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<https://doi.org/10.18297/etd/2763>

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MAGNETIC FIELD-INDUCED INTRAMOLECULAR
CYCLIZATION AS A TRIGGER FOR NANOPARTICLE-
BASED DELIVERY SYSTEMS

By

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B.S., University of Louisville, 2012

M.S., University of Louisville, 2015

A Dissertation

Submitted to the Faculty of the

College of Arts & Sciences of the University of Louisville

in Partial Satisfaction of the Requirements

for the Degree of

Doctor of Philosophy in Chemistry

Department of Chemistry

University of Louisville

Louisville, Kentucky

August 2017

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A Dissertation Approved on

July 11, 2017

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ACKNOWLEDGEMENTS

First and foremost I would like to thank my research advisor, Dr. Michael Nantz. He knew me as a lowly sophomore, taking his organic final and offering me sour patch kids, and I never thought I would read a “work for me” post-it when choosing an advisor. He pushed me more as a chemist than I ever thought possible, and I am extremely grateful to be one of his last PhD advisees (seriously...no more!).

Thank you to my committee members. One of the first courses in graduate school was bioinorganic chemistry, a special topics course in inorganic chemistry taught by Dr. Buchanan, and I am thankful that he helped spark my interest in connecting biology with chemistry. I am thankful to have collaborated with Dr. O'Toole and his lab because they taught me how challenging but exciting nanoparticle research can be. Dr. Richter showed me how important teaching is, and I am very thankful that she took the time and energy to be my teaching mentor.

I am grateful to my former lab members, especially Seb who trained me, and Ralph and Stephanie. From the crazy techno music to jousting in the hallway, you made my graduate school career enjoyable. You also provided great leadership in the lab, and I knew I could come to you with any problem, chemistry or otherwise. You gave me the confidence and inspiration to do great things, and I'm so thankful to have worked in lab with you.

I am extremely grateful for the friends and family who have helped me throughout my time in graduate school. To my best friend Maggie, who consoled me when my nanobots weren't working and shared in the celebration when they finally did. To my surrogate family PR, Danya, Neal and Noah, who provided a chance for me to recharge whenever I was home. To my mom, who was my constant

cheerleader. To my dad, who kept me knowledgeable about Kentucky politics but also knew how to give me a good laugh. To my teammates on Cardinal Masters, who were so supportive, both in and out of the pool. And finally, I give thanks to God who gave me the abilities and opportunities to pursue all that I have done.

ABSTRACT

MAGNETIC FIELD-INDUCED INTRAMOLECULAR CYCLIZATION AS A TRIGGER FOR NANOPARTICLE- BASED DELIVERY SYSTEMS

Sara Katherine Biladeau

July 11, 2017

Magnetic nanoparticles (MNPs) are used in a variety of applications, including as agents for magnetic resonance imaging, generation of local hyperthermia, and as platforms for drug delivery. Iron-based MNPs are often coated with a shell, such as silica or gold, to increase biocompatibility for drug delivery applications. Many MNPs used for cancer therapy rely on either an internal trigger, such as a difference in pH, or an external trigger, such as light or an alternating magnetic field (AMF), to cause release of a payload, typically a chemotherapeutic drug. Internal triggers are appealing because drug release can be targeted to a tumor environment, but a major drawback is untriggered release, or release prior to reaching the targeted area. To address the problem of untriggered release, researchers have explored the use of thermally responsive triggers on iron oxide nanoparticles. In these cases, payload release occurs as a result of local hyperthermia that is induced by application of an alternating magnetic field. NP-linker-drug motifs containing an azo functionality or a substructure prone to retro-Diels Alder reaction are examples of thermally responsive triggers. This thesis work expands AMF-mediated drug delivery by demonstrating a new mechanism for substrate release: intramolecular cyclization of a linking tether between payload and nanoparticle. Described is a linker system (LS) fitted with a thiol moiety, a secondary amine, carbonate

functionality, and a payload. Attachment of the linker system to gold-coated iron oxide nanoparticles delivers a thermally responsive drug delivery system (Fe@Au-LS-drug). On exposure to an AMF, the magnetic nanoparticles generate heat that powers cyclization of the amine onto the carbonate for payload release to occur. The cyclization mechanism was confirmed by synthesis and testing of an amine-free linker to rule out carbonate hydrolysis as the mode of payload release. Testing the system with a water-soluble fluorophore as payload showed 40% release in response to AMF application with minimal release from the amine-free linker under identical conditions. Payload release could be increased to nearly 100% by addition of PEG-coated iron oxide nanoparticles as a means to increase local hyperthermia. This work shows for the first time that the highly flexible process of intramolecular cyclization can serve in conjunction with magnetic iron-gold nanoparticles as a delivery system trigger for externally triggered applications.

Chapter 1 reviews the role of iron oxide nanoparticles in delivery applications and summarizes the challenges of drug delivery using various internally and externally responsive linkers. Chapter 2 describes different methods for gold-coated iron oxide nanoparticle preparation and the challenges of such core-shell syntheses. Chapter 3 focuses on the synthesis of an amine-based linker system designed for AMF-mediated payload release. Synthesis of the analogous control linker is also described. Chapter 4 describes studies using an AMF to cause payload release from Fe@Au-LS NPs. Chapter 5 presents all experimental procedures and the characterization data for key intermediates, as well as a brief synopsis of previous synthetic work unrelated to this project.

