**UTILIZATION OF GOLD CONTAINING NANOMATERIALS FOR TARGETED RADIOTHERAPEUTIC
AND DIAGNOSTIC APPLICATIONS**

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RADIOTHERAPEUTIC AND DIAGNOSTIC APPLICATIONS

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For my parents, who nurtured me into the man I am today and provided such stunning
examples of selfless love.

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LIST OF ABBREVIATIONS

<u>Abbreviation</u>	<u>Definition</u>
MRI	Magnetic resonance imaging
CT	computed tomography
LET	Linear energy transfer
DNA	Deoxyribonucleic acid
EPR	Enhanced permeability and retention
DMPS	2,3-dimercapto-1-propanesulfonic acid
DMSA	Dimercaptosuccinic acid
SRIM/TRIM	Stopping range of ions in matter/transport of ions in matter
SPECT	Single photon emission computed tomography
PET	Positron emission tomography
TETA	1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid
CB	Cross-bridged
IT	Intratumoral
GRP	Gastrin-releasing peptide
DTPA	diethylene triamine pentacetic acid
ACS	American Chemical Society
THPAL	Tris-hydroxymethyl phosphine alanine
DPBS	Dulbecco's phosphate buffered saline
UV-Vis	Ultraviolet-visible
ICRSCID	Imprinting control region severe combined immunodeficient
NAA	Neutron activation analysis
TEM	Transmission electron microscopy
DLS	Dynamic light scattering
ID	Injected dose
RES	Reticuloendothelial system
MURR	University of Missouri Research Reactor
NO ₂ A	1,4,7-Triazacyclononane-1,4-diacetic acid
BSA	Bovine serum albumin
TLC	Thin layer chromatography
IV	Intravenous
IP	Intraperitoneal
PEG	Polyethylene glycol
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide)

NHS	N-hydroxy succinimide
AAS	Atomic absorption spectroscopy
DTDTPA	Dithiolated diethylene triamine pentacetic acid
BA	Bis-anhydride
DMF	Dimethyl formamide
NMR	Nuclear magnetic resonance
DI	Deionized
RSA	Rat serum albumin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
bromide	
MWCO	Molecular weight cutoff
XRD	X-ray diffraction
EELS	Electron energy loss spectroscopy
TPP	Triphosphosphate
mAb	Monoclonal antibody
GFAP	Glial fibrillary acidic protein
TCEP	Tris(2-carboxyethyl)phosphine
hEGFR	Human epidermal growth factor receptor
DAB	Diaminobenzidine
CNS	Central nervous system

ABSTRACT

Several different investigations are discussed in this dissertation, with the common thread of using gold-containing nanomaterials in nuclear medicine applications. Gold nanoparticles form a non-toxic surface layer that can be easily functionalized with biological targeting agents. The first set of experiments focuses on attempts to develop cancer therapeutic and imaging agents using the radionuclides $^{198/199}\text{Au}$. Gold nanoparticles with both passive (gum arabic coated) and active (bombesin) targeting are examined. Additionally, the possibility of attaching a chelate to bind the PET imaging radionuclide ^{64}Cu with DTDTPA is explored.

The second area of emphasis focuses on developing a nanoparticle capable of retaining the radioactive decay daughters of the *in vivo* α generator ^{225}Ac . This generator system produces 4 successive α emissions in its decay chain. However, daughter products escape and cause renal toxicity. A LnPO_4 nanoparticle coated with gold can both retain the energetic daughter products in the decay chain and be easily functionalized to deliver radiation to biologically relevant targets *in vivo*.

Nanoparticle technology allows for the combination of multiple therapeutic and imaging modalities by a single platform. Unlike conventional approaches, the multi-atom nature of nanoparticles allows for delivery of large, varied payloads to biological receptors. The nanomaterials discussed here can theoretically be used for any combination of PET/SPECT imaging, MRI contrast, or α/β^- radiotherapy.

CHAPTER 1:

INTRODUCTION TO NANOPARTICLE BASED RADIATION THERAPY

1.1 Cancer Epidemiology

Great strides have been made in the diagnosis and treatment of a wide variety of cancers. Relative survival rates of all cases have risen to 67% for cancers diagnosed between 2001 and 2007, compared with only 45% for cancers diagnosed between 1975 and 1977. Despite this progress, cancer remains the second leading cause of death in the United States, accounting for nearly one in every four deaths.¹ Globally, cancer incidence is expected to rise 75% by 2030.² Early diagnosis and detection of cancer increases the success of therapeutic regimens. Unfortunately, many types of cancer will metastasize, creating tumors in distant parts of the body prior to diagnosis. Neoplastic cells can lie dormant for decades before developing into tumors.³ The presence of metastatic disease drastically decreases survival. For example, primary breast cancer tumors are currently cured with a 98.6% success rate, as measured by the relative 5 year survival fraction. Regional spread of tumor cells via the lymphatic system lowers survival expectancy to 83.8%. In contrast, only 23.3% of breast cancer patients with distant, metastatic spread of cancer survive five years after diagnosis.⁴

1.2 Current Oncology Therapies

Current cancer treatment falls into three basic categories: surgery, chemotherapy, and radiotherapy. Most patients diagnosed with cancer undergo some sort of surgery to remove large, primary tumors. When the tumor is localized, surgical resection effectively treats a wide variety of tumors. Unfortunately, a number of factors can render a tumor inoperable. The tumor may be connected to or embedded within a vital organ such as the heart or brain or the tumor may have metastasized to distant sites, rendering removal of the original tumor ineffectual. Finally, the tumor may not have adequate margins of neighboring healthy tissue to be safely removed. Even a small remaining neoplastic fraction will quickly regenerate the removed tumor mass. Surgery represents an effective, if invasive, treatment for isolated solid primary tumors.

Inoperable tumors, metastases, and dormant circulating tumors require other methods of treatment. Chemotherapy, the use of cytotoxic chemicals to attack neoplastic cells, can be effective as a standalone therapy or in combination with surgery and/or radiation to prevent the spread and recurrence of surgically removed neoplasms.⁵ Most chemotherapeutic agents act non-specifically on rapidly dividing cells which include tumor cells, bone marrow, intestinal cells, and hair follicles. The effect of chemotherapeutics on healthy tissues causes numerous side effects associated with cancer treatment, including decreased immune function and anemia from bone marrow destruction, nausea and vomiting from intestinal cell toxicity, and alopecia (hair loss) from the loss of hair follicles.^{6,7,8}

Radiation therapy is a method often used either as a standalone treatment or in combination with surgery and/or chemotherapy. The two predominant types of radiotherapy currently in clinical use are external beam radiotherapy and brachytherapy. External beam radiotherapy uses either x-rays, protons, or α particles to destroy the target cancer cells, often guided by advanced imaging techniques such as magnetic resonance imaging (MRI) and computed tomography (CT).⁹ However, external beam radiotherapy disrupts healthy tissue as it traverses the path to the tumor, causing harmful side effects. Brachytherapy uses implantable “seeds” containing a radioactive isotope. These seeds are placed in the immediate vicinity of the tumor and deliver a continual dose until the source is removed or decays. Implantable brachytherapy seeds are particularly common and effective in treatment of inoperable prostate cancer.¹⁰ Brachytherapy seeds also have the potential to shift *in vivo*, delivering dose to healthy tissue or causing a radiological hazard if excreted from the body. Large doses to normal tissue result in undesirable side effects such as incontinence and impotence.¹¹

1.3 Targeted Radiotherapies and Types of Therapeutic Radiation

In order to simultaneously improve cancer patient outcomes and quality of life, more specific therapeutic modalities must be pursued. Side effects would be drastically reduced, if not eliminated, by development of radiotherapeutic compounds that spare healthy tissue while delivering cytotoxic doses to tumor cells. Currently, there are two targeted radiotherapy compounds approved by the FDA for human use. BEXXAR utilizes the β^- particle emitted from ^{131}I in treatment of follicular lymphoma while Zevalin

incorporates the β^- emitting radionuclide ^{90}Y for treatment of non-Hodgkin's lymphoma.^{12,13}

There are two distinct types of radiation used for therapeutic purposes. Photons, β^- particles, and Auger electrons deliver low linear energy transfer (LET) radiation while protons and α particles deliver high LET radiation. Low LET and high LET radiation differ drastically in their properties and mechanism of action and therefore their clinical applicability. Both types of radiation destroy cancer cells by inducing double strand breaks in cellular deoxyribonucleic acid (DNA). This type of lesion proves difficult for the cell to repair and leads to fatal chromosomal aberrations.¹⁴ Low LET radiation acts primarily by generating reactive oxygen species as a result of ionization events. Because low LET radiation acts, at least in part, via these intermediaries, their efficacy can be decreased based on external factors such as cell oxygenation or cell cycle considerations.^{15,16} Cells which are hypoxic or anoxic require 2-3 times more activity of low LET radiation to produce the same level of toxicity.¹⁷ Dividing cells are able to repair DNA damage, including double strand breaks, during the S phase of mitosis when chromosomes are being duplicated. They are least likely to successfully repair DNA lesions during the mitotic, or M phase.¹⁵

In contrast, high LET radiation creates double strand breaks by acting directly on DNA. As a result, the effects of high LET radiation depend less on external factors. The path length between ionization events closely approximates the distance between cross-sectional base pairs in the DNA molecule.¹⁵ Only a few traversals of the nucleus is

required to generate a cell kill as opposed to the thousands necessary in β^- therapy.¹⁸ Thus, α and proton therapy can cause tumor cytotoxicity with only a fraction of the activity required in β^- therapy, i.e. α therapy has a much higher relative biological effectiveness. Because high LET radiation deposits its energy in a small, localized space, it limits collateral damage to healthy tissue. Unfortunately, this effect also limits the scope of α therapy to small micrometastases, disseminated/blood-borne neoplasms, and film-like peritoneal tumors.¹⁹ Crossfire effects from the longer range of β^- particles provide a better option for treating large, solid tumors which cannot be surgically resected or treated with chemotherapy/external beam radiotherapy.²⁰

The bifunctional chelate approach represents a major paradigm for radiopharmaceutical research in both α and β^- therapy.²¹ In this approach, a radiometal is stably sequestered by a chelating agent bound to a targeting molecule such as a peptide or antibody. The chelate must be able to bind the radiometal quickly relative to the radionuclide half-life in order to maximize radiochemical yield. It also must form a very stable bond (both thermodynamically and kinetically) that will remain impervious to attack by the various proteins and salt concentrations found in the biological environment.

1.4 Nanoparticles for Cancer Therapy

Recently, nanoparticles have emerged as an exciting alternative to molecular chelating agents for delivering radioisotopes in cancer therapy. The wide swath of

constructs that falls under the umbrella of nanoparticles possess a number of exciting properties for delivery of both radioactive and non-radioactive therapeutic agents. Nanoparticles possess extraordinary versatility, where the same platform can be radically changed by modifying size, shape, and surface characteristics of the particle. These changes alter the pharmacokinetic profile of the nanoparticle. *In vivo* characteristics such as biodistribution, excretion pathway, and drug release timing can all be tuned by slight changes in particle composition.²²

Many important processes in the body are regulated by receptors which are present in very low concentrations, yet have a very high affinity for their associated substrate. These low receptor concentrations make them difficult to target for therapeutic and imaging purposes.²³ Using a nanoparticle rather than a molecular bifunctional chelate can drastically increase the dose delivered to each receptor site. While a traditional molecular chelate typically binds one radiometal, a single nanoparticle can deliver multiple radioactive atoms to a single receptor site. Additionally, the size of the nanoparticle makes it possible to link multiple targeting vectors onto a single particle. Emerging data on tumor heterogeneity suggests that variations between tumor cells even within a single tumor type may frustrate attempts to effectively target all of the tumor cells. Incorporating multiple targeting mechanisms onto each particle holds the potential to broaden the effect of targeted radiopharmaceuticals.²⁴

Nanoparticles possess some degree of innate targeting towards tumors *in vivo*.

The remarkable growth rate of tumors requires recruitment of neovasculature to provide sufficient nutrients to the neoplasm. Tumor cells often release large amounts of vascular epithelial growth factor to promote angiogenesis and vascular permeability. As a result of the increased permeability, large constructs such as nanoparticles and liposomes are filtered from the bloodstream into the interstitial space surrounding the tumor. In normal tissue, these species are typically removed from the interstitial space by the lymphatic system. However, in tumor tissue the lymphatic system is largely disabled, increasing the residence time of the nanoparticle in the interstitial space. This combination of leaky, permeable vasculature and disabled lymphatic function is known as the enhanced permeability and retention (EPR) effect.²⁵

The increased size of nanoparticle constructs relative to bifunctional chelate platforms presents unique challenges. While increased passive tumor targeting occurs via the EPR effect, specific targeting is hindered. Large nanoparticles have difficulty extravasating out of the vascular space, potentially limiting application to tumors with vascular targets, blood-borne neoplasms, and peritoneal cancers. Various techniques such as mild hyperthermia can improve nanoparticle extravasation on clinically relevant time scales.²⁶

Much of the work on nanotechnology has focused on gold nanostructures. While gold colloids were known and used in medieval times as a stain, monodisperse gold nanoparticles were first routinely synthesized in the modern era by Turkevich, *et al.*

in 1951.²⁷ Their procedure produces spherical gold nanoparticles of diameters from 10-20 nm in aqueous solution. Ionic gold (Au^{+3}) is reduced by citrate with heat to neutral gold. Brust later pioneered an organic phase gold synthesis and solvent transfer method by modifying the surface groups on the nanoparticle. This method involves reduction of ionic gold by sodium borohydride in toluene with tetraoctylammonium bromide capping agent.²⁸ Due to its relative inertness and facile functionalization, gold makes an attractive surface coating for other nanoparticle materials. For instance, the surface plasmon resonance of gold provides a visual marker for MRI active, gold coated Fe_3O_4 particles.²⁹

1.5 *In vivo* α generator therapy

Alpha generator therapies use radionuclides with four (^{223}Ra , ^{225}Ac) or five (^{227}Th) α particles in their decay chain. Decay chains for ^{227}Th and ^{225}Ac are shown in Figures 1a³⁰ and 1b. Currently there are two barriers to development of clinically relevant *in vivo* α generators. The first challenge consists of producing a reliable and sufficient supply of the radionuclides. Secondly, a means of sequestering the radioactive daughter products *in vivo* must be devised to prevent toxicity to non-target tissue.

Figure 1.5.1 A and B: Simplified decay schemes for ^{227}Th and its parent radionuclide, ^{227}Ac (A) and for ^{225}Ac (B).

Currently, ^{225}Ac is available from a ^{229}Th generator produced at Oak Ridge National Laboratory.³¹ The ^{229}Th comes from legacy stockpiles of ^{233}U developed during the cold war era and produces no carrier added ^{225}Ac . Alternative strategies for production of ^{225}Ac include using stockpiled ^{226}Ra seeds in a $^{226}\text{Ra}(p, 2n)^{225}\text{Ac}$ reaction. This reaction has been used with proton energies of 16.8 MeV on a 0.0125 mg target for 7 hours with a current of 10 μA to produce 78 kBq of ^{225}Ac .³² This method also produces ^{224}Ac , which is decayed away before separation and ^{226}Ac , which has a negligible yield at 16.8 MeV. A third method for production of ^{225}Ac involves the spallation reaction on ^{232}Th . Cross section data obtained at 800 MeV proton energy indicate that 10 day irradiations at the Los Alamos Neutron Science Center facility will be capable of producing 740 GBq of ^{225}Ac . A non-negligible amount of ^{227}Ac (0.25%) will also be produced. An additional 13 GBq of no carrier added ^{225}Ac could be made available from simultaneous production of the ^{225}Ra parent.³³ Further studies are required to determine if the ^{227}Ac impurity is sufficient to cause long term radiation damage *in vivo*.

Figure 1.5.2: Purified sample of 1.85 GBq of $^{225}\text{AcCl}_3$. Alpha particles from the sample cause the glass vial to radioluminesce.

Thorium-227 and ^{223}Ra are primarily available in small quantities as decay products of ^{235}U and ^{227}Ac . Both the United States and Europe possess modest stockpiles of ^{227}Ac . The ^{227}Ac parent can also be produced with good yield from the $^{226}\text{Ra}(n,\gamma)^{227}\text{Ac}$ reaction.³⁴ Successive neutron capture on ^{227}Th can produce ^{228}Th and ^{229}Th , the parent isotope for ^{225}Ac .³⁵

Beyond radionuclide availability, the emission of multiple α particles presents unique challenges for specific delivery of therapeutic radiation. Namely, the chemical behavior of daughter radionuclides often differs radically from that of the parent radionuclide. For example, the bivalent ^{223}Ra decays to the unreactive noble gas radon while the trivalent ^{225}Ac decays to the monovalent francium. Chemical behavior along the decay chain of *in vivo* generators switches from cationic to anionic and back again, frustrating attempts to contain the radionuclides within a single chelate. The recoil energy of the daughter radionuclides presents an equally daunting challenge. Each α -particle emission creates a recoil energy of >100 keV (four orders of magnitude greater than the energy of a typical covalent bond) on the daughter radionuclide. As a result, daughters chelated by traditional molecular species will not remain bound to the bioconjugate at the targeting site.³⁶

The *in vivo* α generator ^{223}Ra provides an example of an effective targeted α therapy generator for treatment of metastatic bone cancer.³⁷ Radium-223 chloride (Alpharadin) has been granted Fast Track designation by the U.S. Food and Drug Administration for the treatment of hormone-refractory prostate cancer in patients with

bone metastases.³⁸ It is effective because radium mimics calcium *in vivo*, providing a high affinity for bone. Further, the daughter products either have short half-lives (< 5 minutes) or have a high affinity for bone (^{211}Pb , $t_{1/2} = 36 \text{ m}$). Translation of *in vivo* α generators to anything besides metastatic bone cancer with a calcium mimic will require a different mechanism for both delivering and retaining the radioactive daughters in the target tissue.

Other methods under investigation for sequestration of α emitting daughters involve chelation with DOTA or analogous macrocyclic chelators and incorporation into liposomes/polymerosomes, fullerenes, or nanocrystals. In the case of ^{225}Ac based therapeutics, ^{213}Bi daughter migration to the kidney represents the dose limiting factor. Scavengers such as 2,3-dimercapto-1-propanesulfonic acid (DMPS) and dimercaptosuccinic acid (DMSA) or non-radioactive Bi competition mitigate, but do not effectively eliminate this limitation.³⁹

Another challenge is that labeling efficiency for molecular chelates depends on the ratio of chelate to ^{225}Ac . Examples from literature studies typically exhibit either high synthetic yield or high specific activity, but not both. In order to achieve high synthetic yield (>97%), Essler *et al.* use huge excesses of chelate/peptide conjugates (6,700 conjugates per ^{225}Ac atom). The resulting low specific activity (72 MBq/ μmol) would be suitable only for receptors that are in high abundance *in vivo*.⁴⁰ Effectively targeting receptors with low *in vivo* concentrations requires high specific activity. Song *et al.* produce higher specific activity bioconjugates (4.44 GBq/ μmol) but at the expense

of labeling efficiency (12.0%).⁴¹ Given the limited supply of ^{225}Ac and the importance of receptors with low concentrations, development of a high yield, high specific activity synthesis is paramount.

Single and multi-layer liposomes are under evaluation for their ability to sequester ^{225}Ac and its α -emitting daughters. Unfortunately, preparations of single wall liposomes suffer from both low ^{225}Ac incorporation (6.4%) and poor daughter retention (^{213}Bi <10%).⁴² Further, single wall liposomes struggle to quantitatively retain the ^{225}Ac parent radionuclide. Despite the lofty sequestration predictions of theoretical models, experimental retention of ^{225}Ac and ^{213}Bi appears much lower. Active loading of liposomes increases ^{225}Ac incorporation to 73%, though ^{225}Ac retention only reaches 81%.⁴³ Despite the higher synthetic yield, ^{213}Bi retention does not improve. Recent theoretical work postulates ^{213}Bi retention as high as 80% in multi-walled polymerosomes. The proposed polymerosomes require a large bulk (800 nm in diameter) to achieve this level of retention.⁴⁴ Such large particles would not extravasate out of the vasculature and would not capitalize on the enhanced permeability and retention effect.^{45,46} A final strategy for ^{225}Ac sequestration involves incorporation into fullerenes. This method exhibits a low synthetic yield (1%) and little to no ^{221}Fr daughter sequestration.⁴⁷

A final method for retaining ^{225}Ac and its α -emitting daughter radionuclides directly incorporates Ac into the crystal structure of a nanoparticle.^{48,49} Due to isomorphism between Ac and the lanthanides, Ac co-crystallizes with lanthanide

phosphates. Both Ac and the lanthanides (with the exception of cerium) almost exclusively form +3 cations. Additionally, the light lanthanides are large enough to approximate the ionic radius of Ac. Incorporating Gd into the crystal provides a sufficient paramagnetic moment for separation of the nanoparticles with a NdFeB magnet.⁴⁹ Addition of a Au surface layer facilitates surface modification, prevents Gd leakage *in vitro*, and prevents *in vivo* reactivity. Ranges of recoiling ²²⁵Ac daughters in gold calculated by the stopping range of ions in matter/transport of ions in matter (SRIM/TRIM) program are shown below in Table 1.5.1.⁵⁰

Table 1.5.1: Range and kinetic energies of ²²⁵Ac daughter products

Daughter	Decay energy (MeV)	Daughter energy (keV)	Range in gold (nm)
²²¹ Fr	6.3	112	11.3
²¹⁷ At	7.1	129	12.5
²¹³ Bi	5.9	109	11.3
²⁰⁹ Pb	8.4	158	12.0

In addition to Alpharadin, there are two other current clinical trials of *in vivo* α generators involving ²²⁵Ac. The first phase I study is currently recruiting patients at Memorial Sloan Kettering Cancer Center in New York. This clinical trial utilizes the humanized antibody HuM195 against the CD33 protein, found in acute myeloid leukemia. In the study, ²²⁵Ac is chelated to DOTA, which is then bound to HuM195.⁵¹ The second study, organized by M.D. Anderson Cancer Center, uses the antibody lintuzumab for treatment of acute myeloid leukemia.⁵²

CHAPTER 2:

GOLD NANOPARTICLES FOR THERAPY OF UNRESECTABLE PROSTATE CANCER

2.1. Introduction

2.1.1. Production, Separation, and Nuclear Properties of Radiogold

Prostate cancer is the most common form of cancer in males, with 241,000 new cases estimated to be diagnosed in 2012.¹ Many of these tumors are not eligible for surgical resection. Methods such as radiotherapy/brachytherapy successfully treat these neoplasms or shrink them to the point where they can be surgically resected.

