Development and Characterization of Targeted Iron Oxide Nanoparticles for Pancreatic Tumor Therapy

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Submitted in Partial Fulfillment of the Prerequisite for Honors in Chemistry

May 2013

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Abstract:

The challenge of specificity remains a great obstacle in modern cancer therapeutics. Pancreatic cancer patients, with a survival rate of less than 5%, are particularly reliant on the specificity of drug therapies due to late diagnosis and non-resectable tumors. This study investigates the targeting of an iron oxide nanovehicle with the novel monoclonal antibody, CHO31.1, to the surface antigen on pancreatic tumor cells, GPA33. This nanovehicle is part of a larger project focused on the development of iron oxide nanoparticles loaded with atoms of $^{10}$B for selective irradiation of tumors via boron neutron capture therapy (BNCT). Success of this therapy depends on effective concentrations of SPIO nanoparticles within tumor cells for tracking within MRI. Conjugation of antibodies to the surface of Ferrotec EMG304® particles was investigated through 3 different routes: 1) hydrazone bond formation 2) hinge thiol attachment and 3) carbodiimide coupling. Coupling methods were characterized with UV-visible spectroscopy, chemiluminescent dot blot, and SDS-page electrophoresis. Efficient binding of particles to the antigen target was investigated through a pull down assay with lysates of CAPAN-2 pancreatic cells expressing the target antigen, GPA33. Surface protein was quantified with a BCA assay and zeta potential measurements of the targeted particles were obtained. MRI in vitro assays were performed with the targeted particles. Particles were successfully and reproducibly coupled to antibodies via carbodiimide coupling. Total protein content on the surface of particles showed that uncoupled particles persisted in solution. Efficient binding of GPA33 target was shown in the pull down assay with CAPAN-2 lysates. In-vitro assays of particles did not show significant uptake by cancer cells. Future work to investigate the effects of surface charge and aggregation of targeted nanoparticles should be performed.
Acknowledgments:

About 3 years ago, I wandered into Nancy Kolodny's office seeking an opportunity to do research. I was hardly familiar with the word “research” as the closest I had been to a lab outside of introductory chemistry was watching Law and Order or CSI. Since that time, I have been fascinated and humbled by the research process and the questions that can be answered. This has been an empowering journey in which friends and advisors helped me develop an exciting project, and ultimately my confidence as a researcher.

I would like to thank all of those who helped me with this thesis. Primarily, Nancy Kolodny: your encouragement and guidance allowed me to understand independent research. From my a cappella concerts to weekly lab meetings, your constant support has shaped my Wellesley experience. I feel honored to have listened to your last lecture and been a part of your legacy at Wellesley College.

To Nolan Flynn, your influence both as a research advisor and as a professor opened up new ways to be excited about chemistry and about life. To Drew Webb, your brilliant insight taught me the art of reducing research questions to a series of experiments and I look forward to continued development of this skill in the future. To Jim Moyer, for fielding my questions, giving brilliant advice, and humoring every new career path I dream up. To Jon Rose, for all of your help with MRI over the years.

To all of my labmates, past and present, who inspired me in and out of lab: Hatice Yayla ’11, Adriane Otopalik ’11, Weiya Mu ’11 your leadership will always be the gold standard in my mind and I miss our summer adventures after my first year. To Jasmine Rana ’12, thank you for keeping lab classy and fun and for all of your hard work on this project. To Raji Nagalla ’14, Alice Liao ’15, and Kara Indrelie ’14 for collaborating with me on this project. I appreciate our conversations and periodic data sharing that got us closer to our combined goal. To Becca Mclain ’13, and Stephanie Schmitt ’13 thank you for making lab a happy place and reminding me that EGBAR 😊 To my fellow thesis buddies, Palig Mouradian ’13 and Rachel Parker ’13 we made it!—I am so proud of our hard work. To Ilana Pollack ’13, Bridget Begg ’13, Tina Truong ’13, Harini Natarajan ’14, Maria Jun ’14 thank you for helping me think about this project in a biological context and for all of your work in developing and perfecting assays that brought this project to a new level.

Last, but not least, I would like to thank my parents, Fran and Darrell, and my family that has given me tremendous support and listened attentively as I droned on about what has been the most empowering experience I have had at Wellesley. Thank you will never be enough.
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1. Introduction

For the past two decades, the primary non-surgical treatment for many types of metastatic cancer has been intravenous cytotoxic chemotherapy. These drugs target rapidly dividing cells leading to unwanted cytotoxicity in the rapidly dividing healthy cells of hair follicles, the gastrointestinal tract, and bone marrow. Patient side effects include alopecia, gastrointestinal symptoms, and myelosuppression (decreased ability of bone marrow to produce blood cells). While mortality has gradually decreased for various types of cancer, pancreatic cancer maintains a positive trend in incidence and mortality. The late detection of pancreatic cancer leaves patients, often hopelessly, reliant on the specificity of drug therapies: fewer than 15% of patients present with resectable tumors and only 4% of pancreatic cancer patients live four years beyond diagnosis. In addition to chemotherapy, the hallmark of cancer treatment, non-surgical regimens currently approved for pancreatic cancer include radiation therapy and targeted drug therapies. In the last decade, the medical and scientific communities have seen a dramatic increase in the role of targeted therapies for cancer. Targeted therapies, including small molecule inhibitors and monoclonal antibodies, exploit differences between cancerous and healthy cells. The goal of our study is to develop a targeted therapy for pancreatic cancer consisting of a conjugated iron oxide nanoparticle and monoclonal antibody.

This study is a continuation of six years of research and development of the proposed nanoparticle complex for pancreatic cancer therapy (Figure 1). The core is a superparamagnetic iron oxide (SPIO) nanoparticle with functional
coatings of gold or silica as biocompatible and chemically understood surfaces for further conjugation. The monoclonal antibody targeting agent is covalently attached to the surface along with an adjunct therapeutic agent of the polymer, poly-D-glutamate-D-lysine (PGL), loaded with atoms of the isotope $^{10}\text{B}$ for boron neutron capture therapy (BNCT).\textsuperscript{13}

Figure 1. Schematic for all-in-one nanovehicle for targeted pancreatic tumor therapy. Iron oxide cores coated with silica or gold are functionalized with polyethylene glycol (PEG). Covalent conjugation of anti-biotin and targeted mAb CH031.1 to PEG surface is achieved through direct linking via EDC/NHS coupling. Biotinylated, $^{10}\text{B}$ loaded polymers combine with anti-biotin antibodies for concentrated delivery of $^{10}\text{B}$ for BNCT.

Previous work on this project has focused on coating of iron oxide cores with gold, silica, and polyethylene glycol, characterized through TEM and MRI relaxivity studies.\textsuperscript{8,9} In this previous research, covalent attachment of monoclonal antibodies to iron oxide nanoparticles was not investigated for in vitro targeting of pancreatic tumor cells. This study develops covalent conjugation of the monoclonal antibody CH031.1 to iron oxide cores and characterization of targeting with in vitro magnetic resonance imaging. A theoretical and literature overview of the monoclonal antibody targeting agent along with MRI theory is presented in the following introduction.
1.1 Iron Oxide Nanoparticles as Contrast Agents

Nanoparticles are ideal drug carriers due to their high surface area to volume ratio providing a large area for adsorption of a variety of moieties for targeted drug delivery. The depth of research interest in nanoparticles stems from their ability to deliver a large therapeutic payload, to have specific pharmacokinetic properties, and to be functionalized with highly specific moieties for targeted delivery.\textsuperscript{14} The ideal size of nanoparticles (10-100 nm) allows for endocytosis and delivery of medicines.\textsuperscript{15} Iron oxide nanoparticles are a growing field of research due to their superparamagnetic properties and possibility for tracking within MRI. Recently, nanoparticles functionalized with the FDA approved antibody, Trastuzumab, were targeted toward breast cancer cells for diagnosis of breast cancer. Results showed significant uptake of the iron oxide nanoparticles to the breast cancer cells as opposed to healthy cells and effective concentrations for imaging.\textsuperscript{16} The use of superparamagnetic iron oxide (SPIO) nanoparticles as MRI contrast agents is a result of their interaction with water protons in a magnetic field.

1.1.1 Nuclear Magnetic Resonance\textsuperscript{17,18}

Magnetic resonance imaging (MRI) is a technique that relies on nuclear magnetic resonance (NMR) to develop high-resolution images of soft tissues. NMR is dependent on spin characteristics of nuclei of atoms with an odd number of protons, neutrons, or both. These NMR active nuclei possess non-zero spin quantum numbers and a magnetic moment.
Table 1. NMR Active Nuclei. The above table lists NMR active nuclei due to nonzero spin quantum numbers resulting from an odd number of protons, neutrons, or both.