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CHAPTER 1

IRON OXIDE NANOPARTICLES IN DRUG DELIVERY SYSTEMS

- 1.1. INTRODUCTION
- 1.2. PREPARATION METHODS
- 1.3. SURFACE MODIFICATIONS
- 1.4. DRUG DELIVERY CHALLENGES
- 1.5. HYPOTHESIS

1.1. INTRODUCTION

Nanoparticles (NPs) can be prepared using a variety of different organic or inorganic materials, including lipids,¹ gold,² and iron oxide.³ Common applications of NPs include water purification,⁴ catalysis,⁵ and as a platform for drug delivery.⁶ To be most effective *in vivo*, NPs must have a hydrodynamic size between 10-100 nm.⁷ The kidneys filter NPs smaller than 10 nm, and the immune system removes NPs greater than 100 nm via phagocytosis. Of particular interest to us are iron oxide NPs, which are made of Fe₃O₄ or the oxidized form γ -Fe₂O₃. Both of these oxides are magnetic. Researchers have exploited the magnetic properties of Fe₃O₄ NPs in a variety of ways, particularly in magnetic resonance imaging,⁸ induction of hyperthermia,⁹ and drug delivery.⁴

Magnetic resonance (MR) imaging is a common practice for non-invasive examination of the body. Similar to proton nuclear magnetic resonance, MR imaging relies on the alignment, excitation, and subsequent relaxation of the protons of water molecules within the body. Protons of water molecules within different tissues have different relaxation times, which allows for contrast between tissues within the body. After excitation, relaxation occurs in the x-, y-, and z-axes. Contrast agents can be administered to give a better image of certain tissues by changing the relaxation time of the protons. Gadolinium chelates alter the longitudinal (T₁) relaxation rate of surrounding water molecules along the z-axis. At low concentrations, they increase the relaxation time of T₁. This provides positive enhancement, which leads to greater contrast, meaning a brighter spot (Figure 1.1a).¹⁰ Iron oxide NPs less than 30 nm alter the transverse (T₂) relaxation rate along the xy-axis of surrounding water molecules.¹¹ They provide negative contrast, and are seen in the image as a dark spot (Figure 1.1b).¹²

Figure 1.1. MRI contrast agents. a. Murine SV11 brain tumor immediately before (left) and after (right) gadolinium-based contrast injection. Adapted with permission from *J. Immunol.* 2000, 165, 7293-7299. Copyright 2000 The American Association of Immunologists, Inc. b. Iron oxide NP accumulation in murine lymphoma. Adapted with permission from *NMR Biomed.* 2004, 17, 484-499. Copyright 2004 John Wiley and Sons.

Another appealing quality of iron oxide NPs is their ability to generate local hyperthermia upon exposure to an alternating magnetic field (AMF). An AMF is created by an alternating current, which is a current that reverses its direction many times a second at regular intervals. Currents produce magnetic fields, and when a current alternates its direction, the direction of the magnetic field also flips. Once a current is established, the current forms a magnetic field and the magnetic NPs align with it. The current is not static though; it constantly flips 180°, which oscillates the magnetic field. Because the NPs are magnetic, they will attempt to realign with the field as it oscillates, and their movement generates local hyperthermia, as seen in Figure 1.2.

Figure 1.2. Methods of local hyperthermia generation upon exposure to an AMF.

Heat from exposure to an AMF is generated through a hysteresis loss, which is the reversal of the NPs as a whole. This can be broken down into two different models: Néelian relaxation and Brownian relaxation. Néelian relaxation is due to the individual movement of each nanoparticle dipole, whereas Brownian relaxation is due to the physical rotation of the nanoparticle itself.¹³ The heat generated is quantified as specific loss power (SLP) or specific absorption rate (SAR). These terms are interchangeable because both are measurements of heat dissipation per unit mass of magnetic nanoparticles in an AMF.¹⁴ This value can vary depending on a variety of factors, such as NP size,¹⁵ viscosity of supernatant,¹⁶ magnetic field strength and frequency,¹⁷ concentration,¹¹ and polydispersity.¹⁸

Local hyperthermia is especially important in some forms of cancer therapy. Solid tumor cells have poor vasculature that leads to hypoxia and low pH, making cancerous cells more susceptible to hyperthermia.¹⁹ Increased temperatures in or near tumor cells, generally between 40 °C and 45 °C, can lead to apoptosis, or cell death. This

rise in temperature does not affect most healthy cells because their structured vasculature allows for better heat dissipation (up to 20 times increased blood flow in healthy tissue versus 2 times increased blood flow into cancerous cells).¹³ In addition, healthy cells produce heat shock proteins on exposure to local hyperthermia. Heat shock proteins (HSP) allow proteins to refold after denaturation caused by a rise in temperature, or heat shock. Cancerous cells overexpress heat shock proteins and present them on the surface of the cell, which then are easily lysed by killer T-cells.¹⁹ Finally, local hyperthermia as an adjuvant therapy increases a cell's susceptibility to radiation treatment post AMF application, as seen in Table 1.1. If the temperature within a cell reaches above 45 °C, cell ablation occurs. At this temperature, both healthy and cancerous cells are affected, and prolonged exposure causes necrosis.²⁰

Tumor	Treatment	Patients	Effect with hyperthermia (complete remission)	Effect without hyperthermia (complete remission)
Lymph-nodes of head and neck	Radiotherapy	41	83%	41%
Breast	Radiotherapy	306	59%	41%
Lung	Chemotherapy	44	68%	36%
Bladder	Radiotherapy, Surgery	102	94%	67%

Table 1.1. Various tumor treatments with or without adjuvant thermotherapy. The dual therapy shows an increase in number of patients in complete remission after primary treatment with adjuvant hyperthermia treatment versus without hyperthermia. Adapted with permission from *Ann. Oncol.* **2002**, *13*, 1173-1184. Copyright 2002 Oxford University Press.

The first study utilizing localized hyperthermia generation for prostate cancer was done in Germany over 10 years ago.²¹ Johannsen *et al.* injected iron oxide NPs with an

aminosilane-type shell into the prostate of a 67 year-old man with locally recurrent prostate cancer and exposed him to an AMF (Figure 1.3). The temperature of the prostate rose between 40 °C and 48.5 °C during the first treatment. Six weeks after NP injection, AMF treatment was repeated without new injection and the intraprostatic temperature was between 39.4 °C and 42.5 °C.

