Developing a Silica-Coated Iron Oxide Nanovehicle for Antibody-Targeted Cancer Therapy

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Developing a Silica-Coated Iron Oxide Nanovehicle for Antibody-Targeted Cancer Therapy

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Submitted in Partial Fulfillment of the Prerequisite for Honors in Biological Chemistry

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Abstract

Due to their small size, nanoparticles (NPs) are a suitable platform for building the next-generation targeted therapy for cancer. The high surface-to-volume ratio and well-studied surface chemistry of NPs allow for efficient loading of treatment and targeting moieties, which would maximize drug delivery and ensure high tumor specificity, all the while sparing healthy tissue. Silica-coated superparamagnetic iron oxide (SPIO) nanoparticles, a type of magnetic resonance imaging (MRI) contrast agent, combine the small size of nanomaterial with the benefits of MRI to provide a non-invasive method for tracking NPs inside the human body. Relaxivity studies of NPs revealed that T2 relaxivity of SPIO NPs were suitable for clinical use, at above 100 mM⁻¹s⁻¹. Previous efforts successfully synthesized silica-coated SPIO NPs with a diameter of approximately 23 nm and a shell thickness of 4 nm using a reverse micro-emulsion method. Current studies focus on attaching a monoclonal antibody (As33), which targets glycoprotein A33, a tumor antigen that is over-expressed in certain pancreatic cancer cells, onto silica-coated NPs. Evidence of cellular particle uptake using fluorophore-labeled, As33-conjugated silica-coated NPs was observed in pancreatic cancer cells that over-expressed A33 in both 2D and 3D cell culture. In order to study live cells, an MR imaging phantom was designed and constructed to enable quantification of cellular particle uptake via MR imaging and ICP-OES.
Acknowledgements

“You may not control all the events that happen to you, but you can decide not to be reduced by them.” –Maya Angelou

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1 Introduction

By the beginning of the twenty-first century, cancer had become the second leading cause of death in the United States, trailing only behind heart disease.\textsuperscript{1} With a five-year survival rate of less than 6%, pancreatic cancer is one of the deadliest cancers.\textsuperscript{2} It is considered, for the most part, incurable. The poor prognosis of pancreatic cancer is a combination of the difficulties of detection in the early stages and the fact that current therapeutic options are unable to improve long-term survival rates. Moreover, available treatments target both healthy and cancerous cells, causing many undesired side-effects ranging from nausea to severe neurotoxicity.

This study is a part of the efforts at Wellesley College to develop a new form of targeted cancer therapy using nanoparticles (NPs), specifically, for treating pancreatic cancer. However, the design of our nano-vehicle has theranostic potential and can be modified easily to accommodate the needs for treating other types of cancers or individual patients. The properties of the NPs offer several advantages as a promising drug delivery system. First of all, NPs can enter cells via endocytosis.\textsuperscript{4} Ranging between 10–100 nm in diameter, the high surface-to-volume ratio of NPs provides a platform for efficiently attaching targeting and therapeutic moieties. With superparamagnetic iron oxide (SPIO) as their core, NPs can also act as a magnetic resonance imaging (MRI) contrast agent that allows the vehicle to be tracked non-invasively in the body. To avoid early clearance by lymphocytes, NPs are coated prior to
further functionalization (Figure 1). Previous work to coat NPs with gold encountered roadblocks in creating a complete coating on the bare SPIO cores. Particles with incomplete coating were difficult to functionalize and the silica coating was explored as an alternative. Silica-coated SPIO NPs are non-toxic to cells and are cheaper to make than gold-coated NPs, which give them an additional economic advantage over gold-coated NPs.

Three types of molecules are used to functionalize the NP surface (Figure 1). A monoclonal antibody (CHO31.1 or As33) is linked via a polyethylene glycol (PEG) polymer to the NP to enable the targeting of glycoprotein A33 (GPA33), a protein over-expressed on >95% of colorectal cancers and >50% of pancreatic cancer cells. Moreover, boronated poly-GL peptides are attached as the therapeutic element to allow for boron-neutron capture therapy (BNCT). By providing a source of thermal neutrons (neutrons with 0.025 eV or lower) to a non-radioactive isotope of boron, $^{10}\text{B}$, alpha radiation is released in a reaction as follows:

$$^{10}\text{B} + \frac{3}{2}\text{n} \rightarrow [^{11}\text{B}] \rightarrow \frac{4}{2}\alpha + \frac{3}{2}\text{Li} + 2.79 \text{ MeV}$$

(1)

Although alpha radiation is highly lethal, alpha particles move rather slowly and their range of damage is limited to a distance approximately equal to the diameter of a cell. Once entering the cell, radioactive-boron would kill cancerous cells while sparing the healthy tissue, as tumor cells will have accumulated more boron than normal cells.

Previously, silica-coated SPIO NPs were successfully synthesized to have a diameter of approximately 50 nm and a shell thickness of 10-20 nm using a reverse micro-emulsion method. Relaxivity studies revealed that the T2 relaxivity of SPIO cores were above 100 mM$^{-1}$s$^{-1}$, indicating that they were suitable to be used in the clinic. Preliminary experiments by Nagalla, using fluorophore-labeled, As33-conjugated silica-coated NPs, showed some evidence of cellular particle uptake in pancreatic cancer cells that over-expressed GPA33.
The goals of this study focus on quantifying cellular uptake of antibody-conjugated silica-coated SPIO NPs in pancreatic carcinoma cells via bioimaging methods. MR imaging phantoms were designed and built to study live cells using MRI. A pH-dependent fluorophore was used to label antibodies to enable tracking of NPs during receptor-mediated endocytosis under a fluorescence microscope. The following sections present a theoretical and literature review of the use of SPIO NPs in MR imaging as well as the endocytosis of NPs.

1.1 Fundamentals of Magnetic Resonance Imaging

MRI relies on the theory of nuclear magnetic resonance (NMR) to create high contrast images indicative of the chemical environment and spatial composition of a material of interest. This overview will explore elements of nuclear magnetic resonance relevant to image formation, followed by a summary of using contrast agents in MRI to optimize image contrast.

1.1.1 Theory of Magnetic Resonance

The theory of magnetic resonance is dependent on the interaction between an applied magnetic field and a nucleus that possess spin. Spin is an intrinsic property of a nucleus. MR-active materials have a non-zero nuclear spin angular momentum ($I$), which results from an odd number of protons, neutrons, or both in the nucleus. Spin is dependent on atomic composition. The resonance absorption and relaxation properties of spin vary from nucleus to nucleus, and are affected by the presence of a magnetic field and the environment surrounding the nucleus.
Using the quantum mechanical model, a non-zero spin gives rise to non-degenerate spin energy states in the presence of a magnetic field, whose energies are quantized. The number of spin states is given by:

\[
\text{# Spin states} = 2(I) + 1
\]  

Almost all elements in the periodic table, with the exception of argon and cesium, have some NMR-active isotope, but most are not abundant enough for detection.\textsuperscript{14a} \textsuperscript{1}H is the most abundant isotope for hydrogen (99.9%). With its abundance in mobile water molecules in the body, \textsuperscript{1}H is an ideal atom for MRI. Since \( I = \frac{1}{2} \) for protons, Equation [2] indicates that protons have two spin states, which are designated as \(+\frac{1}{2}\) and \(-\frac{1}{2}\). Energy spacing between two spin states depend on the gyromagnetic ratio (\(\gamma\)) of a nucleus, which is unique to the nucleus, as well as the strength of the external magnetic field (\(B_o\)) (Figure 2). The frequency associated with this energy difference between two states is known as the Larmor frequency (\(\omega\)):

\[
\omega = \gamma B_o
\]  

This energy difference \(\Delta E\) is used to produce an NMR signal by absorbing radiofrequency (RF) electromagnetic radiation at an appropriate resonance frequency \(\nu\):

\[
\Delta E = h\nu = \gamma \left(\frac{\hbar}{2\pi}\right) (1 - \sigma)B_o
\]  

where \(\sigma\) depends on the bonding environment.
Although a rigorous mathematical description of spin requires quantum mechanical principles, a tangible explanation of how NMR-active materials interact with a magnetic field can be provided by the classical (Newtonian) model. A nucleus with spin has a magnetic dipole moment \( \mu \), where the nucleus can be considered to gyrate about an axis at a constant velocity. Without \( B_o \), the \( \mu \) of nucleus precesses at the Larmor frequency and points in random directions, producing a net magnetization of zero. However, in the presence of \( B_o \), nuclear magnetic moments align in a direction with or against the direction of the external magnetic field (Figure 3A). Nuclei with \( \mu \) aligned with \( B_o \) are in the lower energy spin state, while nuclei whose \( \mu \) aligned against \( B_o \) are in the higher energy spin state (Figure 2). In the presence of \( B_o \), although the net magnetization in the transverse plane is zero, the interaction (known as the Zeeman interaction) between the nuclei and \( B_o \) is constant and non-zero, with the component of the vector in the direction of \( B_o \) remaining constant with time. Therefore, nuclei with magnetic moment in alignment with \( B_o \) are in the energetically favorable state in comparison to nuclei that aligned against \( B_o \).