While many atoms on the periodic table have isotopes that are NMR active, hydrogen is most commonly used for NMR due to its isotopic abundance and high concentration in biological systems.

There are two independent schools of thought that explain the phenomenon of magnetic resonance: the quantum mechanical model and the classical model. The quantum mechanical model explains magnetic resonance as arising from spin energy states that are not degenerate in the presence of a magnetic field. The number of spin energy states are described by the below equation which depends on the spin angular momentum quantum number \( S \) which is \( \frac{1}{2} \) for \(^1H\).

\[
\text{# energy states} = 2S + 1
\]  \hspace{1cm} (1)
The number of spin energy states is described by the above equation and depends on the spin angular momentum quantum number (S). There are two spin energy states for $^1\text{H}$. Interaction between the main magnetic field and the spin magnetic moment causes non-degenerate spin energy states. The energy difference between the two spin states is equal to energy at the resonance frequency. The resonance frequency depends on the gyromagnetic ratio and the strength of the magnetic field.

The difference in energy between spin energy states in the presence of a magnetic field ($B_0$) is equal to energy at the resonance frequency. When energy at this precise frequency is applied to the sample it causes excitation to higher spin energy states. This resonance frequency is also referred to as the Larmor frequency ($\omega$) and varies with the magnetic field ($B_0$) and gyromagnetic ratio ($\gamma$) according to the Larmor equation:

$$\omega = \gamma B_0$$

(2)

1.1.2 T1 and T2 Relaxation and Image Construction$^{17,18,19}$

While the quantum mechanical interpretation of NMR is useful in understanding the origin of the resonance frequency, the classical interpretation allows for understanding signal generation through relaxation of nuclear magnetic moments. The classical model describes the magnetic moments as precessing in the presence of a strong external magnetic field. When this external magnetic field is absent, nuclear magnetic moments point in random directions and cancel each other.
out. In the presence of a strong external field, nuclear magnetic moments align
either with or against the external magnetic field. Magnetic moments precess due to
a torque exerted on them by the external magnetic field.

![Precession of nuclear magnetic moments](image)

**Figure 3. Precession of nuclear magnetic moments.** In the classical
interpretation of nuclear magnetic resonance, nuclear magnetic moments precess
due to a torque exerted by the external magnetic field. Nuclear magnetic moments
align with and against an external magnetic field.

The frequency at which the nuclei precess in the presence of an external magnetic
field is the Larmor or resonance frequency.

Production of MR signal depends on the disruption of precessing magnetic
moments through the application of a radiofrequency (RF) pulse. The RF pulse is
applied so that it matches the energy difference between spin states (Figure 2) or
the exact precessional frequency of the proton as understood through the classical
interpretation. Application of the RF pulse perpendicular to the vector of the
external magnetic field at the resonance frequency causes excitation. A 90° pulse
will “flip” the magnetic moment into the xy-plane (Figure 4).
Figure 4. Application of RF Pulse to Flip Magnetic Moment. A 90° pulse will flip the nuclear magnetic moment into the xy-plane.

After the radiofrequency pulse is turned off, two simultaneous relaxation processes occur: T1 and T2. These two relaxation processes correspond to the recovery of the z-component of the nuclear magnetic moment to the axis of the external magnetic field (T1) and dephasing of transverse magnetic moments in the xy-plane (T2). These two time constants are also termed spin-lattice relaxation (T1) and spin-spin relaxation (T2). They are governed by the Bloch equations which relate the magnetic moments in the vertical ($M_z$) and transverse ($M_{xy}$) planes to the T1 and T2 time constants.

\[ M_z(t) = M_z(0) \left( 1 - e^{-\frac{t}{T_1}} \right) \]  

\[ M_{xy}(t) = M_{xy}(0) \left( e^{-\frac{t}{T_2}} \right) \]

T1 relaxation is the time constant that governs the return of longitudinal relaxation to the z-axis. From the classical perspective, T1 relaxation can be described as the return of the magnetic moment vector to the z-axis (Figure 5). In the quantum mechanical interpretation, this type of relaxation is the time constant for 63% of excited nuclei to relax to the ground state. Energy is transferred from the
excited spin state to the lattice of surrounding nuclei at the Larmor frequency. Therefore, efficient and quick relaxation occurs when a sufficient number of the surrounding nuclei are moving at the Larmor frequency. This energy transmission to surrounding molecules at the Larmor frequency is termed stimulated emission because it is stimulated by the rate of rotation and tumbling of surrounding molecules. Stimulated emission is the greatest contributor to T1 relaxation, however spontaneous emission occurs at a small probability proportional to the cube of the emitted frequency ($\nu^3$).

Figure 5. Longitudinal T1 Relaxation. The return of the magnetic vector in the xy-plane to the z-axis in the classical interpretation of T1 relaxation.

The T1 time constant is influenced by a variety of factors including temperature, solution viscosity, molecular size, structure, and magnetic field strength (Table 2). The more molecular motion at the Larmor frequency, the faster the T1 longitudinal relaxation.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>T1, 0.5 T (msec)</th>
<th>T1, 1.5 T (msec)</th>
<th>T2 (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>210</td>
<td>250</td>
<td>80</td>
</tr>
<tr>
<td>Liver</td>
<td>350</td>
<td>500</td>
<td>40</td>
</tr>
<tr>
<td>Muscle</td>
<td>550</td>
<td>870</td>
<td>45</td>
</tr>
<tr>
<td>White matter</td>
<td>500</td>
<td>780</td>
<td>90</td>
</tr>
<tr>
<td>Gray matter</td>
<td>650</td>
<td>900</td>
<td>100</td>
</tr>
<tr>
<td>Cerebrospinal fluid</td>
<td>1,800</td>
<td>2,400</td>
<td>160</td>
</tr>
</tbody>
</table>

*Estimates only, as reported values for T1 and T2 span a wide range.

Table 2. T1 and T2 Relaxation Times for Various Tissues and Magnetic Field Strengths.
T2 or spin-spin relaxation differs from T1 in that it measures a decay of the transverse magnetic moments rather than a change in spin energy states. It is the time required for 63% of the transverse magnetization to decay. This decay is a result of two processes: 1) spin-spin interactions and 2) inherent inhomogeneity (ΔB) in the magnetic field. Spin-spin interactions lead to dephasing of the magnetic moment in the xy-plane because local nuclei create their own magnetic fields and cause different precessional frequencies for proximal nuclei. These different frequencies allow spin magnetic moments to become out of phase and cancel each other out over time. The variations in magnetic field homogeneity also contribute to dephasing in the xy-plane for the same reason as spin-spin interactions. Relaxation due to spin-spin interactions is termed T2 while relaxation due to local field inhomogeneities and spin-spin interactions are termed T2*. These two time constants can be measured independently and are related by the following equation:

\[
\frac{1}{T_{2*}} = \frac{1}{T_2} + \gamma \Delta B
\]

T2 times depend largely on how different spins interact and because of this, the increase in compactness of the tissue can shorten the T2 times as seen in Table 2.

*Image Construction*¹⁷,¹⁸,¹⁹

Signal intensity and spatial information are developed and optimized through the use of specific pulse sequences (Figure 6) that describe the pattern of RF pulses and gradient magnetic fields that are administered to a sample for imaging. In the common spin-echo pulse sequence, a 90° pulse is administered
followed by a $180^\circ$ pulse creating signal intensity termed an “echo”. The signal intensity decays and is termed the free induction decay (FID).

**Figure 6. Spin Echo Pulse Sequence.** In the first line of the sequence, the pulses are shown (first a $90^\circ$ then $180^\circ$ RF pulse). The time to echo (TE) and time to repetition of the sequence (TR) are shown above and below this line. The FID is along with the three gradients comprise the 4 bottom lines: slice-select ($G_z$), phase encoding ($G_y$), and frequency encoding ($G_x$).

Variation of times to repetition of the pattern (TR) and times to echo (TE) govern the sequence and allow for T1 and T2 weighted images according to the equation below:

$$\text{Signal Intensity} \propto N(H)e^{\frac{TE}{T_2}}(1 - e^{\frac{TR}{T_2}}) \quad (6)$$

T1-weighted images have short TE and short TR to maximize signal from spin-lattice relaxation. T2-weighted images have long TE and long TR to maximize the signal from transverse relaxation.

