscript{50} Once nanoparticles are injected into the bloodstream, they are almost instantly covered by opsonins that tag them as “foreign” for phagocytosis by macrophages. This results in rapid clearance of nanoparticles from the bloodstream, preventing them from reaching the site of action. In order to overcome this issue, multiple solutions have been tried, but by far the most successful and most popular is termed as
‘PEGylation’. PEGylation involves the introduction of poly(ethylene glycol) or PEG chains on the surface of nanoparticles. Presence of the PEG layer forms a hydrophilic barrier, reducing adsorption of opsonins. This allows the nanoparticles to remain “hidden” from the MPS, significantly extending their circulation half-life. Thus, we decided to use PEGylated PLGA nanoparticles as our primary delivery system. Paclitaxel was chosen for the drug loaded nanoparticles since taxanes (Abraxane, for example) are used to treat M-TNBC, although the metastatic phenotype exhibits increasing resistance to paclitaxel. Improved drug delivery via nanoparticles could potentially improve cytotoxicity in M-TNBC. Paclitaxel acts by binding to tubulin and stabilizing the microtubules during mitosis, leading to cell death.

Our lab uses the emulsion-solvent evaporation process for nanoparticles synthesis. In brief, this involves formulation of an oil-in-water emulsion with the polymer dissolved in the oil phase (organic solvent), followed by evaporation of the oil phase to form solid nanoparticles. An earlier publication from our lab describes the introduction of surface functional groups on nanoparticles by Interfacial Activity Assisted Surface Functionalization (IAASF). This particular method is applicable to the solvent-emulsion evaporation technique of nanoparticle formulation. After the oil-in-water emulsion is formed, if a diblock co-polymer is introduced into the emulsion, its hydrophobic portion will align within the oil phase while the hydrophilic portion will align in the water phase. This expected orientation can be utilized to our advantage by the introduction of the required functional group on the hydrophilic portion of the polymer that will align outwards. We used IAASF for the introduction of PEG with terminal maleimide groups on the nanoparticle surface.

Thiol-Maleimide chemistry has been commonly used for hetero-functional crosslinking of proteins. Here we sought out to use thiol-maleimide chemistry to conjugate antibodies to nanoparticles. Free thiols are not found on a native IgG in its most stable configuration. Due to their reactive nature, thiol groups are often introduced on the IgG structure for chemical conjugation reactions. There are multiple ways through which
thiols can be introduced on an IgG including lysine modification by Traut’s reagent, lysine modification by N-succinimidyl-S-acetylthioacetate (SATA), and reducing the hinge disulfides using 2-mercaptoethanolamine. In our case, we used 2-immunothiolane (Traut’s Reagent) to introduce a reactive thiol group on the lysine amino acids on the IgG structure. It must be noted, however, that not all lysines are amenable to modification, simply because they may be embedded in a hydrophobic region that is not exposed to the environment. The thiolated antibodies are then reacted with the maleimide functionalized PLGA nanoparticles to form a stable thiol-maleimide bond.

Figure 8: Schematic of antibody-conjugated nanoparticles

1.9. Specific Aims

The specific aims of this thesis are as follows:

**Specific Aim 1**: Development and optimization of anti-perlecan antibody conjugated nanoparticle platform for drug delivery to breast cancer metastasis.

**Specific Aim 2**: *In vitro* evaluation of the perlecan-targeted nanoparticles followed by preliminary *in vivo* studies.
2. Methods

2.1. Materials

PLGA (50:50, 0.55-0.75Dl/g) was purchased from LACTEL Absorbable Polymers (Birmingham, AL). PLA-PEG-Maleimide (Al119) and PLGA-Rhodamine (AV011) was purchased from PolySciTech (West Lafayette, IN). Polyvinyl alcohol (PVA, 30,000-70,000 MW), Coumarin-6, sucrose, 2-immunothiolane hydrochloride, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma (St. Louis, MO). Paclitaxel was purchased from Phytogen Life Science (B.C, Canada). Borate buffer stock was purchased from Alfa Aeser (Ward Hill, MA). Control Isotype IgG (Cat. No. 401114) was purchased from Calbiochem (Billerica, MA). The materials for SDS/PAGE were obtained from Bio-Rad (Hercules, CA). HPLC grade organic solvents were obtained from Fisher Scientific (Pittsburgh, PA). All cell culture media and buffers (including phosphate buffered saline or PBS) were purchased from Corning (Tewksbury MA) or Life Technologies (Carlsbad, CA) unless otherwise specified. Deionized (DI) water was available through university resources.