Figure 1.3. Alternating magnetic field applicator for human use (MFH300F, MagForce[®] Nanotechnologies GmbH, Berlin). Adapted with permission from *Int. J. Hyperthermia* **2005**, *21*, 637-647. Copyright 2005 Taylor and Francis Group.

Local hyperthermia can be effective for cancer treatments, but it is often difficult to completely eradicate the tumor, especially in the case of prostate cancer, where the distribution of NPs is poor. Despite the initial shortcomings, Johannsen *et al.*²² continued

to evaluate the quality of life after iron oxide NPs hyperthermia treatments. They found no significant impairment of the quality of life. The coated iron oxide NPs had no systemic toxicity, but, as seen before, the distribution of NPs within the prostate was poor. This created hotspots within the tumor, up to 55 °C, and thus necessitated that the power of the AMF be significantly lowered compared to other studies (i.e., 4-5 kA/m for prostate versus 10-14 kA/m for brain tumors) to limit discomfort and skin reactions. Of the 15 most commonly used FDA-approved nanotherapeutics in the past 20 years, few involve using only iron oxide NPs, and none appear to use hyperthermia as the foremost method for cancer eradication (Table 1.2).

Trade Name	Formulation	Indication	Initial Approval Date
Exparel	Pegylated Adenosine Deaminase	Severe Combined Immunodeficiency Disease*	1990
Oncospar	Pegylated Asparaginase	Acute Lymphoblastic Leukemia	1994
Doxil	Liposomal Doxorubicin	Kaposi's Sarcoma, Ovarian Cancer, Metastatic Breast Cancer*	1995
Abelcet, Amphotec, Ambisome	Liposomal Amphotericin B	Fungal Infections	1995, 1996, 1997
Copaxone	Glutaramer Acetate	Multiple Sclerosis	1996
Ferumoxsil	Siloxane-Coated Iron Oxide	Oral Contrast Agent	1997
PegIntron	Pegylated Interferon α -2b	Hepatitis C	2001
Pegasys	Pegylated Interferon α -2a	Hepatitis B, C	2002
Neulasta	Pegylated Filgastrim	Chemotherapy-induced Neutropenia	2002
Abraxane	Albumin Bound Paclitaxel	Advanced lung, pancreatic, breast cancers	2005
Feraheme	Carbohydrate-Coated Iron Oxide	Anemia of Chronic Kidney Disease	2007
Depocyte	Liposomal Cytarabine	Lymphomatous Meningitis	2007
Exparel	Liposomal Bupivacaine	Post-operative Pain	2011
Sienna+	Dextran-Coated Iron Oxide	Sentinal Lymphnode Mapping*	2011
Marquibo	Liposomal Vincristine	Adult Acute Myelogenous Leukemia	2012

Table 1.2. Most commonly used FDA-approved nanotherapeutics; highlighted entries show use of iron oxide NPs. * indicate approval by European Regulatory Agency but not FDA. Adapted with permission from *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* **2017**, 9, 1416. Copyright 2017 John Wiley and Sons.

Current nanotherapeutics and clinical studies focus on delivery of chemotherapeutic drugs in a NP formulation.²³ Many clinicians believe that NP formulation of drugs will decrease systemic toxicity as well as increase efficacy. To date, only the former has been proven true. In the case of NP-mediated hyperthermic treatment of cancer, it appears likely that a combination of local hyperthermia and drug delivery will be necessary for optimal cancer eradication – this is the basis of the present thesis work.

Iron oxide NPs are an ideal platform for drug delivery. However, direct binding of drugs to iron oxide NPs is not common because of the limited reactivity of the NP surface. As a result, iron oxide NPs are often coated with a shell. In one example, Mattingly *et al.*²⁴ used a surrounding cationic lipid bilayer to host small molecules, such as doxorubicin (Dox), an anti-cancer agent (Figure 1.4). Efficacy of this system was explored using *in vitro* studies on MCF-7 breast cancer cells. After incubation at 37 °C for 2 hours, the magnetic liposomes released Dox and caused >90% cell death. Other common coatings include silica or gold. Core-shell NPs of this type will be discussed in greater detail later in this chapter.

Figure 1.4. Magnetic liposomes for drug delivery. Dox release and >90% cell death was seen after incubation at 37 °C for 2 hours. Adapted with permission from *Langmuir* **2015**, *31*, 3326-3332. Copyright 2015 American Chemical Society.

Several researchers have exploited the local hyperthermia generated by iron oxide NPs upon exposure to an AMF as a mechanism (i.e., trigger) for drug release. A popular coating for iron oxide NPs is mesoporous silica (Figure 1.5). Thomas *et al.*²⁵ designed mesoporous silica NPs with zinc-doped iron oxide as the core. A “thread” (consisting of *N*-(6-*N*-aminohexyl)aminomethyltriethoxysilane) was chemically attached to the inside of the pores, followed by drug loading within the pores of the silica NPs. The pores then were capped with cucurbit[6]uril, which is a macrocycle that interacts electrostatically with the “thread” molecule to prevent drug release. Upon exposure to an AMF, the generated local hyperthermia helped the caps to overcome their attraction to the “thread” molecules, and the drug then escaped the pores.

Figure 1.5. Mesoporous silica NPs for drug delivery. Doxorubicin is loaded into pores that then were sealed by addition of a “cap” (4). Upon exposure to an AMF (5), the cap is displaced and the drug is released. Adapted with permission from *J. Am. Chem. Soc.* **2010**, *132*, 10623-10625. Copyright 2010 American Chemical Society.

In another example, thermoresponsive hybrid nanogels were prepared by Cezar *et al.*²⁶ This group designed a ferrogel from alginate and iron oxide NPs to be solid at room temperature (Figure 1.6, left). After exposure to an AMF (Figure 1.6, right), the hydrogel deforms. The biphasic hydrogel was loaded with mitoxantrone, an antineoplastic agent, through ionic interactions with alginate. Deformation of the hydrogel led to diffuse drug release with cumulative therapeutic doses up to 95 μg .