The difference between NMR spectroscopy and MR imaging is spatial encoding of signal. This spatial information is obtained through variation of the
magnetic field over the sample to distinguish between protons in different positions. This variation in magnetic field is obtained through a gradient magnetic field causing unique Larmor frequencies of protons in different positions.

\[ \Delta E = h\nu = \gamma \left(\frac{h}{2\pi}\right)B = \gamma \left(\frac{h}{2\pi}\right)(B + q\Delta B_q) \]  

The effect of the gradient is shown in Equation 6 as \( \Delta B_q \) in which \( q \) specifies the direction of the gradient \((x,y,z)\) that is added to the main magnetic field in every distinct position. Three gradients are used to extract spatial information: 1) frequency-encoding or readout gradient \((G_x)\), 2) phase-encoding gradient \((G_y)\) and 3) slice-select gradient \((G_z)\). The slice-select gradient, as its name suggests, selects a slice and differentiates protons along the z-direction. The steeper the slice-select gradient, the thinner the slices that can be selected from the sample. The frequency-encoding gradient sets a gradient magnetic field across the selected slice to obtain spatial information in one slice. The last gradient, phase-encoding, is used to write the data from the image into a region termed k-space. The number of phase encoding gradients is related to the number of pixels in the image-- adding more phase encoding gradients will increase the resolution of the image.

1.1.3 Iron Oxide as a MRI Contrast Agent

Superparamagnetic iron oxide (SPIO) contrast agents create local field inhomogeneities in MRI samples which are key to their contrast in T2*-weighted images. Common SPIO contrast agents are maghemite \((\text{Fe}_3\text{O}_4)\) and magnetite \((\text{Fe}_5\text{O}_4)\). These contrast agents exist in a crystal lattice structure containing different
domains with unpaired electrons (paramagnetic). In the presence of an external magnetic field, these domains align their magnetic moments with the external magnetic field. However, when these contrast agents are developed as nanoparticles, rather than bulk material, the domains do not exist because they are larger than the size of the nanoparticle itself. Instead they lack the dividing domains and have an even greater net magnetic moment in the B_0 direction. This property makes these nanoparticles superparamagnetic.

Due to the superparamagnetic nature of the iron oxide nanoparticles, they possess their own internal magnetic field. This magnetic field can be likened to a tiny bar magnet possessing magnetic field lines that decrease in strength with distance from the particle. These local magnetic fields cause inhomogeneity in the magnetic field of the samples. As water protons diffuse by the nanoparticles they “feel” different magnetic field strengths. Those nuclei closer to the particles will be

Figure 7.20 Aligning multiple magnetic domains in magnetite or maghemite material. A) The divisions are present in bulk material but B) on the nano-scale, particles lack divisions in the crystal lattice structure and have a stronger magnetic moment in the B_0 direction causing their superparamagnetic behavior.
in a stronger magnetic field than those farther away. This difference in magnetic
field in the local area causes the transverse magnetic moments to rapidly dephase
leading to signal loss in T2*-weighted images. This signal loss is seen as a darkening
in images and can give information about the location of the particles within the
system.

1.2 Targeted Therapy: Small Molecule Inhibitors

Current advances in development of targeted therapies for pancreatic cancer
have been minimal and have not significantly affected clinical outcomes. FDA
approved in 2005, the only targeted drug therapy currently used as treatment for
pancreatic cancer is Erlotinib, better known by its commercial name, Tarceva
(Figure 8). Tarceva blocks cancer cells from dividing by targeted inhibition of
epidermal growth factor receptors on the cell membranes.

![Chemical structure of Tarceva (Erlotinib).](image)

Figure 8. Chemical structure of Tarceva (Erlotinib). A targeted EGFR tyrosine
kinase inhibitor developed by Pfizer

Epidermal growth factor receptors (EGFR) are transmembrane glycoproteins that
are over-expressed on many types of cancer cells. EGFR sits on the surface of cells
and is activated by binding of specific ligands. This over-expression allows for
targeted inhibition of the cancer cell growth. Phase III trials performed with
Tarceva indicated its effectiveness in combination with the chemotherapeutic agent
gemcitabine. In a trial of 569 patients, the number of pancreatic cancer patients
who lived beyond one year and had progression-free survival was significantly
greater with Tarceva in combination with gemcitabine, as opposed to gemcitabine alone. While the results of the study with Tarceva were statistically significant, they were overall clinically marginal. Further advancement in the development of targeted therapies for pancreatic cancer remains pivotal in optimizing treatment of the disease.

### 1.2.1 Targeted Therapy: Monoclonal Antibodies

Monoclonal antibodies are another therapy targeted against proteins over-expressed on the surface of cancer cells and have proven a promising approach to targeted cancer therapy. Antibodies are proteins that bind specific antigens and consist of heavy and light chain regions converging in variable regions for antigen binding (Figure 9).

![Structure of Antibody Molecule](image)

**Figure 9. Structure of Antibody Molecule.** Antibodies have two identical light chain regions (24-25 kD) and two identical heavy chain regions (55-70 kD). Chains are connected and given further structure and stability by disulfide bonds. Fab portions consist of heavy and light chain domains converged for antigen binding while Fc portions consist of only heavy chain domains.

In 1975, Köhler & Milstein developed techniques for producing monoclonal antibodies, making it possible to produce large quantities of identical antibodies directed against specific antigens. Monoclonal antibodies are monospecific in
antigen binding because they are developed from identical immune cell clones. This is in direct contrast to polyclonal antibodies which are made from different immune cell parents and vary in antigen targets. What makes monoclonal antibodies such an attractive cancer therapy is the monovalent affinity that allows them to respond to not only a specific antigen, but a specific portion or sequence on the antigen, commonly referred to as an epitope. The widely used procedure for developing monoclonal antibodies differs from conventional antibody production through the use of hybridoma technology. Immortal hybridoma cells are formed by fusion of B lymphocytes with myeloma cells which are then grown in selective medium. These hybridoma cells produce specific murine antibodies and the cells with the desired specificity are screened for and isolated.

Figure 10. Production of Monoclonal Antibodies. Conventional antibody production differs from monoclonal antibody production in the development of hybridoma (hybrid myeloma) cells. Hybridomas producing antibodies of the desired specificity are cloned for large production of the monoclonal antibody.

Using this well-known technique (Figure 10) for large production of murine monoclonal antibodies, several antibody therapies were investigated for cancer
therapeutics. In order to avoid immunogenic human-anti-murine-antibody (HAMA) responses in humans, a chimeric form of antibodies was developed. Chimeric antibodies combine conserved regions from the human antibody IgG and variable antigen binding sites from murine antibodies to produce a less immunogenic therapy. In the last twenty-five years, six monoclonal antibody therapies have been FDA approved for clinical cancer therapy (Table 3). Rituximab was the first monoclonal antibody approved for cancer therapy.

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Target</th>
<th>Clinical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rituximab</td>
<td>Chimeric</td>
<td>CD20</td>
<td>NHL</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td>Humanized</td>
<td>Erb B2</td>
<td>Breast</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>Humanized</td>
<td>VEGF</td>
<td>Colorectal</td>
</tr>
<tr>
<td>Alectuzumab</td>
<td>Humanized</td>
<td>CD52</td>
<td>CLL</td>
</tr>
<tr>
<td>Cetuximab</td>
<td>Chimeric</td>
<td>EGFR</td>
<td>Colorectal</td>
</tr>
<tr>
<td>Panitumumab</td>
<td>Human</td>
<td>EGFR</td>
<td>Colorectal</td>
</tr>
</tbody>
</table>

**Table 3. FDA Approved Monoclonal Antibody Cancer Drug Therapies.** Twenty-five years of research into monoclonal antibodies has yielded 6 clinical targeted agents for four types of cancer.

The mechanism of cancer cell destruction by monoclonal antibodies is antibody dependent cell cytotoxicity (ADCC). ADCC is activated by binding of antibodies to targets on the cell surface (Figure 11).
Figure 11. Antibody Dependent Cell Cytotoxicity\textsuperscript{29} ADCC begins with binding of the monoclonal antibody to the antigen presented on the surface of the cell. Natural killer cells targeted to FC portions lyse tumor cells. Antigen debris is taken up by antigen presenting cells and displayed on the membranes of these cells for the production of more antibodies by B cells.