Radiogold possesses favorable properties for β^- therapy of tumors. Production of radiogold occurs via neutron irradiation. Only one stable isotope of gold, ^{197}Au , occurs in nature, making enrichment unnecessary. Neutron absorption by natural gold produces a large amount of ^{198}Au and a smaller fraction of ^{199}Au . A summary of their nuclear properties is shown below in Table 2.1.1.

Both gold radionuclides have approximately a 3 day half-life and release a β^- particle. Lower ratios of $^{198}\text{Au}/^{199}\text{Au}$ (higher production of ^{199}Au) are produced using a

Table 2.1.1: Properties of $^{198/199}\text{Au}$ ^{53,54}

Radionuclide	Half-Life (d)	β^- energy (keV)	γ energy (keV)	σ_{thermal} (b)	$\sigma_{\text{epithermal}}$ (b)
^{197}Au	Stable			1550	98.7
^{198}Au	2.70	719	412 (96%)	26,000	
^{199}Au	3.14	453	158 (40%)		

high thermal/epithermal flux and long irradiation times. The high energy γ ray at 411.8 keV, which occurs with 96% abundance in ^{198}Au , presents a major drawback for radiotherapy purposes.⁵⁴ This γ ray delivers a whole body radiation dose to healthy tissue in the patient. In contrast, ^{199}Au has an ideal imaging γ ray of 158 keV (40% abundance).⁵⁴ Current single photon emission computed tomography (SPECT) equipment designed to image the 140 keV γ ray from $^{99\text{m}}\text{Tc}$ will easily detect the ^{199}Au γ with little or no modification. Monte Carlo N-Particle analysis of these two γ rays in a standard reference man indicates that the ^{198}Au γ gives a whole body dose 2.37 times higher than the ^{199}Au γ .⁵⁵ The non-specific, whole body dose can disrupt normal function and lead to carcinogenesis in other parts of the body.

No carrier added ^{199}Au , which will provide a lower whole body γ dose, can be produced by neutron irradiation of ^{198}Pt (7.16% natural abundance).⁵³ The $^{198}\text{Pt}(n,\gamma)^{199}\text{Pt}$ reaction has a high cross section (3.8 barn thermal, 56 barn epithermal) and the ^{199}Pt decays into ^{199}Au with a half-life of 30.8 minutes.⁵³ Solvent extraction with ethyl acetate or cation exchange can be used to separate Au(III) ions from their Pt(IV)

counterparts in the dissolved target. After irradiation, the Pt target is dissolved in a glove box using 500 μL of boiling aqua regia in a glass liquid scintillation vial. The resulting solid is redissolved in 500 μL of 1 M HCl and again evaporated to dryness. The solid is prepared for extraction by addition of 500 μL of 3 M HCl and transfer to a plastic V-bottom vial. Extraction occurs through addition of an aliquot of ethyl acetate (300 μL), vortexing, and pipetting of the ethyl acetate layer. This process is repeated until adequate yield is achieved. After extraction, the ethyl acetate is evaporated and the remaining impurities are digested with 300 μL of 35% H_2O_2 . The gold is then redispersed in 0.05 M HCl for use. The separation process has high yield (>90%) and radiochemical purity (>99%).^{56,57}

2.1.2 Chemical Reactivity of Gold

Chemically, gold exhibits three easily accessible oxidation states. Most commonly, gold exists as solid, neutral gold. Au^0 has historically been used in the production of coins, hence its designation as one of the unreactive coinage metals. Under harsh conditions, such as immersion in hot aqua regia, gold will oxidize to the Au^{+3} state in the form AuCl_4^- . Reduction of AuCl_4^- to form nanoparticles is favorable, with a standard reduction potential of 1.002 V from AuCl_4^- to Au^0 . Partial reduction of AuCl_4^- to AuCl_2^- (0.93 V) produces Au^{+1} which can then be further reduced to Au^0 (1.154 V).⁵⁸

While all three gold oxidation states are currently being investigated for potential therapeutic application, ionic gold shows instability in biologically relevant

media. Reduction to colloidal gold is seen in many Au^{+3} and Au^{+1} compounds due to the body's natural redox potential.^{59,60} Stabilizing Au^{+3} *in vivo* presents an even more daunting challenge in light of proteins evolved to chelate trivalent metal ions such as Fe^{3+} . For this reason, many groups are using neutral gold in nanoparticulate form for therapy. Au^0 remains inert *in vivo* and, given the proper surfactant, can be highly water soluble. Gold nanoparticles are among the most studied and consequently best understood nanomaterials.

2.1.3 Production, Nuclear, and Chemical Properties of ^{64}Cu

While ^{199}Au provides SPECT capability, many applications call for the higher spatial resolution inherent from positron emission tomography (PET) imaging. This imaging requires a positron emitter such as ^{64}Cu . Copper-64 decays by β^- (38.4%), β^+ (17.8%), and electron capture (43.8%) decay. Copper-64 can be produced from the $^{64}\text{Ni}(p,n)^{64}\text{Cu}$ reaction on an enriched ^{64}Ni target at modest cyclotron energies (12 MeV). The reaction and separation via ion exchange chromatography produce ^{64}Cu with high radionuclidic purity (>99%).⁶¹ The decay scheme of ^{64}Cu is shown in Figure 2.1.2.

Figure 2.1.1: Simplified decay scheme of ^{64}Cu .

Ionic copper is more stable than ionic gold, and can be chelated with a number of different ligands. When chelated, copper exists predominantly in the +2 oxidation state. Nitrogen based, macrocyclic chelating agents such as 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA) effectively bind Cu^{2+} *in vitro*, but show instability *in vivo*. The crossbridged analog to TETA, CB-TE2A, adds copper slowly but exhibits high stability *in vivo*.⁶² Substituting phosphonic acid arms for the acetic acid arms of the TE2A system increases the kinetic speed of Cu^{2+} incorporation while retaining high *in vivo* stability.⁶² Other radionuclides of copper also may prove useful for radiotherapy and imaging, with ^{67}Cu functioning as a β^- therapeutic and ^{60}Cu having potential as a PET radionuclide.

2.1.4 Specific Aims

The first portion of this work assessed the ability of non-specifically targeted nanoparticles to serve as brachytherapy agents. In these experiments, the gold nanoparticle surface is passivated by the biocompatible surfactant gum arabic. Nanoparticles are injected intratumorally (I.T.) and monitored for retention in tumor tissue over a 1 month period. Rather than painfully inserting hundreds of macroscopic seeds into the prostate, a single injection of nanoparticles delivers a prodigious number of microscopic seeds directly to the tumor.

The second portion of this work evaluates a specifically targeted β^- therapeutic consisting of $^{198/199}\text{Au}$ nanoparticles coated with starch and directed *in vivo* by a

truncated bombesin targeting agent. Targeting efficacy is monitored by multiple injection pathways in comparison to untargeted nanoparticles and bombesin-targeted bifunctional chelate constructs. Bombesin targets the gastrin-releasing peptide (GRP) receptors in cells. These GRP receptors are over expressed in prostate cancer cells, with as many as 44,000 receptors per PC-3 prostate cancer cell.⁶³ While the full length bombesin peptide consists of 14 amino acids, truncated peptides based on amino acids 7-14 show improved targeting and pharmacokinetics as a result of amino acid substitution or modified chelation/spacer groups.⁶⁴ The full length bombesin peptide (Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂) is shown in Figure 2.1.1.

Figure 2.1.2: Structure of the 14 amino acid bombesin peptide.

The third portion of this work focuses on development of a ⁶⁴Cu containing gold nanoparticle. Radiometal chelation with non-macrocyclic compounds such as diethylene triamine pentacetic acid (DTPA) can prove unstable *in vivo*.⁶⁵ Binding multiple DTPA chelates to a single nanoparticle could provide a sandwich style structure which would sufficiently stabilize the ⁶⁴Cu. This development would generate a PET imaging agent with the passive targeting advantages of a nanoparticle.

2.2 Gum Arabic Gold Nanoparticle Experimental

2.2.1 Materials

All chemicals were obtained from Sigma-Aldrich and were at least American Chemical Society (ACS) grade. Materials were used as received without further purification or modification. All animal experiments were conducted according to the procedures set forth by the Institutional Animal Care and Use Committee of the University of Missouri-Columbia.

2.2.2 Gum Arabic Gold Nanoparticle Synthesis

Gum arabic (2.94 mg) was dissolved in 6 mL of 18 M Ω water. NaAuCl₄ (100 μ L, 0.1 M) was added to the gum arabic solution in a 20 mL glass scintillation vial. Tris-hydroxymethyl phosphine alanine (THPAL, 3.9 mg) was dissolved in 116 μ L of water. The dissolved gum arabic solution was heated under reflux, then 60 μ L of THPAL solution was added. A deep purple color forms immediately upon addition of the THPAL. The nanoparticle solution was heated for one minute, then cooled with stirring for 30 minutes. The solution was neutralized to physiological pH by addition 1 M NaOH and HCl. Dulbecco's phosphate buffered saline (DPBS) was added to buffer the sample and restore the volume to 6 mL. The nanoparticle solution was diluted by taking 100 μ L of solution and mixing with 900 μ L of water for Ultraviolet-visible (UV-Vis) measurements on an Ocean Optics USB 2000 spectrometer.

2.2.3 Biodistribution and Animal Studies

Biodistribution studies were performed by Lisa Watkinson and Terry Carmack under the supervision of Prof. John Lever and Dr. Jeff Smith at the Harry S Truman Memorial Veterans Affairs Hospital. The non-radioactive gum arabic coated nanoparticles (30 μ L) were injected I.T. into randomly selected female imprinting control region severe combined immunodeficient (ICRSCID) mice (n=7) with average body mass of 23.01 ± 1.76 g. Human PC-3 prostate cancer xenografts were grown in the mice prior to injection with average tumor volume of 0.052 ± 0.029 g. PC-3 cells were obtained from American Type Culture Collection and were maintained by the University of Missouri Cell and Immunobiology Core Facility. Control mice (n=7) were injected with 30 μ L of DPBS solution I.T. Over the next month, tumors were measured using external calipers to estimate tumor volume. Additionally, animal weight was monitored as an indicator of overall health. If an animal experienced drastic weight loss, it was euthanized and its organs harvested. After 30 days, the surviving mice were sacrificed and their organs harvested (bladder, kidney, small intestine, large intestine, liver, lung, pancreas, spleen, muscle, and tumor) for analysis by neutron activation analysis (NAA). Each organ was freeze dried and loaded into a 2/5 dram (1.48 mL) polyethylene vial before co-irradiation with gold standards. Samples were allowed to decay for three hours before counting.

2.3 Gum Arabic Gold Nanoparticle Results and Discussion

Gum arabic coated gold nanoparticles exhibited a plasmon resonance absorption maximum at 539 nm. This absorption corresponds with the absorption of gum arabic nanoparticles previously measured by scanning transmission electron microscopy (TEM) of ~15 nm in diameter. Previous dynamic light scattering (DLS) measurements indicate that these 15 nm nanoparticles have a hydrodynamic diameter of 85 nm and a zeta potential of -24.5 mV.⁵⁷

During the experiments, two mice from the nanoparticle treatment group and two mice from the DPBS control group showed significant weight loss and were euthanized before the end of the thirty day trial period. Within the surviving mouse population, nanoparticles injected into the tumor showed long residence times, with $80.0 \pm 18.7\%$ of the injected dose (ID) retained over the 1 month time period. Nanoparticles which did not remain in the tumor were excreted primarily via the reticuloendothelial system (RES). Next to the tumor, the highest organ uptake following IT injection occurred in the liver, with $1.4 \pm 0.4\%$ ID present. Uptake in the spleen ($0.11 \pm 0.01\%$ ID) is also indicative of the RES clearance pathway. The total amount of gold detected in the collected organs accounted for 83.9% of the initial ID. Undetected gold nanoparticles were presumably present in small amounts in the remainder of the animal or excreted over the one month study period. The average minimum detectable amount of gold in tissue via the NAA measurements was approximately 10 ng. The biodistribution of the particles is shown in Figure 2.3.1.

Figure 2.3.1: Gum arabic coated gold nanoparticles present in SCID mice one month following I.T. injection in PC-3 xenografts measured by NAA.

Non-radioactive gum arabic coated gold nanoparticles are retained in the tumor to a much higher degree than radioactive nanoparticles. Previous therapy studies done by Chanda, *et al.* utilizing ^{198}Au gum arabic nanoparticles indicate that only $19.9 \pm 4.2\%$ of the ID was retained in the tumor after one month.⁶⁶ In the radioactive study, most of the tumor tissue was necrotic at this point, which was not observed with the non-radioactive nanoparticles. The difference may possibly be explained through differences in clearance between the necrotic tissue found in mice treated with radioactive

nanoparticles and the viable tumor tissue found in mice treated with non-radioactive nanoparticles.

Tumor sizes between groups receiving DPBS and non-radioactive gold nanoparticles showed no statistically significant difference in tumor volumes or mass (p-value = 0.1258). In the group receiving gold nanoparticles, tumor volume averaged $0.65 \pm 0.21 \text{ cm}^3$ while in the group receiving PBS, tumor volume averaged $0.49 \pm 0.43 \text{ cm}^3$. After sacrifice, tumors from the treatment group had an average mass of $0.888 \pm 0.193 \text{ g}$ while tumors from the PBS group had an average mass of $0.704 \pm 0.225 \text{ g}$. These results are shown in Figures 2.3.2 A and B.

Gum arabic coated ^{198}Au nanoparticles have shown tremendous potential to shrink tumors in unresectable prostate cancer.⁶⁷ However, it had not been rigorously established that this tumorcidal effect was due to the radioactivity present rather than to some innate cytotoxicity of the gold nanoparticles. While some gold nanoparticles show cytotoxicity, this most often occurs in cationic particles of $<2 \text{ nm}$ diameter.⁶⁸ In this case, cytotoxicity results from the small positively charged nanoparticle disrupting the negatively charged cellular membrane.⁶⁹ Membrane disruption inhibits the cell's ability to maintain homeostasis and regulate its environment, ultimately leading to cell death.

Figure 2.3.2 A and B: Tumor volume (A) and Tumor mass (B) after treatment with gum arabic gold nanoparticles. No significant difference was observed between mice treated with DPBS and mice treated with non-radioactive gum arabic coated gold nanoparticles.

The gum arabic nanoparticles used in the experiment show no statistically significant cytotoxic effect on the tumor. Additionally, the nanoparticle group showed

no additional weight loss or other symptom relative to the DPBS group. Two mice from each group were sacrificed early as a result of deteriorating health and tumor associated weight loss. Therefore, radioactivity from ^{198}Au is responsible for the tumor shrinking properties seen in I.T. injections, rather than any chemical properties of the nanoparticles.

2.4 Starch-Bombesin Gold Nanoparticle Experimental

2.4.1 Materials and Radiogold Production

All chemicals were obtained from Sigma-Aldrich and were at least ACS grade unless otherwise noted. Materials were used as received without further purification or modification. Water (18 M Ω) was produced from an in-house system. Bombesin 7-14 (hereafter referred to as simply bombesin) was purchased from Anaspec. Radiogold was produced by irradiating a 0.75 mg gold foil at the University of Missouri Research Reactor (MURR) for 6 hours in a thermal neutron flux of 8×10^{13} n/cm²/s. After irradiation, the quartz vial containing the gold target was washed of contaminant radioactivity and opened in a glove box where it was dissolved in 800 μL of aqua regia. The aqua regia was evaporated and the gold dissolved in 400 μL of 1.0 M HCl. This HCl was evaporated and the gold reconstituted in 800 μL of 0.05 M HCl.

2.4.2 ^{64}Cu -Bombesin Conjugate Synthesis

[^{64}Cu - 1,4,7-Triazacyclononane-1,4-diacetic acid (NO2A)-bombesin] conjugates

were synthesized by Prof. Jeff Smith's group at the Harry S Truman Veterans Affairs Hospital.⁷⁰ The synthesis was conducted by adding $^{64}\text{CuCl}_2$ (260 MBq, 2.83×10^{-11} mol, MDS Nordion) to a plastic tube containing bombesin-NO2A conjugate (20 μg) and 250 μL of 0.4 M ammonium acetate. The pH of the reaction mixture was adjusted to ~ 7 by the addition of 2 mM NaOH then incubated (80°C, 1 h). Fifty microliters of 10 mM DTPA solution was added to scavenge unbound radioactivity. The radiolabeled conjugates were then purified by reverse phase high performance liquid chromatography and collected into 100 μL of 1 mg/ml bovine serum albumin (BSA) stabilizing agent with 25 μg ascorbic acid. Figure 2.4.1 shows the synthetic conjugate.⁷⁰

Figure 2.4.1: Molecular structure of ^{64}Cu -NO2A-Bombesin conjugate

2.4.3 Starch Gold Nanoparticle Synthesis

Starch (12.5 mg) was dissolved in 6 mL of 18 M Ω water. NaAuCl_4 carrier (95 μL of 0.1 M solution in water) and $\text{H}^{198}\text{AuCl}_4$ (190 MBq in 225 μL of 0.05 M HCl) were added to a 20 mL glass scintillation vial. The solution was neutralized by adding 10 μL of 1 M NaOH, then heated under boiling conditions. THPAL solution (30 μL , 0.1 M) was added

and the solution was heated for an additional minute. The solution was cooled with stirring for 30 minutes then analyzed by UV-Vis spectroscopy. Two mL of this solution was placed on a size exclusion column (300 mg Sephadex G-100 resin in 7 mL water prepared in a 9 cm Bio-Rad Poly-Prep polyethylene column) and eluted with 2 mL water. The 2 mL of eluent collected had an activity of 12 MBq. The solution was diluted with DPBS to achieve an activity concentration of 1.9 MBq/mL. Gum arabic particles were synthesized as a comparator using the method described in 2.2.2 and similarly characterized.

2.4.4 Bombesin Conjugation

Thioctic acid-linked bombesin was synthesized by Dr. Raghuraman Kannan.⁷¹ The thioctic acid-bombesin (3.9 mg) was dissolved in 2 mL ethanol. Five hundred μ L of the starch nanoparticles after size exclusion were added to 1 mL of the bombesin solution and allowed to stir overnight. The nanoparticles agglomerated into a pellet overnight. The supernatant was removed and the pellet was washed 3x with 1 mL of ethanol to remove any excess bombesin. The bombesin peptide is soluble in ethanol while the nanoparticle-bombesin conjugates are not. Each washing consisted of vortexing, centrifuging, and decanting the remaining supernatant via pipette. The pellet was then washed in a similar manner 3x with 18 M Ω water to remove any unconjugated nanoparticles.

After washing, particles were analyzed by UV-Vis spectroscopy to confirm a change in gold surface environment following the reaction. The sample was redispersed in 500 μ L of dimethyl sulfoxide and 1.5 mL of DPBS for a total volume of 2 mL and an activity of 9.4 MBq. The pH of the solution was adjusted to between 7-8 with 1 M NaOH and HCl. Nanoparticles were then diluted with DPBS to achieve the desired activity concentration of 1.9 MBq/mL.

2.4.5 Nanoparticle Characterization

Nanoparticles were characterized via UV-Vis spectroscopy as in 2.2.3. Starch nanoparticles were analyzed by radio- thin layer chromatography (TLC) to quantify the amount of gold incorporated into the nanoparticle using a Bioscan B-AR-2000-2. Two μ L of nanoparticle solution was placed at the bottom of a silica TLC plate and developed in methanol with 2 drops of concentrated HCl. Due to their large size, the gold nanoparticles remain at the origin while ionic gold travels up the TLC plate with an R_f of 0.66. Synthetic yield was determined from the relative peak areas. The peaks were integrated with the provided WinScan software. Nanoparticles were analyzed both before and after passage through the size exclusion column to confirm the removal of unbound gold.

