Functionalizing Silica Coated Iron Oxide Nanoparticles for Imaging and Targeted Treatment of Pancreatic Cancer

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Abstract

The treatment of pancreatic cancer remains a challenge in the biomedical community. Over half of cases are diagnosed after the cancer has spread to other tissues, and in these cases the 5-year survival rate is 2%. The non-specific nature of currently available chemotherapies has spurred interest in developing vehicles to deliver potent therapies specifically to tumors. Nanoparticles (NPs) present an ideal vehicle due to their high surface area, small size, and low toxicity. This project explored the functionalization of silica-coated superparamagnetic iron oxide (SPIO) NPs with polyethylene glycol (PEG) and antibody moieties targeting pancreatic cancer tissue. Attachment of multiple moieties was explored using orthogonal surface chemistries, including protein thiolation with 2-iminothiolane. Quantification and detection of conjugated moieties was investigated using infrared spectroscopy. In vitro techniques were also developed to quantify SPIO NP detection, targeting, and cellular internalization, using magnetic resonance imaging (MRI) and confocal microscopy. The results of this study show promise for the selective treatment of pancreatic cancer using silica-coated SPIO NPs. Techniques developed in this work pave the way for understanding how these NPs interact with the body at the cellular level.
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Every gardener knows that under the cloak of winter lies a miracle ... a seed waiting to sprout, a bulb opening to the light, a bud straining to unfurl. And the anticipation nurtures our dream. --Barbara Winkler

There are no foolish questions, and no man becomes a fool until he has stopped asking questions. ~Charles Proteus Steinmetz

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1.0 Introduction

The treatment of pancreatic cancer remains a challenge in the biomedical community. While one of the rarest forms of cancer, this disease is the fourth most deadly. Over half of cases are diagnosed after the cancer has spread to other tissues, and in these cases the 5-year survival rate is 2%.\(^1\) The remote location of the pancreas, behind the stomach in the abdomen, leads to many challenges in diagnosis and treatment. Current therapies for pancreatic cancer include the Whipple surgical procedure, radiation, and chemotherapy.\(^2\) These options are invasive and/or cause extensive damage to healthy tissues, and quality of life is often severely diminished for survivors.\(^3\) Thus, there is a need for other therapeutic options. This thesis project works towards creating such a treatment, using nanoparticles (NPs) to selectively treat pancreatic cancer while preserving healthy tissues.

Nanoparticles (NPs), each about 1/1000 the width of a human hair, are an emerging tool for targeted delivery of therapeutics. A teaspoon of these particles, at the typical concentration of $10^{16}$ particles per milliliter, contains the surface area of Wellesley’s academic quad, providing “real estate” to attach large quantities of therapeutics and targeting components to direct the therapy to the cancer.

This work uses the platform of silica-coated iron oxide NPs to build a multifunctional therapy traceable via magnetic resonance imaging (MRI). In addition to serving as a vehicle for therapeutic moieties, the iron oxide NP core allows for tracing of NPs for tumor localization and monitoring. Visualization is based on magnetic resonance imaging (MRI) of superparamagnetic iron oxide nanoparticles (SPIO NPs).
1.1 Principles of Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) uses the nuclear magnetic resonance (NMR) of water protons to non-invasively gather information about the chemical environment and spatial organization of a material of interest. The low-energy electromagnetic radiation required for MRI makes it ideal for imaging biological systems. This section describes the theory of NMR and MRI, followed by its application to samples containing superparamagnetic iron oxide (SPIO) nanoparticles (NPs).

1.1.1 Nuclear Magnetic Resonance

NMR is based on the difference in energy between quantum mechanical spin states that occurs in nuclei with non-zero spin quantum numbers in the presence of an external magnetic field; this phenomenon is known as the Zeeman Effect. Elements whose atoms have an odd number of protons, neutrons, or both, exhibit these properties. These nuclei are known as “NMR active” and possess magnetic dipole moments (MDMs), which align with or against an external magnetic field. The isotopes $^{13}$C, $^{31}$P, $^{14}$N, and $^{19}$F are used for various biological applications of NMR and MRI. However, $^1$H, composing 63 percent of biological matter due primarily to the abundance of water, is the most ubiquitous nucleus in nature with a non-zero spin quantum number and the key to MRI analysis of biological samples.

Protons have two spin states, derived from their spin angular momentum quantum number (I) of $\frac{1}{2}$, as per Equation 1. The two spin states are degenerate in
energy in the absence of an external magnetic field, causing each state to be equally populated and indistinguishable.

\# \text{Spin States} = 2(I) + 1 \quad \text{(Equation 1)}

Protons in a sample exposed to an external magnetic field will have a slight excess of atoms in the energetically favored spin state aligned with this field. The energy spacing between the two spin states depends on the gyromagnetic ratio ($\gamma$)\textsuperscript{1} unique to the nucleus of each element and the strength of the external magnetic field ($B_0$) (Eqn. 2).\textsuperscript{4} Because this energy is very small compared to available thermal energy at room temperature, a large population of protons and/or a very strong magnet is required to create a detectable population difference.\textsuperscript{5}

$$\Delta E = h\nu = \gamma \left(\frac{h}{2\pi}\right) B_0 \quad \text{(Equation 2)}$$

Looking now at NMR through the lens of the classical model, we can consider vectors representing the proton MDMs defined above, which orient randomly in the absence of a magnetic field resulting in no net MDM (Figure 1). When an external magnetic field is introduced along the z-axis, all proton MDMs in a sample will align axially such that the net MDM is oriented in the same direction as the magnetic field.

\textsuperscript{1}$\gamma_H = 42.58$ MHz/Tesla.
Figure 1. Magnetic dipole moments of NMR active atoms in the A) absence and B) presence of an external magnetic field. The magnetic field is directed to the right of the page in B.²

In addition to their axial orientation, proton MDMs also classically precess about the axis of the external magnetic field at the Larmor frequency ($\omega$) (Eqn. 3). Also known as the resonance frequency, this intrinsic parameter occurs in the radiofrequency range and corresponds to the difference in energy between hydrogen’s quantum mechanical spin states.

$$\omega = \gamma B_0$$  \hspace{1cm} (Equation 3)

Resonant radiofrequency (RF) radiation is used to manipulate the precession of proton MDMs. An RF pulse applied perpendicular to the net proton MDM aligned with an external magnetic field along the z-axis generates a magnetic field in the xy plane. This smaller field causes the net MDM to “flip” away from the z-axis and towards precession in the transverse plane (Figure 2). The strength and duration of the RF pulse determines the degree of proton MDM excitation.⁶ Receiver coils detect the raw signal of current induced by changes in the transverse magnetic field.³ The observed decay of transverse magnetization when the RF pulse is removed provides

² Graphics from http://www.schoolphysics.co.uk
information about the chemical and physical environment surrounding these protons and thereby about the samples in which these atoms exist.

Figure 2. Use of radiofrequency (RF) pulse to manipulate net proton MDM. An RF pulse applied perpendicular to the external magnetic field (B₀) generates a magnetic field B₁. The net proton MDM (M₀) is forced from precession around B₀ to around B₁, in a path following the gray arrow in a rotating frame of reference.

Neighboring sources of moving charge, such as electrons, generate local magnetic fields, affecting the relaxation of proton MDMs. Figure 3 shows the decay of transverse magnetization (Mₜ) and the recovery of axial magnetization (Mₐ) that occurs during relaxation. The receiver coil records the free induction decay (FID) of current, proportional to the loss of Mₜ. MRI uses spatial encoding to generate an image from this signal.
Figure 3. Transverse and axial relaxation following an excitatory RF pulse. Note loss of transverse magnetization ($M_T$) and gain of axial magnetization ($M_Z$) as the proton MDMs realign with and against the external magnetic field. The corresponding free induction decay (FID) recorded by the receiver coil is shown over time.

1.1.2 Relaxation Parameters of Nuclear Magnetic Resonance

$T_1$, $T_2$, and $T_2^*$ relaxation time constants are intrinsic values that characterize the relaxation of the nuclear MDMs in a particular material.\(^5\) $T_1$ is the time required for a 63 percent recovery of the net MDM in the axial direction and is due to spin-lattice relaxation, the transfer of energy to neighboring particles tumbling at the Larmor frequency (Eqn. 4). $T_2$ is the time required for the loss of 63 percent of the net transverse MDM and is due to spin-spin relaxation, the dephasing of MDMs in the $xy$ plane (Eqn. 5). $T_2^*$ also characterizes transverse relaxation due to spin-spin relaxation but additionally includes the contribution of magnetic field inhomogeneity that is present in all samples, no matter how well shimmed the external magnetic field of the MR instrument.\(^4\)
\[ T_1 \text{ signal intensity} = SI_{T1} = SI_{T1(0)} \left( 1 - e^{-TE/T1} \right) \quad \text{(Equation 4)} \]

\[ T_2 \text{ signal intensity} = SI_{T2} = SI_{T2(0)} \left( e^{-TE/T2} \right) \quad \text{(Equation 5)} \]

SPIO NPs, as their name implies, are superparamagnetic, and thus act as tiny bar magnets in an external magnetic field, introducing local inhomogeneities that expedite T$_2^*$ relaxation. SPIOs have relatively little effect on T$_1$ relaxation and thus MR imaging in this study focuses on T$_2$ characteristics.