Figure 1.6. Biphasic hydrogel for drug release. After synthesis, the gel remains solid (left), but upon exposure to an AMF, the hydrogel deforms and releases its payload. Adapted with permission from *Adv. Healthc. Mater.* **2014**, 3, 1869-1876. Copyright 2014 John Wiley and Sons.

1.2. PREPARATION METHODS

Iron oxide NPs can be prepared in a variety of ways. One simple, highly used method is co-precipitation of iron salts.²⁷ This method relies on the addition of the iron salts $\text{Fe}^{2+}/\text{Fe}^{3+}$ in a 2:1 ratio to a basic aqueous medium to yield Fe_3O_4 . The method is straightforward, is done within minutes, and gives high yields of NPs. However, poor shape control, oxidation, and aggregation are all problems associated with the co-precipitation method. Riaz *et al.*²⁸ explored the size and shape of iron oxide NPs in relation to pH by varying the amount of sodium hydroxide in solution. At low pH (pH = 2), the NPs had diameters of 25 nm, while at high pH (pH = 10), the NPs had diameters of 100 nm. At pH = 8, the Fe_3O_4 NPs were non-uniform, varying 50-100 nm in diameter. A former colleague in the Nantz group, Dr. Stephanie Mattingly, measured particle surface charge by varying washing steps after NP synthesis and isolation and then examined the NP zeta potential.²⁹ Different washing methods, such as dilute hydrochloric acid versus neutral water, significantly altered the resultant NP surface charges. Rinsing once with neutral water after the co-precipitation method gave anionic NPs, while rinsing with dilute hydrochloric acid gave positively charged NPs, as might

be expected. NPs that have a surface charge tend not to aggregate because of ionic repulsion. When the NPs were rinsed multiple times with neutral water, as is often described in Methods sections of literature preparations, the NPs had a neutral charge, which can lead to aggregation. To help achieve monodispersion and avoid aggregation, a thermal decomposition method was developed.³⁰ This method is performed in non-aqueous media and coats the NPs with an organic ligand, which decreases aggregation. NPs made in this way also have a very narrow size distribution, good shape control, and relatively high yields. Unlike the co-precipitation method, the reaction requires high temperatures (e.g., 210 °C-320 °C), an inert atmosphere, and longer reaction times. Also, because the reaction is not performed in water, further steps are necessary to make the resultant NPs water-soluble.³¹ Other methods for NP synthesis include microemulsion³² and hydrothermal syntheses.³³

1.3. SURFACE MODIFICATIONS

In addition to the need for shells to enable convenient substrate attachments, other problems have been noted using “naked” iron oxide NPs. Without surface modification, iron oxide NPs are degraded into ferrous Fe (II) and ferric Fe (III) by the liver or spleen³⁴ or cause oxidative stress, as seen in *in vivo* studies by Chahinez *et al.*³⁵ Surface modifications allow for biocompatibility, and often include organic ligands, such as polyethylene glycol (PEG)³⁶ and chitosan,³⁷ or inorganic coatings, such as silica³⁸ or gold.³⁹ These modifications change the iron oxide surface, which allows for the attachment of a variety of molecules, such as drugs, targeting agents, or antibodies.

Silica is widely used to modify iron oxide NPs because it is hydrophilic, which aids in aqueous solvation, it helps prevent aggregation through electrostatic repulsions, and it provides a surface that is more amenable to attachment of molecules.⁴⁰ One of the most popular methods to coat iron oxide with silica is the Stöber method³⁸ in which the iron oxide nanoparticles are dispersed in a water/ethanol solution. Formation of the silica shell happens after hydrolysis of a silica precursor, such as tetraethoxysilane (TEOS), followed by condensation of the siloxane onto the surface of the iron oxide NPs. This method is attractive because the silica shell thickness can be fine-tuned based on the amount of alkoxy silane added.⁴¹ Recent examples of silica-coated iron oxide NPs in drug delivery include work by Hwang *et al.*,⁴² Moorthy *et al.*,⁴³ and Zhang *et al.*⁴⁴

Gold nanoparticles are also widely employed in drug delivery because gold is an inert noble metal with excellent biocompatibility. Gold is becoming popular as a shell coating of iron oxide NPs principally for this reason, although the ease of attachment of substrates via thiol methodology is another strong driving force.⁴⁵ Iron oxide-gold core-shell (Fe@Au) NPs can be synthesized in a few ways, namely a bilayer method via aqueous or thermal decomposition, or a multilayer method using a “glue” molecule to attach a gold shell.³⁹ In the aqueous bilayer method, the iron oxide core is combined with chloroauric acid (HAuCl₄) and the gold is reduced chemically. Common reducing agents include sodium borohydride,⁴⁶ citrate,⁴⁷ and ascorbic acid.⁴⁸ A second common bilayer method is thermal decomposition, where hydrophobic iron oxide NPs are combined with oleic acid, oleyl amine, and gold (III) acetate, then heated in a high boiling organic solvent.⁴⁹ This method does require further modification to the shell to make the Fe@Au NPs water-soluble. Though prevalent in the literature, bilayer methods are challenging

because of the difficulty of gold deposition on the iron oxide core, as well as the formation of gold nanoparticles (GNPs) in the process of shell formation. To overcome this challenge, researchers have explored the use of molecules that strongly attach to both iron oxide and gold to serve as a “glue” that binds the metals together. Often, this method involves using gold nanoseeds in the form of amine-terminated molecules as nucleation points onto which the gold can reduce. After synthesis, ligands that can bind to the gold shell, namely amines or thiols, both of which form strong bonds with gold,⁵⁰ are added to coat the NP surface.