2.2. Preparation of Maleimide Functionalized PLGA Nanoparticles

Maleimide functionalized PLGA nanoparticles loaded with paclitaxel and 6-coumarin or rhodamine were synthesized as described in an earlier publication.\textsuperscript{55} PLGA (30 mg) and Coumarin-6 (100 µg) or PLGA (25mg) and PLGA-rhodamine (5mg), along with paclitaxel (5mg) were dissolved in 1 ml of chloroform. An oil-in-water emulsion was formed by emulsifying the polymer-drug solution in 8 ml of 2.5% w/v aqueous PVA solution by probe sonication (18–24 W; Sonicator XL, Misonix, Melville, NY) for 5 minutes over an ice bath. The diblock copolymer polylactide-polyethylene glycol with terminal maleimide functionalization (PLA-PEG-Maleimide; 8 mg) was dissolved in chloroform (200µl) and added drop-wise to the above emulsion with stirring. The emulsion was stirred for 16 -18 hours under ambient conditions followed by 1 hour under vacuum to remove the residual chloroform. Nanoparticles were washed twice by ultracentrifugation (35,000 rpm for 35 min at 4°C, Optima XPN-80, Beckman Palo Alta,
CA) and reconstitution in DI water. The final nanoparticle dispersion was then stored at 4°C until conjugation reaction on the same day.

2.3. Preparation and Purification of Antibodies

Expi293 Expression System by Life Technologies (Carlsbad, CA) was used for the expression of human IgG antibodies. Affinity purification of antibodies was carried out using Protein A Plus (Pierce, Rockford, IL) followed by buffer exchange into tris buffered saline containing 5mM EDTA. Zeba™ Spin Desalting Columns (87769, Pierce Biotechnology, Rockford, IL) were used for the buffer exchange step. Antibody stocks were stored at -20°C in single use aliquots until use. Once thawed, the samples were placed at 4°C for short-term storage. Quality control evaluation involved resolution via SDS/PAGE for the reduced and non-reduced samples.

Flow cytometry was carried out to confirm binding to whole cells. The antibodies were incubated with LM2 cells at a concentration of 100nM for 1 hour at 4°C on a rotating platform (Barnstead International, Dubuque, IA). The cells were then washed three times using FACS Buffer (PBS containing 0.5% BSA, 2mM EDTA and 0.05% sodium azide). Alexa 647 goat anti-human secondary antibody (Life Technologies, Carlsbad, CA) was added and the cells were incubated at 4°C for one hour on a rotating platform. Finally, the cells were washed 3 times, re-suspended and placed on ice until analysis by flow cytometry (BD LSRFortessa).

2.4. Optimization of Antibody Thiolation

300 µg of purified antibody (Clone 6, AM6 or isotype IgG control) was thiolated in 0.1M borate buffer (pH 8) with 5mM EDTA. The thiolation reaction was first optimized to ensure the formation of a thiol-maleimide bond as well as minimize changes to the binding affinity of the antibodies. A 20 – 200 fold molar excess of the thiolating reagent 2-iminothiolane (also known as Traut’s reagent) was used for the reaction. The samples were placed on a rotating platform for 2 hours at room temperature, followed by desalting (Zeba™ Spin Desalting Columns 87769, Pierce Biotechnology, Rockford, IL)
into PBS with 5mM EDTA. Number of thiols per antibody was determined using Amplite fluorimetric thiol assay kit by AAT Bioquest (Sunnyvale, CA). Any effects on antibody binding were tested using flow cytometry as described under subheading 2.3.

2.5. Conjugation of Antibody to Nanoparticles

Once the thiolated antibodies were desalted, the samples were immediately added to the nanoparticle dispersions and placed on a rotating platform at 4°C to allow the conjugation reaction to take place overnight. Next morning, nanoparticles were washed once by ultracentrifugation (35,000 rpm for 35 min at 4°C) and dispersed in 5ml DI water containing 30mg sucrose (lyoprotectant). The final dispersion was probe sonicated (30 seconds, 6-9W) on an ice bath and centrifuged at 1000RPM for 5min to pellet any large aggregates. The supernatant was then lyophilized (Labconco, FreeZone 4.5, Kansas City, MO). The lyophilized product was stored in a desiccator at 4°C until use.