1.1.3 Forming an MR Image

Building on the principles of NMR, we will next explore how this phenomenon is used to form two-dimensional MR images. There are many methods to acquire an MR image, the simplest of which is a Spin Echo. The collection of a FID from a slice of an object of interest requires the use of magnetic field gradients, which are applied sequentially (Figure 4).
Figure 4. Pulse sequence to generate a Spin Echo MR image. RF line indicates timing of the excitatory and refocusing pulses; following three rows describe gradient encoding to acquire spatially localized data. Recording by receiver coil synced with refocusing of transverse MDM to acquire largest signal.\(^3\)

The slice-encoding gradient (Gs) is used to change the net magnetic field in a slice by \(\Delta B_q\), giving the subset of water protons within a unique range of resonance frequencies (Eqn. 6).

\[
\Delta E = h\nu = \gamma \left( \frac{h}{2\pi} \right) (B_0 + \Delta B_q)
\]  
(Equation 6)

A soft 90° RF pulse is then applied to excite the protons in this region of interest, forcing them to precess in the transverse plane, as shown in Figure 2. Due to \(T_1\) and \(T_2\) relaxation, the excited proton MDMs will begin to dephase and revert to precession about the z-axis once the RF pulse is removed.

A 180° refocusing RF pulse is then applied to “flip” all proton MDMs about the transverse plane.\(^5\) As they relax back towards precession around the axis of the

\(^3\) http://www.medical.siemens.com
external magnetic field and begin to precess in phase once again, the frequency-encoding gradient (Gf) is applied and FID signal is collected by the receiver coil for one value of the phase encoding gradient (Gp). This captures the “echo” of transverse magnetization, and the peak in signal marks the echo time (TE). Successive echoes are collected at different values of Gp; the duration of each repetition of the pulse sequence is marked by the repetition time (TR) parameter. The MR data are then fourier transformed to acquire an image. In summary, a spin echo MR image is generated using slice, frequency, and phase encoding to spatially encode the MR signal, which provides the spatial resolution to form a two-dimensional MR image of a material.

As discussed in the previous section, each material has a unique $T_1$ and $T_2$ relaxation time constant. MR imaging parameters are weighted to selectively collect information about either relaxation characteristic. This study uses $T_2$-weighting to capture contrast enhancement by SPIO NPs. A long TE and TR generate a $T_2$ weighted MR image.

1.1.4 MRI with Exogenous Nanoparticle Contrast Agents

As mentioned in Section 1.2, iron oxide NPs act as exogenous contrast agents for MRI by increasing magnetic field inhomogeneity and thereby expediting transverse relaxation of neighboring water protons. Like proton MDMs, SPIO NPs magnetically align with an external magnetic field. Iron oxide is ferromagnetic and contains many magnetic domains that remain aligned after the removal of an external magnetic field, preserving the magnetization of the material. SPIO NPs, with
an iron oxide core less than about 10 nm in diameter, have only one magnetic domain and therefore very different magnetic properties. Each SPIO NP's domain will align with an eternal magnetic field, just like those of individual domains within bulk iron oxide (Fig. 5). However, SPIO materials will revert completely back to random magnetic orientation when the external field is removed, unlike iron oxide materials greater than 10 nm, which retain some residual magnetization. Their superparamagnetic properties make SPIO NPs nonmagnetic in the absence of an external magnetic field, a key attribute for in vivo use.

![Diagram of Ferromagnetic and Superparamagnetic properties](image)

**Figure 5. Ferromagnetic and superparamagnetic properties of iron oxide.** NPs less than about 10 nm exhibit superparamagnetic properties, with a single magnetic domain and reversible magnetization, whereas ferromagnetic particles remain magnetized after the external magnetic field is removed.\(^7\)

SPIO NPs decrease the MR signal observed for a given TE.\(^3\) Localized presence of SPIO NPs manifests as darker regions in \(T_2^*\) weighted MR images (Figure 6B). The efficacy of SPIO NPs as contrast agents is quantified by the
concentration dependent decrease in $T_2$ time of a sample, which is determined by collecting FIDs at many different TEs and fitting the recorded $M_T$ signal to Equation 5 (Fig. 6A). A molar relaxivity value, $r_2$, is obtained for different types of NPs by graphing the inverse of the $T_2$ time constant versus the concentration of iron in the sample, which is proportional to the concentration of NPs (Equation 7)(Fig. 6C). This value varies with the composition of moieties attached to the surface of the SPIO NP.3

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

(Equation 7)

\[ (y = mx + b) \]
Figure 6. Obtaining Relaxivity values for silica coated SPIO NPs. A) Transverse MR signal decay shown with increasing echo time (TE). The curve that shows the least decay corresponds to the pure water sample. B) T\textsubscript{2}-weighted MR image showing the cross section of phantom containing water suspensions of two types of silica coated NPs (type 1 and 2) at three different concentrations: i: 1:7.5 dilution, ii: 1:15 dilution, iii: 1:30 dilution. Contrast achieved by NPs indicated by image darkening with increased concentration. Bright sample on far left is nH\textsubscript{2}O control. C) A representative plot of T\textsubscript{2} relaxation rate (R\textsubscript{2}; s\textsuperscript{-1}) versus iron concentration (Fe; mM), obtained by ICP-OES. The slope of this line is r\textsubscript{2}, or molar relaxivity of the sample of NPs.

1.2 Functionalization of SPIO NPs

Additional components are often attached to SPIO NPs to improve their biocompatibility, stability, and efficiency as contrast agents. These moieties can also
afford diagnostic and treatment applications. The $r_2$ value of a type of NP is directly proportional to its Magnetic polarization (M), or the magnitude of its magnetization vector (Fig. 7A). Doping SPIO NPs with zinc increases $r_2$, as does the incorporation of manganese.\textsuperscript{8,9} Larger SPIO NPs show higher $r_2$ values both in vivo and in vitro.\textsuperscript{10} Increased $r_2$ enhances the sensitivity of SPIO NPs as a therapeutic/diagnostic tool (Fig. 7B).

**Figure 7. Relaxivity characteristics SPIO based NPs.** A) Relaxivity versus magnetization (M) of SPIO NPs doped with various transition metals. Particle diameter shown in gray scale. B) Core size and relaxivity values of FDA approved SPIO NP therapies.\textsuperscript{6,9}
To further expand the capabilities of SPIO-based NPS, researchers have tethered additional components to the surface for multi-modal imaging. Positron emitting moieties allow for positron emission tomography (PET) and fluorophores afford near infrared fluorescence (NIRF) spectroscopy in vivo; these modalities complement the MRI facilitated by the NMR active SPIO core. Attachment of multiple components must be conducted using orthogonal chemistries to limit side reactions and improve control. This functionalization chemistry is divided into three main categories: peptidic, thiol, and click chemistry (Fig. 8). Peptidic chemistry uses the peptide bond to fasten components to the NP surface. Thiol chemistry links an electron rich maleimide to a reduced thiol via a Michael addition. Finally, click chemistry connects a terminal azide and an alkyne in a cycloaddition reaction.

**Figure 8. Linker chemistries to tether functional moieties to the surface of NPs.** Peptidic chemistry creates a peptide bond to connect proteins to the NP surface; thiol chemistry uses a Michael addition to a maleimide to create a sulfur-carbon covalent bond, or oxidizing conditions to form a disulfide bond; click chemistry involves the cycloaddition process of an azide and an alkyne.
In addition to the covalent chemistries discussed above, small proteins can also be used to attach moieties in an orientation-dependent manner. Bacterially derived or recombinant Proteins A and G have a high affinity for the fragment crystalline (Fc) region of immunoglobulin antibodies and can be used to achieve orientation specific antibody attachment for immunotargeting. 11-13 Other methods of conjugating antibodies to the NP surface are described by E. White (2013). 14 These diverse chemistries allow NP functionalization for applications from the diagnosis to treatment of disease.

1.3 Interaction with Biological Systems and Toxicity

There is a myriad of possibilities for multifunctional SPIO NPs in medicine, but the NPs’ biocompatibility must also be considered when designing a viable clinical product. Iron oxide has a very low biological toxicity, most likely due to the high concentrations of iron carried in the body. 11 LD₅₀ values obtained for mice, rabbits, and dogs 4 lead to an estimation of human toxicity above 60 milligrams per kilogram body weight. 15 Commercial SPIO NP contrast agents, used in the clinic for almost twenty years, are routinely administered below 1 mg Fe/kg body weight.