1.4. DRUG DELIVERY CHALLENGES

Drug delivery systems utilize a stimulus to deliver a drug at a specific time and location within the body to avoid side effects due to systemic distribution. A common chemotherapeutic agent used in the treatment of cancer is Dox, which induces apoptosis in replicating cells.⁵¹ The cells that are affected the most are rapidly growing cancer cells, but rapidly growing non-cancerous cells are affected as well. When administered intravenously, common side effects include hair loss and digestive issues. A serious side effect of Dox in large quantities (i.e., 500-600 mg/m²) includes myocardial toxicity, which can lead to congestive heart failure or death.⁵² Because of these limitations, better spacial and temporal drug delivery is the next step in cancer treatment. Precise delivery of drugs to cancer cells, so that healthy cells are minimally affected, reduces the side effects seen with systemic distribution. Because of this, the Dox dosage loaded onto a nanoparticle can be decreased because healthy cells are not destroyed as they were with systemic distribution. In what we believe will be a key advance, Dox tethered to iron

oxide NPs can be used in combination with thermotherapy – cancer cells are more susceptible to chemotherapy after thermotherapy.¹⁹

Scheme 1.1. Common conjugations of Dox. Dox can react with a primary amine ($R, R' = \text{carbon}$), hydrazine ($R = \text{NH}, R' = \text{carbon}$), or acyl hydrazine ($R = \text{NH}, R' = \text{acyl}$) to form an imine, hydrazone, or acyl hydrazone, respectively.

Dox attachment to NPs can be through ionic interaction or covalent bonds.⁵³ Of particular interest to us is covalent attachment. One way to load Dox onto NPs conveniently is through its C(13) carbonyl moiety (Scheme 1.1). A hydrazine compound can react with Dox to form a hydrazone linkage, which cleaves under acidic conditions. This is an ideal internal stimulus, since the area surrounding cancer cells is slightly more acidic, pH 5-6, than physiological conditions, pH 7.4.⁵⁴ Aryal *et al.*⁵⁵ attached an equimolar amount of PEG-thiol and DOX-acyl hydrazone to gold nanoparticles (Figure 1.7a) and showed 80% Dox release after 5 hours at pH 5.3. Mohammad *et al.*⁵⁶ attached Dox to Fe@Au via cysteamine to form an imine (Figure 1.7b), which are cleaved in acidic conditions as well. Release of Dox is seen, but only 56% is seen at the extremely low pH of 2.1 after 3 hours. They also performed Dox release studies at pH 2.1 using

AMF at various power settings, where the highest setting (430 Hz) released 72% Dox after 3 hours.

Figure 1.7. a. Dox attached to gold NPs via acyl hydrazone linkage. After 5 hours at pH 5.3, 80% release was seen. Adapted with permission from *J. Mater. Chem.* **2009**, *19*, 7879-7884. Copyright 2009 Royal Society of Chemistry. b. Dox attached to Fe@Au NPs via imine bond. At extremely low pH with application of an AMF at 430 Hz, 72% release was seen. Adapted with permission from *J. Colloid Interface Sci.* **2014**, *434*, 89-97. Copyright 2014 Elsevier.

Despite the appeal of internally triggered drug release, it is not ideal. In each of the above “internal release” studies, Dox escape from the carrier was seen, even at physiological pH (10% for Aryal and 30% for Mohammad). This problem of untriggered release is critical. If NPs are injected into patients intravenously, they passively pool in the tumor though the enhanced permeation and retention (EPR) effect. Enhanced permeation is caused by leaky vasculature of the tumor cells, and retention is due to poor

lymphatic drainage.⁵⁷ The pooling process is not instantaneous; it takes time for blood to circulate and NPs to retain near the tumor. As the NPs circulate, though, they release drugs in a systemic type fashion rather than only near tumor cells. The advantage in using a delivery system is lost. The payload released during circulation may or may not kill cancer cells as intended (i.e., they are likely to affect other rapidly growing, healthy cells, as seen in systemic distribution), meaning more drug must be loaded onto the NPs to deliver the initial intended dosage to the target area. This, in turn, would lead to a greater concentration of untriggered release systemically, making this delivery system only slightly better than systemic chemotherapeutic distribution.

Because an internal trigger cannot be easily controlled, the use of an external trigger, such as an AMF, is needed for precise payload release. One such method is the use of a thermally labile group that would release payload upon triggering. In recent years, researchers have explored the azo functionality^{58,59} as well as a retro-Diels Alder reaction^{60,61} for payload release, as discussed below.

The azo functionality decomposes at high temperatures, generally above 150 °C.⁶² The mechanism for azo decomposition is through a radical reaction, as outlined in Scheme 1.2.⁶³ There are 3 stages in a radical reaction: initiation, propagation, and termination. Radical formation is initiated by heat generated upon NP exposure to AMF, creating a carbon and nitrogen radical (Scheme 1.2a). In the next step, a new radical is formed and nitrogen is expelled (Scheme 1.2b). Termination is caused by the combination of two free radical molecules to form a new bond (Scheme 1.2c, d).

Scheme 1.2. Thermal decomposition of azo functionality. a. Initiation of radical formation is caused heat to form a carbon and nitrogen radical. b. The nitrogen radical reacts to expel N_2 and a new carbon radical. c, d. Termination occurs when radicals combine to form new bonds. Adapted with permission from *Int. J. Chem. Kinet.* **1984**, 16, 767-779. Copyright 1984 John Wiley and Sons.

Researchers have already explored the use of thermally labile linkers attached to iron oxide NPs for payload release. Riedinger *et al.*⁵⁸ first explored the distance at which an azo moiety would decompose when iron oxide NPs were exposed to an AMF. For ease of detection, they initially used fluoresceineamine, then Dox after optimization. Each step of the linker was built onto the NP, as opposed to complete synthesis first, with attachment to the NP last.