2.6. Physiochemical Characterization of Nanoparticles

Hydrodynamic diameter and zeta potential of nanoparticles in DI water were determined using DelsaTM Nano C (Beckman Coulter Inc., Fullerton, CA, USA). Amplite™ Colorimetric Maleimide Quantitation Kit was used for maleimide quantification. To determine paclitaxel loading, nanoparticles were dispersed in methanol (1 mg/ml) and incubated overnight at room temperature. The dispersion was then centrifuged at 13,000RPM for 10 min and the supernatant was processed for HPLC analysis. Coumarin-6 and/or Paclitaxel loading was determined using an HPLC method previously described.56

2.7. Confirmation of Antibody Conjugation and Loading

One milligram of nanoparticles (Clone 6 conjugated, AM6 conjugated, Isotype IgG conjugated and blank nanoparticles) was dispersed in 40µl DI water and analyzed for protein concentration using bicinchoninic acid assay (Pierce BCA Protein Assay, Rockford, IL). In order to confirm the formation of a thiol-maleimide bond, we used gel electrophoresis. One milligram of nanoparticles was resuspended in 25µl DI water. To
each tube, 25μl of 95:5 2X Gel Loading Buffer:2-Mercaptoethanol (reduced samples) or 25μl of 2X Gel Loading Buffer (non-reduced samples) was added. The samples were placed in a water bath at 90°C for 10 min, centrifuged at 13,000RPM for 10 min and the supernatants were resolved on a 4-10% SDS/PAGE Criterion Precast Gel (Bio-Rad, Hercules, CA) along with the respective antibody controls.

2.8. Cell Culture

Luciferase expressing LM2 cells were a generous gift from Dr. Deepali Sachdev, University of Minnesota. LM2 cells were derived from spontaneous lung metastases isolated from mice carrying MDA-MB-231 tumors. LM2 cells between passages 2-10 (following initial passages in our lab) were used for all studies. They were cultured in minimum essential medium (MEM) with 10% v/v Fetal Bovine Serum (FBS) and 1% v/v penicillin/streptomycin in a humidified atmosphere with 5% CO2. M12 cells were a gift from Dr. Jann Sarkaria, Mayo Clinic. M12 cells have been expanded from a patient-derived xenograft of melanoma brain metastasis. M12 cells between passages 5-10 were used for studies. They were cultured in RPMI with 1X insulin-transferrin-selenium (ITS), 10% v/v FBS and 1% v/v penicillin/streptomycin in a humidified atmosphere with 5% CO2.

2.9. Cell Uptake of Antibody Conjugated Nanoparticles analyzed by HPLC

Coumarin-6 loaded nanoparticles were used for this study. LM2 cells were seeded at 50,000 cells per well in a 24 well plate and allowed to adhere overnight. Cells were washed once with PBS and fresh media was added. The cells were incubated at 4°C for 1 hour with Coumarin-6 loaded nanoparticles at a final concentration of 100μg/ml to allow binding to the target (n=3 or 4). At the end of 1 hours, the media was replaced with fresh media and the cells were placed at 37°C to allow internalization. The cells were lysed at 60 minutes post addition of fresh media using 100μL RIPA. Part of the cell lysate (30μL) was used to determine protein concentration by BCA assay and the rest was processed for HPLC analysis. Coumarin-6 was extracted into 0.5mL methanol overnight, the samples
were centrifuged at 13,000 RPM for 10 min and the supernatant was analyzed for coumarin-6 concentrations using HPLC.  

2.10. **Cell Uptake of Antibody Conjugated Nanoparticles analyzed by Flow Cytometry**

Rhodamine labelled nanoparticles were used for this study. 200,000 LM2 or M12 cells suspended in 150µL FACS buffer were incubated with 50µg suspension of nanoparticles on a rotating platform at 37°C for 1 hour (n=2). At the end of one hour, the cells were washed twice using FACS Buffer, fixed using 4% v/v formaldehyde in FACS Buffer, washed once followed by analysis using flow cytometry. Flow cytometry analysis was carried out using BD LSRFortessa H0081. In order to normalize data to rhodamine loading, the fluorescence of the initial nanoparticle suspensions was measured using the IVIS Spectrum *in vivo* Imaging System (Caliper Lifesciences, Hopkinton, MA).