Various parameters contribute to the pharmacology of SPIO NPs, including size, coating, surface charge, and distribution of charge. For non-targeted chemotherapeutic NPs, localization to tumor tissue is dependent on the enhanced permeability and retention (EPR) effect, which is due to the increased vascularization of cancerous tissue. 11 The blood half-life of NP therapies must

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4 Iron oxide LD₅₀ (grams iron oxide per kilogram body weight) for mouse: 5 g/kg; rabbit: 1 g/kg; dog: 0.7 g/kg.
therefore be long enough to realize this passive accumulation in the tumor, which typically takes over six hours.

The body has two primary response mechanisms to remove foreign matter such as NPs: the renal system, which clears particles less than 10 nm through the kidneys, and the innate immune system, which uses macrophages to capture particles greater that 100 nm in size for disposal through the lymph system.\textsuperscript{11} Macromolecules greater than 50 kDa are known to best demonstrate the EPR effect, but are also typically greater than 100 nm in size, making them vulnerable to non-specific uptake by macrophages. Building multifunctional SPIO NPs targeted towards cancerous tumors requires balancing the competing factors of contrast capability and therapeutic efficacy.

The role of physical characteristics of NPs in regulating their interaction with the body has been extensively studied.\textsuperscript{10,16-22} Particles between 20 and 40 nm in size are non-toxic to macrophage and tumor cells at concentrations up to 200 μg/ml.\textsuperscript{10} For particles of similar size and charge, surface coating dictates NP interactions with cells and proteins.\textsuperscript{21} Silica coated SPIO NPs do not stimulate the secretion of inflammatory cytokines or disrupt mitochondrial function in macrophages or dendritic cells, providing evidence that this core/shell combination is biocompatible.\textsuperscript{20} Polyethylene glycol (PEG), a hydrophilic polymer, effectively shields SPIO NPs from the innate immune system, allowing the EPR effect and targeting moieties to deliver these NPs to malignant tissue.\textsuperscript{17,18} The effects of this moiety are dependent on the length of PEG used, where a hyperbolic trend describes the optimal PEG length, with 5000 Da PEG yielding the most stealthy particles.\textsuperscript{17,23}
At the macromolecular level, NPs exposed to biological fluids adsorb serum proteins, which form a protein corona whose composition and kinetics of formation have been computationally and experimentally explored. Del’Orco and colleagues (2010) used experimentally derived parameters to model the kinetics of corona formation on NPs in blood.\textsuperscript{24} The model accounted for the binding of three critical blood proteins: high-density lipoprotein (HDL), human serum albumin (HSA), and fibrinogen.\textsuperscript{24} These researchers concluded that the 70 nm polymer-based NPs tested take four to twenty-eight hours to acquire a stable corona when delivered intravenously and exposed to this cocktail of proteins in the blood. Implementation of this simple model for other systems would facilitate a greater understanding of the physiological interactions that may determine the efficacy of NP therapeutics.

Cellular internalization is another critical component of therapeutic NP delivery and depends strongly on the magnitude and spatial distribution of charge on the NP surface.\textsuperscript{21} The cell membrane is negatively charged, and thus, by Coulomb’s Law, highly positively charged NPs should be most effective at generating binding events. However, NPs encounter many other potential binding “partners” en route to the target tissue and exposed positive charges, such as primary amines, increase non-specific binding, inhibiting effective NP delivery (Fig. 9A). In addition, exposed charges stimulate the formation of a more extensive protein corona around NPs, further impeding tissue targeting.\textsuperscript{11, 22} Negatively charged particles are identified and cleared by the spleen, reducing circulation time for NPs to find their target tissue. This evidence points to neutral NPs as the ideal for targeted therapies. However, Townson and colleagues (2013) found that obstructed positive charges.
protected by larger PEG molecules, can produce strong binding and internalization while minimizing non-specific uptake (Fig. 9B).\textsuperscript{11, 21}

![Diagram of NP surfaces with PEG and PEI](image)

**Figure 9. Spatial arrangement of charges on the NP surface determine cellular internalization.** A) Exposed primary amines on polyethylene imine (PEI) increase non-specific uptake. B) Amines shown in blue are shielded by polyethylene glycol (PEG) and show strong tissue targeting when functionalized with targeting moiety, with limited non-specific internalization.\textsuperscript{20}

The effect of NPs on body tissues is known to vary.\textsuperscript{11, 22} Internalization of iron oxide NPs may alter cell fate, but that impact is cell-type specific.\textsuperscript{11} Tissue specific targeting is therefore particularly apt for this therapeutic modality and allows for treatment and monitoring of metastatic disease in addition to primary tumors.
1.4 Project Overview

This thesis work focuses on building multifunctional silica-coated iron oxide nanoparticles for imaging and targeted cancer therapy. The silica shell disguises the NPs from the body’s innate immune system and affords easy attachment of treatment and targeting components. The adaptability of these nanoparticles in a “plug and play” model makes them ideal for targeted cancer therapy. This project aims to integrate imaging and targeting modalities and optimize the detection and tracing of these NPs in tissue using MRI. Proton magnetic resonance imaging (MRI) is employed in this study to quantify the $T_2$ contrast enhancement achieved by SPIO NPs in agarose suspension. Endocytosis of NPs is evaluated by attaching a pH dependent fluorophore to the silica surface and imaging treated cells using confocal microscopy.
2 Materials and Methods

2.1 Building Multifunctional Silica-Coated SPIO NPs

2.1.1 Silica coating of SPIO NPs (ME12)

Silica-coating of Ferrotec™ SPIO NPs and subsequent characterization via MRI, ICP-OES, and TEM was performed by Alice Liao (Wellesley College ’15).25

2.1.2 Conjugation of Antibody to NP

2.1.2.1 Thiolation of Antibody for Attachment to PEG-silane Linker

Samples of human IgG purified from plasma (Athens Research and Technology) and monoclonal antibody 31.1 (Andrew Webb) were concentrated using a VirTis Sentry 3L lyophilizer and thiolated using Traut’s reagent, 2-iminothiolane•HCl (Thermo Scientific 26101) according to manufacturer’s specifications. Thiolated antibodies were isolated using a 7k MWCO desalting spin column (Thermo Scientific 89892;). Absorbance at 280 nm, measured using the NanoDrop spectrophotometer, confirmed the recovery of IgG.

2.1.2.2 Sulfhydryl Quantification via Ellman’s Reagent

The number of thiol groups per antibody was assessed using Ellman’s reagent, 5,5’-dithiobis-(2-nitrobenzoic acid) (Thermo Scientific 22582) (Fig. 10). Solutions of 0-1.5 mM cysteine•HCl•H2O (Pierce 44889) were used to generate a standard curve for quantification of thiols with this colorimetric indicator. Manufacturer’s protocol was optimized for a 28 µl scale assay, using 5 µl thiolated protein, 3 µl 3.3 mM Ellman’s reagent, and 20 µl 0.1 M pH 8 sodium phosphate
buffer chelated with 1 mM EDTA. NanoDrop, Cary 50, and Molecular Devices’ SpectraMax 190 UV-Visible spectrophotometers were used to measure absorption of the reaction product at 412 nm for each sample. The SpectraMax 190 instrument produced the most precise results while requiring only a 4 μl volume and thus is optimal for reading this assay (see section 3.2.2).

Figure 10. Schematic of thiolation of primary amine via 2-iminothiolane.

2.1.2.3 Conjugation of Protein to Mal-PEG-Silane Linker and to NP

Thiolated antibody was coupled to Maleimide-PEG-Silane overnight at 4 °C in a 1:10 molar ratio. Unconjugated polymer was removed using a 7 kDa molecular weight cut off desalting column (Pierce) per manufacturer’s protocol. The linker-antibody complex was tethered to the silica coated SPIO in NP excess the following day for 24 hours before purification. Fully conjugated NPs were collected by magnet and washed twice with PBS.
2.2 Characterization of NPs

2.2.1 NP Relaxivity via ICP-OES and MRI

MRI contrast capability of SPIO NPs was evaluated by obtaining a molar relaxivity value for the material, the slope of the plot of $1/T_2$ relaxation time against iron concentration (Fig. 6 Section 1.1.4).

The iron concentration of stock NPs was determined via ICP-OES. Samples were digested overnight in aqua regia (1:1 w/v; OmniTrace Ultra (Fisher Scientific), diluted to 2% nitric acid, and filtered through a 0.2 mm HT Tuffryn syringe filter (Acrodisc) prior to analysis. Iron (40, 4, .4, .04, 0.004 ppm) and gold standards (100, 10, 1, 0.1, 0.01, 0.001 ppm) (Sigma Aldrich) were prepared in 2% nitric acid (Fisher Scientific) and used to generate the standard curves for experimental determination of iron and gold concentrations in the NP samples. Optical measurements were taken at 267.6 nm and 238.2 nm to determine the concentration of gold and iron respectively using an Optima 7000 DV ICP-OES spectrometer.