The FC portion of the antibody that is opposite the antigen-binding site serves as a target for natural killer cells (NK). Binding of FC receptors on NK cells with the monoclonal antibody target for cancer cells triggers the release of perforin and granzymes, enzymes that lyse the tumor cell. The cell debris from the lysed cells is taken up by antigen presenting cells which present antigens and epitopes on the surface to allow for B cells to develop antibodies in response to these antigens. A prolonged memory of antibodies is developed and cytotoxic T lymphocytes are raised against the target antigens.\textsuperscript{29}

While ADCC is the proposed mechanism for monoclonal antibody immunotherapy, there is no evidence that unconjugated monoclonal antibody therapies of human cancer induce large tumor infiltration by leukocytes. While ADCC does induce a variable degree of tumor destruction that results in antigen presentation and generation of directed cytotoxic T-cells, there is little published evidence that ADCC contributes to clinical responses. This varying therapeutic potential is ultimately non-curative.\textsuperscript{30} Due to this fact, researchers have
investigated conjugated drugs, toxins, radionuclides, and nanoparticles that use monoclonal antibodies as a targeting agent and adjunct therapy.\textsuperscript{30} Despite the relatively large size of antibodies (about 150 kDa), they remain useful targeting agents due to their high affinity for targets usually with dissociation constants ($K_D$) on the nanomolar scale (Figure 12).\textsuperscript{14} Our study investigates an iron oxide nanoparticle conjugated with the monoclonal antibody CHO31.1.

\[ [Ag - Ab] \rightleftharpoons [Ag] + [Ab] \]

\[ K_D = \frac{[Ag][Ab]}{[Ag - Ab]} \]

**Figure 12. Dissociation constant for antigen-antibody complex.**\textsuperscript{31}

The dissociation constant is the equilibrium constant for the dissociation of the antigen-antibody complex based on the concentrations of the antigen and the antibody binding sites at equilibrium.

**1.2.2 Monoclonal Antibody CHO31.**

Targeted therapies rely on specific differences between healthy and tumor cells and monoclonal antibodies directed against over-expressed, steady targets on cancer cells are ideal. The cell surface glycoprotein A33, a prime monoclonal antibody target, is expressed almost exclusively on epithelial cells of the gastro-intestinal tract.\textsuperscript{32} The glycoprotein A33 (GPA33) (Figure 13) is also expressed on 95% of colorectal cancers, 63% of gastric cancers, and 50% of pancreatic cancer cells.\textsuperscript{33} The exclusive expression and lack of secretion of the antigen into the blood stream allows for a steady target for a monoclonal antibody.\textsuperscript{34} In a brown stain of multiple
cell lines, GPA33 was seen expressed with high intensity per cell on the CAPAN-2 pancreatic cancer cell line.5

Figure 13. Cartoon Structure of GPA33. A33 is a 43 kDa glycoprotein antigen on the surface of tumor cells. GPA33 is a member of the junctional adhesion molecule (JAM) family. Members of the JAM family are characterized by two extracellular Ig-like domains (V and C₂ type), conserved cysteine residues in the extracellular domain, and a single transmembrane domain. The Ig-like domains are similar in sequence and structure to the chains of immunoglobulin molecules.

Several hybridoma cells lines have been prepared with tumor extracts containing tumor associated antigens by International Bioimmune Systems (IBS). Antibodies developed from these hybridoma cells were tested for immunoreactivity and the antibody 31.1 proved to be highly selective in immunohistochemical staining of both primary and metastatic colon cancer specimens. The development of 31.1 and its chimeric form was patented by Jeffrey Fasick of IBS. The use of monoclonal antibodies for immunotherapy requires the ability to produce large quantities for human administration. In order to obtain Federal Drug Administration (FDA) approval, it is necessary that the antibody preparation have as few contaminants as
possible. Chinese Hamster Ovary (CHO) cells provide both high-level expression and the ability to grow under serum free conditions thus reducing the number of contaminants in the final antibody solution. Monoclonal antibody, CHO31.1, was shown to target a specific epitope on GPA33 by Jeffrey Fasick through tests with point-mutated forms of the A33 antigen.\(^{37}\)

1.3 Conjugation of Nanoparticles to Antibodies

The use of monoclonal antibody-targeted nanoparticles has necessitated a variety of conjugation methods to optimize targeting. Coupling strategies fall into two categories: (1) physical adsorption and (2) covalent binding. Various conjugation methods have already been attempted on gold nanoparticle surfaces in association with our project (Figure 14).

![Figure 14. Conjugation methods previously tested on gold surfaces.](image)

Electrostatic adsorption of CHO31.1 to the surface of gold nanoparticles was characterized by UV-visible spectroscopy by Choi, ’11 (A).\(^6\) Covalent conjugation to gold nanoparticle surfaces via a dithiol-PEG-hydrazine linker (Webb, Sun pers. comm.) (B).

Nonspecific electrostatic adsorption of CHO31.1 antibodies to the surface of gold nanoparticles was characterized by UV-visible spectroscopy by Choi.\(^6\) Physical adsorption has the disadvantages of both random orientation on the nanoparticle
surface and competition with other proteins in the environment for surface adhesion.\textsuperscript{38} Conjugation of CHO31.1 to the surface of gold nanoparticles was also investigated via a PEG linker (Webb, Sun personal communication). Coupling of the glycosylated CHO31.1 antibody to 11nm gold nanoparticle surfaces was achieved via a bifunctional dithiol-PEG-hydrazine (SensoPath Technologies Inc. MW=708.97) according to the protocol by Kumar et al, 2008.\textsuperscript{39} Previous coupling of antibodies in association with our project was tested on gold nanoparticle surfaces (Figure 14). Due to fundamental differences between gold and iron oxide or silica surfaces, alternate conjugation methods were explored for the iron oxide conjugation to mAb CHO31.1. A summary of common conjugation methods follows.

1.3.1 Random mAb Orientation Strategies

Covalent conjugation methods are ideal but vary in their ability to achieve site-specific attachment for optimizing orientation of Fab portions on the nanoparticle surface. Random orientation mAb coupling strategies include: (1) EDC/NHS coupling, (2) formation of a Schiff base, (3) formation of an isourea derivative, and (4) primary amine-amine crosslinking.\textsuperscript{38}

1.3.1.1. EDC-NHS Coupling

The most commonly used covalent conjugation method for antibodies to nanoparticles involves carbodiimide crosslinking (Figure 15).\textsuperscript{40} The cross-linker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), reacts with carboxylated NPs in the presence of N-hydroxysuccinimide (NHS) to form amine-reactive NHS esters. EDC is a zero-length cross linker because it adds no atomic distance between the two groups being linked.
Figure 15. Carbodiimide coupling of nanoparticles to antibodies. Coupling via EDC/NHS results in the formation of O-acyl-isourea which has the capacity for intramolecular attack. Addition of N-hydroxysuccinimide (NHS) allows for nucleophilic attack of the electrophilic carbonyl for formation of a stable intermediate to couple with amino groups on lysine chains of antibodies. Preparation of carboxylic acid coated SPIOs can be achieved through carboxylic acid terminated PEG or derivatization of amino groups functionalized on the surface of particles via succinic or glutaric anhydride. A disadvantage of EDC/NHS conjugation of antibodies to nanoparticles is nonspecific orientation of the antibody on the surface of nanoparticles as it will couple directly to lysines in any portion of the antibody.

1.3.1.2 Schiff Base

Formation of a Schiff base, or carbon-nitrogen double bond, occurs via combination of aldehydes and primary amines of the two groups to be linked. Reduction of the Schiff base intermediate with sodium borohydride (NaBH₄) yields stable secondary amines via an amide bond between nanoparticle and antibody (Figure 16). An advantage of this procedure is that it can yield site-specific attachment if aldehydes are formed from oxidation of glycosylated Fc-portions of the antibody so that Fab-portions are opposite nanoparticle binding. Non-site-specific attachment would result from coupling of aldehydes on the surface of nanoparticles with free amines on lysines of the antibody.
Figure 16. Formation of a Schiff Base. Site specific attachment via the Schiff base due to oxidation of sugars on the Fc portion of the antibody. Reduction with NaBH₄ results in formation of stable secondary amines conjugated via an amide bond.

1.3.1.3 Isourea Derivative
Another conjugation method frequently used in literature is via the formation of an isourea derivative. This method again couples amines on the antibody to the nanoparticle surface. In the case of the isourea derivative it is possible to couple hydroxy groups on the surface of nanoparticles with amines on the antibody using an activator (Figure 17). Typically cyanogen bromide (CNBr) is used to activate hydroxyl groups for addition. An advantage of this method is the ability to perform it under aqueous conditions, eliminating the need for organic solvents and protecting the antibody that is conjugated.

Figure 17. Formation of isourea derivative. Hydroxy groups on the surface of the nanoparticle are activated by cyanogen bromide for addition of amines on lysine groups of antibodies. The isourea derivative is the final conjugated product.
1.3.1.4 Amine-Amine Crosslinking

Direct primary amine-amine crosslinking is the last commonly used non site-specific conjugation method for antibodies to nanoparticle surfaces. This method requires activation of amines on the surface of nanoparticles with a cross linker for addition to antibodies. A disadvantage of this method is the formation of large polymeric conjugates of nanoparticles linking together via the crosslinker.