2.11. **Exocytosis of Antibody Conjugated Nanoparticles**

LM2 cells were seeded at 50,000 cells per well in a 24 well plate and allowed to adhere overnight. Cells were washed once with PBS and fresh media was added. The cells were incubated at 37°C for 2 hours with Coumarin-6 loaded nanoparticles at a final concentration of 100μg/ml. At the end of 2 hours, the cells were washed once with and fresh media was added to allow exocytosis. The cells were lysed at 0, 15, 30 and 60 minutes post addition of fresh media using 100µL RIPA. Part of the cell lysate (30µL) was used to determine protein concentration by BCA assay and the rest was processed for HPLC analysis. Coumarin-6 was extracted into 0.5mL methanol overnight, the samples were centrifuged at 13,000 RPM for 10 min and the supernatant was analyzed for coumarin-6 concentrations using HPLC.  

2.12. **Competition Experiment**

LM2 cells (200,000) in suspension, were incubated at 4°C with 1µM (final concentration) of antibodies (or no antibody in case of control) for 1 hour on a rotating platform. At the end of 1 hour, the cells were washed three times using FACS buffer and re-suspended in
fresh FACS buffer. 50µg antibody conjugated nanoparticles (Rhodamine labelled) were added to the respective antibody treated/control tubes. The cells were once again incubated at 4°C for 1 hour on a rotating platform. Finally, the cells were washed three times using FACS buffer, fixed using 4% v/v formaldehyde in FACS Buffer and washed once. Flow cytometry analysis was carried out using BD LSRFortessa H0081.

2.13. Cytotoxicity Study
LM2 cells were seeded at 2500 cells per well in a 96-well plate and allowed to adhere overnight. Treatments (free paclitaxel or paclitaxel loaded nanoparticles) were added the next day at 10nM, 30nM and 50nM equivalent paclitaxel concentrations. The treatments were incubated for 6 hours or 24 hours, followed by removal of the treatments and addition of fresh media. Cell survival was analyzed at 72 hours using CellTiter 96 AQ
deous One Solution Cell Proliferation Assay (Promega, Madison, WI).
3. Results

3.1. Optimization of Antibody Thiolation

Introduction of free thiols in the IgG molecules was carried out using 2-immunothiolane, which reacts with primary amine groups to form a reactive sulfhydryl group. A number of groups have used this strategy in the past, but the ratio of the thiolating reagent to antibody is critical and must be optimized for each antibody. We optimized the thiolation reaction in terms of the reaction buffer and the concentration of the thiolating reagent, holding the reaction time as a constant. Two different buffers were tested – borate buffer at pH 8 and Tris-buffered saline at pH 7.5. Since Tris buffer has free amine groups, the number of thiols introduced was variable even at the same concentration of the thiolating reagent (results not shown). In order to obtain reproducible results, all further reactions were carried out in borate buffer. Different molar ratios of the thiolating reagent (to the antibody) were tested – 20X, 50X, 100X and 200X. The number of thiols introduced per antibody at each ratio, is shown in figure 9A. Since roughly two thiols/antibody molecule were introduced by a 200-fold excess of the thiolating reagent, we selected that concentration for nanoparticle conjugation. In addition to optimized conjugation to nanoparticles, we also ensured that thiolation does not affect antibody binding to its antigen. We carried out a simple flow cytometry based evaluation of the thiolated antibody at 200X. As depicted in figure 9B, there was no change in the binding affinity of the antibody upon thiolation.
3.2. Physiochemical Characterization of Nanoparticles

Average hydrodynamic size of nanoparticles was determined to be ~200 nm as measured using DLS. Zeta potential was in the range of -15 mV. Presence of antibody did not influence the particle size or zeta potential to a significant extent. We used Interfacial Activity Assisted Surface Functionalization (IAASF)\(^5^3\) to introduce maleimide groups on the surface of nanoparticles. The maleimide content on nanoparticles was confirmed prior to conjugation of the antibody and was found to be ~ 710 maleimide groups per nanoparticle. Paclitaxel encapsulation efficiency was greater than 90% and loading was 120 µg/mg of nanoparticles. Antibody concentration was determined by BCA to be between 7-10 µg/mg of nanoparticles, which translates into approximately 17 antibodies/nanoparticle (Table 1).
Table 1: Physicochemical characterization of nanoparticles