Although the two populations of nuclei are approximately equal, there is a slight excess of atoms (~ten in a million) in the energetically favored spin state, which creates a net magnetic moment (the sum of individual magnetic moments precessing \textit{out of phase}) in the direction of \( B_o \) at

\[ \text{Figure 2: Zeeman Effect.} \] In the absence of an external magnetic field \( B_o \), protons in the two spin states (up or down) have the same energy (Left). However, in the presence of \( B_o \), protons with spin up (in alignment with \( B_o \)) are of lower energy than protons with spin down (opposing the direction of \( B_o \)). Moreover, there are more protons in the lower energy state. The difference in energy between the two states (\( \Delta E \)) is dependent on the strength of \( B_o \).
the Larmor frequency (Figure 3). The exact number of atoms in each spin state is governed by the Boltzmann distribution:

$$\frac{N_{\text{upper}}}{N_{\text{lower}}} = e^{\frac{\Delta E}{kT}}$$  \[5\]

where $N_{\text{upper}}$ and $N_{\text{lower}}$ are the number of nuclei in the upper and lower energy spin states, $\Delta E$ as the difference in energy between the spin states, $k$ as the Boltzmann constant, $T$ as temperature. This small excess of atoms in $N_{\text{upper}}$ in comparison to $N_{\text{lower}}$ is critical to produce a non-zero signal. Since $\Delta E$ is very small in comparison to available thermal energy at room temperature, a very strong magnetic field is needed to create a detectable energy difference.

**Figure 3.** Each proton precesses about the direction of the external magnetic field $B_0$. A) If $B_0$ is in the direction of the z-axis, the protons can be considered to be tracking two “cones”, one in the direction with or against the direction of $B_0$. B) Since there are more protons in the direction of $B_0$ than against, in a rotating frame of reference (where x and y can be of any value), there is a nonzero vector $M$ of constant magnitude in the direction of $B_0$.

To produce and detect an NMR signal, the magnetic moment must be perturbed from its equilibrium state (the direction of $B_0$) to the transverse plane, where signal is recorded. The application of RF pulse at Larmor frequency creates a small magnetic field precessing at that frequency. The net magnetic moments that were precessing out of phase around the direction of $B_0$ now begin to precess in phase around the small magnetic field created by the RF pulse (Figure 4).
This causes the longitudinal magnetic moment to spiral into the transverse plane. In a rotating frame of reference, the longitudinal magnetic moment “flips” toward the transverse plane. The angle by which it flips is a function of the strength and duration of the RF pulse. The angle is known as “θ”; when θ=90° all the magnetic moments “flip” into the transverse plane.

After the application of the RF pulse, the RF coil monitors the precession of the magnetic moment as it returns to equilibrium via $T_1$ and $T_2$ relaxation. Changes in the transverse magnetic field induce a current, which produces sinusoidal decaying signals known as free induction decays (FIDs). These signals are then Fourier transformed and spatially encoded into an MR image. More details of MR image formation are discussed in 1.1.3.

1.1.2 $T_1$ and $T_2$ Relaxation

The two relaxation parameters, $T_1$ and $T_2$, characterize two separate and simultaneous processes by which the magnetic moment returns to equilibrium. In the quantum mechanics world, excited nuclei return to lower energy state. In the classical explanation, the nuclear magnetic
moments that precess in phase around the magnetic field become out of phase. T1, known as spin-lattice relaxation, characterizes the recovery of the magnetic moment \( M_z \) along the direction of \( B_o \), (Figure 5A). T1 is the time required for the recovery of 63\% of \( M_z \) to return to the direction of \( M_z(0) \).

T2, known as spin-spin relaxation, on the other hand, measures the loss of phase of the magnetic moments in the transverse plane \( M_{xy} \). It is the time required to lose 63\% of \( M_{xy} \) from \( M_{xy(0)} \) (Figure 5B). Mathematically, T1 and T2 are time constants in the Bloch equations which characterize the two processes:

\[
M_z(t) = M_z(0) \left( 1 - e^{\frac{t}{T_1}} \right) \quad [6]
\]
\[
M_{xy}(t) = M_{xy(0)} \left( e^{\frac{t}{T_2}} \right) \quad [7]
\]

Nevertheless, the rates associated with each exponential process are not the same; T2 decay occurs faster than T1 recovery.\(^{14a}\)

There is an additional relaxation time constant known as \( T_{2\ast} \), which includes the effects of spin-spin relaxation as well as the contribution of magnetic field inhomogeneity present in all samples (Figure 5C):

\[
\frac{1}{T_{2\ast}} = \frac{1}{T_2} + \gamma \Delta B \quad [8]
\]
Superparamagnetic materials are T2-contrast agents and have important effects on T2* relaxation. In the presence of an external magnetic field, they act as small magnets and cause local field inhomogeneity. SPIO NPs increase the rate of T2* relaxation of the water protons located close to the particle core, effectively decreasing local T2* relaxation time.
1.1.3 Image Formation

There are many pulse sequences (protocols used to specify timing and strength of applied RF and magnetic field gradient pulses) that could be used to acquire MR images. All pulse sequences have two main parameters: Time to Repetition (TR) and Time to Echo (TE). TR is the time between the RF pulses that initiate a pulse sequence, whereas TE is the time between an excitation pulse and time at which a signal is collected from a FID. Unlike T1 and T2, which are intrinsic properties of the material, TR and TE can be adjusted to achieve signal that have more or fewer contributions from T1 or T2 relaxation processes:

$$signal\ intensity \propto N(H) e^{-\frac{TE}{T_2}}(1 - e^{-\frac{TR}{T_1}})$$  \[9\]

where $N(H)$ is the number of mobile protons present. T1-weighted images generally have a short TE and a short TR to maximize the effects of longitudinal relaxation. Comparatively, T2-weighted images tend to have longer TE and TR, to capture the effects of transverse relaxation.

Since SPIOs have little effect on T1 relaxation, the following discussion will focus on T2-weighted image formation. To produce an MR image, proton frequencies are localized to different regions of space by applying gradients. Gradients are small linear perturbation superimposed on $B_0$, so that the exact magnetic field, $B_i$, is linearly dependent on the location of protons inside the magnet. Three gradients are used to specify spatial information in three dimensions (3D), one in each axis. Each physical gradient has one or more of the functions for obtaining an image: slice selection, phase encoding, and frequency encoding. These functional gradients are applied sequentially (Figure 6). To begin, a gradient coil localizes RF excitation to a region of space using a slice-selecting gradient along one of three dimensional axes (x, y, and z). This RF pulse has a narrow range of frequencies,

---

1 T1 and T2 relaxation times are intrinsic properties of a material. However, in the presence of contrast agents, T1 and T2 can be altered.
which allow for the excitation of a “slice” of tissue with the matching Larmor frequency. Along the two remaining axes, a phase-encoding gradient is applied after the RF pulse, while a frequency-encoding gradient is applied during readout and the formation of the echo.

The simplest pulse sequence is a Spin Echo, where a $90^\circ$ excitation RF pulse is applied to flip the magnetic moment into the transverse plane (Figures 6 and 7). At time $\tau$, a $180^\circ$ pulse inverts the magnetic moment and refocuses the dephasing transverse magnetic moment. Since T2 relaxation is an intrinsic property, only signal decay due to spin-spin interactions are refocused. The point of maximum rephrasing occurs at $2\tau$. This point of rephrasing is known as an echo. The signal is measured at $2\tau$, known as TE, to form T2-weighted images. The decay function of the signal can be fit to obtain the time constant T2:

$$S_{I_{T2}} = s_0 (e^{-\frac{TE}{T2}})$$

$$\ln(S_{I_{T2}}) = -\frac{TE}{T2} + \ln(s_0)$$
\[ \ln(S_{T_2}) = \left(1 - \frac{1}{T_2}\right)TE + \ln(s_0) \]

The final step is to Fourier transform the signal intensity values from the time to the frequency domain for image formation.

1.1.4 SPIO as MRI contrast agents

Most iron oxides are ferromagnetic. NPs with diameter greater than 10 nm contain multiple magnetic domains separated by domain walls (Figure 8). These domains align with or against the direction of \( B_0 \), and retain some residual magnetization even after \( B_0 \) is removed. For NPs smaller than 10 nm in diameter, it is less energetically favorable to form domain walls. In other words, it costs more energy to create a domain wall than to support a single domain.\(^{16}\) As a result, these NPs smaller than 10 nm in diameter form a domain on their own, and are known as superparamagnetic. SPIO NPs align their domains with or against the direction of \( B_0 \) in the presence of an external

---

Figure 7. Radiofrequency component of Spin Echo Pulse Sequence (Adapted from Kolodny, 2013).\(^{14\text{b}}\) Multiple 180° pulses follow a 90° pulse to refocus the transverse magnetic moment in the transverse plane, forming an echo. The time to echo (TE) is the time between application of the RF pulse and the application of the 180° pulse.
magnetic field. When $B_o$ is removed, the magnetic domains of SPIO NPs revert completely back to random directions, and have no dependence on how many times the NPs had been exposed to a $B_o$. In the absence of $B_o$, SPIO are nonmagnetic.

![Diagram of magnetic fields](https://via.placeholder.com/150)

**Figure 8.** Ferromagnetic nanoparticles (NP) contain multiple domains while each superparamagnetic NP is a domain on its own. In the presence of an external magnetic field $B_o$, these domains lined in the direction of $B_o$.

When SPIO NPs are inserted into an area of interest with mobile water protons, they act as bar magnets in the presence of an external magnetic field. SPIO NPs create their own local magnetic field, which increases local field inhomogeneity. This in turn results in the increase of the rate by which the transverse magnetic moment of the nearby water protons dephases in an area. The shortening $T2^*$ relaxation time of water protons decreases signal, and causes the area to darken. Thus, a contrast agent works by increasing the contrast level of the area of interest to beyond its intrinsic level of contrast. The darkening of image is concentration dependent. The more iron oxides that are present in the area of interest, the darker the image.