$T_2$ relaxation time constants for serial dilutions of the NPs (0.3, 0.15, 0.07, 0.04 mM iron) were determined by collecting MRI data at serial dilution values of TE for NPs in agarose suspension. MR images of NP samples in water, agarose, and cell suspension were collected using a 400 MHz Bruker Avance NMR spectrometer (9.4 T, 2.4 G/cm/A gradient strength, vertical bore). For agarose suspension, NP samples were mixed with molten 2% w/v agarose, in a 37 °C walk in incubator, transferred to separate 7-inch, 3 mm diameter NMR tubes (Wilmad Glass) and allowed to set at room temperature for one hour. Cells treated with NPs were suspended in agarose for imaging in the same manner.
A custom sample holder (built by Stephanie Huang, Wellesley College ’12) was used to suspend these samples in the bore of the magnet for imaging. A maximum of 7 NP samples were image at one time. Tuning and matching was first conducted manually, followed by an auto tuning and matching tripilot scan using the shim file “JRSHNPP” (created by Jasmine Rana, Wellesley College ’12). This preparatory scan generated two images with axial orientation and one in sagittal view (Fig. 11). Paravision 4.0 software’s “MSME-T$_2$-map” pulse sequence was used to generate the MR images for analysis and consisted of the following parameters: FOV of 4 x 4 cm with 0.5 mm slice thickness; TR = 3062 sec; TE = 10 - 600 ms at 10 ms intervals; 60 echoes, 1 average, 128 x 128 matrix. Acquisition required approximately 9.5 min and generated three axial images (Fig. 11B).

![Figure 11. MR images from Tripilot scan JRSHNPP. A) Sagittal image, showing length of NMR tube containing agarose suspension. B) Axial image, taken midway along the NMR sample tube, showing 5 samples in phantom holder.](image)

Using the image sequence analysis (ISA) tool in Paravision 4.0, a 3 mm diameter region of interest (ROI) was selected for each sample. The decay of signal intensity within the ROI for each sample (Fig. 6, Section 1.1.4) was fit to Equation 7
to obtain T<sub>2</sub> values for each sample. 1/T<sub>2</sub> values were plotted against concentration of iron in each sample to determine the r<sub>2</sub> values of ME11 and ME12 NPs.

### 2.2.2 Infrared Spectroscopy

A Bruker alpha Pt attenuated total reflectance-fourier transform infrared (ATR-FTIR) spectrometer was used to detect silica coating and PEGylation. Spectra of silica coated, PEGylated, SPIO NPs and mixtures of mPEG-silane and Mal-PEG-silane were collected and analyzed. NP samples were prepared by drying water suspensions of NPs in a chemical fume hood. PEG mixtures were prepared in ratios of 1:1 and 2:1 in ethanol at 0.01 mg/μl and then dried for three days before IR analysis. The following instrument parameters were used to collect spectra: a 4-acquisition background scan; 256 acquisitions per sample at 1 cm<sup>-1</sup> resolution; spectra collected over 500-4000 cm<sup>-1</sup> range. OPUS 7.0 software and Microsoft Excel were used to display and analyze spectra.

### 2.2.3 TEM (Protocol in Appendix IA)

Imaging of NPs using Transmission Electron Microscopy (TEM) was conducted on the JEOL TEM at Harvard Medical School to gauge NP size and morphology. NPs were mounted on Formvar carbon grids with SiO lattice fringes by inverting the grids onto a droplet of undiluted NPs from the synthesis, allowing them to adhere. These grids were then dried overnight and subsequently imaged using a tungsten filament JEOL TE microscope under the following parameters: 80, 100 kV, 40,000-150,000x magnification.
2.2.4 PEG Assay

A 96-well format barium chloride, iodine assay for PEG optimized by Harini Natarajan (Wellesley College ’15) was conducted using serially diluted standard solutions of 5 kDa methoxyPEG (mPEG)(0-10 mg/ml) to find the concentration of PEG in the NP sample.\textsuperscript{26} 1/50 and 1/100 dilutions of sample were analyzed to ensure PEG concentrations in the appropriate range.

2.2.5 Chemiluminescent Dot Blot

Approximately 10 μg samples of protein conjugated to NPs were spotted onto a nitrocellulose membrane and allowed to dry for confirmation of antibody-NP conjugation. The membrane was incubated on a rocker for 1 hour in blocking buffer (5 g Bio-Rad blotting grade blocker in tris buffered saline (TBS) and 0.1% tween) and then another hour in the presence of (1:1000 dilution in blocking buffer) anti-biotin antibody (HRP conjugated anti-biotin, Webb Lab). The unbound antibody was removed with washing buffer (0.1% tween in TBS) in 4 washes. The membrane was then dabbed dry and sprayed with HyGlo\textsuperscript{TM} Quickspray luminol/peroxide solution, then visualized using the Bio-Rad “chemiluminescent blot” program on the Bio-Rad Imaging System. Protocol adapted from the Webb Lab.

2.2.6 Characterizing Amine-Reactive pHrodo-Green Fluorophore

Absorbance and fluorescence spectra of pHrodo-green (Life Technologies) in solutions of varying pH were collected using Molecular Devices SpectraMax M3 microplate spectrophotometer. Known pH solutions (pH 2-8) were prepared using
HCl and NaOH. Solutions were loaded into 96-well clear bottom fluorescence plate (Corning Costar 3603). Because pHrodo-Green did not remain well suspended in aqueous solution for over ten minutes, frequent vortexing was necessary to achieve uniform aliquots. Spectrophotometer was operated according to protocol in instrument manual to instrument under the following parameters: Absorbance spectra 400-600 nm with 10 nm steps were collected after auto-mixing for 5 seconds; Fluorescence spectra were collected using 490 nm excitation and the same spectral parameters as for absorbance measurements. Precision was set to 7 reads/well and PMT sensitivity to automatic; other settings were left at default. The samples were auto-mixed once before starting data collection.

2.3 In Vitro Studies of NPs (Protocols in Appendices IB-IE)

2.3.1 Cell Culture and Treatment

(adapted from cell culture protocol in the Webb lab)

CAPAN-2 and BxPC3 cells were maintained in T-25 flasks suspended in RPMI complete media (Sigma Aldrich). Cells were seeded on #1.5 glass cover slips (Corning) at a density of 100,000 or 50,000 cells per well in 6-well cell culture plates (Falcon). These cultures were incubated at 37° C, 5 % CO₂ until approximately 65% confluent at which time treatment was initiated. Cells were washed once with room temperature PBS and then treated for 90 minutes with As33@pHrodo (4 and 10 μg/ml). Post treatment, cells were washed twice with PBS and then fixed and mounted for visualization as per the immuno fluorescence protocol described in Appendix IC.
2.3.2 Confocal Microscopy

Cellular internalization was detected using a Leica SP5 confocal microscope equipped with 488 Argon and 405 diode lasers (for pHrodo and DAPI excitation respectively), in addition to a broad spectrum metal halide light source. For DAPI, emitted light 430-550 nm was collected; for pHrodo-Green, emitted light between 515 and 600 nm was collected. Quantum yield of pHrodo was higher than for Dapi, and corresponding adjustments were made to gain and offset settings when imaging. The pinhole was set between 1 and 8 AU depending on the fluorophore and sample. Three frame averages were taken per image and resolutions from 512x512 to 1024x1024 pixels were used. Vertical image stacks were collected using twenty steps between top and bottom of detectable fluorophore (approximately 25 μm).

In vitro fluorescence was also detected using Nikon 80i fluorescence microscope, with broad spectrum metal halide lamp excitation. Preset DAPI settings in Nikon software NIS ELTs were used to capture DAPI fluorescence.
3 Results and Discussion

This project optimized new techniques to functionalize silica-coated SPIO NPs to achieve tissue targeting. The following goals were achieved:

♦ A framework was developed to detect multifunctionalization of SPIO NPs via IR spectroscopy.

♦ Immunotargeting was explored using a stochastic thiol linkage to conjugate antibodies to the NP surface.

♦ Directionally specific attachment was performed via recombinant protein A/G, whose high affinity for the constant portion of immunoglobulins allows for indirect, precise, NP-Antibody assembly.

♦ A cellular assay was developed to test MRI detection in vitro, using commercially available gold-coated SPIO NPs conjugated with targeting antibody.

♦ Tissue specific targeting and the mechanism of cellular NP internalization were studied by attaching the pH dependent pHrodo-Green fluorophore to targeting antibody As33 and observing endocytosis via confocal microscopy.

The discussion below expands on the results of building multifunctional silica-coated SPIO NPs and assessing their efficacy as contrast agents and vehicles for targeted intracellular chemotherapy.

3.1 Construction of Silica-Coated SPIO NPs

The silica-coated SPIO NPs used in this study were synthesized by Alice Liao (Wellesley College ’15) by coating 12 nm Ferrotec SPIO cores with a 10 nm SiO₂ shell in a microemulsion synthesis adapted from Narita et al 2009 and the thesis work of Jasmine Rana (Wellesley College ’12). Smaller particles have a higher surface area to volume ratio and thus possess a higher loading capacity for PEG. The silica-coated SPIO NPs used in this study were 30 nm in diameter on average,
allowing for dense surface loading and thus potent therapy. These NPs were characterized via MRI and ICP-OES as described in section 1.1.4 and found to possess a molar relaxivity of 176.1 mM$^{-1}$ s$^{-1}$. This value surpasses the bar of commercially available contrast agents (Ferridex 100 mM$^{-1}$ s$^{-1}$, Resovist 151 mM$^{-1}$ s$^{-1}$) and indicates the viability of this type of NP as a multifunctional contrast agent. NPs were stored in ethanol, then dried and resuspended in nanopure water to allow for functionalization and characterization in aqueous solvent.