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>Particle Size (PDI)</th>
<th>Zeta Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank NPs</td>
<td>212 (0.183)</td>
<td>-16.34</td>
</tr>
<tr>
<td>Isotype IgG NPs</td>
<td>206 (0.216)</td>
<td>-11.83</td>
</tr>
<tr>
<td>TW154 NPs</td>
<td>209 (0.213)</td>
<td>-13.22</td>
</tr>
<tr>
<td>AM6 NPs</td>
<td>210 (0.186)</td>
<td>-12.21</td>
</tr>
<tr>
<td>Maleimide Content</td>
<td>~710 Maleimide Groups/NP</td>
<td></td>
</tr>
<tr>
<td>Protein Loading</td>
<td>7-10µg Protein/mg NPs (0.5-1% w/w)</td>
<td></td>
</tr>
<tr>
<td>Protein Encapsulation Efficiency</td>
<td>50-100%</td>
<td></td>
</tr>
<tr>
<td>Presence of Antibody</td>
<td>Confirmed by Gel Electrophoresis (17 antibodies/NP calculated)</td>
<td></td>
</tr>
<tr>
<td>Paclitaxel Loading</td>
<td>120µg Paclitaxel/mg NPs (12%)</td>
<td></td>
</tr>
</tbody>
</table>

3.3. Confirmation of Antibody Conjugation and Loading

Antibody conjugation on nanoparticles was determined to be between 7-10 µg protein/mg nanoparticles. BCA is able to provide information only about presence or absence of protein. In order to confirm covalent conjugation of the antibody, we analyzed the nanoparticles using SDS/PAGE. 2-mercaptoethanol was used to reduce the thiol bonds between the heavy and light chains of the antibody. Nanoparticles were incubated under reducing conditions and the reduced samples were analyzed using SDS/PAGE. The thiol-maleimide bond is resistant to reduction by 2-mercaptoethanol. Thus, when antibody-conjugated nanoparticles are subjected to a reduction step, only half the antibody molecule is expected to be released from the surface of nanoparticles (either the light chain or the heavy chain, depending on which is attached to the particle surface). If no reduction step is present, the antibody should not be released at all. We were able to confirm this in the case of Isotype IgG, Clone 6 and AM6 antibody conjugated nanoparticles as shown in figure 9C. Lanes 5, 7 and 9 represent antibody-conjugated nanoparticles that were reduced and thus we see bands at 25kD corresponding to the antibody light chains. Lanes 4, 6 and 8 represent non-reduced samples – no bands were observed in this case.
3.4. Cell Uptake of Antibody Conjugated Nanoparticles

Uptake of antibody-conjugated nanoparticles was studied in LM2 cells, which have been previously established in our laboratory as a perlecan positive cell line. Presence of antibody improved internalization of nanoparticles into cells as observed by cell uptake data in figure 10B. A one-way ANOVA performed on the data showed a statistically-significant difference ($P=0.001$) between the groups. We saw a 2-fold improvement in uptake of Clone 6 ($P=0.002$) nanoparticles and 2.5-fold improvement in case of AM6 nanoparticles as compared to blank nanoparticles ($P=0.04$).

We also attempted to conjugate antibodies to nanoparticles after 20X thiolation. However, in this case, there was no difference in cell uptake between the targeted and non-targeted groups, as determined by one-way ANOVA ($P=0.07$) (figure 10A). On further examination, we found that even though we obtained similar protein loading on nanoparticles (determined by BCA), the antibody was only physically adsorbed on the particles and not covalently conjugated as determined through gel electrophoresis studies.

![Figure 10: Cell uptake comparing 20X and 200X thiolation of antibodies. A – Cell uptake of nanoparticles conjugated to antibodies with 20X thiolation; B – Cell uptake of nanoparticles conjugated to antibodies with 200X thiolation.](image)

3.5. Cell Uptake of Antibody Conjugated Nanoparticles analyzed by Flow Cytometry

Improved internalization of perlecan-targeted nanoparticles was also verified by flow cytometry. We tested both LM2 and M12 cells in this experiment. For this purpose, we
used PLGA polymer that was covalently conjugated to a fluorescent marker i.e., rhodamine, to prevent leaching of the dye. We observed a statistical difference amongst the groups using a one-way ANOVA (P=0.003). A 2-fold difference was observed between IgG and Clone 6 nanoparticles as well as IgG and AM6 nanoparticles (figure 11A and 11B). There was no significant difference in the uptake between Clone 6 and AM6 nanoparticles in LM2 cells. On the other hand, we observed a 2-fold increase in nanoparticle uptake between IgG and Clone 6 nanoparticles in LM2 and a 3-fold difference between IgG and AM6 nanoparticles in M12 cells (figure 11C and 11D). In case of M12 cells there was a significant (P=0.0009) difference between Clone 6 and AM6 nanoparticles.