2.4.6 Biodistribution and Animal Studies

Biodistribution studies were performed by Lisa Watkinson and Terry Carmack under the supervision of Prof. John Lever and Dr. Jeff Smith at the Harry S Truman

Memorial Veterans Affairs Hospital. Four distinct radioactive agents were injected intravenously (I.V.) into SCID mice bearing human prostate tumor (PC-3) xenografts. PC-3 cells were obtained from the American Type Culture Collection and cultured by the University of Missouri Cell and Immunobiology Core. Mice were prepared by subcutaneous implantation of 10,000,000 PC-3 cells into the right flank. Solid tumors were allowed to develop for 3 weeks. For the first treatment group, 100 μ L of PBS was injected I.V. into four mice followed by intraperitoneal (I.P.) administration of 130 kBq of ^{198}Au gum arabic gold nanoparticles in 100 μ L PBS to determine the biodistribution of passively targeted nanoparticles. An additional four mice received 100 μ L of bombesin peptide 15 minutes before I.P. administration of 130 kBq of ^{198}Au gum arabic nanoparticles in 100 μ L of PBS.

Starch capped nanoparticles consisted of four different injection groups of five mice each. The first group received 100 μ L of PBS I.V. 15 minutes prior to I.P. administration of 130 kBq of starch capped ^{198}Au nanoparticles in 100 μ L of PBS. Group 2 received 100 μ L of bombesin peptide I.V. 15 minutes prior to an identical nanoparticle administration as group 1. The third group received PBS I.V. 15 minutes before I.P. administration of 130 kBq of ^{198}Au starch-bombesin coated nanoparticles in 100 μ L of PBS. The final group received I.V. administration of 100 μ L bombesin peptide 15 minutes before I.P. administration of 130 kBq of ^{198}Au starch-bombesin coated nanoparticles in 100 μ L of PBS.

Additionally, four groups of three mice each received 190 kBq (20 ng) of ^{64}Cu -bombesin conjugate in 100 μL of PBS. Group 1 was injected I.V. with 100 μL of PBS before conjugate administration I.V. The second group received 100 μL of bombesin peptide I.V. before I.V. conjugate administration. Group 3 was injected with 100 μL PBS before I.P. administration of conjugate. Finally, group 4 received 100 μL of bombesin peptide before I.P conjugate injection. All mice were sacrificed 2 hours after injection of activity and dissected. Organs and tissue (blood, heart, lung, liver, spleen, large intestine, pancreas, and tumor) were collected for analysis by γ spectroscopy. A summary of the injections is presented in Table 2.4.1

Table 2.4.1: Summary of bombesin injections

Treatment	# of mice	Pretreatment	Nuclide	Activity (kBq)
Gum arabic NP, I.P.	4	PBS, I.V.	^{198}Au	130
Gum arabic NP, I.P.	4	Bombesin, I.V.	^{198}Au	130
Starch NP, I.P.	5	PBS, I.V.	^{198}Au	130
Starch NP, I.P.	5	Bombesin, I.V.	^{198}Au	130
Starch-BBN NP, I.P.	5	PBS, I.V.	^{198}Au	130
Starch-BBN NP, I.P.	5	Bombesin, I.V.	^{198}Au	130
^{64}Cu -bombesin	3	PBS, I.V.	^{64}Cu	190
^{64}Cu -bombesin	3	Bombesin, I.V.	^{64}Cu	190
^{64}Cu -bombesin	3	PBS, I.V.	^{64}Cu	190
^{64}Cu -bombesin	3	Bombesin, I.V.	^{64}Cu	190

2.5 Starch-Bombesin Gold Nanoparticle Results and Discussion

2.5.1 Starch Gold Nanoparticle Yield and Characterization

Starch coated gold nanoparticles showed a UV-Vis absorbance maximum at 544 nm. After filtration, the solution showed an absorbance maximum at 541 nm. During the synthesis, a small fraction of starch particles will polymerize, creating long chains of linked nanoparticles. These oligonanoparticles represent the longer wavelength absorbance. After filtration, the absorption maximum decreases in intensity due to lower number of particles present and also shifts towards the blue end of the spectrum signifying the smaller size of the remaining particles (Figure 2.5.2). Additionally, ionic gold present in the unfiltered sample remains on the sephadex column, as shown in Figure 2.5.1 A and B.

Figures 2.5.1 A and B: Radio-TLC of starch coated gold nanoparticles before (A) and after (B) filtration. The origin on each plate is at 50 mm and marked by a red line while the solvent front is shown by the line at 95 mm and marked by a blue line.

After conjugation to bombesin, the nanoparticles were washed successively with ethanol and water to remove both unconjugated bombesin and free particles. The activity in the supernatant in each wash is shown in Table 2.5.1.

Table 2.5.1: Activity in each wash of bombesin coated gold nanoparticles

Wash	Activity (kBq)
Initial supernatant	318
1 st ethanol	44
2 nd ethanol	41
3 rd ethanol	74
1 st water	1890
2 nd water	1300
3 rd water	370

The ethanol washes were to remove unconjugated peptide and showed small activity breakthrough, indicating that very few gold nanoparticles were removed in the process. Water washes, meant to remove nanoparticles without bombesin, showed successively decreasing activity in each wash indicating the progressive removal of unconjugated nanoparticles. Removal of both conjugated and unconjugated nanoparticles in the wash would remove a constant fraction of activity rather than a decreasing amount. The final nanoparticle solution showed a UV-Vis absorbance of 0.291 at 561 nm, reflecting the addition of the large bombesin conjugate. UV-Vis Spectra are shown in Figure 2.5.2.

Figure 2.5.2: Shift of the plasmon resonance UV-Vis peak of the nanoparticle after starch addition, filtration, and bombesin addition to the surface.

2.5.2 Gum Arabic Gold Nanoparticle Yield and Characterization

The gum arabic gold nanoparticles gave a peak absorbance at 524 nm.

Nanoparticles were made with 87% yield, as measured by radio-TLC (Figure 2.5.3).

Figure 2.5.3: Radio-TLC of ^{198}Au gum arabic gold nanoparticles. The origin of the TLC plate is at 50 mm (red line) while the solvent front is at 95 mm (blue line).

2.5.3 Biodistribution and Animal Studies

Injections of ^{64}Cu -bombesin showed expected uptake patterns comparable to those previously seen.⁷⁰ Bombesin targets a GRP receptor subtype which is highly expressed in endogenous murine tissue, particularly on the pancreas and large intestine.⁷² As such, these organs are in fact better exemplars of bombesin-specific targeting than the tumor xenograft itself. Human pancreas and large intestine display a different GRP receptor subtype which is not targeted by bombesin. Most of the uptake in the ^{64}Cu conjugate occurs in the pancreas and large intestine, indicating targeting of GRP receptors. Further, after blocking with free bombesin peptide, intestinal and pancreatic uptake show dramatic, statistically significant decreases (at the 95%

confidence level) in both the I.V. and I.P. injection pathways indicating receptor specific targeting. Blocking GRP receptors did not induce a statistically significant change in uptake in the tumor xenograft. Deviations between animals in each injection group are small. Biodistribution data for each organ and injection pathway are shown in Figure 2.5.4.

Figure 2.5.4: Blocking studies of ^{64}Cu -bombesin conjugates using both I.V. and I.P.

injection pathways. Injecting free bombesin peptide before particle injection produced statistically significant differences in pancreatic and intestinal uptake.

Passively targeted ^{198}Au gum arabic coated gold nanoparticles exhibit biodistribution patterns similar to those observed previously.⁵⁷ Because the gum arabic is tightly bound to the gold nanoparticle, it is not possible to coordinate bombesin using

the same chemistry as in the starch nanoparticle case. Without specific targeting, tumor uptake is low after only 2 hours of circulation time. Nanoparticles are cleared primarily through the RES, as evidenced by the large uptake in the liver and spleen.

Biodistribution data for ^{198}Au gum arabic coated particles is shown in Figure 2.5.5 both with and without blocking by unconjugated bombesin peptide.

Figure 2.5.5: Biodistribution of gum arabic coated gold nanoparticles. These nanoparticles show uptake and clearance typical of passively targeted colloidal systems.

Passively targeted starch coated gold nanoparticles mirror uptake observed in the gum arabic system. Primary clearance pathway is through the RES, as evidenced by

similar uptake in liver and spleen. No statistically significant differences in uptake were observed by blocking the GRP receptor with excess bombesin.

Starch binds less tightly to the surface of gold than gum arabic, and can be displaced by a lipoic acid-bombesin conjugate for specific targeting. Unfortunately, this weaker starch binding may also lead to dissociation of the starch from the gold *in vivo* and increased particle aggregation. Addition of bombesin to the starch nanoparticles should increase their uptake by GRP receptors. Average uptake in the pancreas for the starch-bombesin nanoparticles system appears dramatically higher than in the passively targeted nanoparticle case, however the large uptake is due primarily to one mouse which showed >190% I.D./g. A Grubbs' test⁷³ confirmed that uptake in this mouse was a statistical outlier, and results for this mouse were disregarded. After elimination of the outlier, the biodistribution data more closely resembles that of gum arabic nanoparticles than ⁶⁴Cu-bombesin conjugates. The biodistribution data of untargeted starch capped nanoparticles and starch-bombesin capped nanoparticles (after elimination of the outlier) are shown in Figure 2.5.5 with and without blocking with unconjugated bombesin peptide. Differences between the four groups showed no statistical significance after removal of the outlier.

Previous reports indicated that starch-bombesin capped gold nanoparticles exhibited specific, receptor-mediated uptake both *in vitro* and *in vivo*.⁷⁴ *In vitro*, the nanoparticles show variable IC₅₀ values against GRP receptor, from 8.10 ± 0.30 µg/mL for low bombesin loading to 2.45 ± 0.18 µg/mL for high bombesin loading.⁷¹ The study

did not include controls of starch-coated gold nanoparticles, therefore no conclusions can be made about the specificity of the nanoparticle system despite its high affinity for the GRP receptors.

Figure 2.5.5: Biodistribution of starch capped gold nanoparticles. These nanoparticles show biodistribution and clearance through the RES system. Actively targeted starch-bombesin capped nanoparticles show no significantly different uptake *in vivo*.

Error bars in the *in vivo* experiments show wide variability in pancreatic uptake consistent with a single outlier that was not excluded. While the ^{64}Cu -bombesin conjugate confirms that bombesin can be an effective targeting agent for the GRP

receptor, bombesin was ineffective in the starch-capped gold nanoparticle system. While there is variance in starch size based on the plant source, potato starches tend to consist of a 24-30 glucose oligomer chain.⁷⁵ Given the large size of the nanoparticle surfactant and the small size of the linker (a 5 carbon chain from lipoic acid), the targeting portion of bombesin may have been covered by the starch and not exposed to GRP receptors *in vivo*. Lipoic acid-bombesin conjugates do not fully displace the starch surfactant, but merely attach to the surface displacing a portion of the attached starch. The remaining starch molecules may sterically hinder bombesin-GRP receptor interactions, blocking the specific targeting. Additionally, the amino acids of the bombesin peptide may interact with the starch molecules themselves, further decreasing the amount of targeting sequences accessible to GRP receptors. It is unlikely that the bombesin separates from the nanoparticles as the nanoparticles do not revert to their pre-conjugation solubility. Additionally, a change in the UV-Vis absorbance would appear after dissociation, which is not observed. Another potential explanation for the poor *in vivo* targeting is rapid clearance of the nanoparticles through the RES. The bombesin conjugates do not circulate for a sufficient time to bind to the GRP receptor, which would explain the discrepancy between the high *in vitro* binding and the low *in vivo* binding.

Future experiments should focus on the effect of different bombesin conjugation approaches on GRP receptor uptake. Longer polyethylene glycol (PEG) linkers are available from a variety of sources, and can easily be linked to the peptide. Other, less

bulky and easily displaced surfactants are also available. The citrate reduction of the Turkevich procedure creates biocompatible nanoparticles that can be further functionalized with lipoic acid linkages. Citrate-capped nanoparticles are unstable towards aggregation *in vivo*, however, they can be passivated by addition of a PEG group linked to lipoic acid.⁷⁶ An alternative system would utilize a citrate capped gold nanoparticle linked to a commercially available lipoic acid-PEG-COOH linker. The bombesin peptide would then be attached via 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/sulfo N-hydroxy succinimide (NHS) chemistry.⁴⁹

2.6 ⁶⁴Cu-DTDTPA Gold Nanoparticle Experimental

2.6.1 Materials and Measurements

All chemicals were at least ACS grade and purchased from Sigma-Aldrich and used as received unless otherwise noted. High purity (>18 MΩ) water was produced from an in-house generator. TEM images were obtained on a JEOL 1400 transmission electron microscope (JEOL Ltd, Tokyo, Japan). TEM samples were prepared by placing 5 μL of gold nanoparticle solution on a 300 mesh carbon-coated copper grid and allowing the solution to stand for five minutes. Excess solution was removed carefully and the grid was allowed to dry for an additional five minutes. The average size and distribution of the gold nanoparticles synthesized were determined by the processing of the TEM

image using Adobe Photoshop (with Fovea plugins). The hydrodynamic diameter and the zeta potential were obtained using a Zetasizer Nano S90 (Malvern Instruments Ltd. USA) using a dilute (0.08 g/L) aqueous solution. The UV-Vis absorption spectra were recorded at room temperature using Varian Cary 50 UV/Vis spectrophotometers. The absorption measurements were performed on dilute colloidal gold nanoparticle solution in disposable cuvettes with a 10 mm path length. Estimation of gold concentration in conjugates was performed using furnace atomic absorption spectroscopy (AAS) with a standard curve spanning 0–40 µg/L (Perkin–Elmer AAnalyst 800 ThGA graphite furnace). Quality-control materials (duplicates, spikes, and instrument-calibration verification) were within appropriate ranges. Dithiolated diethylene triamine pentacetic acid (DTDTPA) and nanoparticle synthesis, characterization, and cell studies were performed by Dr. Ajit Zambre (sections 2.6.2-2.6.5 and 2.6.9). Radiolabeling, radiostability, radiosynthesis, and data analysis for animal studies were performed by Mark McLaughlin (sections 2.6.6 – 2.6.8). Biodistribution and animal studies were performed by Lisa Watkinson and Terry Carmack under the supervision of Prof. John Lever and Dr. Jeff Smith at the Harry S Truman Memorial Veterans Affairs Hospital (section 2.6.10).

2.6.2 DTDTPA Synthesis

DTPA (0.78 g), acetic anhydride (0.75 mL) and anhydrous pyridine (1.02 mL) were mixed and heated to 70°C for 18 hrs in a 5 mL glass V-bottom vial. The resultant mixture was cooled to room temperature and further chilled to 4°C. The precipitate

was collected by filtration, purified by repeated washing with acetic anhydride followed by anhydrous ether. The washed precipitate was filtered and dried under vacuum to obtain 0.63 g, 91% of DTPA-bisanhydride (DTPA-BA).⁷⁷

DTPA-BA (0.5 g, 5.6×10^{-3} mol) was dissolved in 10 mL of dimethylformamide (DMF) in a 100 mL round bottom flask with constant heating at 70°C. Aminoethanethiol (0.25 g, 1.23×10^{-2} mol) was dissolved separately in another flask with 8 mL of DMF and 0.44 mL of triethylamine. This solution was then added to the round bottom flask containing DTPA-BA with constant stirring and the reaction mixture was heated to 70 °C overnight. Subsequently, the reaction mixture was cooled to room temperature and placed in an ice bath. The resulting white residue (triethylamine hydrochloride) was filtered. The filtrate was then concentrated at reduced pressure and further mixed with chloroform to form a white precipitate. This precipitate was filtered and washed thoroughly with chloroform to remove impurities. The precipitate was dried under vacuum to obtain white DTDTPA (0.49 g, 86%) and characterized by nuclear magnetic resonance (NMR).⁷⁸

Figure 2.6.1: Structure of DTDTPA.

2.6.3 Synthesis of DTDTPA Gold Nanoparticle Conjugates

DTDTPA functionalized nanoparticles were prepared by dissolving 200 mg (5.1×10^{-5} mol) of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ in 120 mL of methanol in a 500 mL round bottom flask. In a separate flask, 482 mg (9.4×10^{-5} mol) of DTDTPA was dissolved in 40 mL of methanol and 2 mL of glacial acetic acid. This solution was added with continuous stirring to an aqueous solution of gold salt to produce an orange solution. To this mixture, 190 mg (5 mmol) of NaBH_4 dissolved in 14 mL of water was added under vigorous stirring at room temperature. Immediately after addition of NaBH_4 , the solution became dark brown followed by appearance of black flocculate. The resultant mixture was allowed to stir for 1 hour at room temperature before addition of 5 mL of 1M HCl. This black solution of gold nanoparticles was then centrifuged at 7000 RPM for 20 minutes. The supernatant was removed and the particles were washed twice with 0.01 M HCl, using the same centrifugation parameters. The particles were further washed successively with water followed by diethyl ether. The resulting black powder of Au nanoparticle-DTDTPA was dried under vacuum and stored at -20°C . As needed, the particles were readily dispersed in 0.01 M NaOH and were stable against aggregation for over a month as determined by UV-Vis spectroscopy.

2.6.4 Chelation of Cu^{2+} with Gold-DTDTPA Nanoparticles

Copper was added to Au-DTDTPA at room temperature with continuous stirring. A 5 mL solution of 10 mg/mL Au-DTDTPA was prepared by dissolving nanoparticles in 5

mL of 1 M NaOH, then neutralized to pH = 7.0 with 1 M HCl. Ten mg of CuCl₂ was added to this solution with continuous stirring. The formation of precipitate was immediately observed. The reaction mixture was stirred for 5 minutes to produce a brownish black residue which was collected by centrifugation. The residue was washed with deionized (DI) water thoroughly to remove any unreacted copper salt and dried under vacuum. The amount of copper required to saturate the chelate was monitored by titrimetric analysis utilizing atomic absorption spectroscopy.

2.6.5 Titrimetric Analysis of Copper on Gold-DTDTPA Nanoparticles:

The amount of Cu²⁺ ions required for complete saturation of Au-DTDTPA was determined by AAS measurements. The titration was performed by successive addition of an aqueous solution of CuCl₂ (2 mg - 30 mg) to 10 mg of colloidal Au-DTDTPA dispersion at room temperature with continuous stirring. After the reaction was complete the reaction mixtures were collected in individual centrifuge tubes and centrifuged at 5000 RPM for 5 minutes. The pellets were resuspended in DI water, centrifuged, and the supernatants decanted. All the supernatants and pellets were analyzed for copper content by AAS.

2.6.6 ⁶⁴Cu Isotope Production and Specific Activity Analysis

Targets were irradiated as previously described⁷⁹ by Tom Brossard using approximately 12 MeV protons on a GE PETtrace cyclotron at MURR with a 99% enriched ⁶⁴Ni target. Isotope yield measurements were determined using either a γ

spectrometer (Canberra model 1510, Meriden, CT) and/or a radioisotope dose calibrator (Capintec CRC-10, Pittsburgh, PA). The γ spectrometer was used to detect radionuclidic impurities. Specific activities were determined as described previously for ^{64}Cu by titration of $^{64}\text{CuCl}_2$ with the macrocyclic chelator TETA.⁷⁹ Briefly, aliquots of varying amounts of TETA were added to ^{64}Cu and the complexation yield monitored by radio-TLC. The samples were spotted on C-18 plates and the plates developed using 1:1 methanol:10% ammonium acetate with two drops of concentrated HCl. CuCl_2 remains at the origin whereas complexed copper in the form of $\text{Cu}(\text{TETA})^{2-}$ migrated with $R_f = 0.9$. The minimum TETA concentration for which 100% labeling occurred was assumed to be equal to the concentration of Cu^{+2} present.

2.6.7 Radiochemical Synthesis of ^{64}Cu Gold-DTDTPA Nanoparticles

Five mg of Au-DTDTPA was dissolved in 500 μL of 1 M NaOH to give a 10 mg/mL solution. This solution (20 μL) was added to 750 μL of a buffer solution consisting of 0.4 M ammonium acetate adjusted to pH = 4 using HCl and sonicated for 30 minutes. Radioactive $^{64}\text{CuCl}_2$ (MURR cyclotron facility, 81 MBq) was received in 150 μL of 0.1 M HCl. Forty-five μL of $^{64}\text{CuCl}_2$ (27 MBq) was added to the Au-DTDTPA/buffer solution and placed in a boiling water bath for 30 minutes. Unlabeled copper was removed from solution by eluting the labeled nanoparticles through a chelex 100 cation exchange resin (Bio Rad) column with 18 M Ω water. The column was prepared by dispersing 300 mg of resin in 7 mL of water and depositing the slurry in a Bio Rad Poly-Prep 9 cm column.

Excess liquid was allowed to pass through the slurry and the prepared column was then washed twice with 2 mL of PBS. The nanoparticle solution (1.25 mL) was loaded onto the column and eluted with 5 mL of PBS. The effluent and column were counted on a γ spectrometer using the 511 keV peak to determine labeling yield. Nanoparticle solution was adjusted to pH = 7.5 using NaOH and HCl, then diluted to a volume of 2.5 mL using PBS.