The relaxivity of commercially available GoldMag Dextran NPs (GoldMag Nanobiotech Co.) was also obtained to determine their contrast capability and found to be 292 mM$^{-1}$ s$^{-1}$. These particles, which consist of gold-coated SPIO NPs further functionalized with a shell of carbohydrate, are over 100 nm in diameter. T$_2$-weighted MR images were collected for four agarose suspensions of these NPs at varying concentrations (Fig. 12A).

![Image of MRI of GoldMag NPs](image)

**Figure 12. MRI of GoldMag NPs: a step towards relaxivity measurements.** A) Image of 0.5 mm slice of GoldMag samples (0.3, 0.15, 0.07, 0.04 mM iron) suspended in 2 percent agarose. B) Relaxivity curves: the raw T$_2$ relaxation times of each suspension of GoldMag in 2% agarose (w/v) plotted against echo time (TE). Sample of agarose without GoldMag was used as a negative control and appears as the uppermost curve in the graph (pink).
Relaxivity was determined as outlined in Section 1.1.4, using transverse signal intensity values from MRI (Fig. 6A, 13A) and iron concentrations of each sample, determined by ICP-OES (Fig. 6C 13A). The observed molar relaxivity value of GoldMag Dextran ($r_2 = 292 \text{ s}^{-1} \text{ mM}^{-1}$) is strikingly high in comparison to that of the silica coated SPIO NPs used in this project ($r_2 = 176 \text{ s}^{-1} \text{ mM}^{-1}$) (Figure 13C). The disparity in contrast capability of these NPs may be explained by the difference in NP core size (size of GoldMag Dextran core is unknown), or by the filtration step prior to ICP-OES. GoldMag Dextran NP samples were filtered to remove aggregates prior to ICP-OES iron concentration determination and thus underestimated the iron concentration in imaged NP solutions, producing an artificially high relaxivity value. In the future, samples will be filtered prior both MRI and ICP-OES to eliminate this source of error.
Figure 13. Iron and gold concentration measurement and relaxivity determination of GoldMag Dextran NPs. Solutions of A) Iron (40, 4, .4, .04, .004 ppm) and B) gold standards (100, 10, 1, 0.1, 0.01, 0.001 ppm) were used to generate standard curves for ICP-OES determination of iron concentration in the four NP samples imaged using MRI (Fig. 1A). C) The molar relaxivity of GoldMag, \( r_2 = 292 \text{ s}^{-1} \text{ mM}^{-1} \), is the proportionality constant of the linear regression fit to the plot of \( T_2 \) relaxation rate for each NP sample against its unique iron concentration as determined by ICP-OES.

Zhang and colleagues (2011) characterized uncoated GoldMag NPs to determine concentrations necessary to achieve contrast; these scientists found a lower limit of 3 ug/ml to detect contrast in water suspension.31 GoldMag Dextran NPs too, have shown the capability to act as effective contrast agents in cell free environments. While much larger than the silica-coated SPIO NPs functionalized in this study, at over 100 nm, GoldMag Dextran NPs are viable as \( T_2 \) contrast agents. Gold-coated SPIO NPs thus served as comparable substitutes for silica-coated SPIO NPs when optimizing cellular assays of MRI detection.6
3.2 Functionalization of Silica-Coated SPIO NPs

3.2.1 Functionalization with PEG

PEG confers NPs with biostability, hydrophilicity, and reduced immune response and is thus ubiquitous in NP functionalization. PEG with a molecular weight of 5 kDa was chosen in this study as a “Goldilocks” length to maximize the benefits listed above while also optimizing SPIO contrast capabilities and minimizing non-specific cellular uptake.\textsuperscript{8, 10, 23} FTIR spectroscopy was used to determine the composition of mixtures of mPEG-silane and Mal-PEG silane as a first step towards detecting and characterizing multifunctionalization of NPs.

Each type of PEG linker appears to have a distinct IR spectrum between 500 and 4000 cm\textsuperscript{-1} (Fig. 14). A sharper sp\textsuperscript{3} carbon-hydrogen peak at around 2900 cm\textsuperscript{-1} and silica-oxygen stretch at 1085 cm\textsuperscript{-1} appear in the Mal-PEG-silane spectrum, along with a clear carbonyl peak at 1710 cm\textsuperscript{-1}. Sharper signatures reflect more restricted vibrational motions, as present in the oscillatory carbonyl stretch, whereas broader features indicate less constrained motions, and greater intermolecular forces.
Figure 14. Distinct FTIR spectra of A) mPEG silane and B) Mal-PEG-silane and their chemical structures. Relevant peaks are labeled with their corresponding vibrational motions. Spectra from 500 to 1000 cm\(^{-1}\) reflect the average of 256 acquisitions.

Jasmine Rana (2012) found similar signatures in IR spectra of mPEG-silane.\(^6\)

Compared side by side, these three peaks distinguish mPEG-silane from Mal-PEG-silane, either by their shape or their magnitude. (Table 1). Experiments by Cauda and colleagues (2010) describe the unique FTIR spectra of PEGylated mesoporous silica NPs coated with silane PEG of different lengths, providing further support for the use of IR to identify moieties on the NP surface.\(^32\)

Table 1. Differential IR signatures of Mal-PEG-Silane and mPEG-Silane. Samples of 5 kDa PEG of each type were analyzed by FTIR spectroscopy. 256 acquisitions were performed per sample and averaged data was analyzed.

<table>
<thead>
<tr>
<th>IR Signature</th>
<th>Wavenumber (cm(^{-1}))</th>
<th>Mal-PEG-Silane</th>
<th>mPEG-Silane</th>
</tr>
</thead>
<tbody>
<tr>
<td>sp(^3) C-H stretch</td>
<td>2900</td>
<td>Sharp</td>
<td>Broad</td>
</tr>
<tr>
<td>C=O stretch</td>
<td>1710</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Si-O stretch</td>
<td>1080</td>
<td>Sharp</td>
<td>Broad</td>
</tr>
</tbody>
</table>
Mixtures of mPEG-silane and Mal-PEG-silane were subsequently prepared to test whether FTIR could distinguish relative amounts of each PEG in a sample. Samples were mixed thoroughly in a droplet of ethanol and allowed to dry prior to FTIR analysis (Fig. 15). A two-fold excess of either variety of PEG was detectable via the carbonyl peak, showing promise for this technique as a means to differentiate the relative conjugation of two PEG moieties and, therefore, potentially discern the attachment of two functional groups to the NP surface. Data in this study and recent literature suggest that FTIR is a viable method to differentiate the composition of PEG on the surface of NPs. However, larger samples of NPs are necessary to evaluate the feasibility of this technique to determine the composition of PEG conjugated to the silica surface of SPIO NPs, as the shell and NP core may confound spectral results.
Figure 15. IR spectra differentiate Mal-PEG-Silane and mPEG-Silane mixtures with A) 2:1 ratio and B) 1:2 ratio of the linker molecules, respectively. PEG samples were mixed in a droplet of ethanol and dried in a chemical fume hood prior to IR analysis. Spectra reflect the average of 256 acquisitions.

3.2.2 Functionalization with IgG

Functionalization of silica coated SPIO NPs with antibodies is critical to achieve immuno-targeting towards cancerous tissue. Eugenia White (Wellesley College ‘13) successfully attached monoclonal antibody CHO 31.1 to the SPIO surface via a carbodiimide linkage. Here, human IgG was used as a prototype to
test the viability of a thiol linkage between a maleimide (Mal-) functionalized PEG linker molecule and a thiolated antibody.

Immunoglobulin G (IgG) was thiolated using Traut’s reagent (2-iminothiolane) at a 1:10 ratio. A colorimetric quantification of sulphydryl groups using a cysteine•HCl standard curve was used to optimize conjugation to approximately one Mal-PEG linker per antibody (Fig. 16). Standard solutions showed non-linear variation in the absorbance at 412 nm, reflected by a poor correlation coefficient. This variation may be an artifact of the spectrometer used to read the solutions, and based on the concavity of the data, thiolation estimates from a linear fit to absorbance values of the standard solutions may be taken as an underestimate. The extent of thiolation exceeded expectations, resulting in over six sulphydryl groups per antibody, as estimated from the linear fit to the standard curve (Fig. 16). To minimize antibody conjugation to more than two PEG linkers, subsequent thiolation reactions with were performed at a 5:1 molar excess of Traut’s reagent.
Figure 16. Sulphydryl Quantification of thiolated IgG. A) Solutions of Cysteine•HCl were used to generate a standard curve to B) quantify the number of thiol groups added per human IgG molecule. Reactions with Ellman’s reagent were measured in triplicate for absorbance at 412 nm using a NanoDrop spectrophotometer. Averaged data are presented.