![Figure 11: Cell uptake of antibody-conjugated nanoparticles in metastatic cell lines. A – Histogram depicting flow cytometry data in LM2 cells (Grey-Blank nanoparticles, Black-IgG nanoparticles, Red-Clone 6 nanoparticles, Blue-AM6 nanoparticles); B - Histogram data depicted in quantitative format in LM2 cells; C - Histogram depicting flow cytometry data in M12 cells (Grey-Blank nanoparticles, Black-IgG nanoparticles, Red-Clone 6 nanoparticles, Blue-AM6 nanoparticles); D - Histogram data depicted in quantitative format in M12 cells.](image-url)
3.6. Exocytosis of Antibody Conjugated Nanoparticles

Presence of a targeting ligand on nanoparticles is not only expected to improve internalization into cells but could also improve retention of nanoparticles. This would allow for maintaining sustained concentrations of the drug within the cells, thus improving the cytotoxic effect. We observed an increase in the amount of nanoparticles retained within the cell due to conjugation of nanoparticles to Clone 6 antibody. The data depicted in figure 12 has been normalized to the amount of nanoparticles at the initial time point and shows that targeted nanoparticles have a lower rate of exocytosis.

![Exocytosis of Antibody Conjugated Nanoparticles](image)

Figure 12: Rate of exocytosis of Clone 6 and Isotype IgG nanoparticles tested in LM2 cells

3.7. Competition Experiment

In order to confirm that the improved uptake of antibody-conjugated nanoparticles could in fact be attributed to specific binding, we conducted a competition experiment. LM2 cells were pre-treated with high concentrations of antibody at 4°C so that the free antibody can block the antibody-conjugated nanoparticles from binding to the target i.e., perlecan. Free Clone 6 and AM6 antibodies, but not the isotype control IgG, reduced the cellular uptake of respective antibody conjugated nanoparticles. The low reduction in uptake in this competition experiment may be attributed to the fact that the antibody concentrations in the pre-treatment step may not have been not close to saturation. As a
result, there still may be perlecarn epitopes available for binding with the targeted nanoparticles.

![Graph showing reduction in nanoparticle uptake upon pretreatment with respective antibodies at 4°C]

**Figure 13:** Reduction in nanoparticle uptake upon pretreatment with respective antibodies at 4°C

### 3.8. Cytotoxicity Study

The final goal of this project was to develop a formulation that is able to target and kill metastatic tumor cells. Thus, we conducted a cytotoxicity study to evaluate tumor cell kill with antibody-conjugated nanoparticles. Five treatment groups were considered – no treatment, free paclitaxel, paclitaxel loaded isotype IgG nanoparticles, paclitaxel loaded Clone 6 nanoparticles, and paclitaxel loaded AM6 nanoparticles. Maximum cell-kill was observed in the case of Clone 6 and AM6 nanoparticles at all concentrations. We also observed an improvement in cell kill with paclitaxel in control nanoparticles relative to free paclitaxel. The difference between cell kill across different groups reduced with increasing concentration of paclitaxel because the IC50 for paclitaxel is 2.4 nM for MDA-MB-231 cells. At concentrations higher than 10 nM, paclitaxel was killing the cells effectively, irrespective of the formulation.
Figure 14: Cytotoxicity in LM2 cells on treatment with free drug paclitaxel and paclitaxel loaded nanoparticles for 6 hours tested on day 3 post-treatment.
4. Discussion

Breast cancer takes the lead in the National Cancer Institute’s (NCI) database on funding for different types of cancer from 2007 through 2013. Close to $600,000,000 has been spent on breast cancer research each year. This has resulted in improved diagnosis and advanced therapeutics, which, in turn, have significantly improved the survival rates of breast cancer. However, M-TNBC patients still have only a few treatment options available to them. In addition, most patients develop resistance to these therapies.

Using phage display, our laboratory has successfully developed two antibodies that bind to M-TNBC cells with varying affinities. Clone 6 and AM6, as the antibodies were termed, bind to perlecan – a glycosylated cell membrane protein overexpressed on M-TNBC cell lines as well as on CTCs isolated from in vivo tumor models. We hypothesized that Clone 6 and AM6 could be used as ligands for targeting metastatic breast cancer.