2.6.8 *In Vitro* Stability of Gold-DTDTPA and ^{64}Cu Gold-DTDTPA Nanoparticles

In vitro stability studies of ^{64}Cu -Au-DTDTPA were performed by challenging 0.5 mL of ^{64}Cu -Au-DTDTPA with water, PBS, cysteine, or rat serum albumin (RSA). Nanoparticle conjugates were synthesized as in 2.6.7 using 5 μL of 10 mg/mL nanoparticles in 495 μL of water. Each of these solutions then received ~ 17 MBq of $^{64}\text{CuCl}_2$. Addition of 500 mL of water, 2x PBS, 0.2 M cysteine, or 0.2 M RSA gave final concentrations in each vial of PBS or 0.1 M. The stability was measured by monitoring the UV-visible absorbance and radio-TLC at 0, 24, and 48, and 120 hours. Radio-TLC was performed with C18 plates (Whatman) in a developing solution of 1:1 by volume solution of methanol:10% ammonium acetate.

2.6.9 *In Vitro* Cytotoxicity Measurements

In vitro cytotoxicity was evaluated using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay kit (Promega Corporation). Cells at the exponential growth phase (1×10^5 cells/mL) were placed in a flat-bottom 96-

well polystyrene-coated plate and incubated in a CO₂ incubator at 5% CO₂ and 37 °C for 24 hours. A series of concentrations (0, 1, 2.5, 5, 10, 20 and 40 µg/mL) of Au-DTDTA and Cu-Au-DTDTA were added to wells in quadruplet. After incubation for an additional 24 hours, 10 µL MTT (5 mg/mL in PBS) was added to each well and kept for 24 hours. Formazan crystals produced by living cells were dissolved in 100 µL of isopropanol added to each well plate and kept in the dark at 25°C for 18 hours. The intensity of developed color was measured by micro plate reader (Dynastic MR 5000) operating at 570 nm wavelength. Wells with complete medium, nanoparticles, and MTT, but without cells, were used as blanks. Untreated cells were considered 100% viable.

2.6.10 *In Vivo* Biodistribution Measurements

Biodistribution and animal studies were performed by Lisa Watkinson and Terry Carmack under the supervision of Prof. John Lever and Dr. Jeff Smith at the Harry S Truman Memorial Veterans Affairs Hospital. Evaluation of pharmacokinetics of ⁶⁴Cu-Au-DTDTA was conducted in normal CF1 mice. Nanoparticle conjugates (1.48 MBq in 100 µL of PBS) were injected via the tail vein (n=3). Animals were sacrificed at 0.25, 0.5, 2, 4, and 24 hours post injection by cervical dislocation. The following organs were collected for measurement on a well counter: tail, blood, heart, lung, liver, spleen, stomach, large intestine, small intestine, kidney, urine, feces, muscle, bone, bladder, brain, pancreas, and carcass.

2.7 ⁶⁴Cu Gold-DTDTPA Results and Discussion

Au-DTDTPA nanoparticles fail to show an absorption maximum associated with the plasmon resonance. The spectrum shows a small absorption hump around 525 nm that corresponds with the expected plasmon resonance peak in both the Au-DTDTPA nanoparticles and the Cu-Au-DTDTPA nanoparticles. These absorption spectra are shown in Figure 2.7.1.⁸⁰

Figure 2.7.1 A and B: UV-Vis absorption spectra of Au-DTDTPA (A) and Cu-Au-DTDTPA (B).

TEM analysis shows that the nanoparticles have a metallic diameter of ~3 nm. Some variability is present, with nanoparticles as large as 20 nm in diameter. This size is typical of nanoparticles made via the Brust procedure.²⁸ Figure 2.7.2 shows a characteristic TEM of the nanoparticles.

Figure 2.7.2: TEM showing a characteristic Au-DTDTPA nanoparticle dispersion

Hydrodynamic diameter and zeta potential are shown in Table 2.7.1. The large hydrodynamic diameter relative to the small metallic diameter is also expected. Zeta potential values that are highly positive or negative indicate the nanoparticles should be stable towards aggregation.⁸¹ The saturation curve as measured by AAS is shown in Figure 2.7.3. At low levels of added copper, synthetic yield is constant. Addition of more than 4.72 mg of copper saturates the 10 mg of Au-DTDTPA used and leads to decreasing yield. This saturation was confirmed using radio-TLC and ⁶⁴Cu.

Table 2.7.1: DLS properties of Au-DTDTPA and Cu-Au-DTDTPA

Construct	Hydrodynamic Diameter (nm)	Zeta Potential (mV)
Au-DTDTPA	133.6	-40.63
Cu-Au-DTDTPA	149.3	-55.06

Figure 2.7.3: Copper saturation of Au-DTDTPA as measured by AAS

Cu-Au-DTDTPA nanoparticles monitored in water, PBS, and rat serum show constant plasmon resonance peak location and intensity as monitored by UV-Vis spectrometry. The constant peak indicates that the nanoparticles are stable towards aggregation. Nanoparticles monitored in cysteine show a consistent wavelength of maximum absorption, but decreased absorption intensity over the monitoring period. The peak completely disappears by 120 hours. Cysteine contains a sulfur side chain, which can displace the thiolated DTDTPA chelating agent from the surface of the gold nanoparticle. Without the protection provided by the DTDTPA ligand, the nanoparticles likely aggregate and the plasmon resonance peak disappears. Figure 2.7.4 shows the decrease of the Cu-Au-DTDTPA plasmon resonance over time.

Figure 2.7.4: Stability of Cu-Au-DTDTPA in cysteine. UV-Vis spectroscopy of Cu-Au- shows decrease in plasmon resonance over time, ultimately disappearing at 120 hours.

Radio-TLC measurements indicate the same process. Initially, radio-TLC shows one large peak at the origin for Cu-Au-DTDTPA samples in each biological mimic solution. Over time, an increasing amount of activity is seen in the cysteine solution at an R_f of 0.2, indicating that the activity is being displaced from the gold nanoparticle. After 96 hours, roughly half of the activity has been displaced from the particle surface. Because of the stability of the Cu-DTDTPA complex, we conclude that the loss of activity is indicative of the loss of chelate from the Au nanoparticles. Figures 2.7.5 A and B show

a characteristic radio-TLC and the cysteine solution radio-TLC after 96 hours, respectively.

Figure 2.7.5 A and B: Stability studies of ^{64}Cu -Au-DTDTPA via radio-TLC. These chromatograms are characteristic of ^{64}Cu -Au-DTDTPA in water/PBS/rat serum (A) and after 96 hours in cysteine (B). Activity travels up the TLC plate as the chelate is displaced from the nanoparticles. The origin is at 50 mm and the solvent front at 100 mm.

While the instability in cysteine solution is troubling, significant dissociation only occurs at high concentrations of cysteine and after multiple half-lives of ^{64}Cu . Mammalian levels of cysteine typically vary from 20-100 μM ⁸² whereas the nanoparticle conjugate was challenged with a solution of 0.2 M cysteine. After ~8 half-lives (96 hours), 50 % of the activity remains bound to the gold nanoparticles. While an ideal construct would exhibit no dissociation, the nanoparticles are likely stable enough to warrant further study. First, the concentration of cysteine used to test stability is over 1,000 times higher than what is observed *in vivo*. Lower concentrations of cysteine will likely cause less significant dissociation. As imaging times with ^{64}Cu are often on the order of 24-48 hours,⁸³ the stability of our complex after 48 hours warrants further study.

In vitro cytotoxicity measurements with SKBr3 breast cancer cells indicate that the Au-DTDTPA nanoparticles are well tolerated at low concentrations. However, above 10 $\mu\text{g/mL}$, cells showed decreased survivability relative to controls. Figure 2.7.6 shows the effect of Au-DTDTPA and Cu-Au-DTDTPA on breast cancer cells.

Figure 2.7.6: Cell viability studies of breast cancer SKBr3 cells

Nanoparticles prepared for biodistribution studies were labeled with a chemical yield of 94% as determined by activity load on the cation exchange column. Unbound $^{64}\text{CuCl}_2$ was completely retained on the column. *In vivo*, the $^{64}\text{Cu-Au-DTDTPA}$ showed uptake consistent with passively targeted nanoparticle constructs. Initially, activity appears primarily in the liver, then decreases as the nanoparticles are cleared through the RES or hepatobiliary system and are excreted in the feces. Approximately 1/3 of the injected activity clears through the feces after 24 hours. Activity present in the kidney likely represents $^{64}\text{Cu-DTDTPA}$, as the nanoparticle construct itself is likely too large to enter the glomerular filtrate and over 95% of free ^{64}Cu binds proteins found in serum.⁸⁴

Figures 2.7.7 A and B: Uptake of ^{64}Cu -Au-DTDTPA nanoparticles as %ID/organ (A) and %ID/g (B). Biodistribution shows uptake similar to untargeted, gum arabic coated nanoparticles.

Additionally, the negative charge on the nanoparticle further limits filtration and urine excretion.⁸⁵ Figures 2.7.7 A and B show uptake as %ID/organ and %ID/g, respectively.

Stability of the ^{64}Cu -Au-DTDTPA nanoparticle system under the observed conditions is inconclusive. While nanoparticles are expected to clear via the RES and hepatobiliary system, free ^{64}Cu also clears via the hepatobiliary system with excretion in the feces after 24 hours.⁸⁶ The large uptake in the liver could indicate uptake through either of these routes. Uptake in the spleen, primarily due to RES clearance, is lower than in the case of the passively targeted gum arabic gold nanoparticles. This difference could be explained by a number of factors, including partial dissociation of ^{64}Cu from DTPA or differences in nanoparticle surface charge, size, or hydrophilicity.

A dual label experiment would conclusively determine the stability of the ^{64}Cu -DTDTPA-nanoparticle conjugate. Comparing the biodistribution of gold nanoparticles synthesized from $^{198/199}\text{Au}$ and the attached ^{64}Cu -DTDTPA would establish the viability of the system. Similar biodistributions for ^{64}Cu and $^{198/199}\text{Au}$ indicate that the conjugate remains largely intact while significantly different biodistributions indicate that the ^{64}Cu does not remain attached to the nanoparticle *in vivo*. If the system is stable, it would warrant future study. The small size of the nanoparticles indicates they may show high tumor uptake via the EPR effect.⁸⁷ Finally, specific targeting agents such as bombesin could be added to target a particular type of tumor rather than relying on passive uptake.

CHAPTER 3:

GOLD SHELL NANOPARTICLES FOR in vivo ^{225}Ac α -GENERATOR

3.1 Chemistry of ^{225}Ac and the Lanthanides

Chemical interest in the actinides focuses primarily on their wide variety of oxidation states and unique f-orbital interactions. These properties of actinides give them a wide range of colors and facilitate their use as catalysts.⁸⁸ Despite also filling f-orbitals, the lanthanide series shows much less oxidation state variety. All of the lanthanides primarily form the 3+ oxidation state, with the exception of Ce, which also easily forms the 4+ state.⁸⁹ As the first actinide, the electronic structure of Ac does not heavily populate the 5f orbital. Because of this, its chemical behavior more closely resembles the lanthanides rather than the rest of the actinide series. Actinium easily loses three electrons to form the 3+ oxidation state.

Additionally, relativistic effects further contribute to the similarity between Ac and the lanthanides, in particular the early lanthanides. Due to relativistic effects, the atomic radii of Ac (in particular the s shells) are more contracted than what would otherwise be expected. Therefore, the difference in size between Ac and La is smaller

than indicated by non-relativistic size calculations. These relativistic effects are also responsible for the surprising stability of high oxidation states found in many of the actinides.⁹⁰

Previous studies incorporated ^{225}Ac into LaPO_4 nanoparticles by a process of precipitation from organic solution. Phosphates of the lanthanides are highly insoluble in water, with pK_{sp} values ranging from 25.51 (Nd) to 26.57 (Sm).⁹¹ Ions of La^{3+} and $^{225}\text{Ac}^{3+}$ mixed with phosphoric acid produce a precipitation of monodisperse nanoparticles with 44% radiochemical yield. This particle system is capable of retaining ~50% of the ^{221}Fr daughter and 99.9% of the ^{225}Ac parent.⁴⁸ Conjugation to biological targeting agents occurs via an ionic linkage between 6-aminohexanoic acid and surface phosphate. Unfortunately, the complex synthesis involves inert atmosphere conditions and reproducibility issues. Presumably, the ionic linkage remains stable in the aqueous *in vivo* environment due to the association of residual organic compounds with the surface of the nanoparticle. An aqueous synthetic pathway, which could reliably be used under normal atmospheric conditions, would be desirable.

3.2 Nanoparticle Synthesis and Characterization Experimental

3.2.1 Materials and Equipment

All chemicals were used as received from Sigma-Aldrich unless otherwise noted and were ACS grade or higher. Water used was purified using an in house purification

system to 18 M Ω or higher. Radioactivity measurements were performed with a γ spectrometer consisting of a high purity germanium detector and a Canberra Industries multichannel analyzer. Full width at half maximum resolution of the detector was 0.7 keV at 218.2 keV. Detector dead times were typically <1% and count rates were automatically corrected for the detector dead time by the γ spectroscopy software. A regenerated cellulose dialysis membrane with a 10 kDa molecular weight cutoff (MWCO, Spectra/Por, 128106) was used to purify nanoparticle dispersions. The dialysis membrane was washed of preservatives with 18 M Ω water before dialysis against 18 M Ω water. A 0.4 T NdFeB magnet (Supermagnet #31, United Nuclear) was used for magnetic separation of Gd containing nanoparticles from solution. Nanoparticles were characterized by TEM (JEOL 1400), NAA, x-ray diffraction (XRD, Scintag X2), and electron energy loss spectroscopy-TEM (EELS-TEM, Zeiss Libra 120). All ^{225}Ac processes were performed at Oak Ridge National Laboratory.

3.2.2 Purification of $^{225}\text{AcCl}_3$

Actinium-225 chloride was prepared as previously described from a stock of ^{229}Th .⁹² Briefly, ^{225}Ra and ^{225}Ac are separated from the parent ^{229}Th using two columns of MP1 (Bio-Rad Laboratories) anion exchange resin in sequence with 8 M HNO_3 . The ^{225}Ac is further separated from ^{225}Ra using two sequential columns of AG50-X4 cation exchange resin. Radium was eluted with 1.2 M HNO_3 while ^{225}Ac was eluted with 8 M HNO_3 . Separated ^{225}Ra was reprocessed as needed to provide additional ^{225}Ac .

Actinium separation was performed by Dr. Rose Boll and Karen Murphy at Oak Ridge National Laboratory.

Samples of ^{225}Ac in 1.2 M HNO_3 were evaporated to dryness on a hot plate with a continuous flow of air in a V-bottom vial. The dried salt was redispersed in 1 M HCl and evaporated to dryness under the same conditions. Finally, the sample was redispersed in 0.1 M HCl and transferred to a 1 mL V-bottom vial for synthesis.

3.2.3 Synthesis of $\{\text{La}_x\text{Gd}_{1-x}\}\text{PO}_4$ nanoparticle cores

Lanthanide phosphate nanoparticles were made by modifying a methodology developed by Buissette *et al.*⁹³ Briefly, aqueous solutions of 0.1 M $\text{LaCl}_3 \cdot 7 \text{H}_2\text{O}$ and $\text{GdCl}_3 \cdot 6 \text{H}_2\text{O}$ were mixed in a 1 mL V-bottom vial with magnetic spin vane. The amount of La and Gd added was varied to give $\{\text{La}_x\text{Gd}_{1-x}\}\text{PO}_4$ nanoparticles where $x = 0, 0.25, 0.5$, and 1 with a total addition volume of 100 μL . For biological preparations, $\sim 190 \text{ MBq}$ of $^{225}\text{AcCl}_3$ in 50 μL 0.1 M HCl was added to the lanthanide mixture. Preparations for *in vitro* retention measurements and yield studies used 7.4-9.3 MBq of ^{225}Ac . Preparations for characterization purposes were synthesized without ^{225}Ac addition.

Next, 200 μL of 0.1 M sodium tripolyphosphate (TPP) was added to give a clear, colorless solution with a Ln:Na-TPP molar ratio of 1:2. If the solution remained turbid after addition of Na-TPP, it was aspirated with small (10 μL) additions of Na-TPP until the solution appeared clear. The resulting solution was then capped and heated at 90°C for 3 hours in a Thermo-Scientific heat bath with a custom made aluminum heat block.

The resulting turbid, white solution of nanoparticles was transferred into a 10 kDa dialysis tube and purified against 18 M Ω water overnight. The nanoparticles were removed via pipette, producing 2.4 mg of monodisperse particles of ~4 nm diameter which were characterized by TEM, NAA, and XRD.

3.2.4 Layering of $\{La_xGd_{1-x}\}PO_4$ nanoparticles

Layers of $LnPO_4$ were added to the crystalline nanoparticle cores by a process of oriented attachment. Nanoparticle cores were collected in a 1.5 mL microcentrifuge vial centrifuged at 3,000 g for 3 minutes. The supernatant was decanted via pipette, and nanoparticles were redispersed into a solution containing 400 μ L 0.05 M Na-TPP with 0.05 M $LaCl_3$ and/or 0.05 M $GdCl_3$. Lanthanides were added to give layer composition of $\{La_xGd_{1-x}\}PO_4$ where $x = 1, 0.5, \text{ or } 0$ in a total volume of 200 μ L. This nanoparticle suspension was then sonicated for 10 minutes in a bath sonicator, vortexed, and heated for an additional 3 hours in a capped 1 mL V-bottom vial at 90°C to add a layer of $LnPO_4$. The layering procedure was repeated to deposit two or four layers of $LnPO_4$ on top of the core nanoparticle. The final thick, white solution of nanoparticles was then purified by dialysis overnight against 18 M Ω water. Non-radioactive analogs of the nanoparticles were characterized by EELS-TEM and NAA.

3.2.5 Gold Shell Addition

Dialyzed $LnPO_4$ nanoparticles (~12 mg) were evenly divided between three 5 mL V-bottom vials (4 mg each) with spin vanes. Next, 300 μ L of 0.1 M sodium citrate was

added to each vial followed by enough 18 M Ω water to bring the final volume to 2 mL. The vials were then sonicated for 10 minutes in a bath sonicator and heated to 90°C. Over the course of 25 minutes, 2.5 mL of 1 mM NaAuCl₄ (ph = 4) was added dropwise, during which time the solution turned the deep purple-red color associated with gold nanoparticles. The solution was heated uncapped for an additional 30-45 minutes and cooled to room temperature. The gold coated nanoparticles were placed next to the NdFeB magnet overnight for separation. The supernatant was decanted via pipette and magnetically active nanoparticles collected for further analysis. Both supernatant and separated particles were counted on a γ -spectrometer after establishment of equilibrium to determine separation yield. Figure 3.2.1 shows a purified sample of gold coated nanoparticles following magnetic separation.

In this dissertation, nanoparticle compositions consisting of a core of varying amounts of lanthanide phosphates will be presented with the cation fraction in braces followed by the counteranion. If a radioactive dopant is present, it will be presented in parentheses. For example, a half LaPO₄, half GdPO₄ nanoparticle core with an ²²⁵Ac dopant will be presented as {La_{0.5}Gd_{0.5}}(²²⁵Ac)PO₄. Additionally, other layers are named using the convention core@shell. Antibodies or linkers attached to the gold surface will be named with a dash followed by the antibody or linker. Thus, a nanoparticle with the aforementioned core, four shells of a lanthanide phosphate, an exterior gold layer, and monoclonal antibody (mAb) 201b antibody would be named {La_{0.5}Gd_{0.5}}(²²⁵Ac)PO₄@4 LnPO₄@Au-mAb 201b.

Figure 3.2.1: $\{La_{0.5}Gd_{0.5}\}({}^{225}Ac)PO_4@4\ GdPO_4@Au$ nanoparticles after overnight magnetic separation

3.2.6 Layering Yield Measurements

$La-140$ and ${}^{159}Gd$ radiotracers were produced at MURR, employing the pneumatic tube irradiation facility. $La(NO_3)_3 \cdot 6\ H_2O$ (5.8 mg) was irradiated for 15 min, generating $\sim 28.7\ MBq$ of ${}^{140}La$. Similarly, 2.31 MBq of ${}^{159}Gd$ was produced by irradiating 6.2 mg of $Gd(NO_3)_3 \cdot 6\ H_2O$ for 45 minutes. $\{La_{0.25}Gd_{0.75}\}PO_4$ nanoparticles in solution as synthesized in 3.2.3 were centrifuged and the supernatant removed. They were redispersed in 400 μL of 0.05 M HCl containing radiotracer ${}^{140}La$ and transferred to

a 5 mL V-bottom vial. Na-TPP (800 μ L, 0.05 M) was added and the solution heated in a capped vial for 3 hours at 90°C, the layered nanoparticles were moved to a liquid scintillation vial and placed by a NdFeB magnet for separation overnight. Following separation, the supernatant fraction opposite the magnet was decanted via pipette and collected. The supernatant and the nanoparticle fractions were counted on a γ spectrometer using identical geometries to determine the fraction of ^{140}La in each component. Nanoparticle composition of gold-coated analogs was measured via NAA at MURR to confirm layering yield. Briefly, three samples of 10 μ L of nanoparticles were dried down in a 2/5 dram polyethylene vial. Vials were heat sealed, then irradiated for 15 seconds, and allowed to cool for 1 minute. The composition of the $\{\text{Gd}_{0.75}\text{La}_{0.25}\}\text{PO}_4@4\text{ LaPO}_4\text{ shells}@Au$ layered nanoparticles was determined by standard comparator NAA using ^{140}La (10 μg), ^{159}Gd (10 μg), and ^{198}Au (1 μg). Standards were irradiated with the samples in the pneumatic tube system at MURR (neutron flux = of $8 \times 10^{13} \text{ n/cm}^2/\text{s}$) for 15 seconds. Each sample was allowed to decay for 60 seconds, then counted for 30 seconds on a high purity germanium detector.