1.3.2 Site-Specific mAb Directional Orientation Strategies

An optimized approach to coupling of antibodies to nanoparticles involves directed conjugation so that Fab-portions of the antibody are directed opposite the nanoparticle conjugate for enhanced antigen binding. A summary of site-directed conjugation methods follows.

There are four main strategies for site directed conjugation of antibodies to nanoparticle surfaces: (1) formation of a hydrazone bond at carbohydrates on the Fc-portion of antibodies, (2) attachment to thiols in the hinge region, (3) multi-step attachment via the avidin-biotin affinity, and (4) novel approaches with click chemistry. These methods are site specific so as to orient the antigen-binding site away from the nanoparticle surface.

1.3.2.1 Hydrazone Bond

Hydrazone bonding to oxidized carbohydrates on the Fc-portion of glycosylated antibodies can be achieved through a heterobifunctional linker with a hydrazine functional group for binding aldehydes on the antibody. Formation of hydrazone bonds at the carbohydrates on the Fc region of CHO31.1 was achieved in
previous work on this project via a dithiol-PEG-hydrazone (Figure 14). The thiol portions of this linker bind to gold surfaces via disulfide bonds. A challenge in using this method for iron oxide surfaces was the difficulty and high cost of synthesis of a heterobifunctional linker with the appropriate functionalities for conjugation.

1.3.2.2 Hinge Thiol

Another method for site-directed conjugation is through targeting of the hinge region of antibodies. Attachment to thiols in the hinge region requires mild reduction of hinge disulfide bonds to attach via a maleimide linker (Figure 18).

**Figure 18. Hinge thiol addition of antibody to nanoparticle.** Antibody is first mildly reduced to obtain thiol groups for conjugation to maleimides on the surface of nanoparticles.

A disadvantage of this procedure is the difficulty in mild reduction of the antibody while preserving immunoreactivity.

1.3.2.3 Avidin-Biotin

The affinity between the avidin protein and cofactor, biotin, has also been widely explored in site directed attachment of antibodies. This multi-step conjugation requires functionalization of the Fc-portion of the antibody with biotin, addition of the tetrameric avidin, and subsequent addition of biotinylated nanoparticles. This conjugation has been investigated previously for this project and a disadvantage proved to be loss of sample after multi-step conjugations.
Further disadvantages of this procedure include the large size of avidin (66-69 kDa) and possibility for aggregation of particles due to crosslinking in available binding sites of the avidin molecule.

### 1.3.2.4 Click Chemistry

A novel technique for addition of antibodies to nanoparticles is through use of click chemistry as opposed to previously described bioconjugations. Click chemistry refers to modular chemical conjugations through simple reactions with high efficiency. The click reactions have advantages over previously described bioconjugations due to their specificity, efficiency, and ability to occur under mild aqueous conditions. The Huisgen azide-alkyne cycloaddition or “click” reaction is commonly used in the functionalization of iron oxide nanoparticles. Thorek compared the conjugation efficiency of this click reaction with carbodiimide chemistry. Results indicated that the copper-catalyzed click reaction of mAb to iron oxide nanoparticles yielded approximately 6.85 antibodies per SPIO, whereas carbodiimide crosslinking resulted in only 2.77 antibodies per SPIO nanoparticle. Coupling methods vary in ease, cost of attachment, efficiency, and site specificity. Optimization of these parameters is necessary in future development of antibody-targeted nanoparticles.
2. Materials and Methods

The research comprising this thesis includes both synthesis of monoclonal antibody conjugated nanoparticles and characterization of the particles. Both are described in detail in the following section.

2.1 Synthesis

Three synthetic techniques for the conjugation of monoclonal antibody CHO31.1 to the surface of iron oxide nanoparticles were investigated: conjugation via a hydrazone bond, via hinge thiol reduction, and via carbodiimide chemistry.

2.1.1 Conjugation via Hydrazone Bond

Ferrotec EMG304® (4.5% v/v, 10nm average size) iron oxide nanoparticles were conjugated to mAb CHO31.1 through a linker of (3-aminopropyl)-trimethoxysilane (APTMS, Sigma Aldrich 97%) and a dithiol-PEG-hydrazine (Sensopath Technologies).

![Figure 19. Schematic of hydrazone bond for conjugation of mAbCHO31.1 to Ferrotec EMG304®.](image)

The amine to sulfhydryl crosslinker, Sulfo-MBS (m-Maleimidobenzoyl-N-hydroxysulfosuccinimide, Pierce Biotechnology), served to join the two linkers in the conjugation. Ferrotec particles were functionalized with APTMS with reagent
concentrations of 0.5 mg Fe (determined by ICP-OES), 250 µL APTMS, and 5 mL nanopure water (18 MΩ). Solutions were sonicated with probe sonicator (Branson Sonifier 250) for 60 seconds and mixed on overhead shaker overnight. Particles were washed by centrifugation for 30 minutes at 13,000 rpm (accuSpin™ Micro R, Fisher Scientific) with nanopure water and resuspended in coupling buffer of phosphate buffered saline (PBS pH=7.2) for reaction with Sulfo-MBS. Sulfo-MBS was added to functionalized nanoparticles at 1mM concentration for a 30 minute incubation before removing excess by centrifugation as above. Dithiol-PEG-hydrazine (Sensopath Technologies) was added in excess to the functionalized nanoparticles and removed by centrifugation after a 2-hour incubation.

Monoclonal antibody CHO31.1 was prepared according to Kumar et al. Glycosylated portions of the antibody were oxidized with sodium meta-periodate (NaIO₄) and aldehydes were detected with Purpald® (Sigma Aldrich). Antibodies were prepared by adding 10 ml of 100 mM sodium meta-periodate (Pierce) to 100 ml of 1 mg/ml antibody solution in 100 mM sodium phosphate buffer and incubated in the dark for 10 min at RT. The reaction was quenched by adding 500 ml of PBS pH=7.2. Oxidized mAb CHO31.1 was incubated with previously functionalized nanoparticles for 2 hours before excess antibody was removed by centrifugation for 30 minutes at 13,000 rpm (accuSpin™ Micro R, Fisher Scientific).

2.1.2 Conjugation via Hinge Thiol

Coupling of mAb CHO31.1 to Ferrotec EMG304® was investigated through mild reduction of human IgG with mercaptoethylamine (MEA) (Thermo Scientific) and dithiothreitol (DTT) (Sigma Aldrich). Oxidation was performed with varying
concentrations of both reducing agents for comparison. Various reagent concentrations were used for reduction of 1 mg/mL solutions of human IgG. IgG was diluted in reaction buffer (20-100mM Na$_3$PO$_4$, 150mM NaCl, 1-10mM EDTA, pH 7.2) and reacted with DTT and MEA for varying times at 37°C before termination by cooling. Samples were characterized with SDS-page gel electrophoresis and a coomassie blue protein stain (Figure 24).

2.1.3 Coupling via Carbodiimide Chemistry

Coupling via carbodiimide chemistry was investigated through use of the carbodiimide, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and the reagent N-hydroxysuccinimide (NHS). Ferrotec EMG304® was functionalized through two different routes to achieve a carboxylic acid terminal group for activation via EDC/NHS: 1) through a carboxylic acid terminated PEG, 2) through APTMS and glutaric anhydride.

Ferrotec EMG304® was functionalized with a silane-PEG-carboxylic acid (NanOCS MW=5000) by combining 0.5 mg Fe (concentration determined by ICP-OES) and 15 mg PEG in 5 mL nanopure water (18 MΩ) for overnight incubation on overhead shaker. Nanoparticles were washed by centrifugation (13,000 rpm 30 minutes) and resuspended in 1 mL activation buffer (0.1M MES, 0.5M NaCl, pH 6.0). To the washed nanoparticles, 0.4 mg EDC (2mM) was added with 0.6 mg (5 mM) NHS and incubated for 15 minutes at room temperature. After incubation the samples were washed by centrifugation and resuspended in coupling buffer (PBS pH 7.2). Either Human IgG or mAb CHO31.1 was added to the activated
nanoparticles (for best results 0.3-0.5 mg antibody). Samples were incubated for 2 hours at room temperature and washed by centrifugation before testing for presence of antibody via chemiluminescent dot blot.

An alternate method of achieving a carboxylic acid terminus on the surface of iron oxide nanoparticles was also attempted via functionalization with APTMS and glutaric anhydride (Sigma Aldrich). Ferrotec EMG304® nanoparticles were functionalized with APTMS by adding 0.5 mg Fe (concentration determined by ICP-OES) to 250 µL APTMS in 5 mL nanopure water. Particles were washed by centrifugation and added to 250 mg glutaric anhydride (Sigma Aldrich) and sonicated before incubation overnight on the overhead shaker (Wrist Action® Shaker, Burrell). After incubation particles were washed by centrifugation and reacted with EDC/NHS and mAb CH031.1 via the same methods as above.