The quality of contrast that MR contrast agents provide is quantified by $T2$ molar relaxivity, $r_2$, which is dependent on concentration of the contrast agent and the decrease in $T2$. By collecting FIDs at different TE's and determining $T2$, $r_2$ of SPIO NPs can be calculated as follows:

$$\frac{1}{T2} = r_2[Fe^{2+/3+}]$$

[12]
There are many advantages of SPIOs over other-metal based NPs, as iron may be easily integrated into tissue physiology, depending on its ability to interact with a cell membrane and enter the cell. On inside a cell, iron oxide can be metabolized, stored and transported through tissue via ferritin, transferritin, and homosiderin. The resultant iron can be incorporated into the iron pool for other uses. In fact, rats subjected to 100 mg Fe^{2+/3+} per kg had no identifiable side effects, although effective contrast can be achieved with a much lower dosage.

1.2 **Cellular Uptake of SPIO NPs**

In order for SPIO NPs to perform their functions in drug delivery, they must first enter the cell. The phospholipid bilayer of the cell membrane controls what goes in and out of a cell. While some molecules, such as water, can enter or leave the cell membrane freely, small polar or charged extracellular material (i.e. amino acids and metal ions) use protein or ion channels with the expenditure of energy to enter cells, while macromolecules as well as NPs, depend on endocytosis (see below). Cell membrane properties, such as surface charge, fluidity, receptor type, rate of receptor production, and characteristics of NPs, including their size, shape, surface charge, coating, and conjugated ligand, all affect how particles interact with a cell. The interaction between conjugated ligand and cell-surface receptors for cell targeting is known as receptor-mediated endocytosis.
1.2.1 Pathways of Vesicle Transport

Although there are many transport pathways in the cell (as shown in Figure 9), there are three main pathways used for NPs to enter a cell: pinocytosis, caveolae-dependent endocytosis (CDE), and clathrin-mediated endocytosis (CME). Pinocytosis is the predominant method of uptake of large NPs and microparticles. It is the method by which a cell engulfs extracellular contents and fluid, and can occur without direct interaction between NPs and the cell membrane. CDE, on the other hand, forms vesicles between 50–1000 nm via cell membrane invaginations for the uptake of anionic NPs and NPs >500 nm in diameter. Vesicles formed from CDE enclose sphingolipids and cholesterol, and bind to associated proteins to form microdomains that contain cationic lipids. It is the predominant method of particle cellular uptake in endothelial and muscle cells. Another well-studied form of endocytosis is clathrin-mediated endocytosis (CME), which occurs in all nucleated

![Figure 9. Transport Pathways in Cells.](image-url)
cells in vertebrates. Clathrin is a protein with three light and three heavy chains that form a three-legged structure called triskelion. Along with clathrin-associated protein components with different functions, known as “assembly peptides”, clathrin assembles into complexes of hexagons and pentagons that form coated pits on the cytoplasmic surface. Clathrin-coated vesicles are around ~100 nm in diameter and are the primary vesicles involved in receptor-mediated endocytosis, which could be used for targeting specific cells.22b, 23

1.2.2 Receptor-mediated Endocytosis

Whereas non-specific NP-cell interactions are induced by cargo of specific sizes, which could be a few large NPs or a cluster of small NPs, specific NP-cell interactions depend on the binding between a receptor and its ligand. The internalization of ligand-conjugated NPs also allows the targeting of NPs to specific cells.23b, 23c Some membrane proteins, such as low-density lipoproteins (LDL), have receptors that are constitutively concentrated in clathrin-coated pits, where vesicles form. These pits can contain as high as 70% LDL receptors, and all pits together can take up to 0.4 to 3.8% of cell surface area, with an average of 2%.24 Due to the amount of receptor at the pits, materials enter cells rapidly via receptor-mediated endocytosis (10–50 %/min). Other receptors become concentrated in clathrin pits upon ligand binding. Specific peptide sequences, known as “internalization motifs,” act as the entrance tickets for extracellular materials and specify where these materials will locate in the cell. For example, the same amino acid sequence could be used to get through cell membrane and target the trans-Golgi network, though the precise mechanism remains unknown.25 Moreover, it is unclear whether coated pits form before or after receptor recruitment. Nevertheless, there are some general patterns in “internalization motifs” despite an absence of a stringent primary sequence for internalization.26 The signaling peptides tend to contain a hydrophilic
stretch of acidic residues and aromatic residues, especially tyrosine, though there are leucine and lysine-based cytoplasmic targeting sequences. For ligand-conjugated NPs to enter targeted cells, they must be located at receptor-rich areas on the cell membrane. Initially, NPs must adhere to the cell surface. After overcoming resistive forces (including electrostatic, Van der Waals forces, and more), the remaining thermodynamic energy must counteract the elastic recoil force of the cell membrane to allow for membrane wrapping and forming a vesicle to engulf the NP at the site of adhesion. The precise mechanism of vesicle budding and fusion is explained by the vesicular transport hypothesis.

1.2.3 Particle-Cell Interactions during Receptor-Mediated Endocytosis

There are many factors that affect NP-cell interactions during receptor-mediated endocytosis. Typically, clathrin coats generate buds that are 50–250 nm in diameter, which explains why the upper limit diameter of NPs entering the cell via this pathway is 200 nm. For most clinical purposes, the NPs used are above 100 nm in size. However, studies have shown that NPs with a diameter smaller than 100 nm are generally more readily taken up into the cell than NPs larger than 100 nm, with an optimal diameter of 50 nm. In fact, silane-PEG coated magnetic iron oxide NPs with a diameter of 40 nm showed greater accumulation in murine tumors than those that were half the diameter. It is likely NPs smaller than 25 nm are too small to trigger CME, and a larger number of NPs are required to initiate internalization.

The cell type with which NPs interact can interfere with the ideal diameter for NP uptake. While embryonic fibroblasts preferentially take up gold NPs around 25 nm in diameter, tumor cell lines, such as HeLa cells, preferentially internalize 50 nm NPs. This difference is due to the cells’
dependence on different vesicular transport pathways. For example, macropinocytosis is absent in brain capillary endothelial cells.\textsuperscript{33}

The shape of NPs also matters. In some studies, rod-shaped gold NPs were found to enter cells more slowly than spherical ones, perhaps due to kinetics as it takes longer for the cell membrane to wrap around a rod.\textsuperscript{34} However, in studies using different materials, the highest internalization rate of hydrogel particles in HeLa cells was for rods with an aspect ratio (a value obtained from dividing the length of the major axis by the width of the minor axis) of three, rather than spherical ones.\textsuperscript{35} Moreover, molecular dynamics simulations of uptake of ligand-conjugated NPs reported higher efficiency for rod-shaped than spherical NPs.\textsuperscript{36} On the other hand, sharp edges on NPs tend to suppress endocytosis, as they may interfere with membrane wrapping.\textsuperscript{36}

Surface characteristics and charge can be tuned to improve internalization of NPs. Cellular uptake of NPs could be improved by the presence of a cell penetration peptide (CPP), which induces local clotting at the cell membrane after penetration that served as new binding sites.\textsuperscript{37} Cationic NPs, even in the absence of a CPP, can induce CME by interacting with the anionic head groups of the phospholipids that make up the cell membrane.\textsuperscript{38} Membrane charge, although different between monolayer cell culture and \textit{in vivo}, can affect the ability of NPs to interact with the cell membrane surface. Moreover, NPs coated with dichain cationic emulsifiers (DMAB) showed higher cellular uptake than single-chain cetyltrimethylammonium bromide (CTAB)- or dodecyltrimethylammonium bromide (DTAB)-modified ones.\textsuperscript{20b} Ionic interactions between lipids and cationic NPs allow for rapid uptake, which provides a promising platform for gene delivery as RNAi mandates the cytoplasmic targeting.\textsuperscript{39} Anionic NPs, unlike cationic ones, rely on adsorptive endocytosis. Bare and silica (SiO\textsubscript{2})-coated iron oxide NPs have hydroxyl groups on the surface, making them slightly negatively charged. SiO\textsubscript{2}-coated iron oxide NPs of different sizes have been used to label human macrophage and dendritic cells, even more efficiently than dextran-coated ones.\textsuperscript{20b} In addition to
cellular uptake, surface modifications can be used to achieve other functions. Cobalt ferrite magnetic NPs with a SiO$_2$ shell-core modified with fluorescein isothiocyanate (FITC) and rhodamine B isothiocyanate (RITC) dyes enable dual-modular imaging via optical and MR imaging.\textsuperscript{40} Other protein-based surfactants, such as transferrin, demonstrated increased internalization, biocompatibility, and most importantly, greater inhibition of tumor growth in prostate cancer cells when conjugated to therapeutic-based NPs.\textsuperscript{20b} With the help of surfactants, NPs can even cross the blood brain barrier (BBB). PEGylated Fe$_3$O$_4$ NPs covalently conjugated to lactoferin (Lf), which binds to Lf receptors present on cerebral endothelia cells, were show to successfully move NPs into the brain via receptor-mediated transcytosis.\textsuperscript{41}

To enable active targeting of NPs to specific cells, antibodies, especially monoclonal antibodies (mAb), provide the potential to concentrate cytotoxic agents in tumor cells, while sparing healthy tissue. SPIO NPs modified with mAb (muj591) have been shown to target prostate-specific membrane antigen (PSMA) and significantly decrease T2 in PSMA-positive cells.\textsuperscript{42} There are, nevertheless, several disadvantages of using mAb, including premature drug release or antigen shedding, as the presence of serum proteins and enzymes, especially those released by our immune system, may bind and disrupt the integrity of mAb on the NP.\textsuperscript{43} When drug and linker are both hydrophobic, mAb-conjugated NPs could also suffer from aggregation which results in reduced circulation time.\textsuperscript{44} As a result, the mAb used for targeting purposes must be chosen carefully to achieve the ideal targeting efficiency.