Layering efficiency of GdPO_4 was determined similarly using ^{159}Gd radiotracer. Because the magnetic properties of Gd preclude magnetic separation of layered and non-layered GdPO_4 nanoparticles, the layered nanoparticles were allowed to precipitate. The supernatant was decanted via pipette and the activity of the nanoparticles and supernatant were measured on a γ spectrometer. Gd yield was

confirmed by measuring the ratio of Gd:La in the $\{\text{Gd}_{0.5}\text{La}_{0.5}\}\text{PO}_4@4 \text{ GdPO}_4 \text{ shell}@Au$ via NAA and comparing to the theoretical ratio.

3.2.7 *In Vitro* Stability Studies

$\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4@4 \text{ GdPO}_4@Au$ nanoparticles with PEG surface functionalization (3.4.2) were monitored for their stability against aggregation *in vitro* using UV-Vis spectroscopy. Samples (0.5 mg) were kept in 1 mL of either DI water, PBS, or BSA for one month. The absorption was measured from 350-700 nm three times a week to determine stability of both the wavelength and intensity of the surface plasmon resonance band.

3.2.8 *In Vitro* Retention Testing

Retention of the ^{225}Ac decay products was quantified *in vitro* via dialysis. The ^{225}Ac nanoparticles (14 mg) were placed in a dialysis tube and suspended in 400 mL of 18 M Ω water. The dialysis tube was allowed to equilibrate for at least 3 hours (four half-lives of the longest lived α -emitting daughter, ^{213}Bi), before the initial measurement and between each subsequent measurement. A 5 mL aliquot of the dialysate was taken for radioactivity measurement each day over the course of 20 days (two ^{225}Ac half-lives). Each dialysate sample was counted immediately to determine ^{221}Fr leakage via the ^{213}Bi activity and then re-analyzed the following day to determine the level of ^{225}Ac in the aliquot using the ^{221}Fr peak. Counts were corrected for decay during sample transport, decay during the counting period, and the decreasing dialysate volume throughout the

monitoring period. The corrected activity was compared to the level of ^{225}Ac activity at the time of separation to determine the ^{221}Fr and ^{225}Ac retention. Both the 218 keV γ ray from ^{221}Fr and the 440 keV γ ray from ^{213}Bi were used to determine ^{221}Fr retention. Because ^{213}Bi does not move across the dialysis membrane,⁴⁸ all of the ^{213}Bi in the dialysate originates from decay of ^{221}Fr present in the dialysate.

Figure 3.2.2: $\{\text{La}_{0.5}\text{Gd}_{0.5}\}(\text{}^{225}\text{Ac})\text{PO}_4@4\text{ GdPO}_4@Au$ nanoparticles loaded into 10 kDa MWCO membrane for *in vitro* daughter retention studies

3.2.9 Layer Leaching Studies

Nanoparticles cores were synthesized as in 3.2.3. One shell of either $^{159}\text{GdPO}_4$ or $^{140}\text{LaPO}_4$ was added as in 3.2.4 using radiotracers produced as described in 3.2.6. The nanoparticles were placed in a dialysis membrane and dialyzed for three hours against 1 L of 18 M Ω water. The dialysate was then removed and replaced with a new 1 L of water. Nanoparticles were dialyzed overnight, then counted on a γ spectrometer. Twenty mL of the dialysate was also collected and counted. The dialysate count was corrected for dilution and counting time then compared to the particle activity.

3.2.10 XRD Studies

Nanoparticles for XRD studies were prepared as in 3.2.3 with compositions of LaPO_4 , $\{\text{La}_{0.25}\text{Gd}_{0.75}\}\text{PO}_4$, $\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4$, and GdPO_4 . Samples were dialyzed and collected in a liquid scintillation vial and dried over low heat (50°C). The mass of the resulting solid was measured, then ground into a fine powder with a mortar and pestle. The powder was mixed with silicon standard 640c from the National Institute of Standards and Technology and ethanol. Five drops of this mixture was deposited on a zero diffraction silicon plate and allowed to evaporate. Each sample was scanned for one hour from 2θ values of 15 to 60 degrees. Detailed scan parameters are found in Appendix 2.

3.2.11 MRI Relaxivity Measurements

MRI relaxivity measurements were performed by Dr. Lixin Ma. Nanoparticles were centrifuged and redispersed in 18 MΩ water at a concentration of 1 mg/mL. This concentration corresponds to a Gd concentration of 0.424 mg/mL (2.70 mM). Relaxivity values were measured in a field strength of 7 T at room temperature. Calculations were done assuming these concentrations and mean particle diameter of 26 nm (determined by TEM).

3.3 Nanoparticle Characterization Results and Discussion

$\{La_xGd_{1-x}\}PO_4$ nanoparticle cores of four different compositions were successfully synthesized. Each exhibits a size of 3-4 nm as confirmed by TEM. Figure 3.3.1 shows a characteristic collection of nanoparticles.

Rietveld refinement of XRD patterns indicated that $LaPO_4$ nanoparticles exhibited the rhabdophane phase consistent with the description of Buissette *et al.*⁹³ However, the compositions $\{La_{0.5}Gd_{0.5}\}PO_4$, $\{La_{0.25}Gd_{0.75}\}PO_4$, and $GdPO_4$ exhibit the anhydrous monazite crystal phase.⁹⁴ Nanoparticles consisting of $LaPO_4$ but synthesized in organic media also exhibit the monazite phase.⁴⁸ XRD measurements indicate nanoparticle grain sizes of 4.04 nm for $LaPO_4$, 2.79 nm for $\{La_{0.5}Gd_{0.5}\}PO_4$, 2.91 nm for $\{La_{0.25}Gd_{0.75}\}PO_4$ and 3.11 nm for $GdPO_4$. These patterns, along with the accompanying Rietveld refinement, are shown in Figures 3.3.2 A-D. Pure $LaPO_4$ and pure $GdPO_4$

exhibited larger grain sizes than their mixed lanthanide counterparts. While the La^{3+} and Gd^{3+} ions are similar enough to co-crystallize, the co-crystallization process is retarded relative to the single element crystal.

Figure 3.3.1: $\{\text{La}_x\text{Gd}_{1-x}\}\text{PO}_4$ nanoparticle cores exhibit sizes of $\sim 3\text{-}4\text{ nm}$

Size measurements of the nanoparticles using TEM match the XRD grain size values, indicating that the core particles consist of a single crystalline phase. NAA of $\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4$ core nanoparticles after magnetic separation indicates a La to Gd molar ratio of 1.11 ± 0.03 . NAA indicates the same ratio in the $\{\text{La}_{0.25}\text{Gd}_{0.75}\}\text{PO}_4$ system is 0.33 ± 0.02 .

Layering $\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4$ with GdPO_4 occurs with a chemical yield of 99%. This yield, combined with a core Gd:La ratio of 0.90, results in a theoretical Gd:La for the

$\{La_{0.5}Gd_{0.5}\}PO_4@4\ GdPO_4$ shell nanoparticle of 7.91. The experimental ratio as measured by NAA is 7.5 ± 0.9 . Nanoparticle growth after each shell addition was monitored by TEM and is shown in table 3.3.1. These $\{La_{0.5}Gd_{0.5}\}PO_4@4\ GdPO_4$ shell nanoparticles have a mean diameter of $22 \pm 8\ nm$. After one day of leaching in $18\ M\Omega$ water, 99.96 ± 0.06 of the tracer activity in the Gd shell remained attached to the core nanoparticle.

Figure 3.3.2 A-D: XRD patterns and Rietveld refinement for $GdPO_4$ (A), $\{La_{0.5}Gd_{0.5}\}PO_4$ (B), $LaPO_4$ (C), and $\{La_{0.25}Gd_{0.75}\}PO_4$ (D). Red lines indicate the calculated spectra while the blue line represents the experimentally measured data.

Table 3.3.1: Nanoparticle growth of $\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4@4 \text{ GdPO}_4 \text{ shell}@Au$

Particle System	Diameter (nm)
$\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4$ Core	5.0 ± 1.5
$\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4@1 \text{ shell GdPO}_4$	7.8 ± 2.8
$\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4@2 \text{ shells GdPO}_4$	9.9 ± 2.6
$\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4@3 \text{ shells GdPO}_4$	13.3 ± 1.8
$\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4@4 \text{ shells GdPO}_4$	22.4 ± 7.7
$\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4@4 \text{ shells GdPO}_4@Au$	26.8 ± 4.9

Radiotracer experiments indicate that LaPO_4 shells add with a 91% chemical yield. Utilizing this layering yield and a core composition with La:Gd ratio of 0.33, the expected La:Gd ratio of a $\{\text{La}_{0.25}\text{Gd}_{0.75}\}\text{PO}_4@4 \text{ LaPO}_4$ nanoparticle is 5.17. Experimental measurements with NAA indicate that the actual nanoparticle has a La:Gd ratio of 5.1 ± 0.7 . TEM indicates that the $\{\text{La}_{0.25}\text{Gd}_{0.75}\}\text{PO}_4@4 \text{ LaPO}_4$ nanoparticle has a mean diameter of $19.9 \pm 6.5 \text{ nm}$. Leaching studies in $18 \text{ M}\Omega$ water indicate that 99.94 ± 0.13 % of the La tracer in the shell remains attached after one day. Overnight magnetic separation occurs with a 89% yield.

Each shell addition process utilizes identical amounts of Ln and TPP. An epitaxial growth mechanism with this reaction scheme would generate successively thinner shells of equal volume. Thus, each successive shell would generate a smaller change in

diameter of the nanoparticle. However, this phenomenon is not observed (Table 3.3.1). Given these observations, the nanoparticles likely grow through a process of oriented alignment and attachment as recently described by Li *et al.*⁹⁵ In this process, nanoparticle growth occurs when smaller nanocrystals combine in a controlled fashion. Dilute starting material, which is insufficient to create new nanoparticles in solution, will fill in around the attachment point to maintain spherical nanoparticles. The added lanthanide ions and the orthophosphate produced by thermolysis of Na-TPP thus incorporate into the crystal structure. While the GdPO_4 and LaPO_4 layers possess slightly different crystal systems, they both generate near quantitative layer deposition onto the mixed $\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4$ or $\{\text{La}_{0.25}\text{Gd}_{0.75}\}\text{PO}_4$ cores.

Gold shells add easily to all compositions of the nanoparticles. EELS-TEM of gold gives a clear picture of the size of the added shell. A characteristic image is shown below in Figure 3.3.3. Ratios of Au:Ln in the particles based off of a 0.5 nm gold shell on top of a 22.4 nm LnPO_4 nanoparticle (Figure 3.3.3) predict a Au:Ln ratio of 0.65. NAA measures a ratio in the particles of 0.61 ± 0.01 . Nanoparticles consisting of $\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4@4 \text{ GdPO}_4 \text{ shell}@ \text{Au-PEG}$ remain stable against aggregation in all biological mimic solutions tested (water, PBS, BSA) over the course of a month.

Addition of a gold shell onto the surface also raises the possibility of simultaneously creating pure gold nanoparticles. Pure gold nanoparticles would possess identical surface chemistry (including properties such as plasmon resonance, Figure 3.3.4) and similar size/mass to the layered construct, making them difficult to separate.

The gadolinium in the layered structure allows for magnetic separation of the layered construct from any pure gold nanoparticles formed in the reaction. Without separation, pure gold nanoparticles would effectively lower the specific activity of the construct. Targeting agents on the pure gold nanoparticles would compete with therapeutic layered nanoparticles for the limited number of receptor sites *in vivo*, lowering the targeting efficacy of the layered nanoparticles and decreasing the delivered therapeutic dose.

Figure 3.3.3: EELS-TEM of a characteristic $\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4@4 \text{ GdPO}_4 \text{ shell@Au}$ nanoparticle showing the width of the gold surface layer

While lanthanum and gadolinium are both present in the nanoparticle solution, this does not confirm that they are co-crystallized. There could be separate LaPO_4 and GdPO_4 nanoparticles without mixture of lanthanides. This possibility was discounted by

two experiments. First, NAA confirms that magnetically separating the nanoparticles does not enrich the nanoparticles in Gd content. Nanoparticles that contain only LaPO_4 will not be attracted to the magnet. However, La:Gd ratios remain constant after separation. Next, EELS-TEM confirms the presence of all three elements, lanthanum, gadolinium, and gold, in a cluster of particles. Those images are shown in Figure 3.3.5.

Figure 3.3.4: UV-Vis of $\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4@4$ GdPO_4 shell@Au nanoparticles. Absorbance peak occurs at 523 nm

Figure 3.3.5: EELS-TEM confirms the presence of lanthanum, gadolinium, and gold in each nanoparticle. The top left image shows the standard TEM of the same particles.

The synthesis of the gold coated, four LnPO_4 shell system has an overall radiochemical yield of 57.5%. Correcting this value for the decay of ^{225}Ac , the overall chemical yield for incorporation of ^{225}Ac in the nanoparticles is ~76% at the end of the 4 day synthesis. Figure 3.3.6 shows a schematic of a completed $\{\text{La}_x\text{Gd}_{1-x}\}\text{PO}_4@4$ $\text{LnPO}_4@Au$ nanoparticle.

GdPO_4 nanoparticles are currently being investigated as potential MRI contrast agents due to the paramagnetic properties of gadolinium.⁹⁶ The Gd^{3+} ion will possess 7 unpaired electrons, the highest number possible using commonly accessible orbitals. MRI measurements of the GdPO_4 shell system indicate r_1 values of $1.00 \pm 0.01 \text{ s}^{-1} \text{ mM}^{-1}$

and r_2 values of $44.0 \pm 4.2 \text{ s}^{-1} \text{ mM}^{-1}$. The r_2 value of these nanoparticles falls on the lower end of approved contrast agents. MRI contrast requires interaction between the gadolinium and the water medium. We hypothesize that the gold shell in the nanoparticles partially inhibits the action of the large quantity of gadolinium in the nanoparticle. MRI contrast remains a compelling application for the GdPO_4 shell nanoparticles when combined with their therapeutic and potential SPECT capabilities. While unlikely to win approval as a standalone contrast agent, the MRI properties of the $\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4@4 \text{ GdPO}_4 \text{ shell@Au}$ system warrant further study. The collection efficiency with overnight magnetic separation of $\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4@4 \text{ GdPO}_4 \text{ shell@Au}$ nanoparticles is typically on the order of $\sim 90\%$.

Figure 3.3.6: Idealized schematic of a $\{\text{La}_x\text{Gd}_{1-x}\}\text{PO}_4@4 \text{ LnPO}_4@Au$ nanoparticle. The decay of ^{225}Ac and subsequent retention of the ^{221}Fr daughter product are illustrated.

Retention of ^{225}Ac in the nanoparticle depends on the core composition and the number/type of shells added. $\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4$ core nanoparticles without additional shells retain $99.04\% \pm 0.07\%$ of ^{225}Ac throughout the three week observation period. Adding two shells of $\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4$ to this core increases ^{225}Ac retention to $99.42 \pm 0.02\%$. Systems with four shells retain ^{225}Ac to an even higher degree. The $\{\text{La}_{0.25}\text{Gd}_{0.75}\}\text{PO}_4@4 \text{ LaPO}_4 \text{ shell}@Au$ system sequesters $99.97 \pm 0.01\%$ of the ^{225}Ac while the $\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4@4 \text{ GdPO}_4 \text{ shells}@Au$ system retains $99.996 \pm 0.002\%$.

Nanoparticles where La comprises a portion of the core retain a higher proportion of ^{225}Ac than compositions that include only Gd. The increased retention likely results from isomorphism between $^{225}\text{Ac}^{3+}$ and La^{3+} ions. For 8-coordinate configurations, the $^{225}\text{Ac}^{3+}$ has an ionic radius of $1.36 \text{ \AA}$ compared with $1.30 \text{ \AA}$ for La^{3+} and $1.19 \text{ \AA}$ for Gd^{3+} .⁹⁷ Further, ^{225}Ac retention in the $\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4@4 \text{ GdPO}_4 \text{ shell}@Au$ system improves by an order of magnitude over the corresponding 4 LaPO_4 shell system. This difference results from the larger layering yield observed in the GdPO_4 shell addition (larger particles with thicker shells). The ^{225}Ac must diffuse through a longer path to escape the GdPO_4 shells than the LaPO_4 system. Due to the lower overall gadolinium content in the lanthanum shell system, the ratio of Gd:La used in the core of the LaPO_4 shell particles was increased from 1:1 to 3:1 so that the nanoparticles could be magnetically separated following gold shell addition. Though this new $\{\text{Gd}_{0.75}\text{La}_{0.25}\}\text{PO}_4$ core still contains the ^{225}Ac to a high degree, retention was measured

to be slightly lower than the value obtained with the $\{\text{Gd}_{0.5}\text{La}_{0.5}\}\text{PO}_4$ core. A summary of the ^{225}Ac and ^{221}Fr retention values are given in Table 3.3.2.

Table 3.3.2: Retention of ^{225}Ac and its daughter ^{221}Fr in different nanoparticle constructs

Core	Shell Material	# of Shells	^{225}Ac retention ^a	^{221}Fr retention ^b
$\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4$	None	0	$99.16 \pm 0.02\%$	$60.2\% \pm 3.0\%$
$\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4$	$\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4$	2	$99.42 \pm 0.02\%$	$69.2 \pm 1.7\%$
$\{\text{La}_{0.25}\text{Gd}_{0.75}\}\text{PO}_4$	LaPO_4	4	$99.96 \pm 0.01\%$	$79.4 \pm 1.6\%$
$\{\text{La}_{0.25}\text{Gd}_{0.75}\}\text{PO}_4$	GdPO_4	4	$99.98 \pm 0.02\%$	$90.9 \pm 0.9\%$
$\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4$	LaPO_4	4	$99.97 \pm 0.01\%$	$79.9 \pm 1.9\%$
$\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4$	GdPO_4	4	$99.996 \pm 0.002\%$	$89.3 \pm 1.8\%$

^a ^{225}Ac retention after 3 weeks ^b ^{221}Fr retention after 3 weeks

Measurements of ^{221}Fr retention indicate that the sequestration efficiency also depends on the number and composition of shells added to the nanoparticle. Unlike ^{225}Ac , retention of ^{221}Fr also depends to some degree on the time since synthesis. The amount of ^{221}Fr retention typically drops slightly over the course of the first week of observation, then stabilizes during the second and third week. After three weeks, the $\{\text{Gd}_{0.5}\text{La}_{0.5}\}(\text{Ac})\text{PO}_4$ core contains $60.2\% \pm 3.0\%$ of the ^{221}Fr daughter. Retention increases to $69.2 \pm 1.7\%$ after adding two shells of $\{\text{Gd}_{0.5}\text{La}_{0.5}\}\text{PO}_4$. Four shells of LnPO_4 further increases ^{221}Fr retention values. The $\{\text{La}_{0.25}\text{Gd}_{0.75}\}\text{PO}_4@4\text{LaPO}_4@Au$ system initially retains $89.8 \pm 0.5\%$ of the ^{221}Fr . This retention decreases over the course of the first week and stabilizes at $79.9 \pm 1.9\%$. The $\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4@4\text{GdPO}_4@Au$ system

initially retains $97.8 \pm 0.5\%$ of the ^{221}Fr daughter. This retention value also decreases over the course of a week, stabilizing at $89.3 \pm 1.8\%$. Francium-221 retention data is presented in Figure 3.3.7. For comparison purposes, previously reported $\text{La}(^{225}\text{Ac})\text{PO}_4$ nanoparticles synthesized in organic solvents retain only $\sim 50\%$ of the ^{221}Fr daughter.⁴⁸

Figure 3.3.7: ^{221}Fr retention data for the $\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4@4 \text{ GdPO}_4 \text{ shell}@Au$ and $\{\text{La}_{0.25}\text{Gd}_{0.75}\}\text{PO}_4@4 \text{ LaPO}_4 \text{ shell}@Au$ nanoparticle systems

Though the layering efficiencies of both the LaPO_4 and GdPO_4 systems are $> 90\%$, GdPO_4 shells clearly contain the ^{221}Fr daughter to a higher degree than their LaPO_4 shell counterparts *in vitro*. Part of this improved retention can be attributed to the higher chemical layering yield of GdPO_4 . Nanoparticles with GdPO_4 shells are slightly larger and have more marginally more material that the recoiling daughter must pass through to escape. Additionally, the higher electron density of the GdPO_4 creates a slightly higher

stopping power. While daughter products are retained in the nanoparticle, the associated α -particles lose a negligible fraction of their energy in traversing the nanoparticle. A 6 MeV α -particle loses less than 0.2% of its energy in the layered nanoparticle whereas the range of the 100 keV recoiling daughters is ~ 20 nm in bulk LnPO_4 .⁵⁰ Moreover, a portion of the kinetic energy of the daughter particle may be transferred to the entire particle. If a portion of the recoil energy is distributed throughout the highly structured crystalline lattice, the recoiling range of the daughter radionuclides will be significantly decreased.⁹⁸ To some extent, the nanoparticle may be able to recoil as a unit in solution, absorbing some of the daughter recoil energy and retarding the daughter radionuclide's motion out of the center of the nanoparticle. Again, the more massive GdPO_4 shell system would absorb slightly more recoil energy than the lighter LaPO_4 shell system.