2.2 Characterization

Nanoparticles were characterized through a variety of methods to assess success of conjugation and quality of contrast through MRI.

2.2.1 Chemiluminescent Dot Blot

Nanoparticle-antibody conjugates were tested for presence of antibody via chemiluminescent dot blot. The characterization was performed by dotting 5 µl of nanoparticle samples onto nitrocellulose paper (Bio-Rad) and blocked in a blocking buffer (5% w/v Blotting Grade Blocker BIORAD, 1 x TBS 0.05% Tween20) for 1 hour at room temperature. Nitrocellulose paper was then washed with wash buffer (1 x TBS 0.05% Tween20) and incubated with a goat anti human secondary antibody conjugated with horseradish peroxidase (HRP) for 1 hour (1:5000 dilution
in blocking buffer). Samples were washed again with washing buffer after incubation and then sprayed with detection agent of luminol substrate and hydrogen peroxide (HyGLO Quickspray Denville Scientific). Nitrocellulose was imaged with Kodak Gel Logic 200 Imaging System and the Kodak 1D 3.6 software program.

2.2.2 ICP-OES

Concentration of iron was determined by inductively coupled plasma-optical emission spectrometry (ICP-OES Perkin Elmer). Samples were prepared by digestion with 70% nitric acid: equal volumes of sample and nitric acid were combined in 15 mL centrifuge tube and digested overnight. Samples were then filtered (Teffryn 2 µm) and diluted to 2% nitric acid with nanopure water. Standard solutions for iron were prepared from a 1000 ppm iron source (SPEX CertiPrep) to serial dilutions of 0.004 to 40 ppm. Absorption wavelength 238.204 nm was used to obtain intensity values for the standards and sample. Concentrations were calculated from a standard curve.

2.2.3 MRI Studies

After determination of iron concentration through ICP-OES, MRI studies were performed by dilution of particles in a concentration range from 0.03 to 0.3 mM Fe in low-melt agarose (2% w/v Seaplaque™ Agarose) for suspension in 3 mm NMR tubes. Samples were diluted 4 times within the concentration range and 750 µL was suspended into 3 mm NMR tubes (Wilmad Lab-Glass). Sample tubes were placed into a custom, reusable, phantom holder designed by Stephanie Huang ’12.
Figure 20. Phantom Holder for MRI T2 Relaxivity Studies:

A) Sample NMR tubes were inserted into the phantom holder and a cross-sectional MR image is shown. Darker circles have a higher concentration of Fe. B) Diagram of the phantom holder designed by Stephanie Huang ’12.

T2 relaxation times were obtained from MR images (400 MHz Bruker Avance NMR spectrometer, 9.4T, vertical bore, 2.4 G/cm/A gradient strength) with the following parameters: TR = 3062 ms, FOV = 4.00 cm, 1 Avg, MSME-T2-MAP pulse sequence, 60 echoes. T2 values were calculated by fitting an exponential decay curve to a plot of TE and Signal Intensity for each NMR tube using the Image Sequence Analysis (ISA) tool in Paravision as outlined in the Appendix of Yayla. T2 is calculated from the exponential decay curve of TE and Signal Intensity according to equation 6 above.

2.2.4 In Vitro Cellular Studies

In vitro cellular MRI assays were performed with CAPAN-2 and BxPc-3 pancreatic cancer cells according the procedure outlined by Bakhru et al. Cells were cultured at 37°C with RPMI culture media supplemented with 0.2% sodium bicarbonate (Sigma #S8791), 1x antibiotic/antimycotic solution (1000 units/ml penicillin,) and 10% fetal bovine serum (FBS; Hyclone #SH30070.03). For in-vitro
studies, 1x10^6 cells were plated in each well of a 6-well plate and allowed to adhere overnight. Cells were incubated with SPIO conjugates at concentrations of 0.25-.05 mg Fe (determined by ICP-OES) per well for 2 hours at 37° C. After incubation, cells were washed with 2 mL acid wash (PBS, 20 mM sodium acetate, pH 3) x3. Cells were trypsinized (0.25% trypsin) by adding 2 mL trypsin to each well and incubated at 37° C for three minutes. Cell pellets were obtained by centrifugation (3 minutes, 1000 rpm). Pellets were resuspended in PBS (pH 7.2) and diluted in 2% w/v low melting point agarose (SeaPlaque ™). MRI samples were prepared by pipetting 750 µL of cell suspensions into 3 mm NMR tubes (Wilmad). T2 values for cell suspensions were determined through MR images according to the procedure described in the appendix of Yayla.8

2.2.5 Zeta Potential Measurements

Zeta potential measurements were performed on the nanoparticle-antibody conjugates to determine surface charge. Malvern Zetasizer Nano-2S instrument with the Zetasizer software 6.0.1 was used. The SOP sample file used for tests on antibody conjugated iron oxide nanoparticles was TC Reagg Zeta.sop for hematite particles. SOP file parameters are listed in the table below:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dispersant</th>
<th>Dispersant Refractive Index</th>
<th>Dispersant Viscosity (cP)</th>
<th>Material Refractive Index</th>
<th>Material Absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematite</td>
<td>Water</td>
<td>1.33</td>
<td>.8872</td>
<td>2.940</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Table 4. Sample file parameters for zeta potential measurements

Folded capillary cells (Malvern) were washed with 70% ethanol. About 1 mL of sample was pipet into the cell and zeta potential was measured.
2.2.6 SDS Page Electrophoresis

SDS Page electrophoresis was performed with 10% Precise Protein Gels (Thermo #25201). About 2 µg of protein was combined with equal volumes Non-Reducing Lane Marker Sample Buffer (Thermo #39001) and pipet into sample lanes. Gels were run in Tris-Hepes SDS Running Buffer (Thermo #28398) for 1 hour at 150 V. Completed gels were incubated with about 15 mL Coomassie Blue protein stain for 1 hour to stain all protein present.

2.2.7 Pull Down Assay and Western Blot

Cell lysates of CAPAN-2 and BxPc-3 cells were prepared with mammalian protein extract reagent (MPER Thermo). Culture medium was decanted from T75 flasks and 8-9 mL of MPER was added and shaken gently for 5 minutes. Cell lysates were centrifuged to remove cell debris (1000 rpm 3 min.). Supernatant containing proteins of the lysed cells was collected for the pull down assay.

Cell lysates (2 µg) were incubated with antibody-nanoparticle conjugates (0.1-0.5 mg Fe) overnight at 4°C. Lysate and nanoparticles were centrifuged at 13,000 rpm for 1 hour to pellet nanoparticle conjugates. Pellet and supernatant were analyzed through western blot. The supernatant was prepared for SDS gel electrophoresis by combining 10 µl with equal volume Non-Reducing Lane Marker Sample Buffer (Thermo #39001). Pellet was prepared by adding 20 µL of the non-reducing sample buffer to resuspend. Supernatant and pellet were analyzed through SDS gel electrophoresis for 30 minutes at 45 V and then 45 minutes at 150 V. After transfer of the gel to nitrocellulose, western blot was performed with a primary polyclonal A33 antibody (0.1 µg/mL) and a secondary goat anti rabbit
antibody (1:5000).

2.2.8 BCA Assay

The bicinchoninic acid (BCA) assay was used to determine total protein content in antibody-nanoparticle conjugate samples. BCA protein assay kit (Thermo Scientific) protocol was followed for preparation of the working reagent. A concentration series (16-1000 µg/mL) of bovine serum albumin (BSA) was incubated with 200 µL of the working reagent in a 96-well plate. Antibody-nanoparticle conjugates were incubated in triplicate with the working solution and UV absorbances were read with a plate reader.
3. Results and Discussion

3.1 Conjugation of Monoclonal Antibody to Iron Oxide Nanoparticles

Data were collected for three different conjugation methods: hydrazone bond formation, hinge thiol attachment, and carbodiimide coupling.

3.1.1 Conjugation via Hydrazone Bond Formation

Conjugation of monoclonal antibodies to the surface of iron oxide nanoparticles was attempted via hydrazone bond formation (Figure 19). UV visible spectroscopy was performed after functionalization of Ferrotec EMG304 nanoparticles with APTMS and sulfo-MBS. Characteristic absorption of sulfo-MBS is seen at about 285 nm.

Figure 21. UV-visible spectrum of Ferrotec EMG304 nanoparticles conjugated with Sulfo-MBS. Bare ferrotec particles were tracked during conjugation to determine change after addition of sulfo-MBS.
An increase in absorbance after coupling to sulfo-MBS suggests success of coupling.