To improve circulation time of NPs, to thrive in the protein-protein competitions, and to avoid the defensive mechanism provided by the reticuloendothelial system (RES), the most common strategy is to add a particle coating. While albumin can minimize non-specific interactions between NPs and other proteins in the bloodstream, polyethylene glycol (PEG) is often the molecule of choice to minimize interactions between NP and phagocytic cells.\textsuperscript{3} Its protective property is a function of
chain length and surface density, where an initial concentration of 10.6 mol% of PEG could provide a sufficient time window to reach the target. Similar to mAb, the chain length and structure of PEG must be optimized, as they could interfere with cellular targeting.

1.2.4 Intracellular Localization of NPs

Once entering the cell, NPs are kept in endosomal vesicles (with an internal pH 6.5) which mature into late endosomes before fusing with lysosomes (pH 4-5). The acidic contents as well as the presence of digestive enzymes can result in the digestion of NPs. Alternatively, NPs that escape digestion maybe recycled back to the cell surface by trafficking endosomes. Some nanomaterials, especially cationic and basic NPs, are capable of escaping the endosome. A theory known as the “proton sponge effect”, where phase transitions of pH-dependent lipids or peptides result in osmotic swelling of vesicles and disruption of the endosomal membrane, thereby allowing NPs to escape, is used to explain such phenomena. Endosomal escape is necessary for NPs to be delivered to various locations in the cell other than the lysosome, including the cytoplasm (i.e. small interfering RNA, siRNA), the nucleus (for delivery of DNA or chelating agents), and the mitochondria (antioxidants or mitochondrial DNA). Some nanomaterials, such as some carbon nanotubes, can penetrate vesicular or cell membrane to enter the cytoplasm even in the absence of targeting ligand. However, for BNCT, NP escape from the endosome is not necessary. Although the destination of our NPs in the cell remains unclear, a pH-dependent fluorophore activated only at low pH is used to track the SPIO NPs optically as they enter the endosomal pathway.

The interactions between many aspects of the cell and the NP dictate whether and how NPs will interact with the cell membrane, as well as the NP’s destination inside a cell. The design of the
NP must be optimized to achieve appropriate targeting efficiency of NPs for efficient drug delivery to take place.
Materials and Methods

2.1. Synthesis of Silica-coated Iron Oxide Nanoparticles

Silica-coated iron oxide nanoparticles were synthesized using a reverse micro-emulsion method adapted from Narita et al. and thesis work by J. Rana. Cyclohexane (J.T. Baker, >99%) and Igepal® CO-520 (Sigma Aldrich) were mechanically mixed on a magnetic stir plate (Thermolyne Cimarec) for 5-10 minutes and the magnetic stir bar was then removed. An aliquot of aqueous EMG 304® Ferrofluid (Ferrotec) was weighed and added to the cyclohexane-Igepal mixture with sonication. The final cyclohexane-Igepal-Ferrotec solution was sonicated for 5 minutes to establish a reverse micro-emulsion phase, before NH₄OH (Sigma Aldrich 28% w/v) was added to increase the pH of the solution to 11.0±1.0. The pH was measured using universal pH indicator paper (Precision Labs, Inc.). This solution was left on a wrist-action shaker (Burell Scientific) for one hour. Next, tetraethyl orthosilicate (TEOS, Sigma Aldrich, 99.9%) and PEG-silane (Polymer Source, Trimethoxy propyl-terminated PEG methyl ether, 420 MW) were added to the sonicated NH₄OH micro-emulsion mixture. The final solution was left on a wrist-action shaker for 1 to 46 hours before the reaction was terminated by the addition of an equal volume of abs. EtOH (VWR International). The terminated reaction mixture was left on the shaker to continue mixing for one hour, before the sample was placed beside a neodymium magnet (Applied Magnets, N50, 32+ lbs pull force) for collection. The supernatant was decanted and the products (dark brown pellets) were washed three times with abs. EtOH before re-suspension in 18.3 Ω H₂O (nanopure water, nH₂O). Silica-coated NP solutions were then filtered through a 0.45 µm cellulose acetate membrane (VWR International) to remove larger aggregates before characterization. Typical amounts of reagents used are shown below:
2.2 Transmission Electron Microscopy (TEM)

Size and morphology of nanoparticles were examined under a JEOL 1200EX transmission electron microscope (80-100 KV accelerating voltage) at the Department of Cell Biology, Harvard Medical School. Samples were prepared by suspending a formvar 400 mesh CU grid (Ladd Research) upside-down in 1-2 drops of sonicated nanoparticle solution for 15 minutes. The excess liquid on the grids was removed using Kimberly-Clark® Kimwipes™ and the grids were left to dry in air for at least 24 hours. TEM images were taken between 25000 and 64000 x magnification. The nanoparticle sizes were determined using an AMT 2k charged-coupled device (CCD) camera system. The CCD detects the movement of electrical charge from the electrons scattered from the sample and moves this charge to an area where the charge can be converted into a digital value in order to create an image, where the size of the sample is calculated.

2.3 Dynamic Light Scattering (DLS)

Particle size distributions of bare and coated iron oxide nanoparticles were measured using dynamic light scattering (Malvern Nano-ZS), when possible. Samples suspended in 18.3 Ω H₂O were sonicated for 1-2 minute(s) before dilution to a concentration between 6-12 µM. The diluted
solutions were filtered using a syringe filter with a 0.45 µm cellulose acetate membrane (VWR International) to remove dusts and larger aggregates. Size measurements and monodispersity data were collected using the standard operation files SiO2 in water.sop and FexOy.sop (173° backscatter measurement, 120 s equilibrium time, RI=2.940, Abs=0.05 in water at 25 °C) and averaged over three trials of 12 to 16 readings each.

2.4 Fourier-Transform Infrared Spectroscopy (FT-IR)

IR spectra of the bare and functionalized nanoparticles were obtained using a Perkin-Elmer Spectrum One FT-IR spectrometer with a universal ATR sampling accessory (scanning range: 650-4000 cm⁻¹; number of scans: 64-256; resolution: 2.00 cm⁻¹) to independently confirm the presence of silica coating. Stock FeₓOᵧ NPs (~750 µl) suspended in abs. EtOH were centrifuged at 13000 rpm for five minutes. Supernatant was decanted until only 20-30 µL of liquid was left inside the eppendorf tube. Using a glass pipette, the leftover supernatant was mixed with the NP pellet to make concentrated NP slurry. A drop of the resulting viscous NP solution was pipetted over the ATR crystal and left to air dry until the EtOH completely evaporated (5-10 minutes). IR spectra were taken from the solid NP remnants using air as background scan (adapted from H. Yayla thesis).  

2.5 Antibody Conjugation to NP

Monoclonal antibody that targeted GA33 was conjugated onto NP surface using methods adapted from thesis work by R. Nagalla (Wellesley College, ’14).  

2.5.1 Creation of the ProteinA/G-Mal-PEG-Silane Linker

Samples of Protein A/G (ProSpec, PRO-646) reconstituted to 1 mg/L in sterile PBS were thiolated using Traut's reagent, 2-iminothiolane•HCl (Thermo Scientific 26101). Thiolated
protein A/G were then purified using a 7k MWCO desalting spin column (Zeba\textsuperscript{TM} Thermo Scientific 89892) according to manufacturer's instructions, and coupled to 5kDa Maleimide-PEG-silane overnight at 4 °C in a 1:10 (protein A/G to PEG) molar ratio on an orbital shaker (Fisher Scientific), to create a linker-antibody complex. This complex was purified with a second a 7k MWCO desalting column (Zeba\textsuperscript{TM} Thermo Scientific 89892).

2.5.2 Antibody Conjugation to NP via Linker

Immediately after purification, the protein A/G-Mal-PEG-silane complexes were tethered to the silica surface of SiO\textsubscript{2}@NP in linker excess at 4 °C on an orbital shaker overnight. Linker-conjugated silica-coated NPs were then collected by a magnet and washed three times with sterile PBS. The protein A/G@Mal-PEG-silane@SiO\textsubscript{2}@NPs were then analyzed for protein absorbance at 280 nm using a spectrophotometer (NanoDrop 2000, Thermo Scientific) to verify the presence of protein A/G on the NPs and the absence of proteins in the supernatant.

Upon confirmation of linker attachment to the NP surface, monoclonal antibody As33 (mAb provided by Prof. Andrew Webb, Wellesley College) tagged with pHrodo-Green Fluorophore (Life Technologies) was added to the protein A/G@mal-PEG-silane@SiO\textsubscript{2}@NPs in antibody excess. The mixture was left to shake on an orbital shaker for 24 hrs at 4 °C, for protein A/G to bind with the Fc portion of mAb As33. The presence of As33 was indirectly verified via the presence of green fluorescence on the NP pellet (not in supernatant) under a fluorescence dissecting microscope (Leica, M165FC) using a green fluorescent protein (GFP) filter and an Enzyme-Linked ImmunoSorbent Assay (ELISA), which will be discussed in details in section II.6.
2.6 Enzyme-Linked ImmunoSorbent Assay (ELISA) for Antibody Quantification

A modified method based on an Enzyme-Linked ImmunoSorbent Assay (ELISA) protocol developed by Harini Natarajan'15 was used to quantify the amount of As33 present on the surface of the silica-coated NPs. Protein A (1 µg/mL, Life Technologies) in sterile 1X PBS (American Bioanalytical AB11108) was used to coat seven out of eight rows of a 96-well polystyrene assay plate overnight at 4 °C. Protein A acted as the binding partner to the antibodies on the plate (Figure 10). The last row of wells was coated with PBS to provide information about background absorbance levels. The plate was washed three times with 1X TBS (Sigma Aldrich T5912) with 0.05% TWEEN 20 (1X TBST, Sigma Aldrich), and blocked with 1X TBST with 2% dried milk (Bio-Rad 1706404).