3.4 Biological Targeting Experimental

3.4.1 Materials and Measurements

All animal experiments were performed according to either the protocols set forth by the University of Missouri-Columbia Institutional Animal Care and Use Committee approved protocol number 7055 or according to the Institutional Animal Care and Use Committee of the University of Tennessee approved protocol 1502. Chemicals were used as received from Sigma Aldrich unless otherwise noted. Anti-

synaptophysin and anti-glial fibrillary acidic protein (GFAP) antibodies were received from Dako. MAb 201b and EMT-6 tumor cells were provided by Dr. Stephen Kennel. Lipoamide-dPEG₁₂-COOH was received from Quanta Biodesign. Sections 3.4.5 - 3.4.8 and 3.4.10 were performed by Dr. Stephen Kennel and Dr. Jonathan Wall at the University of Tennessee - Knoxville Graduate School of Medicine.

3.4.2 Surface Functionalization

Nanoparticles (12 mg in 10 mL water) were functionalized using a water soluble lipoamide-dPEG₁₂-COOH linker. The structure of the linker is shown in Figure 3.4.1. Two mg of dPEG were added to a solution of 12 mg of {La_xGd_{1-x}}PO₄@4 LnPO₄ shell@Au nanoparticles in 5 mL of PBS, followed by 6 mg of tris(2-carboxyethyl)phosphine (TCEP) reducing agent to cleave the disulfide bond and facilitate citrate displacement. The solution pH was adjusted to 7 with 0.1 M NaOH monitored by pH test strips and the reaction mixture was stirred for 4 hours. Nanoparticles were centrifuged at 3000 g for 3 minutes and the supernatant containing excess dPEG decanted via pipette. Connection of the linker was confirmed by a shift in the plasmon resonance near 540 nm as monitored by UV-Vis spectroscopy before and after the addition of the linker. The surface plasmon resonance is highly sensitive to changes in the dielectric constant at the gold surface. A shift in the plasmon resonance indicates a change in the surface environment of the nanoparticle and is indicative of antibody attachment.

Figure 3.4.1: Structure of lipoamide-dPEG₁₂-COOH linker

3.4.3 Antibody Conjugation

Five separate antibodies (anti-synaptophysin, anti-GFAP, mAb 201b, HuM-195, and panitumumab) were individually conjugated to the nanoparticles using the same basic procedure. Each antibody is an immunoglobulin G type with a molecular mass of ~150 kDa. In a standard conjugation, 0.5 mg of dPEG modified

{La_xGd_{1-x}}PO₄@4 LnPO₄@Au nanoparticles was dispersed in 0.5 mL of PBS in a 1 mL microcentrifuge vial. To this vial, 8 µL of 10 mg/mL sulfo-NHS and 80 µL of 10 mg/mL EDC were added and stirred for 30 minutes. The solution was centrifuged for 3 minutes at 3000 g and the supernatant decanted via pipette to remove excess EDC/sulfo-NHS. The nanoparticles were redispersed in 0.5 mL of PBS and stirred with 0.5 mg of antibody overnight (pH = 7.4). The reaction was quenched with glycine and stirred for 15 minutes. Particles were again centrifuged as above and redispersed in 0.5 mL of PBS containing 5 mg/mL BSA. The mechanism for the amide reaction is shown in Figure 3.4.2.

Figure 3.4.2: Amide bond formation by carbodiimide activation

3.4.4 *In Vitro* Antibody Quantification and Competition Assay

Panitumumab was added to the nanoparticles as in 3.4.3. Nanoparticles were centrifuged and washed with PBS 5 times before determining surface protein concentration via a modified Lowry assay.⁹⁹ Briefly, eleven standards were created with varying amounts of BSA (0-20 µg) for comparison purposes. Each polypropylene tube was filled with the appropriate amount of BSA, 10 µL of PBS buffer, and diluted to 300 µL with DI water. Samples were created by taking 6, 8, or 10 µL of nanoparticle solution, adding PBS to bring the volume to 10 µL, then diluting to a volume of 300 µL with deionized water. Solution A was prepared by mixing 0.5 mL of 1% cupric sulfate and 0.5 mL of 2% potassium tartrate with a solution of 50 mL of 2% sodium carbonate/0.1 M

NaOH. Solution A (1.5 mL) was added to each tube, vortexed, and allowed to stand at room temperature for 10 minutes. Next, 150 μ L of 1 M phenol was added to each tube and immediately vortexed. The tubes were allowed to stand at room temperature for 30 minutes before measuring their absorbance at 750 nm. Absorbance in the nanoparticle samples was compared to the absorbance of the BSA standard to determine protein concentration on the nanoparticles. Nanoparticles with PEG attached were treated similarly and used as controls for the antibody labeled nanoparticle samples. Absorbance from the PEG coated nanoparticles was subtracted from the absorbance of the antibody labeled nanoparticles. The number of antibodies per nanoparticle was calculated by assuming a 150 kDa antibody and a nanoparticle consisting of a 22.4 nm diameter LnPO_4 with a density of 5 g/cm^3 and a 0.5 nm thick outer layer of gold of density of 19.3 g/cm^3 . An example calculation is given in Appendix 3.

Human epidermal growth factor receptor (hEGFR) was coated onto well plates (50 ng hEGFR/50 μ L DPBS per well) and incubated overnight at 4°C. The following day, hEGFR was removed and each well washed with 100 μ L of 1% BSA in PBS. The wells were kept at room temperature for 1 hour before removal of PBS. A series of 11 dilutions of HuM-195, panitumumab, nanoparticle-HuM-195 conjugates, and nanoparticle-panitumumab conjugates with protein concentrations from 0.8 ng/mL – 40 μ g/mL (as determined by Lowry assay) was prepared. Twenty-five μ L of each dilution and a control consisting of PBS was added to the wells in triplicate followed by addition

of 25 μL of panitumumab antibody labeled with 50,000 cpm ^{131}I . The wells were incubated for 4 hours at 37°C , then washed 3x with 150 μL of 1% BSA in PBS. One hundred μL of 0.2 M NaOH was added to each well to release the bound hEGFR. Wells were shaken for 2 minutes at a moderate speed and their contents absorbed onto filters and transferred into polypropylene tubes for counting. The amount of radioactivity absorbed onto the filters was then used to compare the binding activity of the nanoparticle-antibody conjugates to that of the free antibodies.

3.4.5 Radiotracer Antibody Saturation Studies and Biodistribution

MAb 201b was radioiodinated with ^{125}I by the chloramine T method.¹⁰⁰ Briefly, 15 μg of antibody was added to 75 μL of a freshly prepared solution of 50 mM NaPO_4 . Na^{125}I (6 μL , 3 mCi) was added to this solution followed by 75 μL of 4 mg/mL chloramines T in 50 mM NaPO_4 . The solution was mixed with a stir bar for 30 seconds, then quenched by addition of 300 μL of 12.6 mM sodium metabisulfate in 50 mM NaPO_4 . The quenched reaction was dialyzed 2 times versus PBS to remove unbound ^{125}I . Three 500 μL microcentrifuge tubes were set up containing 5, 55, or 250 μg of mAb 201b. Each mAb addition contained 5 μg of radiolabeled antibody and an appropriate amount of cold antibody to provide the correct total mAb mass in a final volume of 100 μL in PBS. Ten μL of 0.5 M sodium phosphate buffer, pH 7.6 was added to each tube.

Nanoparticles (1.5 mg in 600 μL of PBS) were activated as described in 3.4.3. Two hundred μL of this solution was dispensed into each of 3 tubes containing antibody.

The mixture was allowed to react overnight, then quenched by addition of 5 μL of 1 M glycine and stirred for 1 hour. The particles were centrifuged as above and the supernatant sampled for ^{125}I content. The number of antibodies per nanoparticle was quantified by calculating the average mass of an individual particle assuming an inner LnPO_4 shell with a 22.4 nm diameter and a density of 5 g/cm^3 and a 0.5 nm thick outer layer of gold with a density of 19.3 g/cm^3 .

Nanoparticles were centrifuged again, resuspended in 0.5 mL of 5 mg/mL BSA in PBS and sonicated (Branson microprobe) for 2-5 sec to complete dispersal. Another 0.5 mL of BSA/PBS buffer was added to each preparation and 200 μL of the three preparations injected via the tail vein into 4 female BALB/c mice (body mass ~ 20 g) per group for a total of 12 mice. Mice (2 per group) were sacrificed at 1 hr or 24 hrs post-injection and liver, spleen, kidneys and lung were harvested for biodistribution studies. Each organ was counted for ^{125}I , ^{221}Fr , and ^{213}Bi content at ~ 20 minutes post sacrifice and then again after ^{213}Bi equilibrium had been established 3 hrs post-sacrifice.

3.4.6 Biodistribution and *in vivo* Retention Studies with $\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4@4\text{GdPO}_4@Au\text{-PEG-mAb 201b}$

Biodistribution and ^{225}Ac daughter retention assays were done on three separate groups consisting of six female BALB/c mice per group, injected I.V. (tail vein). Groups 1

and 2 were injected with $\{La_{0.5}Gd_{0.5}\}PO_4@4GdPO_4@Au-PEG-mAb$ 201b, while group 3 was treated with $\{La_{0.5}Gd_{0.5}\}PO_4@4GdPO_4@Au-PEG$ nanoparticles as a control. Group 1 mice received 14.6 μg of conjugates with 74 kBq of Ac-225 and $\sim 5 \mu g$ of attached mAb 201b (as measured in 3.4.5) in 100 μL of PBS. Group 2 received an identical amount of targeted nanoparticles but with co-injection of 750 μg of unconjugated mAb 201b as competitor. Group 3 received the same amount of nanoparticles and ^{225}Ac , but with no conjugated antibody. Mice received food and water *ad libitum* in a light/dark cycle environment. Three mice from each injection group were sacrificed at time points of 1 and 24 hours post-injection for biodistribution and *in vivo* retention studies. Mouse lung, liver, spleen, and kidneys were harvested to evaluate the amount of both ^{221}Fr and ^{213}Bi in target organs. Each organ was massed and counted for content of ^{221}Fr and ^{213}Bi in a γ scintillation counter at a specific time post sacrifice and again after the radioisotopes had achieved decay equilibrium (>3 h). Quantities of ^{221}Fr and ^{213}Bi present at the time of animal sacrifice were determined by appropriate decay corrections as previously described.⁴⁸

3.4.7 MicroSPECT/CT Imaging:

Small animal imaging was performed using a Siemens Preclinical Imaging microCAT IIp SPECT dual modality platform on loan to the University of Tennessee. Mice were injected I.V. with approximately 3 MBq of ^{225}Ac in the same three groups and with the same volumes as in 3.4.6. Instead of the large excess described in 3.4.6, an identical quantity of free antibody was injected in group 2, thus the mice in the

competition group had a different ratio of cold competitor to nanoparticle conjugate and competition was not as complete. Animals were sacrificed by overdose of isoflurane at 1 hour post injection and imaged via microSPECT/CT 3 hours post-sacrifice when the ^{225}Ac and its daughters had reached equilibrium. SPECT data for the final images were acquired as previously described.⁴⁸

3.4.8 Effect of Clodronate Liposomes on Biodistribution and Uptake

Biodistribution and daughter retention assays were done using I.V. injections in four groups of mice, consisting of six mice per group. Mice received 14.6 μg of $\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4@4\text{GdPO}_4@Au\text{-PEG-mAb 201b}$ nanoparticle conjugates with 74 kBq of ^{225}Ac and $\sim 5 \mu\text{g}$ of attached mAb 201b in 100 μL of PBS. Group 1 was injected with clodronate liposomes followed by nanoparticle-mAb 201b conjugates. Mice in group 2 were injected with clodronate liposomes followed by a mixture of 500 μg of unconjugated mAb 201b and nanoparticle-mAb 201b conjugates. Group 3 and 4 mice were injected as group 1 and 2, respectively, except that they were not pretreated with clodronate liposomes. Mice were housed with food and water *ad libitum* in a light/dark cycle environment. Three mice from each injection group were sacrificed at 1 and 24 hours post injection for biodistribution and *in vivo* retention studies. A γ scintillation counter windowed for 212 keV (^{221}Fr) and 440 keV (^{213}Bi) was used to evaluate the amount of radioactivity present in the harvested lungs, liver, spleen, and kidneys. Quantities of ^{221}Fr and ^{213}Bi present at the precise time of animal sacrifice were determined by appropriate crossover and decay corrections as previously described.⁴⁸

3.4.9 Electron Microscopy Samples

Mice were anesthetized with an oxygen/isoflurane mixture. Each mouse received 100 μ L of $\{La_{0.5}Gd_{0.5}\}PO_4@4GdPO_4@Au-PEG-mAb$ 201b non-radioactive conjugates prepared as described in 3.4.3. After 45 minutes, each mouse was sacrificed by injection of 4 mL of fixative buffer solution (2% paraformaldehyde, 2% glutaraldehyde, 0.1M cacodylate, hereafter referred to as simply cacodylate) via the catheter as below and euthanized via cervical dislocation. Mouse tissues were embedded in resin, sliced, and fixed for examination on Cu TEM grids (Ted Pella). Electron microscopy studies were performed at the University of Missouri Electron Microscopy Core on a JEOL 1400 instrument.

3.4.10 EMT-6 Therapy Study

Fifteen mice were injected I.V. (tail vein) with clodronate liposomes followed the next day by injection of 80,000 log phase EMT-6 cells in 0.2 mL of PBS. EMT-6 cells form tumor colonies in lung tissue simulating metastatic disease.¹⁰¹ Forty-eight hours after tumor cell injection, 5 mice were injected with 100 μ L of PBS, 5 mice with free mAb 201b mixed with ~ 37 kBq of $\{La_{0.5}Gd_{0.5}\}PO_4@4GdPO_4@Au-PEG-mAb$ 201b conjugates, and 5 mice with ~ 37 kBq of nanoparticle-mAb 201B conjugates. Mice were sacrificed at 7 days post EMT-6 cell injection. Lungs were inflated via tracheal catheter with Bouin's fixative (0.2% picric acid, 2% paraformaldehyde, 40% formalin in water), suspended in fixative for 24 hours and then washed into 70% ethanol/PBS. Paraffin sections were cut,

stained, and counted by a blinded observer to quantitate tumor colony growth.

Statistical significance was calculated at the 95% confidence level using a two tailed Student's T test with unequal variance. Separate samples were fixed in cacodylate buffer and allowed to decay for 6 months before examination on electron microscopy.

3.4.11 *In Vivo* Evaluation of Blood Brain Barrier Penetration with Anti-GFAP and Anti-Synaptophysin

Anti-GFAP and anti-synaptophysin antibodies were added to {La_{0.5}Gd_{0.5}}PO₄@4GdPO₄@Au-PEG nanoparticles as described in 3.4.3. The attachment of the antibodies to the nanoparticles was confirmed with immunohistochemistry. The anti-synaptophysin and anti-GFAP antibody labeled nanoparticles were incubated with normal brain and used as a primary immunochemistry label to target these proteins in normal brain. Secondary antibody biotin and avidin-horseradish peroxidase were used to detect the presence of primary antibody. Diaminobenzadine (DAB) was used as the color agent.

Mice were anesthetized with an isoflurane (1.5%) and oxygen mixture and catheterized via partial dissection in either the femoral or axillary vein using a 0.115 mm outer diameter, 0.10 mm inner diameter catheter (A.P. Extrusion, Salem, N.H.). Mannitol (50 µL) was injected to open the blood-brain barrier. One hundred µL (300 µg) of immunolabeled nanoparticles (anti-synaptophysin or anti-GFAP) were injected intravenously into each mouse. Under terminal anesthesia, mice were sacrificed by

perfusion cacodylate buffer 30 minutes post-injection. Death was ensured via cervical dislocation. The brain, tongue, colon, muscle, heart, spleen, kidney, lung, and liver were harvested. The tissue was prepared for analysis by TEM, light microscopy, and NAA.

3.5 Biological Targeting Results and Discussion

3.5.1 Characterization of Antibody Attachment

The absorption wavelength of the plasmon resonance as measured by UV-Vis spectroscopy steadily increases as larger groups are added to the nanoparticle surface. Citrate coated $\{\text{La}_{0.5}\text{Gd}_{0.5}\}\text{PO}_4@4\text{GdPO}_4@Au$ nanoparticles absorb maximally at 523 nm. After addition of the lipoamide-dPEG₁₂-COOH linker, this absorption peak red-shifts to 529 nm. After antibody addition, the plasmon peak further red-shifts to 537 nm. The changes in plasmon resonance for the addition of the anti-synaptophysin antibody are shown in Figure 3.5.1.

The antibody addition process also causes a drastic increase in the hydrodynamic diameter of the nanoparticles. From an initial hydrodynamic diameter of 101.4 ± 1.5 nm, addition of mAb 201b causes it to grow to 1498 ± 77 nm. Zeta potential also becomes more positive as a result of mAb 201b addition. The nanoparticle surface has been passivated by the addition of PEG and mAb, so that the nanoparticles will not aggregate despite the more neutral zeta potential.

Figure 3.5.1: UV-Vis spectra showing the red-shift in the plasmon resonance as PEG and antibody are added to citrate coated $\{La_{0.5}Gd_{0.5}\}PO_4@4GdPO_4@Au$ nanoparticles.

Table 3.5.1: Hydrodynamic diameter and zeta potential of $\{La_{0.5}Gd_{0.5}\}PO_4@GdPO_4@Au$ -mAb-201b nanoparticles.

Nanoparticle composition	Hydrodynamic diameter (nm)	Zeta potential (mV)
$\{La_{0.5}Gd_{0.5}\}PO_4@GdPO_4@Au$ -citrate	101.4 ± 1.5	-63.2 ± 1.6
$\{La_{0.5}Gd_{0.5}\}PO_4@GdPO_4@Au$ -PEG	382.3 ± 6.5	-56.4 ± 0.1
$\{La_{0.5}Gd_{0.5}\}PO_4@GdPO_4@Au$ -mAb-201b	1498 ± 77	-27.9 ± 2.4

The Lowry assay detected 0.22 mg of HuM-195 and 0.19 mg of panitumumab bound to the nanoparticles. This quantity compares with 0.875 mg of each antibody which was used in the synthesis, giving a labeling yield of 25.1% for HuM-195 and 21.7% for panitumumab. These labeling yields correspond to an average of 4.9 HuM-195 antibodies per nanoparticle and an average of 4.3 panitumumab antibodies per nanoparticle.

Nanoparticle-panitumumab conjugates show binding characteristics similar to the panitumumab antibody. However, the binding affinity of the conjugates, as measured by the IC_{50} value, appears to be roughly 4.5x lower than the free antibody. This difference is likely explained by the loading of multiple antibodies onto the nanoparticle. Binding to hEGFR will only occur through one of the average 4.3 antibodies on the nanoparticle surface. The other antibodies remain, but do not take up receptor sites, hence the apparent decrease in binding affinity. Correcting for this factor brings the binding affinity of the nanoparticle-panitumumab conjugate in line with the free panitumumab antibody. The conjugation process therefore does not decrease the inherent binding ability of the antibody. HuM-195 and nanoparticle-HuM 195 conjugate controls show no affinity for the hEGFR receptor at any concentration. Results from the competition assay are shown in Figure 3.5.2.

Antibody conjugation was also quantified using a ^{125}I radiotracer to label mAb 201b prior to attachment. MAb 201b has high affinity and specificity for thrombomodulin *in vivo*, which is expressed primarily in lung tissue. Actinium-225 was

recovered nearly quantitatively in these antibody saturation experiments, indicating little loss of nanoparticles. Attachment of mAb 201b varied with amount added according to table 3.5.2. The amount of antibody bound approaches a maximum, indicating saturation of the available sites at slightly over 4 antibodies per nanoparticle. The maximum antibody bound per particle corresponds well with the labeling yield seen with panitumumab and HuM-195.

Figure 3.5.2: Nanoparticle-panitumumab conjugates show a decreased binding affinity relative to free antibody due to the binding of multiple panitumumab molecules onto a single nanoparticle. Nanoparticle-Hum 195 conjugates and free Hum 195 antibody show no affinity for the hEGFR receptor used at any concentration.

Table 3.5.2: Labeling efficiency of mAb 201b onto 0.5 mg of $\{La_{0.5}Gd_{0.5}\}({}^{225}Ac)PO_4@4$ GdPO₄ shell@Au nanoparticles

mAb added (μg)	mAb bound (μg)	% bound	mAb/NP
5	4.5	90	0.65
55	26.0	48	3.39
255	32.2	13	4.20

3.5.2 Lung Targeting with mAb 201b

Localization of activity in the lung at 1 hour depends on the amount of antibody used in the preparation. Nanoparticles with a larger amount of antibody bound show higher uptake by lung tissue. This effect remains evident after 24 hours, as shown in Figure 3.5.3.

The %ID of each radionuclide detected in lung tissue does not match. This indicates that the ${}^{125}I$ and ${}^{225}Ac$ daughter products are becoming separated *in vivo*. Further, the ratio of %ID ${}^{225}Ac$ to %ID ${}^{125}I$ detected in depends both on the amount of antibody loaded onto the individual particles and the time since injection. Over a 24 hour period, the ratio increases by roughly a factor of 5. Those ratios are shown in Table 3.5.3.