Nanoparticles have shown significant promise in the field of cancer – Abraxane and Doxil have improved the status of traditional drugs like paclitaxel and docetaxel by reducing their side-effects and improving their efficacy. Even though there are some concerns and questions regarding the fraction of the nanoparticles administered reaching the tumor, the success of Abraxane and Doxil over their traditional counterparts is promising. Nanoparticles may be tailored to the requirements of a specific cancer type in terms of shape, size, material, drug release, and charge. Further, the evidence in favor of the EPR effect provides a strong scientific rationale for nanoparticles being a good choice for cancer therapy.

PLGA was chosen as the polymer of choice due to one primary reason – the degradation products i.e. lactic acid and glycolic acid are naturally found in the body and thus toxicity from the excipients would be minimal. A number of other groups have used PLGA nanoparticles for cancer nanotherapy with minimal toxicity issues. The nanoparticles were grafted on the surface with the block co-polymer PLA-PEG-
Maleimide using IAASF technique. The PEG layer served to prolong their half-life in the circulation by forming a hydrophilic shield on the particles and preventing adsorption of opsonins. The reduction in phagocytosis of nanoparticles by the MPS due to the presence of a PEG layer on their surface has been termed as the “stealth” effect. The maleimide group served as the reactive functional group on the nanoparticle surface.

Clone 6 and AM6 were intended to be used as the targeting ligands for this formulation. In order to do so, we needed to conjugate the antibodies to the surface of nanoparticles and thus ensuring that it is stably bound. Thiol-maleimide chemistry, which has been commonly used for hetero-functional crosslinking of proteins, was used to conjugate the antibodies to nanoparticles. This was carried out via a two-step process; first – introduction of free thiol groups on the antibody, second – conjugation of thiolated antibody to maleimide functionalized nanoparticles.

Thiolation using Traut’s reagent involves an addition reaction where the free amine groups on the antibody are converted to free-sulfhydryl containing groups. These sulfhydryl groups are highly reactive towards maleimide groups. The same thiolation method has been used by multiple groups with different reaction conditions. Anhorn et al used thiolated Trastuzumab for synthesis of antibody conjugated nanoparticles. Using a similar approach as we did, they thiolated the antibody using 50-fold molar excess of 2-immunothiolane and yet observed as high as 40-50% of adsorptive binding. Similarly, Steinhauser et al obtained only 0.7 thiols per antibody using the same protocol, again with some antibody physically adsorbed onto the nanoparticles.

For our initial experiments, we carried out thiolation at 20-fold molar excess of the thiolating reagent, obtaining only 0.4 thiols per antibody. Similar to that described by others, we observed physical adsorption of the antibody rather than covalent conjugation. As a result, we did not see a significant targeting effect in vitro. Thus, in order to avoid adsorption issues and to improve antibody binding to target cells, we optimized the reaction conditions and were able to introduce between 2-3 thiols per antibody. The next
step, conjugation of the thiolated antibody to the functionalized nanoparticles, was fairly simple due to the high specificity and reactivity of thiol-maleimide reaction. Final characterization of the antibody-nanoparticle conjugates revealed a stable bond between the antibody and nanoparticles. The characteristics of nanoparticles were not significantly affected by presence of the antibody likely due to the small number of antibody molecules conjugated to the nanoparticles.

H. Gao et al\textsuperscript{65} showed in their experiments with IL-13 conjugated nanoparticles that targeted uptake takes place primarily via receptor-mediated endocytosis as opposed to unconjugated nanoparticles that are primarily taken up by micropinocytosis (non-specific endocytosis). They observed that targeted nanoparticles are rapidly distributed throughout the cytoplasm of cells, while on the other hand non-targeted nanoparticles end up mostly in the endosomal compartment. Although several pathways of nanoparticle internalization are likely operating in the cell at any given point, presence of a ligand changes the major pathway from micropinocytosis to receptor-mediated endocytosis. Even though our case, perlecan is not a receptor per se, HSPGs have been known to serve as cell surface receptors for the transport of macromolecular cargos.\textsuperscript{66,67}