Scheme 1.3. Thermal decomposition of azo linkage (red) between spacer attached to iron oxide NP (black) and fluorophore (green). Adapted with permission *Nano Lett.* **2013**, *13*, 2399-2406. Copyright 2013 American Chemical Society.

As seen in Scheme 1.3, gallic acid was derivatized with different lengths of PEG for attachment to iron oxide NPs, then the azo functionality was attached followed by attachment of the fluorophore via an amide bond. The length of spacer between the NP and the azo functionality did not significantly change the amount of azo decomposition when held at a constant temperature. When the NP-linker system was exposed to an AMF for 1 hour, the shorter PEG spacer released more fluorophore. It can be inferred from the data that the carbon-based free radicals formed did not combine as seen in Scheme 1.2d. If this were so, the fluorophore would remain on the NP and would not show release. The fluorescence release data for the shorter PEG spacer corresponded to a larger change in temperature compared to the longer PEG spacer (i.e., 40 K vs 10 K difference at 13.5 kA m^{-1} and 334.5 kHz). Their studies also showed a significant local

temperature change was detected 0-3 nm from the surface of the iron oxide NP, with significant exponential decay as the distance increased. More recently, Romero *et al.*⁵⁹ used thermal decomposition of a short linker with an azo group for rapid release of a fluorescent dye, CG633. Upon exposure to an AMF (500 kHz and 15 kA m⁻¹), nearly entire release was seen after only 10 seconds for NPs with a diameter of 20 nm. This technology was used to release a pharmacological agent, allyl isothiocyanate, for neuron excitement. After exposure to an AMF, rapid Ca²⁺ influx was noted, indicative of neuron excitation.

In another strategy, N'Guyen *et al.*⁶⁰ designed a retro-Diels Alder (DA) linker for delivery of a fluorophore, rhodamine, without inducing a significant temperature increase to the bulk solution. At higher temperatures, the synthesized linker undergoes retro-DA reaction. The requisite higher temperature is induced when NPs are exposed to an AMF. The linker design is shown in Scheme 1.4. The linker was synthesized prior to loading on the iron oxide NPs, with installation of the fluorophore performed after linker loading. A phosphonic acid ligand was used for attachment to iron oxide NPs (**4**, Scheme 1.4). A hydrophilic polymer was attached to the linker via a 1,3-dipolar cycloaddition. The azide was attached to the polymer, and the alkyne was attached to the linker. Under typical copper-catalyzed azide-alkyne cycloaddition, the two moieties reacted in 3+2 fashion to form the corresponding triazole (**2**, Scheme 1.4). Rhodamine was attached via DA (**5**, Scheme 1.4) with 85 % attachment confirmed via NMR analysis. The loaded NPs were exposed to AMF (frequency 332.5 kHz, magnetic field strength 11.3 kAm⁻¹) for 10 minutes. Release of 3 % and 5 % for 2.5 x 10⁻³ wt % and 2.5 x 10⁻³ wt %, respectively, could be seen without any significant increase in bulk solution temperature.

Scheme 1.4. Synthesis of a retro-DA linker with hydrophilic polymer attachment via 1,3-dipolar cycloaddition and DA for fluorophore attachment onto iron oxide NPs. Adapted with permission *Angew. Chem. Int. Ed.* **2013**, 52, 14152-14156. Copyright 2013 John Wiley and Sons.

In another example, Hammad *et al.*⁶¹ designed a retro-Diels Alder (DA) linker for delivery of Dox. The linker-drug was attached to $\text{Zn}_{0.4}\text{Co}_{0.6}\text{Fe}_2\text{O}_4@\text{Zn}_{0.4}\text{Mn}_{0.6}\text{Fe}_2\text{O}_4$ core/shell NPs by means of alendronic acid-iron interactions. The zinc-cobalt and zinc-manganese doped NPs have a higher SAR than pure iron oxide NPs, meaning they have enhanced magnetic heat inducing properties and induced local hyperthermia to a greater extent. Their linker design favors formation of a DA product at lower temperatures by

utilizing furan and maleimide as the diene and dieneophile, respectively. As seen with retro-DA linker design by N'Guyen *et al.*, higher temperatures caused the linker to undergo a retro-DA reaction. The requisite higher temperature can be induced when NPs are exposed to an AMF. The linker design is shown in Scheme 1.5. Like with Reidinger *et al.*, the linker was synthesized directly on the iron oxide NPs rather than complete synthesis of the linker prior to attachment. Alendronic acid served as the means for attachment to NPs (**2**, Scheme 1.5) and Dox was attached via an amide bond (**5**, Scheme 1.5) with confirmation of loading via FT-IR analysis. The loaded NPs were exposed to AMF (frequency 1950 kHz, power 6200 W) for 5 minutes. The temperature of the solution reached 50 °C after 5 minutes of exposure to the AMF and remained at 50 °C for 10 minutes after exposure was ceased. Dox release was ~45% after the 5 minutes of exposure to AMF, and release increased to greater than 90% Dox release in the 10 minutes after AMF exposure was stopped. This work with thermally labile linkers ultimately encouraged our pursuit of a short linker that would require significant thermal energy for rapid payload release.

Scheme 1.5. Synthesis of a retro-DA linker onto zinc-cobalt-iron NPs. Adapted from *Colloids Surf., B* **2017**, *150*, 15-22. Copyright 2017 with permission from Elsevier.

1.5. HYPOTHESIS

Combining local hyperthermia treatment with drug delivery by using a single agent to accomplish both tasks is the next generation of cancer treatment. The use of a thermally labile linker attached to biocompatible NPs, specifically Fe@Au NPs, to release a payload based on an external stimulus such as AMF is not prevalent in literature and should be explored in greater depth and breadth. We designed a thermally responsive linker for attachment to Fe@Au NPs which utilized the ability of iron oxide NPs to create a local hyperthermia upon exposure to AMF. The generated local hyperthermia promoted intramolecular cyclization and caused linker cleavage (i.e., drug delivery) as seen in Figure 1.8, thus providing thermotherapy and drug delivery simultaneously. The following chapters discuss work associated with this hypothesis.