After oxidation of the FC portion of mAb CHO31.1 with NaIO4 according to Kumar et al., aldehydes were detected with Purpald®. The characteristic purple color qualitatively indicated the presence of aldehydes on the antibodies (Figure 22).

![Image](image1.jpg)

**Figure 22. Detection of Aldehydes on CHO31.1 with Purpald.** Aldehydes were detected with Purpald on the FC portion of CHO31.1 after oxidation with NaIO4. Purple color indicates presence of aldehydes.

Oxidized antibodies were added to the nanoparticles conjugated with Sulfo-MBS and the conjugates were characterized with chemiluminescent dot blot (Figure 23).

![Image](image2.jpg)

**Figure 23. Chemiluminescent Dot Blot of hydrazone bond conjugated Ferrotec EMG304 and mAb CHO31.1.** A.) bare Ferrotec EMG304 B.) CHO31.1 2 mg/mL C.) uncoupled CHO31.1 and Ferrotec D.) hydrazone bond coupled Ferrotec and CHO31.1
The particles covalently coupled via the hydrazone bond show greater presence of antibody than the uncoupled particles, suggesting successful covalent attachment. Although this method was successful, there was significant product loss after multiple conjugations. Furthermore, multiple expensive products (PEG, sulfo-MBS) were required to obtain the correct terminal functionalizations for attachment.

3.1.2 Conjugation via Hinge Thiol

Conjugation was also attempted through hinge thiol attachment to nanoparticles (Figure 18). SDS page electrophoresis was performed on reduced human IgG to determine fragmentation with different concentrations of reducing agents: MEA and DTT.

**Figure 24. Coomassie Blue stain of SDS Electrophoresis Characterization of Dithiothreitol (DTT) and mercaptoethyamine (MEA) Reduction of Human IgG.** MEA and DTT were added to 1 mg/mL human IgG at the following concentrations. All samples were run with Non-Reducing Lane Marker Sample Buffer (Thermo) M1.) 50 mM MEA 90 minutes M2.) 50mM MEA terminated with iodoacetamide (IAAm) for 90 minutes M3.) 50 mM MEA for 3 hours terminated with IAAm D2.) 100mM DTT for 2 hours D1.) 10 mM DTT for 1 hour D3.) 10 mM DTT for 3 hours M1*.) M1 reaction with reducing lane marker sample buffer (Thermo)
Fragmentation was seen with DTT at a 100 mM concentration incubated for 2 hours (Figure 24 lane D2). The fragment at 78 kD represents half antibody fragments. Due to the time constraints of this thesis, the hinge thiol conjugation was not pursued beyond initial reduction. The simpler conjugation method via carbodiimide was attempted for initial in vitro studies. Future work on optimized orientation of a covalent binding method to iron oxide should be investigated.

### 3.1.3 Conjugation via Carbodiimide

Conjugation via carbodiimide was investigated through functionalization of Ferrotec EMG304 with silane-PEG-carboxylic acid (NanOCS) (Figure 15). Ferrotec EMG304 was first conjugated with human IgG to test the conjugation method and characterized with chemiluminescent dot blot (Figure 25).

**Figure 25. Chemiluminescent Dot Blot of EDC/NHS Reaction with Human IgG.** Conjugation of iron oxide nanoparticles to human IgG via EDC/NHS coupling was characterized with chemiluminescent dot blot. From left to right: 5 μL of 1mg/mL human IgG, Ferrotec covalently coupled with human IgG via EDC/NHS, and Ferrotec electrostatically attached to human IgG were blotted onto nitrocellulose.

Greater intensity for the EDC/NHS coupled product is seen to suggest success of covalent coupling. The same coupling method was then used to conjugate Ferrotec EMG304 nanoparticles to mAb CHO31.1 and characterized again with dot blot (Figure 26).
Figure 26. Chemiluminescent Dot Blot of EDC/NHS Reaction with mAb CHO31.1. Conjugation of mAb CHO31.1 to iron oxide nanoparticles via EDC/NHS coupling characterized with chemiluminescent dot blot. 5 µL mAb CHO31.1, Ferrotec incubated with CHO31.1, and Ferrotec coupled to CHO31.1 via EDC/NHS were dotted onto nitrocellulose.

Greater intensity for the EDC/NHS product was seen for conjugation of CHO31.1 in accord with the IgG results.

This reaction was attempted with APTMS and glutaric anhydride to provide a more cost effective conjugation.

Figure 27. Chemiluminescent Dot Blot Characterization of EDC/NHS Reaction with APTMS and Glutaric Anhydride. Ferrotec EMG 304 nanoparticles functionalized with APTMS and glutaric anhydride for addition of mAb CHO31.1 via EDC/NHS coupling. 5 µL 2 mg/mL CHO31.1, Ferrotec iron oxide nanoparticles conjugated via EDC/NHS with CHO31.1 and Ferrotec iron oxide incubated with CHO31.1 nanoparticles were blotted onto nitrocellulose.

The glutaric anhydride reaction appeared successful from the dot blot. Drawbacks to using this conjugation method include a lack of biocompatibility as these particles lack a hydrophilic PEG coating.
3.2 Characterization of Monoclonal Antibody Targeted Nanoparticles

Targeted nanoparticles were characterized to determine surface charge, efficiency of binding the target GPA33 antigen, and in vitro cellular uptake with MRI.

3.2.1 Zeta Potential Measurements

Zeta potential measurements were performed on the particles to determine surface charge.

<table>
<thead>
<tr>
<th></th>
<th>Zeta Potential (mV)</th>
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<tbody>
<tr>
<td>Bare Ferrotec</td>
<td>-50.7</td>
</tr>
<tr>
<td>Ferrotec conjugated</td>
<td>-15.7</td>
</tr>
</tbody>
</table>

Table 5. Zeta potential measurements for bare and conjugated nanoparticles. EDC/NHS conjugation was performed on the particles and zeta potential measurements were taken with Malvern Zetasizer software.

After the addition of hydrophilic PEG and human IgG, the surface charge of the particles became less negative. Recent research by Chung et al suggests that positively charged silica nanoparticles are easier to internalize into cells.49 These results have been confirmed by Chen et al as the amount of internalized nanoparticles was significantly greater for positively charged than negatively charged or neutral particles.50

The effect of surface charge on the uptake of particles should be more thoroughly investigated in future work. It is promising that the surface charge of the particles becomes less negative after functionalization with antibody.

3.2.2 In-vitro MRI Studies

Initial studies were performed with targeted nanoparticles to assess uptake in vitro. An initial study was performed to determine the effect of acid washing particles from the surface of CAPAN-2 cells. For this assay, 1.5 mg Ferrotec by weight was suspended in complete growth media and an excess of particles was
administered to wells containing 1x10^6 CAPAN-2 cells. After incubation with SPIOs one plate was acid washed and the other was not prior to MR imaging. Results of the acid wash study are shown below:

<table>
<thead>
<tr>
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<th>T2 (msec)</th>
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<tbody>
<tr>
<td>Plain Agarose (A)</td>
<td>50</td>
</tr>
<tr>
<td>CAPAN-2 Acid Washed (B)</td>
<td>46</td>
</tr>
<tr>
<td>CAPAN-2 no Acid Wash (C)</td>
<td>34</td>
</tr>
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</table>

**Figure 28. Acid wash study with bare Ferrotec particles.** (1) Bare Ferrotec particles diluted in complete growth media were incubated with CAPAN-2 cells. (A) plain Agarose (B) acid washed cells (C) non-acid washed cells. (2) T2 times for each sample

As expected, the acid wash removes particles from the cells as shown by the longer T2 of the acid washed sample (B). However, the T2 of the acid washed sample did not return to the T2 of the plain agarose suggesting that not all of the particles were removed from the sample by acid washing.

The first uptake assay was performed with targeted particles developed with synthesis I (Table 6). A set of non-targeted nanoparticles was developed by suspending bare Ferrotec in PBS pH=7.2. This incubation differed from the previous (Figure 28) in that the non-targeted particles were suspended in PBS rather than in complete growth media. Targeted and non-targeted particles were incubated with cells at the same concentration of Fe determine by ICP-OES (0.25 mg). After acid wash of particles from the surface of the cells, the non-targeted, bare Ferrotec, particles stuck to the surface of the cells.
Figure 29. **Cell plates after acid wash with bare Ferrotec particles.** Non-targeted particles stuck to the CAPAN-2 and BxPc-3 cells.

While in the acid wash study this sticking behavior to the surface of cells was not observed, when the bare particles were suspended in PBS for incubation, a visible amount of particles were still stuck to the cells.