![Figure 10. Steps of well treatment in a typical Enzyme-Linked ImmunoSorbent Assay (ELISA) prior to secondary antibody addition. Top: Addition of Protein A (1 µg/mL) in the first seven rows, with PBS as control in Row 8. Bottom: Addition of As33 and NP serial dilutions were added in triplicates in Rows 1-6. Serial dilutions of BSA were added in Row 7, and a fourth replicate of serial dilution of NPs were added in Row 8.](Image)
for one hour at room temperature. Twelve serial dilutions of nanoparticles and mAb As33 (1 µg/mL) were prepared in 1X TBST with 0.1% BSA (Santa Cruz Biotech Sc2323). NP and mAb As33 dilutions were then added to the plate in triplicates. In the seventh row, 1X TBST with 0.1% BSA was added in the absence of NPs or As33 to ensure that the secondary antibody did not cross-react with protein A. The solutions were then incubated at room temperature for one hour and washed three times with 1X TBST before exposure to a secondary antibody, a goat anti-mouse antigen-binding fragment (Fab, Jackson ImmunoResearch 115-036-072) for As33 conjugated with horseradish peroxidase (in 1X TBST with 0.1% BSA) for one hour at room temperature. After incubation, the plate was washed three times with 1X TBST to remove weakly bound proteins. The 3,3',5,5'-tetramethylbenzidine (TMB, Thermo Scientific 34028), a horseradish peroxidase (HRP) substrate, is then added as a staining method, developing a blue color in the solution with HRP activity to indicate the binding of secondary antibody to As33. The reaction was stopped using 2M H₂SO₄ (Thermo Scientific, ACS grade) and absorbance intensity at 450 nm read with a SpectraMax® M3 microplate reader (Molecular Devices). A standard curve of absorbance vs. As33 concentration was created using Beer's Law (A=εcl) and the concentration of As33 in the NP sample was calculated based on this standard curve.

2.7 Uptake of Particles by Cells

2.7.1 Cell Culture

CAPAN-2 and BxPC-3 human primary pancreatic adenocarcinoma cell lines were provided by Prof. Andrew Webb (Wellesley College). For 2D cell culture, tumor cells were grown in RPMI media (Sigma Aldrich) at 37 °C and 5% CO₂ in T-25 flasks until 70-80% confluent.
For 3D cell culture, tumor cells were first grown in T-150 culture flasks until ~70% confluent, before being scraped from the flask and washed twice with PBS. After washing, cells were resuspended in RPMI media without phenol red, antibiotics, fetal bovine serum, but with 0.5% BSA for BxPC3. For CAPAN-2 cells, 2% methylcellulose was included in the previously mentioned media solution to assist with spheroid formation (method based on a protocol developed by Karina Verma '14). 200,000 to 250,000 cells were seeded in each well of the 96-Well Hanging Drop Plate (3D Biomatrix) and incubated at 37 °C and 5% CO₂. Cell counts were performed with a hemocytometer. In the case to create a standard curve of cell counts, both a hemocytometer and an automated cell counter (Moxi Z™, ORFLO) were used for cell counting.

2.7.2 Particle Exposure

As33-conjugated silica-coated NPs or silica-coated NPs at 2.45 nM Fe²⁺/³⁺ concentration were subjected to CAPAN-2 and BxPC-3 cells at three time points of tumor spheroid formation: before formation (immediately after seeding), beginning of formation (5 hrs), after formation (36 hrs) for two hours. An excess of NP cores, at approximately 0.04 mM, was also used as control. Post NP exposure, tumor spheroids were transferred to a 96-well polystyrene assay plate, before being collected into larger cell pellet in a PCR tube. The spheroids were washed twice with PBS before imaging via MRI.

2.8 Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)

The concentration of Fe²⁺/³⁺ and Mg²⁺ ions were determined using an Optima 7000 DV ICP-OES spectrometer and an analysis protocol adapted from Oca-Cassio et al.⁵² An aliquot (1-2 mL) of filtered, diluted FeₓOᵧ NP solution (bare or functionalized) was acid digested with 35% w/w high-purity HNO₃ (OmniTrace Ultra) for a minimum of 48 hours to displace oxygen atoms from the FeₓOᵧ
crystal lattice, releasing free-floating \( \text{Fe}^{2+/3+} \) into the solution. Similarly, a volume excess of 69% high-purity HNO\(_3\) (OmniTrace Ultra) was used to digest cell pellets, after removal of supernatant, for a minimum of 48 hours to release cellular Mg\(^{2+}\) ions into solution. A temporary increase in solution temperature and a color change from rust brown to clear liquid were indicators of progress in the digestion of Fe\(_{x}\)O\(_y\) NPs. A change of clear to light yellow in the acid solution were indicators of cellular digestion. The acid-digested samples were then diluted with nH\(_2\)O to create 5-10 mL of 2% HNO\(_3\) w/w solution of acid-digested NPs or cell pellets in 15 mL conical centrifuge tubes that were previously soaked in 2% HNO\(_3\) overnight. Atomic emission intensities of known concentrations (40 ppm, 4 ppm, 0.4 ppm, 0.04 ppm, and 0.004 ppm) of Fe\(^{2+/3+}\) standard solutions were taken at \( \lambda_{\text{em}} = 238.204 \) nm. For magnesium, Mg\(^{2+}\) standard solutions (1 ppm, 0.1 ppm, 0.05 ppm, 0.01 ppm, and 0.001 ppm) were read at 279.077 nm or 283.204 nm. These values were plotted as a function of [Fe\(^{2+/3+}\)] or [Mg\(^{2+}\)] to create standard curves. Sample [Fe\(^{2+/3+}\)] or [Mg\(^{2+}\)] were calculated by substituting sample signal intensity values collected at \( \lambda_{\text{em}} = 238.204 \) nm, 279.077 nm, or 283.204 nm into the standard curves. Since iron and magnesium standards were analyzed before each batch of samples, a new standard curve was calculated for each batch of samples. A sample standard curve for Fe\(^{2+/3+}\) is shown below:

![Figure 11](image-url)

**Figure 11.** Intensity vs. concentration standard curve of Fe in the iron oxide nanoparticles used in ICP-OES determination of iron concentration in the nanoparticles samples.
2.9 MRI Phantom Preparations and Relaxivity Studies

2.9.1 Nanoparticle Characterization

Fe\textsubscript{x}O\textsubscript{y} NP samples in nH\textsubscript{2}O were prepared by pipetting an equal volume (0.5-1 mL) of 3 serial dilutions (typically 0.03-0.3 mM) of Fe\textsubscript{x}O\textsubscript{y} NP samples in nH\textsubscript{2}O into individual 3 mm OD NMR tubes (Wilmad). Sample NMR tubes were then put into a customized, reusable phantom holder designed by Stephanie Huang ’12 (Figure 12). A typical MR image of the phantom is shown on the right:

![Image of phantom and MR image](image)

**Figure 12.A** Customized reusable phantom designed by Stephanie Huang ’12. The phantom can hold up to eight 3mm NMR tubes at a time. **B** A typical MR image of the phantom containing sample iron oxide nanoparticle solution.

To obtain T2 relaxation times of the NP samples for molar relaxivity calculations, MR images (400 MHz Bruker Avance NMR spectrometer, 9.4 T, vertical bore, 2.4 G/cm/A gradient strength) of the iron oxide NPs samples were acquired with Bruker Paravision 4.0 MSME-T2 Map pulse sequence (TR=3062 s, TE=10, 20, 30,…, 600 ms, 60 echoes, avg=1, 4x4 cm FOV, 128x128 matrix). By fitting an exponential decay curve to a plot of TE vs. signal intensity using the Image Sequence Analysis (ISA) tool in Paravision for each iron oxide NP concentration as outlined in the Appendix of Yayla’s thesis, T2 relaxation times of each sample were determined. The slope of the
line of best fit between $1/T_2$ and $[\text{Fe}^{2+/3+}]$ (determined by ICP-OES as outlined in Section 2.8) is known as the molar relaxivity of the NPs according to the following equation:

$$r_2 = \frac{R_2}{[\text{Fe}]} , \text{where } R_2 = \frac{1}{T_2}$$

Statistical tests of molar relaxivity between slices and various syntheses were computed using SPSS 22.0.

2.9.2 MRI of Cell Pellets

Three customized designs of MRI phantom were built for imaging tumor cell pellets using MRI (Fig. 13). Designs A and B were built for containing individual wells from a 96-well plate.