There is precedent for the use of Ellman’s reagent for sulphydryl quantification during NP functionalization. Bedeneau and colleagues (2007) used a milliliter-scale Ellman’s reagent-based assay to determine thiolation of monoclonal antibodies for attachment to nanoscale liposomes via a similar Mal-PEG linker. The present study transformed this assay to conserve thiolated protein sample, optimizing to a 28 µl reaction per sample (Section 2.1.2.2).

Three spectrophotometers were compared to optimize the reproducibility of the micro-scale Ellman’s assay and optimize sample conservation and accuracy. The NanoDrop and Molecular Devices SpectraMax 190 spectrophotometers (Fig. 17A and 17B respectively) require a 4 µl reaction volume whereas Cary 50 (Fig 17C)
requires at least 750 μl. Three acquisitions were necessary to generate consistent results on the NanoDrop instrument and data reflect the average absorbance (Fig. 17A).

Figure 17. Absorbance at 412 nm of standard Cys•HCl solutions reacted with Ellman’s reagent measured via A) NanoDrop, B) SpectraMax 190 Plate Reader, and C) Cary 50. Linear regression applied and lines of best fit and correlation coefficient shown.

All three instruments generated acceptable standard curves with solutions of cysteine•HCl, leading to the exclusion of the high volume Cary 50 for sample conservation in this assay. Increased standard solutions with thiol concentration between 0 and 0.5 mM were used to compare NanoDrop and SpectraMax 190 spectrophotometers in reading the Ellman’s based assay, as most thiolated protein samples have low sulfhydryl concentrations and thus efficacy in this range was

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critical for assay optimization. Standard deviation was not comparable between instruments, as absorbance data were acquired on a different (unknown) scale for each. The SpectraMax 190 instrument (Fig 17B) optimized sample volume and was able to read absorbance with greater precision. As an automated plate-reader system, it also was more controlled and easy to use and was thus determined to be the best instrument to read the 28 µl sulfhydryl quantification assay developed in this study. With data collection methods optimized, a reliable small-volume Ellman’s reagent based thiol quantification was successfully developed for use as a characterization step in NP functionalization.

After thiolation, targeting antibodies were next conjugated to Mal-PEG linkers for subsequent attachment to silica-coated SPIO NPs. As a proof of concept, thiolated IgG was purified by desalting column (7 kDa cut off) and coupled to Maleimide, Biotin, heterobifunctional PEG (MW 525.6 g/mol). After another round of protein purification, antibody-PEG conjugation was confirmed via chemiluminescent dot blot for biotin (Fig. 18).

Figure 18. Conjugation of IgG to Mal-PEG-Biotin confirmed via chemiluminescent dot blot for biotin. A) biotinylated IgG (positive control); B) Mal-PEG-Biotin linker (negative control); C) purified IgG conjugated to Mal-PEG-Biotin. Anti-biotin antibody @HRP was used for detection, in the presence of hydrogen peroxide and luminol.
This pilot study provides evidence for the efficacy of the antibody-Mal-PEG conjugation system, despite its lack of directional specificity. Attachment of protein A/G via the thiol linkage and subsequent conjugation of a targeting antibody via the strong interaction between Protein A/G and the FC portion of immunoglobulins is one solution to achieve the desired orientation of this functional moiety.\textsuperscript{11}

### 3.2.3 Functionalization with Monoclonal Antibody CHO 31.1

The optimized thiol-Mal-PEG-silane linkage scheme was applied towards conjugating the targeting antibody CHO 31.1 to the NP surface (Fig. 19).

![Conjugation scheme](image)

**Figure 19. Conjugation scheme for attachment of monoclonal antibody 31.1 to silica-coated SPIO.**

Non-specific thiolation of CHO 31.1 was accomplished via Traut’s reagent and detected with Ellman’s reagent as optimized in section 4.2.2. Sulfhydryl quantification showed the presence of on average 0.76 sulfhydryl groups per antibody, less than ideal, but still an adequate proportion for conjugation to Mal-PEG, according to the literature.\textsuperscript{33} Thiolated CHO 31.1 was purified via desalting
column and conjugated to 5 kDa Mal-PEG-Silane. After another round of purification, PEGylation of antibody was analyzed via the barium chloride assay for PEG described in section 2.2.4 (Fig. 20). Even 50 and 100-fold diluted samples showed coloring beyond the range of the assay.

![Figure 20](image)

**Figure 20. Confirmed conjugation of 31.1 to Mal-PEG-Silane via PEG, BaCl assay.** Columns 1-5) Standard curve of 5 solutions containing 0, 1, 2.5, 3.75, 5, 6.25, 7.5, and 10 µg/ml mPEG were performed in replicate. Columns 6 and 7 correspond to 1/50 and 1/100 dilutions of PEGylated CHO 31.1 were analyzed in duplicate as well.

Following collection of NPs by magnet and two washes with PBS, a chemiluminescent dot blot for human protein was used to confirm conjugation of CHO 31.1 to the NP surface (Fig. 21).
Figure 21. Conjugation of 31.1 to silica-coated SPIO NP confirmed via chemiluminescent dot blot for human protein. Anti human antibody conjugated to HRP was used to detect CHO 31.1 in protein samples; positive controls A) IgG and B) CHO 31.1, supernatants from PBS washes C) 1 and D) 2 respectively, and 31.1 conjugated SPIO NP sample.

The conjugation of CHO 31.1 to silica-coated SPIO NPs was achieved via a thiol linkage (Fig. 21). Although this method does not allow control over the orientation of the coupled antibody, previous studies show that it does stochastically guarantee some functional moieties.\textsuperscript{14, 34} Thus the successful functionalization via the thiol linkage observed brings us a step closer to building directed silica-coated SPIO NPs for the targeted delivery of chemotherapy.

3.3 In Vitro Targeting Analysis

Cellular assays were developed to evaluate MRI detection of NPs as well as their targeting and cellular internalization. Modified antibody molecules and commercially available NPs were used to develop these assays, for future use on novel NPs.
3.3.1 NP detection via MRI Using GoldMag@As33 NPs

GoldMag NPs functionalized with monoclonal antibody As33 as described in the literature (by the Webb lab) were added to both CAPAN-2 and BxPC3 cells. As33 also targets glycoprotein A33 expressed on CAPAN-2 and not expressed on BxPC3 cells. Cultures were incubated for 2 hours, pelleted and washed with PBS either at pH 7 or pH 3, then suspended in agarose and transferred to 3 mm NMR tubes for MR imaging. Washed cell pellets from samples treated with NPs were rust colored to the naked eye, indicating the presence of NPs (Fig. 22B). Untreated and cell-free samples showed expected T2 times, confirmed by previous studies. Darkening of the MR image in agarose suspensions of cells treated with SPIO NPs confirms that detection of NPs in cells via MRI is feasible qualitatively (Fig 22A). Reduced T2 time in the cells treated with NPs provides quantitative evidence of MRI detection of SPIO NPs in cellular samples, corroborating acellular MRI studies of SPIO NPs in the literature (Fig. 22C).
Figure 22. MRI detection of GoldMag NPs conjugated with As33 in treated CAPAN-2 and BxPC3 cells. A) MR image of CAPAN-2 (C2) and BxPC3 (B3) cells suspended in agarose and treated with GoldMag@As33 NPs or media (C2/B3 -M). Prior to suspension for imaging, cells were acid washed with pH 3 PBS (C2/B3 –A) or washed with PBS (C2/B3 -P). B) Pelleted CAPAN-2 and BxPC3 cells treated with NPs and washed with PBS. C) T$_2$ time constants (ms) of samples from A).

T$_2$ relaxation was not consistent within or between cell lines. Acid washed BxPC3 cells appear darker via MRI than the corresponding CAPAN-2 cells, but the Neutral PBS washed CAPAN-2 cells appear darker on MRI. Variability in number of cells suspended in agarose most likely explains the inconsistency in T$_2$ times, however, and similar irregularities were observed in previous experiments in this project.$^{14}$ Although preferential NP binding to CAPAN-2 cells, which express target glycoprotein A33, was not observed, detection of in vitro NP contrast was confirmed, even with the sample heterogeneity introduced by cells. T$_2$ relaxation curves show accelerated loss of M$_T$ in samples containing NPs (Fig. 23).
Figure 23. $T_2$ signal decay over time for agarose suspensions of A) BxPC3 (B3) and B) CAPAN-2 (C2) cells treated with with GoldMag@As33 NPs and washed as in Figure 22. Media washed is indicated by (M), acid washed with pH 3 PBS indicated by (A), and PBS washed with (P). Pure agarose suspension (AG) used as a control. Decay of transverse magnetization, $M_T$, observed at successive time points after excitation pulse. Signal decay is accelerated in samples containing NPs.