Figure 1.8. Overall pictorial representation of thesis work. I: Fe@Au NPs, II: Dual NP system of gold NPs and water-soluble iron oxide NPs. Left panel: NPs at physiological temperature (37 °C), no release of payload. Middle panel: Application of AMF to induce local hyperthermia of iron oxide, bulk solution temperature 40-45 °C. Right panel: Payload released from NPs into solution.

CHAPTER 2

CORE-SHELL NANOPARTICLE PREPARATION

- 2.1. INTRODUCTION
- 2.2. GOLD SEEDED IRON OXIDE NANOPARTICLES
- 2.3. CORE-SHELL IRON OXIDE-GOLD NANOPARTICLES
- 2.4. DUAL NANOPARTICLE SYSTEM
- 2.5. CONCLUSION

2.1. INTRODUCTION

Figure 2.1. Gold-coated iron oxide nanoparticle.

Gold-coated iron oxide NPs (Fe@Au, Figure 2.1) can be synthesized in a variety of ways.^{1,2} The medium in which the gold coating is applied can be either aqueous or organic, and the coating can be either deposited directly onto an iron oxide core or indirectly, by application using a “glue” material to aid the coating process. Each approach uses gold in a +3 oxidation state, typically gold (III) chloride hydrate or gold (III) acetate, and a reducing agent, such as sodium borohydride, sodium citrate, or oleyl amine. The reducing agent reduces gold (III) to metallic gold (0), starting with a nucleation step followed by deposition and growth on the iron oxide NP to become the shell.^{3,4} As mentioned previously, coating iron oxide NPs with gold imparts adequate biocompatibility to the resultant NPs and allows for flexible NP functionalization through reactions with sulfhydryl (-SH) compounds. Another common functionalization method includes the use of amines, as discussed by Grabar *et al.*⁵ in their report on 2D arrays of colloidal gold NPs for surface-enhanced Raman scattering. The ready coating by thiols and amines is due to the prominent interaction between amino or sulfhydryl groups and gold. Leff *et al.*⁶ first synthesized and characterized amine-coated gold NPs in the 90s,

and they concluded that the interaction between amino and gold is weakly covalent. This is unlike the thiol-capped gold NPs first synthesized by Brust *et al.*,⁷ who described the sulfhydryl and gold interaction as covalent. More specifically, the interaction between sulfhydryl and gold has been explored at the nanoscale since the discovery of spontaneous assembly of alkanethiols on noble metals.⁸ A recent review by Häkkinen⁹ describes the interaction as: “the sp^3 -type hybridization of sulfur, with two of the hybrid orbitals making covalent bonds to the Au(6s) electrons, as well as the important contributions from Au(d) electrons”.

Prior to gold coating, the magnetic iron oxide core must be synthesized. Methods for this are described in Chapter 1. Magnetic NPs can be made of a variety of materials,¹⁰ including pure iron, cobalt, and nickel, but for the Fe@Au NPs of this work the core is made from iron oxide. Iron oxide NPs consist primarily of magnetite (Fe_3O_4) or its oxidized form maghemite ($\gamma\text{-}Fe_2O_3$). Recent reviews on Fe@Au NPs^{1,11} discuss advances in the synthesis of Fe@Au NPs and claim the core for the majority of Fe@Au NPs described in the literature consist of Fe_3O_4 , although $\gamma\text{-}Fe_2O_3$ is used as well.¹² The process to coat $\gamma\text{-}Fe_2O_3$ is very similar to that of Fe_3O_4 . For example, Siurdyba *et al.*¹³ used a short amino-terminated linker to coat a $\gamma\text{-}Fe_2O_3$ core, followed by gold nanoseed capture strategy and then reduced gold to form a shell, which is quite similar to work described later in this chapter. The following subsection reviews methods of preparation of iron oxide NPs having Fe_3O_4 cores.

Scheme 2.1. Synthetic routes to Fe@Au NPs. a. Direct aqueous method uses reducing agents such as sodium borohydride, glucose, sodium citrate, and ascorbic acid. b. Direct organic method uses reducing agents such as oleyl amine and 1,2-diols. Adapted from *J. Chem. Commun.* **2016**, 52, 7528-7540. Copyright 2016 The Royal Society of Chemistry. c. Indirect method, where the “glue” coating can be added in an aqueous or organic solvent, and the shell formation can use either direct coating method. This “glue” is often an amine-terminated molecule, such as (3-aminopropyl)trimethoxysilane, polyphosphazene, or polyethyleneimine. Adapted with permission from *ACS Appl. Mater. Interfaces* **2013**, 5, 4586-4591. Copyright 2013 American Chemical Society.

The most common and simplest method for direct aqueous Fe@Au NP synthesis involves iron oxide NPs that are gold coated by reaction in the presence of Au (III) and sodium citrate as both a reducing agent and capping agent, as seen in Scheme 2.1a. As the Fe@Au NPs are formed, they are immediately coated with sodium citrate, preventing aggregation. Other reducing agents can be used, such as sodium borohydride addition directly in solution followed by 3-mercaptopropionic acid capping¹⁴ or delivery of sodium borohydride via a micelle if microemulsion method is used,¹⁵ glucose followed by chitosan coating,¹⁶ or ascorbic acid, which acts as both a reducing and capping agent.¹⁷