![Image](image.png)

**Figure 13.** Three customized phantom designs were built by Larry Knowles (Wellesley College) for imaging tumor cell pellets. Design A) contained a two layers with wells that have a diameter of 6.86 mm (comparable with wells in 96 well plates, while Design B) contains a single layer. Design C) was created to hold 0.2 mL PCR tubes. A fully assembled phantom with Design C) is shown in D). E) is a comparison between the middle layer used in Designs A/B vs. C. Scale bar=1 cm.
Design C was built for containing PCR tubes. T2 relaxation times of cell and PBS samples were determined using the Image Sequence Analysis (ISA) tool in Paravision 4.0 following directions outlined in the Appendix of a thesis by H. Yayla. One-Way ANOVA of the T2 relaxation times of the same slice of each sample were performed using SPSS 22.0.
3 Results and Discussion

3.1 Synthesizing Silica-Coated Particles

The diameters of individual Ferrotec© NPs before and after silica-coating formation were measured by transmission electron microscopy (TEM). Darker regions of the micrographs represent NP cores, whereas lighter regions represent silica coating (Figure 14). Although Ferrotec© FeₓOᵧ cores were advertised as 10 nm spheres, they were 15±2 nm (n=20) in size and not spherical in shape (Figure 14A). Silica-coated NPs were 23±4 nm in diameter, with some aggregation (Figure 14B). By approximating the core size as 15 nm, the thicknesses of the silica-coating on the NPs were

![Figure 14. Transmission electron micrographs (TEMs) of A) uncoated iron oxide cores, 15±2 nm (n=26) and B) silica-coated NPs in 2 hr, 23±4 nm (n=22). Images obtained using copper TEM grids coated with Formvar. Blue outline = core, orange outline= silica-coating, yellow arrow = diameter of overall NP, and black arrow = diameter of core.](image-url)
approximately 4 nm.

The coating of Fe₃O₇ NPs was further analyzed using FT-IR spectroscopy. The presence of silica on Fe₃O₇ NPs was confirmed via the presence of a peak around 1080 cm⁻¹ (Si-O stretch), as the bare Fe₃O₇ NPs showed a flat region in spectrum (Figure 15). Uncoated Fe₃O₇ NPs showed two small fluctuations in the CH-sp³ stretch region, which may be due to a coating of oleic acid on the particle surface. However, this fluctuation disappeared when the Fe₃O₇ NPs were coated with silica and only a peak indicating the presence of Si-O bonds remained.

![FT-IR spectra](image)

**Figure 15.** FT-IR spectra of bare Ferrotec© NPs (top), the silica-precursor tetraethylorthosilicate (TEOS) (middle), and silica-coated NPs (bottom). Silica-coated NPs has a peak in the characteristic Si-O stretch region between 1000-1300 cm⁻¹ (N=64).

### 3.2 Antibody Conjugation and Quantification

The presence of As33 on antibody-conjugated SiO₂@NPs was confirmed and quantified using an enzyme-linked immunosorbent assay (ELISA) (Figure 16). Absorbance of the antibody
Figure 17. Color change observed in the ELISA plate for quantifying the number of antibody per nanoparticles (NPs) (See Figure 10). Serial dilutions (with most concentrated solution in Well 1) of As33 antibody standards in triplicates are in Rows A-C. Serial dilutions (with most concentrated solution in Well 1) of As33@silica@NPs in triplicates (Rows D-F) displayed a color change to yellow, indicating the presence of secondary F’ab binding to the NPs. To ensure that the secondary F’ab did not bind nonspecifically to the plate, row G was exposed to TBST buffer with 0.1% BSA (no As33). To quantify background absorbance and to confirm that NPs did not bind non-specifically to the plate, row H did not have any protein A but was exposed to the same serial dilutions of As33@silica@NP as Rows A-C.

A standard at 450 nm was used to create a standard curve, from which As33 concentrations in the NP samples were calculated (Figure 17). A solution of 1.6 x10^{12} NP per mL was the most concentrated

Figure 16. Absorbance values less than 1 from serial dilutions of As33 collected at 450 nm were used to produce a standard curve. This standard curve was used to quantify the concentration of antibody presence in As33@SiO_{2}@NPs dilutions in an ELISA using As33@SiO_{2}@NPs dilutions that had an absorbance between 0.1 and 0.8 (n=3).
As33@SiO$_2$@NP sample used in the ELISA and did not have above background absorbance at $\lambda=450$ nm. The lack of absorbance from the fluorophore conjugated to As33 suggested that the fluorophore on As33 did not affect sample absorbance at this concentration, although work by Nagalla suggested that 20 µM pHrodo fluorophore had a small absorbance at 450 nm.$^{13}$

ELISA results indicated that there was an average of 0.0043 nmol/mL of As33 in the stock As33@SiO$_2$@NP solution ($N=3$). Assuming that each antibody is approximately 150 kDa, there were $2.57\times 10^{12}$ antibodies per $1.64 \times 10^{12}$ NP, which was 1.6 antibodies to each NP two months after the conjugation reaction took place, suggesting stability of the antibody conjugation. DLS measurements showed that As33@SiO$_2$@NPs have a median hydrodynamic radius of 109±7 nm ($N=26$). With an average polydispersity index value (PDI) of 0.421, the nanoparticles had a range of sizes after antibody conjugation (Figure 18). TEM of the nanoparticles confirmed this finding (Figure 19). Moreover, antibody-conjugated NPs seemed to be in found either in clump of 110±20 nm or as singletons at approximately 30 nm each, indicating a degree of aggregation in these samples (Figure 19A). Regular spacing between the NPs suggested the presence of a PEG linker and antibodies, although they do not appear on TEM images (Figure 19A). The diameter of the small clumps were comparable to the hydrodynamic radius of the sample measured using DLS, although it was expected that the diameter measured using TEM to be smaller than that of hydrodynamic radius. This size

![Figure 18. Size distribution of As33@SiO$_2$@NPs measured using dynamic light scattering (DLS). Red and green represent mean ± SD ($n=26$).](image)
difference may be due to the fact that As$_{33}$$@$SiO$_2$$@$NP samples could not undergo sonication before blotting on TEM grids, in an effort to prevent As$_{33}$ denaturation, whereas DLS measurements were performed on filtered and sonicated samples. Nevertheless, some unconjugated silica-coated NPs were also observed under TEM, as spacing was not observed between the NPs (Figure 19B).

![Figure 19](image-url)

**Figure 19.** Transmission electron micrograph of A) As$_{33}$ conjugated silica-coated iron oxide nanoparticles (NPs) and B) un-conjugated nanoparticles. As$_{33}$-conjugated NPs were 32±7 nm (n=25) in diameter and have regular spacing between NPs. Small clumps of As$_{33}$-conjugated NPs are 110±20 nm. Un-conjugated NPs were found in small clumps. Orange arrows point to phosphate salt-covered NPs, whereas blue arrows point to representative single As$_{33}$-conjugated silica-coated NPs. Scale bar = 100 nm (A), 20 nm (B).

### 3.3 Designing a New Phantom

A two-layer well phantom was designed to position multiple NP samples within the sensitive region of the RF coil of the MRI system for relaxivity measurements (Figure 13A). This holder would have allowed for imaging of eight samples simultaneously. However, reproducible data could not be obtained using the two-layer design and a single-layer well holder phantom was built instead to hold only four samples (Figure 13B). Different conditions (Conditions 1-6 in Figure 20A) of solutions with varied iron concentration were used to study the effectiveness of the phantom, to
ensure that T2 relaxation times of the same NP sample (e.g. the yellow dot in Figure 20A) could be measured reproducibly using the phantom. A one-way ANOVA of T2 relaxation times of an Fe₃O₇ NP solution with an [Fe²⁺/³⁺] of 0.436 M revealed a statistically significant difference in its T2 relaxation time when imaged in conditions 1-6 outlined in Figure 20A ($F(3, 8) = 24.092, p < 0.001$). This one-way ANOVA suggested that the iron oxide NPs in different wells were interacting with each other in the presence of an external magnetic field and the phantom was not suitable for imaging multiple NP samples.

**Figure 20.** A) Combination of Fe concentrations of iron oxide NP solutions used for studying the new phantom. B) T2 relaxation time of 0.436 M Fe solution in the two phantom designs.

<table>
<thead>
<tr>
<th>Condition ID</th>
<th>96-Well Phantom (n=3)</th>
<th>PCR Tube Phantom (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>53±0</td>
<td>102±9</td>
</tr>
<tr>
<td>4</td>
<td>93±2</td>
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<td>114±8</td>
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<tr>
<td>8</td>
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<td>112±3</td>
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In order to minimize the effects of NP interaction, the cross-sectional volume of NP solution in the phantom was reduced and the distance between samples was increased by designing a phantom to hold samples in PCR tubes (Figure 13D). A one-way ANOVA of T2 relaxation times of a Fe$_3$O$_y$ NP solution with an [Fe$^{2+/3+}$] of 0.436 M Fe$^{2+/3+}$ did not find a statistical difference between the T2 relaxation times of the sample in all conditions outlined in Figure 7 ($F(5, 13) = 1.388, p =0.291$). The results indicated that at an iron concentration <0.436 M, the phantom could be used study four NP samples. Since NP cellular uptake studies are performed at nanomolar ranges, the new phantom was suitable for studying particle cellular uptake.

### 3.4 Magnesium Content and the Number of Tumor Cells

CAPAN-2 and BxPC-3 cells contain relatively stable levels of Mg$^{2+}$ over time, so the amount of Mg$^{2+}$ ions in a cell pellet is linearly correlated with the number of cells in a pellet formed by cells from the same cell line.$^{53}$ Two wavelengths of observation were compared in ICP-OES to determine

**Figure 21.** ICP-OES determination of Mg$^{2+}$ content in cell pellets of known sizes at two characteristic wavelengths of magnesium: A) $\lambda=279.099$ nm and B) $\lambda=285.024$ nm. $\lambda=285.024$ nm was chosen in subsequent experiments as it had higher sensitivity at lower magnesium concentrations.
the appropriate wavelength for measuring Mg$^{2+}$ content of tumor cells.

Although the R$^2$ value of the standard curve at both wavelengths are well above 0.90 for both cell lines, $\lambda$=279.099 nm occasionally provided intensity values below detection limit at lower Mg$^{2+}$ concentrations. As a result, a wavelength of 285.024 nm was used in subsequent measurement of magnesium content in cells.