Although our \textit{in vitro} data showed at least a two-fold improvement in cell uptake, other groups have obtained as high as a ten-fold uptake with targeted nanoparticles.\textsuperscript{57,58,55} This brings to light the fact that aside from the nature of antibody conjugation to nanoparticles, uptake is affected by other factors such as the intrinsic antibody affinity, the density of antibody on the surface of the nanoparticles and the density of the target receptor on the cell surface.\textsuperscript{68} The intrinsic antibody affinity has been shown to affect the uptake to a certain extent. This has been attributed to the fact that nanoparticles display multiple antibodies on their surface, as a result the cumulative avidity of nanoparticles may not significantly increase with increasing antibody affinity. Such an affinity, beyond which there is no improvement in nanoparticle uptake, has been termed as the threshold affinity. A similar avidity based explanation is also applicable for the density of antibodies on the surface of nanoparticles.
We observed that in the case of LM2 cells where Clone 6 and AM6 nanoparticles did not show a significant difference in uptake despite the 10-fold difference in their affinity values (density of antibodies on the surface remaining the same). However, in the case of M12 cells we observed that AM6 nanoparticles were taken up better than Clone 6 nanoparticles. This could potentially be attributed to the difference in expression levels of perlecan between the two cell lines. If LM2 cells have a higher perlecan expression than M12 cells, even a low affinity antibody would show sufficient avidity effects. This would result in a lower threshold requirement in terms of antibody affinity. On the other hand, M12 cells with a higher affinity threshold would show improvement in nanoparticle uptake until the threshold is reached. Quantitative data regarding the expression levels of perlecan in the two cell lines is required to confirm this possibility.

Following internalization, nanoparticles must be retained long enough such that they are able to release the drug over time to achieve sustained intracellular drug concentrations. We observed that conjugation of the targeted antibody results in an increase in the intracellular retention of nanoparticles. This is attributed to the ability of nanoparticles to “hold on” to a specific receptor inside the cell. As a result, nanoparticles are not rapidly recycled back. Presence of Clone 6 and AM6 improved the fraction of nanoparticles retained within the cells when tested in vitro over a period of 60 minutes.

Considering that our primary aim was to develop a drug delivery system to treat M-TNBC, we evaluated our antibody-conjugated nanoparticles for cytotoxicity in vitro. We used LM2 cells for the study since the nanoparticles had demonstrated improved uptake and retention in that particular cell line. There was a significant difference in the cytotoxic effectiveness between control nanoparticles and targeted nanoparticles that can likely be attributed to an increased cellular uptake and longer retention of targeted nanoparticles and the resulting higher drug concentrations inside the cell. We also observed a difference between free drug and control nanoparticles. This is likely attributed to the fact that nanoparticles are taken up by endocytosis (receptor mediated or
non-specific) resulting in subcellular localization (closer to the target site of action inside the cell).\textsuperscript{69,70} Nanoparticles also enable sustained release of the drug, thus providing a consistent concentration as opposed to the transient increase in drug concentration with free drug treatment. We observed nearly 90\% cell kill with antibody-conjugated nanoparticles at the highest concentration of drug that was tested i.e. 50 nM paclitaxel. Taken together, the above studies provide us with initial evidence for the potential of a targeted delivery system that has improved efficacy towards metastatic triple negative breast cancer.
5. Conclusion

There are currently over 200 trials being conducted for the treatment of M-TNBC all over the world. This provides an insight to the intensive research being conducted in this field. The recent classifications of breast cancer based on genotyping have provided interesting molecular insights into the disease. Keeping in mind the phenotypic diversity in breast cancer, our lab has developed two human IgG antibodies targeted specifically to EMT phenotypic TNBC cells. These antibodies bind with varying affinity to perlecan, an HSPG found in the tumor ECM. The overarching aim of this thesis was to develop perlecan targeted nanoparticles as a therapeutic option for M-TNBC. We were successful in developing a stable nanoparticle formulation using thiol-maleimide chemistry and our results show that the formulation is targeted towards perlecan. The next aim was to evaluate this formulation in vitro and perform preliminary in vivo efficacy studies. The perlecan-targeted nanoparticles demonstrated greater uptake and retention than control non-targeted formulation in metastatic cells in vitro. In addition, targeted nanoparticles loaded with paclitaxel resulted in improved cell-kill in comparison with free drug and the drug-loaded control formulation. Perlecan–targeted anticancer nanoparticles have thus demonstrated significant potential for targeting metastatic breast cancer cells. Based on the exciting in vitro data, we are currently investigating the in vivo efficacy of paclitaxel-loaded, perlecan-targeted nanoparticles in an orthotopic LM2 tumor model. We expect that these promising in vitro results bring us one step closer to improving the prospects of patients presenting with metastatic disease.
6. Bibliography


to the University of Minnesota, Minneapolis, USA. 2016.