Figure 3.5.3: Saturating the nanoparticle with antibody increases targeting and retention in lung tissue at time points of 1 hour and 24 hours. Percent ID/g increases as a function of the amount of antibody used in the synthesis at both time points.

Table 3.5.3: Ratio of %ID/g ^{225}Ac to %ID/g ^{125}I in lung tissue at 1 hour and 24 hours

antibody added (μg)	1 h	24 h
5	0.15	1.50
55	0.97	5.45
250	1.08	4.47

There are a variety of explanations for the discrepancy between %ID ^{225}Ac and %ID ^{125}I that present a complicated picture of *in vivo* interactions. When the amount of antibody on each particle is low, the ratio of %ID of ^{225}Ac to %ID of ^{125}I at one hour is 0.15, indicating that some nanoparticle must be cleaved from antibody and leave the lung. This ratio with moderate and high antibody loading onto the nanoparticles is close to unity at 1 hour, indicating that the radiolabeled antibody and nanoparticle are remaining intact. More than one antibody may bind the nanoparticle to the lung, stabilizing the attachment. This interpretation is consistent with the number of antibodies bound to each nanoparticle. If more than one antibody is present per nanoparticle, cooperative binding ensures that the nanoparticle remains attached to the target site.

At 24 hrs, ^{125}I is nearly gone in all samples. ^{225}Ac is also lost, but at a drastically slower rate. The loss of ^{125}I is also much faster than loss of the free antibody.¹⁰⁰ The increase of %ID ^{225}Ac relative to %ID ^{125}I from the 1 h to 24 h time point indicates that the nanoparticles are being retained in the lung tissue while either the targeting antibody or the ^{125}I label separates from the nanoparticle. Iodine-125 linked by the chloramines T method will cleave once inside the cellular environment and release into circulation.¹⁰² TEM images taken of the lung show the presence of nanoparticles in the lung epithelium (Figure 3.5.4), indicating that the change in ratio likely results from cleavage of ^{125}I from the antibody. Other tissues show similar changes in the %ID ratio

from 1 to 24 hours indicating that the change results primarily from the loss of ^{125}I . This could be confirmed by measuring the thyroid for escaped ^{125}I .

Figure 3.5.4: $\{\text{Gd}_{0.5}\text{La}_{0.5}\}\text{PO}_4@\text{GdPO}_4@\text{Au}$ nanoparticles bound to the vascular side of lung epithelium (A), contiguous with red blood cells (B), and actively being endocytosed (C) into a lung epithelium vesicle (D).

Figure 3.5.5 A and B: Biodistribution of $\{Gd_{0.5}La_{0.5}\}PO_4@4\ GdPO_4\ shell@Au-PEG-mAb$ 201b nanoparticles at 1 hour post-injection (A) and 24 hours post-injection (B).

Given the increases in lung uptake with additional amounts of antibody, $\{\text{Gd}_{0.5}\text{La}_{0.5}\}\text{PO}_4@4 \text{ GdPO}_4 \text{ shell@Au}$ and $\{\text{Gd}_{0.25}\text{La}_{0.75}\}\text{PO}_4@4 \text{ LaPO}_4 \text{ shell@Au}$ nanoparticles were saturated with mAb 201b for biodistribution studies by combination of equal masses of mAb and nanoparticles during synthesis. Targeted nanoparticles in each case were compared with non-targeted, PEG coated nanoparticles and with receptor-blocked mice. Biodistribution results are shown in Figure 3.5.5 A and B at time points of 1 and 24 hours.

In vivo biodistribution experiments of the $\{\text{Gd}_{0.5}\text{La}_{0.5}\}({}^{225}\text{Ac})\text{PO}_4@4 \text{ GdPO}_4 \text{ shell@Au-PEG-mAb 201b}$ nanoparticles demonstrated uptake in the lung consistent with the binding properties of mAb 201b. The nanoparticles exhibit high lung uptake with the antibody conjugate after 1 hour (151 %ID/g). This high lung uptake of nanoparticles drops to 16.8 %ID/g when competed with unconjugated antibody. Thus, the antibody retains its binding affinity and specificity even after conjugation to the nanoparticles. Further, nanoparticle uptake in the lung is primarily through antibody-mediated binding.

In vivo, $69\% \pm 3\%$ of the ${}^{213}\text{Bi}$ from the ${}^{225}\text{Ac}$ generator was retained in lung tissue after 1 hour and $84\% \pm 3\%$ was retained after 24 hours. Similar ${}^{213}\text{Bi}$ retention values were observed in liver (1 hour, $81\% \pm 4\%$; 24 hours, $92\% \pm 1\%$) and spleen tissue (1 hour, $72\% \pm 3\%$; 24 hours, $82\% \pm 16\%$). In contrast to ${}^{225}\text{Ac}$ conjugates made from metal chelation, a very small fraction of the injected dose travels to the kidney. Under 5% of the ID/g of the nanoparticles locate in the kidney after 1 hour and less than 1% after 24

hours. Renal toxicity associated with ^{213}Bi relocation to the kidney typically forms the dose-limiting factor in ^{225}Ac α -generator therapies,¹⁰³ however with $\{\text{Gd}_{0.5}\text{La}_{0.5}\}(\text{}^{225}\text{Ac})\text{PO}_4@4\text{ GdPO}_4\text{ shell}@Au\text{-PEG-mAb 201b}$ nanoparticles only 2.8% of the ^{213}Bi from the injected dose migrated to kidney tissues after 1 hour. After 24 hours, this number further decreased to 1.5%.

A larger dose (*ca.* 3.0 MBq/animal) of ^{225}Ac NPs was imaged using CT/SPECT of the ^{221}Fr γ ray (218 keV, 11.6%). The CT/SPECT images (Figure 3.5.6) clearly show large uptake in the lung for the $\{\text{La}_{0.5}\text{Gd}_{0.5}\}(\text{}^{225}\text{Ac})\text{PO}_4@\text{GdPO}_4@Au\text{-mAb-201b}$ nanoparticles which is in agreement with the biodistribution data. When competed with unconjugated mAb 201b antibody, the images reflect the higher RES uptake in the liver and spleen. Unconjugated, PEG-coated nanoparticles also show high uptake by the RES, further indicating that the lung uptake is antibody-mediated and not due to particulate trapping in the small capillary system of the lung.

Compared with single α -emitting therapies, the use of *in vivo* α generators holds the potential to deliver a much larger biologically effective dose to target tissues. The high, receptor-mediated uptake of nanoparticles in the lung epithelium demonstrates the ability of $\{\text{La}_{0.5}\text{Gd}_{0.5}\}(\text{}^{225}\text{Ac})\text{PO}_4@\text{GdPO}_4@Au$ nanoparticles to deliver ^{225}Ac to a tissue target in the vascular space. The nanoparticle construct improves ^{225}Ac daughter retention relative to both chelate approaches and previous nanoparticle systems both *in vitro* and *in vivo*. *In vivo*, the increase of retention of ^{213}Bi in the target tissue over time results from a combination the intrinsic retention of the nanoparticle and its

Figure 3.5.6: CT-SPECT scans of $\{La_{0.5}Gd_{0.5}\}({}^{225}Ac)PO_4@GdPO_4@Au\text{-}mAb\text{-}201b$ show high uptake in the lung when targeted (left column). When competed with mAb-201b (middle column) and when untargeted (right column), uptake occurs in the RES.

endocytosis into lung epithelium. Daughters later in the decay series, such as ${}^{213}Bi$, are harder to retain because prior decays can move the remaining α -emitting nuclides out of the nanoparticle core towards the nanoparticle surface. From this position nearer the surface, subsequent α decays are more likely to release the daughter nuclide from

the nanoparticle. Thus, the retention values for ^{221}Fr should be higher than those for ^{213}Bi . The amount of ^{213}Bi which relocates to the kidney from other tissues shows marked improvement with the $\{\text{La}_{0.5}\text{Gd}_{0.5}\}(\text{}^{225}\text{Ac})\text{PO}_4@\text{GdPO}_4@\text{Au-mAb-201b}$ system compared to core only nanoparticles. Layered $\{\text{La}_{0.5}\text{Gd}_{0.5}\}(\text{}^{225}\text{Ac})\text{PO}_4@4\text{ GdPO}_4\text{ shell@Au}$ nanoparticles allow only 3% of ^{213}Bi ID to migrate to the kidney after 1 hour and 1.5% after 24 hours. In the core only $\text{La}(\text{}^{225}\text{Ac})\text{PO}_4$ system, 10% of the ^{213}Bi ID relocated to the kidney after 1 hour and 5% after 24 hours.

While the $\{\text{Gd}_{0.5}\text{La}_{0.5}\}\text{PO}_4@4\text{ GdPO}_4\text{ shell@Au-PEG-mAb 201b}$ nanoparticles clear rapidly from the lungs into the liver and spleen, previous strategies used to reduce reticuloendothelial functioning such as treatment with clodronate liposomes could be applied to mitigate the rapid clearance.^{104, 105, 106} Uptake at various time points as a result of injecting mice with clodronate liposomes prior to injection of $\{\text{La}_{0.5}\text{Gd}_{0.5}\}(\text{}^{225}\text{Ac})\text{PO}_4@4\text{ GdPO}_4\text{ shell@Au-mAb-201b}$ or $\{\text{La}_{0.55}\text{Gd}_{0.75}\}(\text{}^{225}\text{Ac})\text{PO}_4@4\text{ LaPO}_4\text{ shell@Au-mAb-201b}$ nanoparticles is shown in Figures 3.5.7 A-D.

Using clodronate liposomes drastically improves target tissue uptake at each time point. The liposomes selectively deplete macrophages, which are responsible for clearing large particulates such as nanoparticles from the bloodstream. At 1 hour, clodronate treated mice exhibit $46.7 \pm 1.8\% \text{ID}$ ($327 \pm 36\% \text{ID/g}$) lung uptake compared with $30.2 \pm 3.6\% \text{ID}$ ($204 \pm 25\% \text{ID/g}$) in saline treated mice. Proportions are similar at the 24 hour and 48 hour time points for $\{\text{Gd}_{0.5}\text{La}_{0.5}\}(\text{}^{225}\text{Ac})\text{PO}_4@4\text{ GdPO}_4\text{ shell@Au}$ nanoparticles. The difference in uptake between mice treated with clodronate and

Figure 3.5.7 A: Nanoparticle uptake in major organs after one hour in mice injected with clodronate liposomes or saline followed by the $\{Gd_{0.75}La_{0.25}\}({}^{225}Ac)PO_4@4 LaPO_4$ shell@Au-201b system.

Figure 3.5.7 B: Nanoparticle uptake in major organs after one hour in mice injected with clodronate liposomes or saline followed by the $\{Gd_{0.75}La_{0.25}\}({}^{225}Ac)PO_4@4 LaPO_4$ shell@Au-201b system.

Figure 3.5.7 C: Change in lung uptake over time in mice treated with clodronate liposomes or saline followed by the $\{\text{Gd}_{0.75}\text{La}_{0.25}\}(\text{}^{225}\text{Ac})\text{PO}_4@4\text{ LaPO}_4\text{ shell}@Au\text{-}201\text{b}$ system.

Figure 3.5.7 D: Change in lung uptake over time in mice injected with clodronate liposomes or saline followed by the $\{\text{Gd}_{0.5}\text{La}_{0.5}\}(\text{}^{225}\text{Ac})\text{PO}_4@4\text{ GdPO}_4\text{ shell}@Au\text{-}201\text{b}$ system.

those treated with saline appears to be almost exclusively due to clearance by the RES. Mice injected with clodronate and $\{\text{Gd}_{0.75}\text{La}_{0.25}\}({}^{225}\text{Ac})\text{PO}_4@4\text{LaPO}_4$ nanoparticles show 7.1% higher uptake in the lung than their saline-injected counterparts. Combined increases in liver and spleen uptake by the saline-injected mice (7.3%) mirror the decrease in lung uptake. The drastic difference in lung uptake after a time as short as 1 hour indicates how quickly the RES clears the nanoparticles from circulation without pre-injection of clodronate liposomes. Unlike many antibodies, mAb 201b quickly localizes to its target tissue due to the vascular availability of the thrombomodulin receptor. This short targeting time allows high uptake even with RES interference. After knocking out the RES, nanoparticle uptake and residence time in lung approaches that of the unconjugated antibody.

In addition to increasing lung uptake, clodronate liposomes also prolong residence time in lung tissue. After 24 hours, only 35% of the activity from $\{\text{Gd}_{0.75}\text{La}_{0.25}\}({}^{225}\text{Ac})\text{PO}_4@4\text{LaPO}_4@Au$ nanoparticles clears from the lung in the clodronate treated mice compared with 81.3% in the saline treated mice over the same time period. $\{\text{Gd}_{0.5}\text{La}_{0.5}\}({}^{225}\text{Ac})\text{PO}_4@4\text{GdPO}_4\text{ shell}@Au$ nanoparticles also show prolonged nanoparticle residence time in lung tissue. After 24 hours, 21.6% of the initial uptake in the lung clears in the clodronate treated mice while 33.1% of the initial activity clears in the non-clodronate treated mice.

The nanoparticle-mAb 201b conjugates show high retention of ${}^{213}\text{Bi}$ in the target lung tissue. After 1 hour, $70.4 \pm 0.8\%$ of the ${}^{213}\text{Bi}$ remains in the lung. This number

increases to $91.4 \pm 0.7\%$ after 24 hours. Electron microscopy of non-radioactive $\{\text{Gd}_{0.5}\text{La}_{0.5}\}(\text{}^{225}\text{Ac})\text{PO}_4@4 \text{ GdPO}_4 \text{ shell}@ \text{Au-mAb 201b}$ conjugates shows the conjugates both lining the vascular space connected to lung epithelium, and internalized into the lung epithelial cells. Figure 3.5.4 further shows NPs during the process of endocytosis into a lung epithelial cell. Retention of daughter radionuclides by the nanoparticles themselves should theoretically remain constant and can be measured as previously described.⁴⁸ As the nanoparticles are internalized *in vivo*, the cell itself augments the inherent capability of the nanoparticles to contain daughter radionuclides, increasing the effective daughter sequestration at the target site.

Therapy experiments using the EMT-6 model system show multiple small lung colonies. Group 1 (PBS) averaged 77.6 ± 34.5 colonies per tissue section area. Group 2 (nanoparticles with competition) showed fewer colonies (57 ± 22.3 colonies per slide). Finally, group 3 (targeted nanoparticles without competition) had drastically fewer colonies with only 20.8 ± 7.5 colonies per slide. Images of a representative section of tissue from each treatment group are shown in Figure 3.7.6. Additionally, Figure 3.7.7 shows a magnified version of the PBS treatment group with labels for tumor colonies and artifactual staining of macrophages/type II alveolar cells.

Figure 3.5.8: Stained slides showing EMT-6 tumor colonies present in lung tissue of mice treated with PBS, $\{Gd_{0.5}La_{0.5}\}({}^{225}Ac)PO_4@GdPO_4@Au$ nanoparticles with competition from mAb 201b, or those same particles without competition.

Figure 3.5.9: A stained slide from the PBS treatment group labeling lung tumor colonies as well as artifactual staining of macrophages and type II alveolar cells.

The EMT-6 therapy experiment shows promising results for future work with more specific antibody targeting. Despite targeting the organ rather than the tumor cells, the treatment group shows clear, statistically significant ($p = 0.019$) decreases in tumor colony growth relative to both PBS and competition from free antibody ($p = 0.020$). Competition with non-radioactive mAb 201b decreases lung uptake by more than a factor of 17. However, the lung in the competition group still receives ~ 1.0 kBq of ^{225}Ac after one hour. This small amount of ^{225}Ac is insufficient to produce a significant ($p = 0.300$) therapeutic effect relative to PBS controls. Higher uptake in the lung in the treatment group (17.3 kBq after one hour) produces the larger therapeutic effect. No abnormal side effects were observed during the 1 week observation period. TEM images from the mice confirm no inflammation in tissues of the kidney as a result of radiation or nanoparticle exposure. Previous experiments with ^{225}Ac and EMT-6 tumors led to pulmonary fibrosis and renal failure from failure to retain ^{213}Bi daughters in the target tissue.¹⁰⁷ Future therapeutic doses could therefore likely be increased without inducing fatal side effects.

3.5.3 Brain Targeting with Anti-Synaptophysin and Anti-GFAP

Utilizing targeted, short range therapeutics based on α particles could revolutionize treatment of tumors in vital organs such as the brain. Due to the vital importance of the brain in regulating bodily function, a number of evolutionary protections have developed to limit the flow of non-essential material into the tissue. While the series of tight junctions and barrier cells that constitute the blood-brain

barrier protect brain tissue from pathogens, they also frustrate attempts to deliver therapeutics to the brain. Difficulty associated with side effects of neurosurgery often precludes surgical resection of brain tumors depending on the location of the neoplasm. Nanoparticles of approximately 20 nm metallic diameter or smaller show the ability to traverse the blood-brain barrier, sparking interest in nanoparticle-based therapies for central nervous system (CNS) disorders such as Alzheimer's disease.^{108,109}

In vitro, nanoparticles bound to antibodies relevant to CNS tissue retain their biological activity. Murine brain tissue sections incubated with anti-synaptophysin labeled $\{\text{Gd}_{0.5}\text{La}_{0.5}\}\text{PO}_4@4 \text{ GdPO}_4 \text{ shell}@Au$ nanoparticles show binding to the basal ganglia and synapses. Surrounding white matter does not show staining, indicating that the antibody retains its biological activity and specificity after binding to the nanoparticle. The staining process involves a number of steps. First, the nanoparticle-primary antibody conjugates bind their antigen receptors in the brain. Next, a secondary antibody contains a peroxidase enzyme and binds to the primary antibody in the tissue section. In peroxide, DAB is converted into a brown, insoluble product and gives a brown stain. Figure 3.5.10 shows the immunohistochemistry of anti-synaptophysin labeled nanoparticles.

Figure 3.5.10: Immunohistochemistry of brain tissue with anti-synaptophysin labeled nanoparticles. Anti-synaptophysin labeled nanoparticles maintain biological activity and specificity after binding to the nanoparticles. Basal ganglia cells with nanoparticles bound are stained by the DAB color agent while adjacent white matter is not.

Similarly, anti-GFAP labeled nanoparticles retain their affinity and specificity for receptors after binding to the nanoparticle surface. Figure 3.5.11 shows immunohistochemistry in normal murine brain. Astrocyte cells show staining by the DAB color agent, while surrounding brain tissue does not. Blood vessels in the brain exhibit artifactual staining as well.

➡ Astrocytes

↪ Blood vessels

Figure 3.5.11: Immunohistochemistry of brain tissue with anti-GFAP labeled nanoparticles. Anti-GFAP labeled nanoparticles also retain their affinity and specificity for biological targets after binding to the nanoparticle surface. Brown areas indicated by large arrows illustrate astrocytes while small arrows illustrate artifactual staining of blood vessels.

For *in vivo* brain receptor experiments, mice were cannulated in the left femoral or axillary vein via partial surgical dissection. The surgical area was first shaved, then dissected using auto-cauterizers to minimize blood loss. This technique ensures that all of the injected dose reaches the bloodstream for consistent, reliable delivery. Utilizing a

catheter also allows for multiple injections using a single point. Figure 3.5.12 shows the catheter inserted into a mouse femoral vein.

Figure 3.5.12: A catheter inserted into the left femoral vein aids injection reproducibility and allows for multiple injections at a single site.

Biodistribution experiments performed with NAA indicate that nanoparticle-mAb conjugates exhibit uptake in the brain. Both anti-synaptophysin and anti-GFAP antibodies show uptake of 0.1 % ID. In each case, the measurement strained MURR's detection limit of NAA for gold of 0.1 ng. However, the low level of detection is

consistent across six mice, lending credibility to the measurement. Unlabeled nanoparticles were not detected in the brain tissue in any of the mice examined.

In order to confirm that the nanoparticle-mAb conjugates are specifically targeting their receptors in the brain and not merely present in the blood vasculature, brain tissue sections were fixed and examined by TEM. Figures 3.5.13 and 3.5.14 show representative images of anti-synaptophysin labeled nanoparticles present at CNS synapses and anti-GFAP labeled nanoparticles present by astrocytes.

Mannitol creates a hyperosmotic environment in CNS arteries, causing water loss in the CNS epithelial cells and stretching the tight junctions.¹¹⁰ TEM images show that the tight junctions of the blood brain barrier have not been disrupted, despite injection of mannitol for that purpose. Because the tight junctions remain largely intact, another mechanism must be responsible for the presence of large nanoparticles in the brain. As in the lung, the nanoparticles most likely extravasate out of the vasculature through an endocytotic mechanism. Vascular receptors similar to synaptophysin and GFAP mediate the endocytosis, importing only those materials necessary to CNS function. Nanoparticles bound to the anti-synaptophysin and anti-GFAP antibodies piggyback on this interaction, and are also endocytosed.

Figure 3.5.13: $\{\text{Gd}_{0.5}\text{La}_{0.5}\}\text{PO}_4@4\text{ GdPO}_4\text{ shell}@Au$ nanoparticles labeled with anti-synaptophysin bind receptors in CNS synapses.

Figure 3.5.14: $\{\text{Gd}_{0.5}\text{La}_{0.5}\}\text{PO}_4@4\text{ GdPO}_4\text{ shell}@Au$ nanoparticles labeled with anti-GFAP bind receptors in astrocytes.