### 3.5 Cellular Uptake

#### 3.5.1 2D Cell Culture

Using CAPAN-2 cells as negative control and excess silica-coated NPs in CAPAN-2 cells as positive control, a decrease in T2 relaxation time in CAPAN-2 cells after exposure to As33@SiO$_2$@NPs compared to that of only CAPAN-2 cells suggest Fe$_x$O$_y$ NPs uptake. To quantify the number of NP uptake per cell, the amount of Fe$^{2+/3+}$ and Mg$^{2+}$ in the sample were determined from ICP-OES. The amounts of Mg$^{2+}$ in the sample were correlated to the number of cells in the sample using standard curves established in Section 3.4. The number of NPs in the sample was calculated by first correcting for the amount of Fe$^{2+/3+}$ in a comparable number of cells (assuming the Fe$^{2+/3+}$ level in each cell did not fluctuate significantly over time) to find the net mass of iron in the sample that is attributed to NPs. Using a mass of 6.4x10$^{-15}$ mg Fe$^{2+/3+}$ per NP (assuming equal amounts of FeO and Fe$_2$O$_3$, as the percentage of FeO or Fe$_2$O$_3$ in the NP core is unknown) and assuming that iron oxide cores are 15 nm spheres, the number of NPs in the sample could be calculated (Personal Communications: Nolan Flynn). After dividing the number of NPs by the number of cells in the sample, approximately 530 antibody-conjugated NPs were found per cell, which is about six times higher than silica-coated NPs uptake in 2-D cell culture even in silica-NP excess (Figure 22B).
Before imaging via MRI, tumor cells were exposed to NPs for two hours before (0 hr), at the beginning of (5 hr), or after (36 hr) tumor spheroid formation. CAPAN-2 cells subjected to NPs with or without silica and antibody showed a large decrease in T2 relaxation time at all three time points of tumor spheroid formation (0, 5, 36 hr). On the other hand, BxPC-3 cells showed a smaller decrease in T2 than did CAPAN-2 cells when the spheroid was grown for 0 or 5 hr in the presence of silica-coated NPs and after spheroid formation in the presence of As33-conjugated SiO$_2$@NPs (Table 2). Even in the absence of As33, silica-coated NPs were able to penetrate into both types of cells. This

<table>
<thead>
<tr>
<th>Conditions</th>
<th>#cells imaged</th>
<th>Net mg of Fe</th>
<th>NP /cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAPAN-2</td>
<td>3.6x10^6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SiO$_2$@NP</td>
<td>3.7x10^6</td>
<td>4.68x10$^{-5}$</td>
<td>90</td>
</tr>
<tr>
<td>As33-SiO$_2$@NP</td>
<td>1.5x10^6</td>
<td>5.02x10$^{-5}$</td>
<td>530</td>
</tr>
</tbody>
</table>

Figure 22. Some evidence of NP cellular uptake was found by exposing 2D cell culture of CAPAN-2 cells to NPs for 1.5 hr and imaging the cell suspension using MRI. A) T2 relaxation times of CAPAN-2 cells, cells exposed to silica-coated NPs, and cells exposed to As33-conjugated silica-coated NPs. B) Average number of NPs per cell calculated assuming the diameter of NP core is 15 nm.

3.5.2 3-D Cell Culture

Before imaging via MRI, tumor cells were exposed to NPs for two hours before (0 hr), at the beginning of (5 hr), or after (36 hr) tumor spheroid formation. CAPAN-2 cells subjected to NPs with or without silica and antibody showed a large decrease in T2 relaxation time at all three time points of tumor spheroid formation (0, 5, 36 hr). On the other hand, BxPC-3 cells showed a smaller decrease in T2 than did CAPAN-2 cells when the spheroid was grown for 0 or 5 hr in the presence of silica-coated NPs and after spheroid formation in the presence of As33-conjugated SiO$_2$@NPs (Table 2). Even in the absence of As33, silica-coated NPs were able to penetrate into both types of cells. This
may be because iron oxide cores were around 15 nm and silica-coated NPs were about 23 nm in diameter. These small sizes could have allowed these NPs to enter cells via non-specific endocytosis or pinocytosis, as some tumor cells tend to take up NPs smaller than 100 nm in diameter, with an optimal uptake diameter of around 45 nm.\textsuperscript{30} Since silica-coated NPs are bigger than NP cores, making them closer to 45 nm, it was expected that more silica-coated NPs could enter cells than bare NPs (assuming minimal contribution from particle surface charge). Moreover, it was expected that more NPs, whether or not silica-coated or conjugated with As33, could enter CAPAN-2 cells than BxPC-3 cells, as CAPAN-2 cells pack more loosely than BxPC-3 cells during spheroid formation, therefore providing more surface area for NP cellular uptake to occur.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>T2 (ms) in CAPAN-2 Cells</th>
<th>T2 (ms) in BxPC-3 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>207.1 200.6 186.5</td>
<td>273.8 250.4 -</td>
</tr>
<tr>
<td>+NP</td>
<td>96.3 119.6 199.9</td>
<td>225.8 186.7 -</td>
</tr>
<tr>
<td>+SiO\textsubscript{2}@NP</td>
<td>48.2 109.7 143.9</td>
<td>97.5 140.2 223.7</td>
</tr>
<tr>
<td>+As33@SiO\textsubscript{2}@NP</td>
<td>75.4 94.5 101.2</td>
<td>130.5 219.1 138.7</td>
</tr>
</tbody>
</table>

Immediately after MRI, cell pellets were digested in HNO\textsubscript{3} for analysis via ICP-OES to quantify NP cellular uptake. As33-conjugated SiO\textsubscript{2}@NPs were not found in either cell line prior to spheroid formation (absence of red bar at 0 hr in Figure 23), although bare NPs and SiO\textsubscript{2}@NPs were present. No NPs were found in BxPC-3 cells at this time point. At the beginning of tumor spheroid formation (5 hr), only As33-conjugated NPs were able to enter CAPAN-2 cells, whereas all three types of NPs, whether or not silica-coated or conjugated with As33, were found in BxPC-3 cells. At 36 hr, a higher amount of As33@SiO\textsubscript{2}@NPs was found in CAPAN-2 cells than BxPC-3 cells after spheroid formation. At this time point, uptake of un-conjugated silica-coated NPs was also observed.
in CAPAN-2 cells, but not in BxPC-3 cells. The amount of silica-coated NPs found in CAPAN-2 cells after spheroid formation suggested that although As33-conjugated SiO$_2$@NPs were able to enter CAPAN-2 cells, the effect may not have been due to the presence of As33 or receptor-mediated endocytosis, but rather because CAPAN-2 cells preferentially take up silica-coated NPs. However,

![CAPAN-2](image1)

![BxPC-3](image2)

**Figure 23.** Particle cellular uptake in CAPAN-2 and BxPC-3 cells determined via ICP-OES. Calculations assumed that each NP has a spherical core of 15nm. Time since seeding is the time at which NP particle cell exposure began.
replicates would be needed to confirm these observations.

MRI results are consistent with ICP-OES results at 36 hr, but not at 0 or 5 hr (Table 2, Figure 23). For the cells exposed to NPs at 36 hr, ICP-OES showed that the CAPAN-2 cells had higher iron concentrations than the BxPC-3 cells and the MR T2 relaxation times for the CAPAN-2 cells were lower for all forms of NPs, as expected. At 0 hr, it was expected that CAPAN-2 cells would have a higher amount of Fe$^{2+/3+}$ than BxPC-3 cells for As33-conjugated NPs, and therefore lower T2 relaxation times. Although it was observed that T2 relaxation times of CAPAN-2 cells were lower than those of BxPC-3 cells for all forms of NPs, ICP-OES results did not show uptake of As33@SiO$_2$@ NPs by the CAPAN-2 cells, or uptake of silica-coated and As33@SiO$_2$@ NPs by the BxPC-3 cells.

A similar discrepancy was observed at the 5 hr time point, where a decrease in T2 of CAPAN-2 cells exposed to SiO$_2$@ NPs and As33@SiO$_2$@ NPs compared to the T2 of just those cells suggested uptake of bare and silica-coated NPs in CAPAN-2 cells, while again, the ICP-OES did not. Moreover, at the 5 hr time point, lower values of T2 in bare NP-treated and As33@SiO$_2$@ NPs-treated BxPC-3 cells were expected in comparison to CAPAN-2 cells, as there was more Fe$^{2+/3+}$ found in BxPC-3 cells via the ICP-OES measurements. As shown in Table 2, however, the T2 values of BxPC-3 cells were not lower than those of CAPAN-2 cells under these conditions.

These discrepancies may have been due to the fact that after MRI and prior to acid digestion, the supernatant from the cell pellets was removed. It is possible that some cells with NP uptake lysed during the process of MRI. The lysis of cells, along with weakly-bound NPs on cell surfaces, could have caused loss of Fe$^{2+/3+}$ during supernatant removal prior to ICP-OES analysis, resulting in a lower Fe$^{2+/3+}$ concentration determined from ICP-OES. In addition, while the total number of NPs and cells in each PCR tube were comparable, spheroid sizes and shape were not equal and are
dependent on how the spheroids were formed. It is possible that, by chance, the way the spheroids settled into a pellet left space for water to pack between each spheroid, increasing T2 of the slice, making the voxel chosen for MRI appear to contain fewer nanoparticles than a voxel in a different PCR tube. Since this study was performed only once, replicates are needed to confirm these results.
4 Discussion and Suggestions for Future Work

This thesis explored the synthesis of antibody-conjugate silica coated \( \text{Fe}_x\text{O}_y \) NPs, tested a design for a new MRI phantom for imaging live tumor cells, and quantified NP cellular uptake via 2D and 3D cell culture. This investigation focused on three areas:

1. **As33@SiO\(_2\)@NPs** were successfully synthesized by building a PEG-protein A/G linker, attaching the linker to silica-coated NPs, and attaching As33 onto the linker. The presence of silica on NP surface was confirmed via FT-IR and the diameter was measured using TEM. The thickness of the silica coating was 2 nm. Approximately two antibodies were found on each NP via ELISA. As33@SiO\(_2\)@Fe\(_x\)O\(_y\) NPs have a range of diameters under TEM and DLS, where the majority of the NPs are 109±7 nm.