3.3.2 Internalization Visualized using Confocal Microscopy of pHrodo, pH-Dependent Fluorophore

Confocal microscopy of pHrodo-Green labeled As33 antibodies and conjugated silica-coated SPIO NPs was used to show an endocytotic internalization mechanism. In a pilot study of endocytosis, untreated CAPAN-2 and BxPC3 cells were treated with DAPI nuclear label, using ProLong Gold mounting media, and imaged using a Nikon 80i fluorescence microscope (Fig. 24). Both cell lines showed localized fluorescence, but lower fluorescence was observed in the BxPC3 cell line. This may indicate a lack of sufficient stain in the samples imaged, or decreased permeability of BxPC3 to DAPI.
Figure 24. DAPI fluorescence localized to nuclei in CAPAN-2 and BxPC3 cell lines. Untreated cells adherent to glass cover slips were fixed and stained with the nuclear label DAPI using ProLong Gold mounting media with DAPI. Images taken at 200x magnification using a Nikon 80i fluorescence microscope.

In parallel, absorbance and fluorescence of pHrodo-Green fluorophore coupled to NH$_2$-PEG-silane were evaluated via Molecular Devices SpectraMax M3 microplate UV-Visible spectrophotometer. Fluorescence spectra were collected using a 490 nm excitation (the closest filter to 505 nm in the plate reader UV-Vis spectrometer (Fig. 25).
Figure 25. A) Absorbance and B) Fluorescence spectra of pHrodo-Green (20 uM) in a pH 4 solution. Spectra were collected via Molecular Devices SpectraMax M3 microplate UV-Visible spectrophotometer. $\lambda_{ex}$ for B was 490 nm.

Fluorescence measurements were confounded by excitation light, yielding a saturated emission spectrum, but an experimental absorbance maximum was observed between 500 nm and 510 nm in an 20 μM solution with a pH of 4 (Fig. 25), consistent with the manufacturer’s specification of 505 nm (Fig. 26).

Figure 26. Manufacturer’s absorption and Emission spectra of pHrodo-Green fluorophore, along the visible electromagnetic spectrum.⁵

⁵ http://www.lifetechnologies.com/content/dam/LifeTech/Documents/spectra/images/pHrodoGreen_lowpH.jpg
Emission spectra of 20 μM pHrodo-Green in solutions of variable pH are shown below, and result from an excitation wavelength of 490 nm (Fig. 27). Solutions were prepared in duplicate and the scatterplot below represents average fluorescence intensity. Saturation around 500 nm in addition to 10 nm step collection resulted in a fragmented spectrum between 470 nm and 510 nm. These spectra do not have sufficient resolution to demonstrate the desired pH dependence of pHrodo-Green.

Figure 27. Average fluorescence intensity of pHrodo Green (20 μM) in solutions of varying pH when excited with 490 nm wavelength light. Spectrum collected via Molecular Devices SpectraMax M3 microplate UV-Visible spectrophotometer.
Fluorophore pHrodo-Green is known to show a non-linear change in fluorescence in response to pH changes, and thus has been faulted as a poor environmental pH sensor. However, pHrodo-Green presents an ideal tool for the purpose of determining internalization, as it shows dramatically different fluorescence between the pH 7 and pH 3, the approximate pH in cell media versus in the late endosome (Fig. 28). Examining emission of well-mixed solutions in the relevant region of the electromagnetic spectrum yielded a clear trend of increased fluorescence with decreasing pH. Quenching effects of NPs were also considered at about a 1:1 molar ratio with pHrodo. No difference in fluorescence was observed between samples with (Fig. 28A) and without (Fig. 28B) NPs, indicating that the NPs used do not quench fluorescence.

Figure 28. Increasing fluorescence of pHrodo-Green with decreasing pH in the A) absence and B) presence of silica-coated SPIO NPs. Spectrum collected via Molecular Devices SpectraMax M3 microplate UV-Visible spectrophotometer. $\lambda_{ex}=490$ nm.
To more easily identify the trend of fluorescence with varying pH, as well as compare fluorescence of pHrodo-Green in the presence and absence of SPIO NPs, single wavelength experiments (end point setting) were conducted, measuring emission at 538 nm (Fig. 29). This acquisition mode also minimized collection time.

![Figure 29. Endpoint fluorescence of pHrodo-Green in the presence of SiO$_2$ coated SPIO NPs. Spectrum collected via Molecular Devices SpectraMax M3 microplate UV-Visible spectrophotometer.](image)

No quenching of fluorescence was observed. In fact, at pH 3, fluorescence was higher in the sample with NPs. Overall, similar fluorescence was observed with and without NPs, boding well for fluorescence of pHrodo-conjugated NPs. These findings agree with previous studies of fluorophore-functionalized silica NPs.\textsuperscript{38} Current work in the field includes the development of more sensitive probes with activation and deactivation capabilities.\textsuperscript{39, 40} The pH dependence of pHrodo fluorescence is clear, established here in an acellular study. Next pH-dependent fluorescence was characterized in cell treated with pHrodo-Green conjugated antibodies (Webb Lab).
Following the optimization of the fixation and nuclear labeling protocol, CAPAN-2 and BxPC3 cells were again cultured on glass coverslips and treated with As33@pHrodo-Green for 1.5 hours. Cells retained normal morphology when cultured on coverslips and after treatment. The treated cells were then fixed and imaged using a Leica TCS SP5 confocal microscope. Excitation and detection settings were optimized for the pHrodo-Green fluorophore, which emits light intensely at 505 nm under 488 nm excitation in low pH environments (pH 3-5). CAPAN-2 cells showed clear co-labeling of pHrodo and DAPI, confirming cellular localization and internalization of As33 antibody (Fig. 30).

Figure 30. Co-labeling of pHrodo-Green and DAPI nuclear stain in CAPAN-2 primary pancreatic tumor cells. A) pHrodo-Green, B) DAPI, and C) overlay image of fixed CAPAN-2 cell incubated with As33@pHrodo-Green for 1.5 hours. Images taken at 200x magnification using Leica TCS SP5 confocal microscope.

Optical sections were taken to visualize localization of pHrodo vertically in the cells (Fig. 31). Strong green coloring, indicating presence of pHrodo-green, is observed in the upper part of the cell (Fig. 31AB), but diminishes closer to the
bottom of the cell where it is adhered to the coverslip (31CD). This observation is explained by the mode of exposure, as As33@pHrodo-Green was administered in cell media and thus would have been internalized from the top of the cell. After 1.5 hours, vesicles containing endocytosed antibodies may not have traveled to the bottom of the cell, resulting in a loss of signal in images taken closer to the coverslip.

![Image](image)

**Figure 31. Z-stack of CAPAN-2 cell shows As33 localized to top of cell.** CAPAN-2 cells were treated with As33@pHrodo, then fixed and mounted with media containing DAPI nuclear stain and imaged using confocal microscopy. Images were taken at a maximum of 8 AU at 200x magnification using Leica TCS SP5 confocal microscope.

BxPC3 cells show similar trends to their CAPAN-2 counterparts, with fewer cells labeled, indicating low non-specific uptake (Fig. 32). Some cells, identified by clear DAPI labeled nuclei, show minimal pHrodo label, indicating lower uptake of
As33@pHrodo. This observation supports As33 targeting toward glycoprotein A33, overexpressed in the CAPAN-2 cell line and not present on the surface of BxPC3 cells.14

In summary, these results show the successful functionalization, feasible MRI detection, and targeting potential of antibody directed silica-coated SPIO NPs towards pancreatic cancer. Attachment of targeting antibodies and PEG was shown, paving the way for the engineering and characterization of multifunctional NPs. Recent literature points to protein-based NP targeting as a viable option in vitro, corroborating the results of this study.41,42 Fluorescent moieties were successfully used to detect targeting and internalization of antibody As33, expanding the applications explored in previously published work.37-39

Additionally, the feasibility of SPIO NP detection via MRI was quantified in vitro and found to be remarkably facile, providing further support for the use of
SPIO NPs as exogenous contrast agents in addition to vehicles for targeting therapy. However, caution must be exercised in translating this multifunctional therapy from the lab to the clinic, as toxicity of SPIO NPs has recently gained ground as a major obstacle to use in humans.\textsuperscript{7,11,21} Multifunctional SPIO NPs hold great promise for diagnostic and therapeutic use in medicine, but further investigation of their interactions with the body, on a cellular and systems level, must be conducted in order successfully translate this versatile vehicle into a diagnostic and/or therapeutic tool in the clinic.
4 Conclusions and Suggestions for Future Work

In this work, I have successfully:

♦ Constructed NPs functionalized with:
  - CHO 31.1
  - PEG

♦ Produced preliminary evidence for the targeting of As33 towards cells expressing target antigen A33.

♦ Produced evidence for the internalization of As33 into pancreatic cancer cells via an endocytotic mechanism.

♦ Developed a micro-scale assay for sulphhydryl quantification.

♦ Optimized a cellular assay to measure MRI detection of SPIO NPs in vitro.

♦ Developed an in vitro targeting assay using confocal microscopy to visualize internalization of the targeting moiety.