3.6 Summary of Findings

A central problem facing *in vivo* α generators has been control of daughter radionuclides. Historically, this lack of retention has led to lethal dose to non-target organs, limiting the efficacy of these systems. The work presented in Chapter 3 elucidates a general solution to the problem of daughter retention through the use of layered nanoparticles. The most successful system consisting of $\{\text{Gd}_{0.5}\text{La}_{0.5}\}\text{PO}_4@4$ GdPO_4 shell@Au nanoparticles contained 90% of the first decay daughter, ^{221}Fr , *in vitro*. *In vivo* results show retentions of > 90% of the third decay daughter, ^{213}Bi , after 24 hours. Retention *in vivo* increases over time as a result of higher percentages of endocytosed nanoparticles. Finally, the nanoparticles can be linked to a variety of different targeting agents for treatment of a wide variety of neoplastic disease.

Given the success of the layered nanoparticle system in containing daughter emissions from ^{225}Ac , the general strategy should be extended to other α -generator radionuclides such as ^{227}Th or ^{223}Ra . Diversifying to these other α -emitters would allow clinical tailoring of α energies and daughter chemistry/half-lives to the specific disease. Preparing a variety of layered nanoparticle formulations which work with α -generator radionuclides will decrease the time required for clinical translation once these radionuclides become available in large quantities.

Chapter 4:

FUTURE DIRECTIONS AND ONGOING WORK

4.1 Other Lanthanides as Radiotherapeutics

4.1.1 Nuclear and Chemical Properties of the Lanthanides

While the nanoparticles examined in this work focused on utilizing lanthanum and gadolinium, a number of other lanthanides should co-crystallize into LnPO_4 nanoparticles. Fortunately, a number of other lanthanides also possess favorable nuclear properties for β^- radiotherapy. A similar synthetic process and gold surface modification should work for a variety of different lanthanides. Moving across the period, the lanthanide contraction causes large disparities in ionic radii. Extremes such as La^{3+} (1.18 Å) and Lu^{3+} (1.00 Å) may not co-crystallize.⁹⁷ However, mixed lanthanide particles may not be necessary if the lanthanide possesses a sufficient magnetic moment to be separated magnetically.

Many lanthanides are under consideration or currently in use for biomedical purposes including ^{149}Pm (β^- cancer therapy), $^{111}\text{ }^{153}\text{Sm}$ (Quadramet, β^- pain palliation in bone metastases), ^{112}Eu (fluorescence imaging probes), $^{113}\text{ }^{159}\text{Gd}$ (Auger electron cancer

therapy), $^{114} \text{ } ^{161} \text{Tb}$ (β^- cancer therapy), $^{115} \text{ } ^{166} \text{Dy}/^{166} \text{Ho}$ (β^- arthritis therapy) $^{116} \text{ } ^{169} \text{Er}$ (β^- arthritis therapy), $^{117} \text{ } ^{175} \text{Yb}$ (β^- bone pain palliation), 118 and $^{177} \text{Lu}$ (β^- cancer therapy).¹¹⁹ Only a few of these ($^{149} \text{Pm}$, $^{166} \text{Ho}$, and $^{177} \text{Lu}$) are available as no carrier added radionuclides. For all of the other radionuclides, only a small percentage of bioconjugates will deliver radiation to the intended target. Incorporating the radionuclides into a nanoparticle would dramatically increase the amount of radioactivity bound to each targeting vector. Radionuclides that are available no carrier added could also use nanoparticle constructs to increase the delivered dose. In order to determine the feasibility of this approach, nanoparticles composed of TbPO_4 , HoPO_4 , and LuPO_4 were synthesized.

4.1.2 Synthesis and Characterization of TbPO_4 , HoPO_4 , and LuPO_4 Nanoparticles

Nanoparticles consisting of TbPO_4 , HoPO_4 , and LuPO_4 were synthesized similarly to 3.2.3. Briefly, 1 mL of 0.1 M $\text{Tb}(\text{NO}_3)_3 \cdot 5 \text{H}_2\text{O}$, HoCl_3 , or $\text{LuCl}_3 \cdot 6 \text{H}_2\text{O}$ was added to a 5 mL v-bottom vial with spin vane and 2 mL of 0.1 M Na-TPP. Each vial was heated for 3 hours at 90°C . Nanoparticles were transferred into scintillation vials and placed by a 0.6 T surface field NdFeB magnet to determine if they could be magnetically separated. LuPO_4 nanoparticles were dialyzed against 18 M Ω water using a 10 kDa MWCO regenerated cellulose membrane overnight, then collected and dried over low heat. Dried particles were crushed using a mortar and pestle, then examined using XRD as described in 3.2.9.

4.1.3 Results and Discussion

TbPO₄, HoPO₄, and LuPO₄ all formed nanoparticle solutions similar to previous LnPO₄ combinations. TbPO₄ and HoPO₄ nanoparticles both showed strong preference towards the magnet and separated cleanly. LuPO₄ nanoparticles on the other hand showed no preference for the magnet and settled uniformly on the bottom of the vial. Of the lanthanides, gadolinium is most often used as a magnetic agent, due to its 7 unpaired f electrons in its preferred 3+ oxidation state. While terbium (6 unpaired electrons in the 3+ oxidation state) and holmium (4 unpaired electrons in the 3+ oxidation state) should exhibit a lower paramagnetic magnetic moment than gadolinium, it still appears sufficient to facilitate magnetic separation. In contrast, lutetium (0 unpaired electrons in the 3+ oxidation state) shows no paramagnetic moment. As a result, ¹⁷⁷Lu would need to be paired with a more magnetic lanthanide such as gadolinium in order to separate the nanoparticles magnetically.

XRD studies indicate that LuPO₄ crystallizes in the anhydrous monazite crystal phase, similar to that observed for GdPO₄ and mixtures of LaPO₄ and GdPO₄. The XRD pattern is shown below in Figure 4.1.1. Rietveld refinement indicates that the grain size of these particles is 1.76 nm, somewhat smaller than the other LnPO₄ systems previously analyzed.

Figure 4.1.1: XRD pattern of LuPO_4 indicates a monazite crystal structure with grain size of 1.76 nm.

4.1.4 Conclusions

A number of lanthanides could easily work in the platform described in Chapter 3. Given the large quantity of ^{177}Lu produced at MURR, investigation of this radionuclide makes particular sense at Missouri. XRD studies indicate that a nanoparticle similar in structure to previously studied constructs is formed under similar conditions. Ongoing experiments involving synthesis of a mixed $\{\text{Gd}_{0.5}^{177}\text{Lu}_{0.5}\}\text{PO}_4$ nanoparticle are in

progress under the direction of fellow graduate student Nick Sobol. Gold coating of the particles should occur analogously and similarly facilitate attachment of biologically relevant targeting agents.

4.2 Potential for ^{227}Th or ^{223}Ra generators

4.2.1 Impetus for ^{227}Th or ^{223}Ra generators

As mentioned in Chapter 1, ^{227}Th and ^{223}Ra are also being considered as potential *in vivo* α generator radionuclides. Further, the higher availability of ^{227}Th and ^{223}Ra avoid the research and clinical trial bottleneck found with ^{225}Ac . While ^{223}Ra as Alpharadin inherently targets bone metastases, incorporation into a LnPO_4 nanoparticle could broaden its clinical applicability. In order to test the ability of LnPO_4 as a carrier for ^{223}Ra , barium was added to a synthesis of $\{\text{Gd}_{0.5}\text{La}_{0.5}\}\text{PO}_4$ nanoparticles as a surrogate for radium. Radium and barium share a common preferred oxidation state (2+) and similar 8-coordinate ionic radii ($\text{Ba} = 1.56 \text{ \AA}$, $\text{Ra} = 1.62 \text{ \AA}$).⁹⁷

Similarly, ^{227}Th should be investigated for its potential to dope into LnPO_4 nanoparticles. Previous work indicates that the lanthanide Ce^{+4} cocrystallizes well with La^{+3} to form fluorescent rhabdophane nanocrystals.¹²⁰ The Ce^{+4} ion should function well as a surrogate for ^{227}Th . Both elements easily access the 4+ oxidation state and have similar 8-coordinate ionic radii ($\text{Ce}^{4+} = 1.11 \text{ \AA}$, $\text{Th}^{4+} = 1.19 \text{ \AA}$).⁹⁷

4.2.2 Barium nanoparticles experimental

A solution of 500 μL of 0.1 M $\text{LaCl}_3 \cdot 7 \text{H}_2\text{O}$, 500 μL of 0.1 M $\text{GdCl}_3 \cdot 6 \text{H}_2\text{O}$, 50 μL of 0.1 M BaCl_2 , and 2 mL of 0.1 M Na-TPP were mixed in a 5 mL v-bottom vial with spin vane. The solution was heated for 3 hours at 90°C .

4.2.3 Ba nanoparticles results and discussion

Nanoparticles with Ba dopants formed large aggregates that would not disperse in solution. It is likely that $\text{Ba}_3(\text{PO}_4)_2$ clumps formed rather than doping into the LnPO_4 nanoparticles. The oxidation state and size mismatch between barium and the lanthanides likely preclude co-crystallization. While it may be possible to dope ^{223}Ra into LnPO_4 nanoparticles on the tracer level, other barium containing nanoparticles such as BaTiO_3 ,¹²¹ BaSO_4 ,¹²² or $\text{BaFe}_{12}\text{O}_{19}$ ¹²³ should be more promising.

4.2.4 Conclusion

Oxidation state and size appear to be the defining factors that influence co-crystallization. While ^{223}Ra may not work well with LnPO_4 , ^{227}Th could likely co-crystallize into LnPO_4 nanoparticles containing a significant fraction of Ce^{4+} ions. Successful doping of Ce^{4+} ions into LnPO_4 nanoparticles has already been demonstrated.¹²⁴ Additionally, thorium doping itself could be done with natural thorium rather than the less common ^{227}Th in initial experiments.

4.3 Future Clinical Applications

While proof of principle targeting with mAb 201b showed high, receptor specific uptake, the thrombomodulin target has little clinical relevance to cancer therapy. Additional antibodies which target receptors overexpressed on tumors should be utilized to determine clinical applicability of the nanoparticle system. As detailed in Sections 1.3 and 1.4, the short range of α emission and large size of nanoparticles limit potential application of the nanoparticle system to small metastases with vascular targets, circulating neoplasms, peritoneal tumors, and intratumoral injections. Utilizing vascular targeting agents such as F3⁴⁰ removes the necessity for nanoparticles to extravasate out of the vasculature.

Another potentially viable strategy to capitalize on the advantages of nanoparticle systems involves targeting cancers of the circulatory system. In particular, a wide variety of leukemias and lymphomas should be treatable with appropriate targeting agents. Targets such as CD20 (the same target used in the clinically approved BEXXAR radiopharmaceutical)¹²⁵ and CD45¹²⁶ are overexpressed on a number of blood-borne cancers. Antibodies that target these receptors, such as 1F5¹²⁷ (CD20) and BC8¹²⁸ (CD45), are currently being investigated in cell pellet and mouse studies with the described LnPO₄ nanoparticle system in collaboration with the Fred Hutchinson Cancer Research Center in Seattle, Washington.

Additionally, tumors of the peritoneum tend to form thin films.¹²⁹ Cancers originating in the peritoneal cavity (ovarian, liver, pancreatic, colorectal, gastric, and peritoneal mesothelioma) have high rates of locoregional metastasis to other locations within the peritoneal cavity.¹³⁰ Administration of nanoparticles directly into the peritoneal cavity would also bypass the need for extravasation out of the vasculature. Additionally, α therapy is ideal for treating the thin cancerous films and metastases found in peritoneal cancers. Panitumumab, an antibody against EGFR conjugated to the particles in 3.4, may be an effective therapy for these peritoneal cancers. Biodistribution studies are currently underway in collaboration with the National Cancer Institute.

Finally, α therapeutic nanoparticles can be injected directly into tumors as a brachytherapy analog. The solution of nanoparticles may perfuse throughout the target tissue, providing better coverage and lower whole body dose than current seed based therapies. More work is needed to determine the degree of perfusion in these therapies as well as the tissue retention of the nanoparticles over time.

APPENDIX

Appendix 1: Total biodistribution of starch-bombesin gold nanoparticles

Compound:	¹⁹⁸ Au-NP (Starch) (I.P.)			130 kBq/100μL
Date:	8/18/2010	Tumor:	PC-3	
Time :	2 Hour	Strain:	SCIDs	5 mice
Blocker:	Saline (I.V.)	Dose Cts.	503838	
<u>Organ</u>	<u>Avg %ID</u>	<u>STDEV</u>	<u>Avg%ID/g</u>	<u>STDEV</u>
Blood	9.83	5.27	7.26	3.20
Heart	0.06	0.06	0.37	0.46
Lung	0.12	0.07	0.46	0.41
Liver	5.34	2.33	4.55	2.21
Spleen	0.85	0.36	9.32	5.72
Stomach	3.87	1.26	13.17	11.59
L Int	5.72	2.88	5.82	3.36
Sm Int	12.93	9.61	10.64	8.63
Kidney	0.42	0.33	1.28	1.21
Urine	0.01	0.01		
Abdominal Fat	31.86	28.55	127.26	115.98
Muscle	0.01	0.01	0.03	0.03
Bone	0.00	0.00	0.05	0.05
Bladder	0.23	0.14	7.81	6.84
Brain	0.00	0.00	0.00	0.00
Pancreas	0.39	0.43	11.05	10.22
Carcass	35.39	19.32	13.67	1.05
Tumor A	0.02	0.02	0.06	0.06

Compound:	¹⁹⁸Au-NP (Starch) (I.P.)			130 kBq/100μL
Date:	8/18/2010	Tumor:	PC-3	
Time :	2 Hour	Strain:	SCIDs	5 mice
Blocker:	Cold BBN (I.V.)		Dose Cts.	503838.2
<u>Organ</u>	<u>Avg %ID</u>	<u>STDEV</u>	<u>Avg%ID/g</u>	<u>STDEV</u>
Blood	12.81	10.87	9.44	6.95
Heart	0.07	0.11	0.61	0.97
Lung	0.58	0.33	2.57	1.71
Liver	7.70	2.06	6.62	1.48
Spleen	0.78	0.45	8.43	7.22
Stomach	6.48	3.20	9.23	4.26
L Int	5.35	1.13	5.49	1.22
Sm Int	10.11	7.29	6.67	3.72
Kidney	0.84	0.38	2.59	1.15
Urine	0.01	0.01		
Abdominal Fat	20.19	16.23	89.28	96.06
Muscle	0.12	0.22	0.70	1.43
Bone	0.01	0.01	0.18	0.36
Bladder	0.23	0.13	6.75	5.80
Brain	0.00	0.00	0.01	0.01
Pancreas	0.92	0.31	22.01	9.07
Carcass	42.13	6.84	11.04	0.44
Tumor A	0.05	0.04	0.17	0.13

Compound:	¹⁹⁸Au-BBN-NP (I.P.)			130 kBq/100μL
Date:	8/18/2010	Tumor:	PC-3	
Time :	2 Hour	Strain:	SCIDs	5 mice
Blocker:	Saline (I.V.)		Dose Cts.	541810.2
<u>Organ</u>	<u>Avg %ID</u>	<u>STDEV</u>	<u>Avg%ID/g</u>	<u>STDEV</u>
Blood	7.10	18.18	3.66	5.36
Heart	0.05	0.08	0.34	0.65
Lung	0.08	0.12	0.33	0.52
Liver	3.71	3.67	2.23	2.78
Spleen	0.43	0.51	6.52	6.98
Stomach	3.07	2.19	4.22	3.34
L Int	2.06	2.13	2.22	2.69
Sm Int	2.79	3.38	1.80	2.36
Kidney	0.22	0.41	0.67	1.08
Urine	3.59	6.42		
Abdominal Fat	42.62	40.30	136.53	135.59
Muscle	0.01	0.03	0.02	0.02
Bone	0.00	0.00	0.05	0.08
Bladder	0.21	0.39	7.74	12.10
Brain	0.00	0.00	0.00	0.00
Pancreas	0.36	2.16	7.79	9.06
Carcass	39.80	30.12	21.84	2.09
Tumor A	0.01	0.01	0.04	0.07

Compound:	¹⁹⁸ Au-BBN-NP (I.P.)			130 kBq/100μL
Date:	8/18/2010	Tumor:	PC-3	
Time :	2 Hour	Strain:	SCIDs	5 mice
Blocker:	Cold BBN (I.V.)		Dose Cts.	541810.2
<u>Organ</u>	<u>Avg %ID</u>	<u>STDEV</u>	<u>Avg%ID/g</u>	<u>STDEV</u>
Blood	10.66	10.27	7.95	6.31
Heart	0.02	0.02	0.08	0.20
Lung	0.27	0.31	1.04	1.62
Liver	3.50	3.49	3.19	2.89
Spleen	0.75	0.78	8.06	12.04
Stomach	2.26	2.50	4.45	6.23
L Int	3.46	3.65	4.06	4.73
Sm Int	5.06	5.12	3.78	3.28
Kidney	0.65	0.91	1.76	3.00
Urine	0.17	0.34		
Abdominal Fat	18.01	22.95	79.45	143.92
Muscle	0.02	0.02	0.08	0.10
Bone	0.00	0.00	0.06	0.07
Bladder	0.09	0.08	1.78	3.13
Brain	0.00	0.00	0.00	0.00
Pancreas	0.56	0.56	9.87	16.93
Carcass	61.58	34.05	9.99	1.69
Tumor A	0.01	0.01	0.02	0.03

Appendix 2: XRD scan parameters

Goniometer=PW3050/60 (Theta/Theta); Minimum step size 2Theta:0.001; Minimum step size Omega:0.001

Sample stage=Multi purpose sample stage; Minimum step size Phi:2.5

Diffraction system=XPRT-PRO

Measurement program=10-60 90 min, Owner=CNMSuser, Creation date=6/30/2011 1:41:07 PM

Scan Axis	Gonio
Start Position [$^{\circ}$ 2Th.]	16.0682
End Position [$^{\circ}$ 2Th.]	59.9802
Step Size [$^{\circ}$ 2Th.]	0.0080
Scan Step Time [s]	59.6900
Scan Type	Continuous
PSD Mode	Scanning
PSD Length [$^{\circ}$ 2Th.]	2.12
Offset [$^{\circ}$ 2Th.]	0.0000
Divergence Slit Type	Fixed
Divergence Slit Size [$^{\circ}$]	0.2500
Specimen Length [mm]	10.00
Measurement Temperature [$^{\circ}$ C]	25.00
Anode Material	Cu
K-Alpha1 [$\text{\AA}$]	1.54060

K-Alpha2 [Å]	1.54443
K-Beta [Å]	1.39225
K-A2 / K-A1 Ratio	0.50000
Generator Settings	40 mA, 45 kV
Diffractometer Type	0000000011010092
Diffractometer Number	0
Goniometer Radius [mm]	240.00
Dist. Focus-Diverg. Slit [mm]	100.00
Incident Beam Monochromator	No
Spinning	No

Appendix 3: Antibody:Nanoparticle Ratio Calculation

Nanoparticle mass:

Mass of individual NP = core volume (core density) + (layer volume) (layer density)

$$\begin{aligned} &= \frac{4}{3}\pi (1.12 \times 10^{-6} \text{ cm})^3 (5 \text{ g/cm}^3) + (\frac{4}{3}\pi (1.17 \times 10^{-6} \text{ cm})^3 - \frac{4}{3}\pi (1.12 \times 10^{-6} \text{ cm})^3) (19.3 \text{ g/cm}^3) \\ &= 4.89 \times 10^{-18} \text{ g/nanoparticle} \end{aligned}$$

Number of nanoparticles:

Number of nanoparticles = mass of nanoparticles/mass of individual nanoparticle

Mass of nanoparticles determined by synthetic conditions

$$\begin{aligned} &= 0.00088 \text{ g} / 4.89 \times 10^{-18} \text{ g} \\ &= 1.8 \times 10^{14} \text{ nanoparticles} \end{aligned}$$

Antibody mass determined by Lowry assay or ^{125}I radiolabeling

Panitumumab antibodies = mass mAb/molecular weight mAb * Avogadro's number

$$\begin{aligned} &= 0.00019 \text{ g} / 150,000 \text{ g/mol} * 6.022 \times 10^{23} \text{ molecules/mol} \\ &= 7.63 \times 10^{14} \text{ antibodies} \end{aligned}$$

Ratio of panitumumab:nanoparticle:

$$7.63 \times 10^{14} \text{ antibodies} / 1.8 \times 10^{14} \text{ nanoparticles} = 4.2 \text{ antibodies/nanoparticle}$$

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Vita

Mark Francis McLaughlin was born February 22, 1987 to Donald and Cheryl McLaughlin. He grew up in Lee's Summit, MO and graduate valedictorian from Lee's Summit High School. Following graduation, he attended the University of Notre Dame in South Bend, IN, earning degrees in chemistry and philosophy. Summer research experiences radiochemistry at the University of Missouri and Brookhaven National Laboratory sparked an interest in radiopharmaceutical research. He returned to Missouri in 2009 to begin work on his Ph.D., defending his dissertation in 2013. Following his doctorate, Mark will attend medical school to gain more insight into the biological basis of disease before pursuing a career as a medical research scientist and clinician.