2. **A new MRI phantom** was designed and built for imaging cell pellets. Although phantoms containing wells from standard 96-well plates were unsuitable for imaging NPs, a holder for PCR tubes designed to encompass the most homogeneous part of the magnetic field was suitable for imaging NPs with a \( \text{Fe}^{2+/3+} \) concentration <0.423 M. Since NP cellular uptake studies use Fe concentrations in the nanomolar range, the PCR phantom was deemed suitable for studying NP cellular uptake.

3. **Some evidence of cellular uptake** of As33@SiO\(_2\)@NP was found in 2D and 3D tumor cell culture of CAPAN-2. A decrease in T2 relaxation time, indicating the presence of Fe\(_x\)O\(_y\) NPs in cells, was found in CAPAN-2 after NP exposure. ICP-OES analysis of Mg\(^{2+}\) and Fe\(^{2+/3+}\) content of cells estimated 2300 NP per CAPAN-2 cell when As33@SiO\(_2\)@NP exposure took place after spheroid formation, whereas only 920 were found per BxPC-3 cell. However, a comparable number of silica-coated NPs were found in CAPAN-2 cells, suggesting non-specific NP uptake.
The number of NPs found in our study is theoretically possible as the NPs take up less than 0.02% of the cell volume and the total number of NPs found inside cells was less than 2% of the available NPs in the environment. Most published studies of NP uptake in cells utilize fluorescence microscopy (e.g. confocal) or a staining method to semi-directly quantify the amount of NPs in the cells, whether in 2 or 3D cell culture.\textsuperscript{54} Whereas NP uptakes have seldom been studied in CAPAN-2 cells, SPIOs have been quantified \textit{in vivo} in BxPC-3 cells using atomic absorption spectrometry or Prussian blue staining.\textsuperscript{55} Although a number of studies did quantify the concentration of the contrast agent (i.e. Fe\textsuperscript{2+/3+}), no known study estimated the number of NPs per cell from these measurements.\textsuperscript{55-56}

The next steps of this research project should investigate other methods of NP characterization. While attempts were made to measure the T2 relaxivity value of silica-coated NPs before antibody-conjugation, R2 remained undetermined as the Fe\textsuperscript{2+/3+} content of NPs may be underestimated by ICP-OES.\textsuperscript{12} ICP-MS may be a potential alternative for quantifying Fe\textsuperscript{2+/3+} contents in silica-coated NPs, if the quantity of NPs produced would allow. Moreover, there is not a method to separate antibody-conjugated NPs from unconjugated ones. Previously work by Nagalla attempted to use a modified gel-electrophoresis for separation, but had limited success as the size differences between antibody-conjugated NPs and silica-coated NPs is quite small.\textsuperscript{13} Perhaps the use of size-exclusion chromatography would be a viable alternative for purifying antibody-conjugated NPs. Moreover, the particle surface charges of silica-coated and antibody-conjugated silica-coated NPs remain undetermined.

The main area of future focus for this study is to repeat the cellular uptake of antibody-conjugated NPs. There are several potential factors responsible for the comparable uptake of silica-coated and antibody-conjugated SiO\textsubscript{2}@NPs in CAPAN-2 cells observed in this study. First, the size of silica-coated NPs is relatively smaller than antibody-conjugated ones. Since NPs smaller than 100
nm tended to have higher degree of NP cellular uptake, the amount of silica-coated NP uptake in CAPAN-2 cells may be due to their smaller diameter.\textsuperscript{30} Second, antibody-conjugated NPs had a range of sizes. Aggregation of NPs may have affected the ability of As33 to bind to GPA33 in order for particle uptake to take place. Moreover, the number of functional GPA33 present on CAPAN-2 cells was decreased due to physical scraping from culture flasks, and would not have recovered at seeding (0 hr) or the beginning of spheroid formation (5 hr). This decrease in the number of functional GPA33 could have resulted in varied uptake of NPs observed in CAPAN-2 cells before (0 hr) and at the beginning of spheroid formation (5 hr). At spheroid formation (36 hr), the number of GPA33 in CAPAN-2 cells was expected to have recovered to normal levels for optimal NP uptake to occur, which led the expected result of As33@SiO\textsubscript{2}@NP uptake in CAPAN-2 cells (Figure 23). On the other hand, the preferred mechanism of NP uptake in BxPC-3 cells remained unclear, although it does not express GPA33. The large amount of NP uptake observed at the beginning of spheroid formation (5 hr) in BxPC-3 cells may have been specific to BxPC3 cells, but it is uncertain without studying NP another in another cell line that does not express GPA33.

To address potential remaining issues, future work should focus on reproducing the synthesis of As33@SiO\textsubscript{2}@NPs and particle cellular uptake results. Since NP cellular uptake is highly dependent on the size of the NP, it may be ideal to use silica-coated NPs with comparable hydrodynamic radius as the As33@SiO\textsubscript{2}@NPs in cellular uptake studies. To conclude definitely that the NPs did not just bound to the cell surface and in fact entered cells, it would also be useful to conduct a control experiment to determine the degree of cellular uptake versus cell binding by performing the same cell uptake experiment at 4 °C. Since endocytosis is a temperature dependent event, NPs should not be able to enter a cell at 4 °C. The observation of NPs in cell digests when NP exposure took place at 4 °C would indicate that a significant amount of NPs remained bound on the cell surface after washing.
Efforts should also be made to explore the degree of NP penetration in tumor spheroids. Since the mAb As33 used in this study is tagged with a pH-dependent fluorophore, it is possible to observe NP cellular uptake via fluorescence microscopy or alternatively, via an iron oxide staining method such as Prussian blue. It may be worthwhile to seed fewer numbers of cells during the spheroid culturing, observe cellular uptake of NPs in different sized spheroids, and compare that with particle uptake in spheroids over growth periods, to ensure that the number of GPA33 on the CAPAN-2 cell surface has recovered for sufficient targeting to occur.

There exist many avenues for the continuation of this project. When mono-dispersed, As33@SiO$_2$@NPs are synthesized, boronated poly-GL peptides could be attached to the silica surface to achieve the therapeutic potential of NPs. Moreover, the quantification of relaxivity values of these NPs post synthesis will determine whether they are indeed suitable for clinical use, as T2 contrast agents currently used in the clinic have a T2 relaxivity of at or above 100 mM$^{-1}$s$^{-1}$. 
Appendix I: Protocol

I.1 Nanoparticle Uptake by Tumor Cells

Materials

- 2 jars of T150 of CAPAN2 or BxPC3 cells, at 70% confluency
- RMPI media without antibiotic, phenyl red, and FBS, with 0.5%BSA
- RMPI media without antibiotic, phenyl red, and FBS, with 0.5%BSA, 2% methyl cellulose
- One 3D Hanging droplet plate for each cell type
- Cell scraper
- Nanoparticle solutions
- Hematocytometer, 20% Trypan blue

1. Use a cell scraper to scrape cells from the flask.
2. Wash cells twice with 10mL of PBS (RT). Centrifuge at 1000rpm for 3min to collect each time.
3. Dilute each T150 flask in 12mL of media. This should give about 1x10^6 cells per mL. Use a hematocytometer to count live cells to make sure and make dilutions as necessary.
4. Resuspend all cells in 5mL of RMPI media without antibiotic, phenyl red, and FBS, with 0.5%BSA (for BxPC3 cells). For CAPAN-2 cells, use RMPI media without antibiotic, phenyl red, and FBS, with 0.5%BSA, 2% methyl cellulose. This will give about ~200,000 cells per 40uL
5. Seed about 200,000 cells per well using RMPI w/o antibiotic, phenyl red, and FBS, with 0.5%BSA
6. Let cells sit in 37 °C incubator, 5% CO₂ for 0hr, 5hr 24hr for formation of spheres
7. During this time, make dilutions for particle exposure so that final concentration of Fe^{2+/3+} in the drop is around 2nM.
8. Incubate cells with NPs for 2hrs by adding the NP solutions of appropriate volume into the droplet individually according to the layer out below. Pink: As_{33}@SiO₂@NP, blue: SiO₂@NP, yellow: NP cores, white: do nothing.

![Layer Out](image)

9. Collect the spheres in a clean 96-well assay plate, by adding 50uL of PBS onto each droplet.
10. Put 8-12 spheroids of exposed to the same conditions into a PCR tube (label/use different colors).
11. Centrifuge the spheroids at 1000rpm for 3 minutes if necessary
12. Remove supernatant.
13. Add 150µL of PBS and flick the pellet around a little. Careful to not destroy the pellet.
14. Centrifuge at 1000rpm for 3min.
15. Repeat steps 11-13 two more times.
16. Ready for MRI.
17. After imaging, remove supernatant above cells and add 150µL of 69% HNO₃ (Omni-grade) to digest cell pellets.
18. Let cell pellets sit in acid overnight. Vortex to mix well if necessary.
19. Ready for ICP-OES.
References