Future directions for this project include:

♦ Confirming the viability of NP-based immuno targeting, repeat internalization study with NPs conjugated with As33 or 31.1.

♦ Building multifunctional SiO2 coated NPs - conjugated with both antibody and pHrodo or antibody and treatment.

♦ Conducting internalization and MRI detection experiments with fully functionalized NPs.
References

1. Chapter 7 Cancer of the Pancreas_SEER survival monograph.


Appendices
Appendix I: Protocols

IA: TEM Imaging

Goldenson Building, Harvard Medical School, Boston, MA

1. Place grid into chamber in probe; align and insert probe into TEM instrument evacuation chamber.
2. When 1 cycle of evacuation has completed, push home into main column of instrument (the imaging chamber is kept evacuated at all times).
3. Tune electron beam to the center of the gross view stage using control knobs.
4. Select magnification of the digital controls to the right of the instrument (start at 40-50kV).
5. View image. Update scale bar before saving images for accuracy.
6. Use "Measure On" to take direct measurements.
7. To save image: select "Click for Final Image."
8. To close down: turn of filament by bringing control knob to zero. Turn off HT (high tension) and then remove probe from instrument to retrieve grid. Always replace the probe in evacuation chamber of the instrument.

Figure I.I. JEOL TEM instrument.
Tips:

- Use the wobble function for automatic focusing.
- FFT corrects for astigmatism
- Round and diffuse light is best for high magnification – alter with \( \Delta \text{def} (xy) \) and objective focus controls.
- Use 100kV for high magnification imaging

IB: Monolayer Cell Culture Protocol in Flasks and on Microscope Cover Slips

General Passaging Preparation

Half an hour prior: Warm RPMI complete media, sterile PBS, and sufficient trypsin in the hood or place in warm water bath.

1. Wash cells with 5 mL PBS to remove debris and dead cells.
2. Add 3 mL trypsin to detach cells.
3. Incubate 3 min in incubator. Tap to detach cells from plastic.
4. Add 8 mL RPMI media and blow over bottom of flask to detach cells.
5. Transfer to 15 mL centrifuge tube and centrifuge to pellet cells 3 min 1000 RPM on DuPont Sorvall TC 6 machine.
6. Draw off supernatant and resuspend cells in RPMI media for seeding.

T-25 flask monolayer culture:

Prepare cells as for passaging.
Seed 2x10^6 - 7x10^6 cells per flask and add 3-5 mL RPMI media. Cells reach confluence in 2-3 days (BxPC3 cell line grows faster than CAPAN-2). Replace media after 3 days if cells not confluent.

Cover slip cell culture Protocol:

Prepare cells as for passaging.
For overnight preparation:
Seed ~ 100,000 cells per coverslip from monolayer culture ~60% confluent (1:15 dilution for t-25).

1. Suspend cells at 10^6 cells/mL and seeded 100 ul via 1 mL serological pipette.
2. Allow to adhere to the glass for 10 min at 37 degrees (incubator)
3. Add 3 ml media slowly
4. Incubate o/n (12-14 hours)
For 1.5 day preparation:
  Seed ~50,000 cells per coverslip (6x per well) from monolayer culture ~60% confluent (1:30 dilution for t-25).

1. Suspend cells at 5 x 10^5 cells/mL and seeded 100 ul via 1 mL serological pipette.
2. Allow to adhere to the glass for 10 min at 37 degrees (incubator).
3. Add 3 ml media slowly.
4. Incubate 15-19 H.

**IC: Cellular Fluorescent Labeling Protocol**

(Courtesy Professor Louise Darling - Wellesley College)

**Cell Fixation:**

1. Check cells for confluence, normal morphology, and health under microscope
2. Wash (#1.5) coverslips in the 6-well plate with 2 ml PBS to remove traces of phenol red and excess treatment.

**On lab bench:**

3. Fix cells in 1 ml 4% Paraformaldehyde (PFA)(Boston BioProducts) for 15 min.
   a. Place “used” PFA in the labeled PFA waste container in chemical fume hood (**warning: PFA is an environmental hazard and must not go down the sink)**.
4. Wash cover slips 3 times with PBS.
5. Apply 10-20 uL (one small drop) of ProLong Gold with DAPI (Life Technologies) to slide and immediately mount coverslip. Allow mounting media to spread to all areas of the coverslip.
6. Seal coverslips with clear nail polish.
ID: Confocal Microscopy Protocol (on Leica SP5 instrument)

Adapted from lab manual written by Jeannie Benton, Beltz Lab, Wellesley College.

• Powering on (green switches on right hand side):
  1. Power source for metal halide light source
  2. PC computer (log in)
  3. Scanner (wait 20 seconds and don’t touch the microscope)
  4. Laser (turn key also)
  5. Open LAS-AF software on the computer. Deselect resonance scanner and click ok.

• Preparation for imaging:
  1. Select No for initialization of automated stage.
  2. Navigate to: Configuration > Laser
  3. Turn on Argon and 405 diode lasers – set to 20 percent laser power.
  4. Navigate to: Acquire
  5. Adjust power of lasers to 50 percent.

• Mount slide on stage, coverslip down, moving the microscope arm appropriately.
• Use broad spectrum excitation (UV - controls on microscope) to achieve Kohler illumination.
• Select Live at the bottom left and adjust smart gain and smart offset knobs on the control panel to the left of the CPU.
• Set pinhole (1-8 AU), resolution (512x512 to 1024x1024), frame averages (3), imaging channel(s), and volume/number of steps (if applicable).
• Select Start to collect image according to specified parameters.
• To power off, reverse the power on instructions, leaving the laser button on for 20 minutes after turning the system off to allow the lasers to cool.
IE: Preparation of Cellular Agarose Suspensions for MRI

1. Seed CAPAN-2 and BXPC3 cells each into 3 t-12.5 flasks to reach confluence overnight at 37 °C (add about thirty to sixty thousand cells if culture doubles every two hours).
2. Confirm confluence in the morning – around 2.5 million cells per flask
3. Remove media and replace with RT treatment media for each cell line: one control with RPMI media only, two with NPs at __mg/ml suspended in media.
4. Incubate for 2 hours at 37 °C. Remove media and wash attached cells with 3 ml RT PBS.
5. Detach cells from flask with 2 ml RT trypsin. Incubate for 5 minutes and tap flask on bench top to loosen cells. [Note: pipette media over inside face of flask to remove as many adhered cells as possible].
6. Pellet the cells for 3 minutes at 1000 rpm at RT (TC6 centrifuge) and remove trypsin/media supernatant.
7. Resuspend pellet in 500 μl RT PBS, pipetting up and down until no clumps visible (uniform cloudy suspension). Move these suspensions to the 37 °C walk in incubator.
8. In 37 °C walk in incubator, prepare 2 % agarose solution in PBS on a hot plate while stirring with magnetic stir bar.
9. Cool agarose to 37 °C and mix 100 μl cell suspension with 900 μl agarose solution.
10. Transfer to 3 mm NMR tubes, filling to the same level for all samples.
11. Allow agarose to set at RT for 20 min prior to imaging.
Appendix II: Supplemental Results

Functionalization With As33 via Protein A/G

NP synthesis: Silica-coated SPIO NPs used in this study were functionalized with As33@pHrodo in an orientation-dependent manner using protein A/G and an out-to-in conjugation scheme as follows. Protein A/G was thiolated as described in section 2.1.2.1 and conjugated to 5 kDa MW Mal-PEG-silane, which was then tethered to the silica surface of the NPs for 30 hours at 4 °C. NPs were washed three times in PBS to remove excess protein A/G. NanoDrop instrument detected no absorbance at 280 nm in the supernatant of the third wash. Finally, As33@pHrodo was attached non-covalently via the affinity of protein A/G for the FC region of antibodies. The last conjugation step was performed for 30 min at 4 °C and followed by collection and washing of NPs with PBS.

These functionalized NPs were then used to treat both CAPAN-2 and BxPC3 cells cultured on coverslips. Each coverslip, cultured overnight with 50,000 cells, was incubated with 10 μl NPs in 1 ml PBS for 2 hours. Cells were then washed and fixed, as described in section 2.3.1 and appendix IC, with mounting media containing DAPI nuclear stain. Confocal microscopy was used to image treated cells for co-localization of DAPI and pHrodo signal (Fig. II.I).
Figure II.I. Confocal microscopy shows internalization of pHrodo-conjugated silica-coated NPs in CAPAN-2 and BxPC3 cells.

pHrodo appears to localize to the cell, and co-localize with the nucleus, indicating internalization. Punctate distribution of pHrodo fluorescence supports internalization into endocytotic vesicles that are low in pH. As gauged by pHrodo fluorescence, NPs appear targeted towards CAPAN-2 cells, which express glycoprotein A33, the target of antigen of As33 antibody. This finding shows both internalization and targeting of NPs conjugated to targeting antibodies. However, the NPs were not fully characterized after conjugation to As33@pHrodo and thus further studies must be conducted to verify these results.