<table>
<thead>
<tr>
<th></th>
<th>T2 (msec)</th>
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<tbody>
<tr>
<td>Plain Agarose (B)</td>
<td>50</td>
</tr>
<tr>
<td>CAPAN-2 targeted (A)</td>
<td>48</td>
</tr>
<tr>
<td>CAPAN-2 non-targeted (C)</td>
<td>----</td>
</tr>
</tbody>
</table>

Figure 30. **Cell uptake with targeted and bare Ferrotec particles.** (A) targeted particles (B) plain agarose (C) non-targeted bare Ferrotec particles suspended in PBS.
A third study was performed to normalize between targeted and non-targeted particles. Targeted nanoparticles were prepared according to synthesis II (Table 6). Non-targeted particles were coated with silane-PEG-carboxylic acid (NanOCS). Equal concentrations of targeted and non-targeted particles were incubated with cells. Cells were acid washed as before and suspended in agarose for imaging. A very slight decrease in T2 was observed for targeted particles of 2ms.

A variety of different variables may be affecting uptake of SPIOs into cancer cells in vitro. Targeted nanoparticles were investigated in order to quantify protein covalently attached to the surface and to determine the binding efficiency of the particles. Particles appear targeted through these characterization methods, however a significant change in uptake is not observed for the targeted particles. Aggregation and surface charge are two variables that may be affecting uptake of particles. Furthermore, the affinity of the CHO31.1 antibody for the target A33 may not be optimal for this work and changing the monoclonal antibody may lead to better results.

3.2.3 BCA Assay

BCA assay was performed to determine total protein content in the targeted nanoparticle samples. Targeted nanoparticles suspended in PBS pH=7.2 were incubated in triplicate with the working reagent (Pierce) for 30 minutes at 37°C. Absorbances were read with a plate reader.
The nanoparticle sample had 200 µg/mL of protein content. A back of the envelope calculation was performed to determine the number of antibodies per particle based on the 4.5% w/v amount of magnetic particles in the Ferrotec EMG304 stock solution. While there is protein on the particles, it appears from the calculation that many of the particles in solution are not conjugated to any antibody. This conjugation should be optimized in future work to obtain better results in MRI in vitro assays.

### 3.2.4 Pull Down Assay

To determine effective binding of nanoparticles with targets, a pull down assay was performed with lysates of pancreatic cancer cells (CAPAN-2, BxPc-3) and the carbodiimide coupled nanoparticles. After incubation of particles with cell lysates the samples were centrifuged and the pellet and supernatant were run
through SDS gel electrophoresis and a western blot to detect presence of GPA33.

Two pull down assays were performed with different samples of targeted nanoparticles that were prepared with the following effective reactant concentrations for synthesis:

<table>
<thead>
<tr>
<th></th>
<th>Synthesis I</th>
<th>Synthesis II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ferrotec EMG 304</strong></td>
<td>0.84 mg/mL Fe</td>
<td>0.84 mg/mL Fe</td>
</tr>
<tr>
<td><strong>Silane-PEG-carboxylic acid</strong></td>
<td>4 mg/mL</td>
<td>4 mg/mL</td>
</tr>
<tr>
<td><strong>EDC</strong></td>
<td>0.5 mg/mL</td>
<td>0.5 mg/mL</td>
</tr>
<tr>
<td><strong>NHS</strong></td>
<td>1 mg/mL</td>
<td>1 mg/mL</td>
</tr>
<tr>
<td><strong>mAb CHO31.1</strong></td>
<td>0.1 mg/mL</td>
<td>0.3 mg/mL</td>
</tr>
</tbody>
</table>

Table 6. Effective reaction concentrations for synthesis of targeted nanoparticles.

Western blots of the pull down assay show a positive control with the CAPAN-2 cells. CAPAN-2 lysates were then titrated with different volumes of targeted nanoparticles and supernatant and pellet were tested. The pellet of the titrated samples showed increased intensity as more SPIO was added to the lysates. This suggests that there is binding of the GPA33 onto the targeted nanoparticles. More efficient binding is shown with synthesis II in Figure 33.
Figure 32. Binding Efficiency of Targeted Nanoparticles Prepared through Synthesis I. Western blot of supernatant (S) and pellet (P) in pull down assay with titrating concentrations of targeted nanoparticle. BxPc-3 cell line is a negative control cell line that does not express the GPA33 antigen

Figure 33. Binding Efficiency of Targeted Nanoparticles Prepared through Synthesis II. Western blot of supernatant (S) and pellet (P) in pull down assay with titrating concentrations of targeted nanoparticle. BxPc-3 cell line is a negative control cell line that does not express the GPA33 antigen
4. Conclusions and Future Directions

The major question in this thesis is this: will targeted nanoparticles be taken into cells at concentrations sufficient to cause signal loss in images? This question was reduced to a series of coupling strategies and tests with in-vitro assays. Three distinct conjugation methods were attempted for SPIO surfaces in this work: (1) hydrazone bond formation, (2) hinge thiol attachment, and (3) carbodiimide coupling. Carbodiimide coupling was pursued further because of its reproducibility and relatively inexpensive cost. Conjugated particles via EDC/NHS coupling were characterized to determine total covalently bonded protein and binding efficiency to GPA33 in cell lysates. After concluding successful attachment, MRI in vitro assays were performed with CAPAN-2 and BxPc-3 cells. Significant uptake was not observed with the targeted nanoparticles and a change in T2 was not seen for these samples.

Future work should investigate the role of aggregation in in-vitro uptake. Kumar et al worked with gold nanoparticles suspended in 2% w/v PEG to prevent aggregation of particles after synthesis. Suspension in PEG may allow for particles to maintain their distance from each other, allowing discrete Ferrotec particles with 10 nm diameter to enter cells. Surface charge should also be thoroughly investigated. While some research argues for more positively charged particles as optimal for uptake, others argue that charges of -15 to -20 mV are optimal to facilitate a receptor mediated interaction rather than non-specific “sticking” to the negative surface of cells. Beyond the role of aggregation and surface charge, more
work should investigate site-specific attachment of antibodies to silica or iron oxide surfaces to obtain better results in vitro.

This project has prepared the basis to study in vitro interactions between targeted iron oxide nanoparticles and cells. A reproducible conjugation method has been developed and can be used to test new antibodies or a different coating. Imaging of cells allows for a quantitative method to determine signal loss from SPIO particles. After uptake has been confirmed with targeted particles at concentrations sufficient for imaging, particles can be more clearly investigated for in-vivo work.

The success of boron neutron captures therapy (BNCT) depends on effective imaging of the nanoparticles in vivo because of the localized irradiation of atoms of $^{10}$B in cancer cells (Figure 1). Future investigation of charge, aggregation, and targeting antibodies will allow for optimization of imaging in-vitro.
5. Appendix

Protocol for In Vitro MRI studies:

1. Capan-2, BxPc-3 cell lines cultured at 37° C and 5% humidity using RPMI-1640 Medium (Sigma R6504) supplemented with 0.2% sodium bicarbonate (Sigma #S8791), 1x antibiotic/antimycotic solution, and 10% fetal bovine serum (FBS; Hyclone #SH30070.03).

2. Count cells with hemocytometer: mix 15 μL cells with 15 μL Trypan Blue (Invitrogen). Pipet 10μL of mixture into hemocytometer (steps shown below): (Otopalik, 2011)

2. Plate 1x10^6 cells in each well of a 6-well plate and allow to adhere overnight

3. Treat cells with SPIO conjugates (0.25-0.5 mg Fe determined by ICP-OES). Pipet up and down to distribute throughout the plate. Incubate at 37° C for 2 hours.

4. After the 2 hour wait, remove media and excess SPIO conjugates by vacuum aspirating the liquid.

5. Treat wells with an acid wash x 2 for 10 minutes each at room temperature (20 mM sodium acetate, PBS, pH=3), and pellet in PBS (100 rpm, 3 min)
6. Trypsinize wells by adding 2-3 mL 0.25% trypsin. Incubate with trypsin at 37° C for 3 minutes.

7. Inspect cells under scope and remove cells still adhered by pipetting up and down within the plate.

8. Transfer cells to 15 mL centrifuge tubes and pellet (3 minutes, 1000 rpm)

9. Vacuum remove the supernatant. Resuspend cell pellets in 250 μL PBS pH=7.2

7. Suspend pellet in 1 mL 2% wt/vol ultra-low melting temperature agarose (Bakhru et al, 2012)

8. Transfer cell suspensions to 3-mm diameter NMR tubes

9. Incubate for 30 min at room temperature to gel the agarose prior to imaging

10. Image according to appendix in Yayla, 2011.


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