One indirect method for gold coating is to use a seeding method that tethers gold nanoseeds, which are GNPs with a diameter of 2-3 nm, to the iron oxide core prior to bulk gold deposition, as seen in Scheme 2.1c. Seeding creates a point of nucleation for the gold deposition to form the shell. An iron oxide core can be functionalized with (3-aminopropyl)trimethoxysilane (APTMS) to give Fe₃O₄@APTMS. Simple mixing of Fe₃O₄@APTMS with gold nanoseeds then enhances nanoseed attachment due to the strong interaction between the amino moiety and gold.^{18,19} In addition, a thiol moiety can be used for gold nanoseed attachment. Researchers have shown greater stability of molecules bound to gold NPs using multiple thiol anchor points. The Mirkin group first explored attachment of a cyclic dithiane-steroid linker to attach DNA onto GNPs.²⁰ Unlike monothiol moieties that the group studied (Figure 2.2a), the dithiane approach (Figure 2.2b) showed decreased susceptibility to displacement using dithiothreitol (DTT), a common reagent used for cleaving the gold-thiol bond. Expanding this concept, the Mirkin group developed an oligonucleotide with three thiol groups attached via an acetal

backbone linker (Figure 2.2c),²¹ which had even greater stability toward displacement via DTT. These syntheses provide robust attachment, but are quite complicated. Both syntheses required multiple steps for DNA derivatization for attachment of the thiol motifs. Sharma *et al.*²² performed a one-step derivatization of DNA for attachment to gold nanoparticles (Figure 2.2d). They reacted amine-modified oligonucleotides with carbon disulfide (CS₂), which formed a dithiocarbamate *in situ*, followed by incubation with gold nanoseeds. This created DNA-modified, coated gold nanoparticles using a bidentate attachment, which showed increased robustness toward DTT in comparison to ligands with monodentate attachment. After only 3 minutes of incubation with DTT, the monodentate DNA-gold (Figure 2.1e) began to aggregate, as seen in the UV-Vis, whereas after 1 hour the bidentate DNA-gold was still intact.

Figure 2.2. DNA modified ligands attached via (a, e) monothiol, (b) dithiol, (c) trithiol, or (d) dithiocarbamate. Adapted from the following sources: *Bioconjugate Chem.* **2000**, *11*, 289-291. Copyright 2000 American Chemical Society. *Nucleic Acids Res.* **2002**, *30*, 1558-1562. Copyright 2002, Oxford University Press. *Chem. Commun.* **2008**, 2140. Copyright 2008, Royal Society of Chemistry.

Another common Fe@Au NP synthesis uses high temperatures and organic solvents to prepare hydrophobic NPs. Wang *et al.*²³ first utilized a thermal decomposition method to form such hydrophobic Fe@Au NPs. In a high boiling solvent, such as phenyl ether at 185 °C, organic soluble iron oxide NPs were added to gold (III) acetate and oleyl amine, which acts as a mild reducing agent and capping agent, followed

by the addition of a 1,2-diol and oleic acid. As seen in Scheme 2.1b, the coated iron oxide NPs are stripped of the coating before gold is reduced onto the surface. Oleyl amine then caps the gold as it is deposited onto the iron oxide until the entire shell is formed. The NPs were purified via centrifugation and resuspended in hexane in the presence of oleic acid and oleyl amine. Alonso-Cristobal *et al.*²⁴ explored the effects of varying the iron oxide:gold (III) acetate ratio and reaction temperature with regards to the gold shell thickness. They found that the ratio did not affect the thickness of the gold shell; rather, a larger amount of gold (III) acetate led to homogeneous nucleation, which created gold NPs rather than coat the iron oxide NPs. This is a key observation that has importance for this thesis work, as discussed in a later chapter. The optimal ratio of iron oxide:gold (III) acetate was 0.05 mmol:3.7 mmol for iron oxide NPs with a mean diameter of 7.5 nm. They also showed that a reaction temperature between 190 °C and 210 °C did not influence the gold shell thickness, but between 230 °C and 270 °C showed significant gold shell thickness, from 3 nm at lower temperatures to 10 nm at higher temperatures.

Though many researchers claim success with a variety of methods, it remains difficult to control deposition of gold onto iron NPs. This is due to the differences in surface energy between iron oxide and gold^{11,25} that oftentimes lead to the undesired co-formation of GNPs. GNPs are caused by autonucleation. They also aggregate once formed, thus complicating methods of purification. Undesired GNPs can be minimized through optimization of the ratio of iron oxide to gold, although too little gold precursor can lead to incomplete coating and thus exposure of the iron oxide NP. Conditions need to be carefully controlled in order to maximize complete gold coating without GNP

formation – this is the key challenge in the field. If GNPs are formed, though, one method of separation from Fe@Au NPs is to use centrifugation to give monodispersed Fe@Au NPs.²³

2.2. GOLD SEEDED IRON OXIDE NANOPARTICLES

We first sought to prepare Fe@Au NPs via gold seeding followed by a Au (III) reduction approach to try to thwart the major problem of GNP formation faced in many of the other methods. Our first idea to make gold-coated iron oxide nanoparticles was based on some previous work done by Dr. Stephanie Mattingly in our lab.²⁶ In this work, she sought to modify the iron oxide nanoparticles by reaction with CS₂ to yield xanthate functionality that would then serve as the “glue” for subsequent small molecule attachment (Scheme 2.2b). Xanthates are typically formed when organic alcohols in base react with carbon disulfide followed by alkylation (Scheme 2.2a). Mattingly investigated functionalization of the oxides on the surface of iron oxide nanoparticles by reaction with excess carbon disulfide. Ultimately, this work was unsuccessful but, upon further research, we discovered related modifications to iron oxide NPs using CS₂ that have been reported and showed promise.²⁷ Instead of reacting naked iron oxide with CS₂ to form a xanthate, Lopes *et al.* functionalized iron oxide NPs with terminal primary amines that then were reacted with CS₂ to form dithiocarbamates (Scheme 2.2c, step 1).

Scheme 2.2. a. Traditional xanthate ester formation. b. Ferric xanthate variation. c. Dithiocarbamate formation (1) with subsequent gold seeding (2).

Lopes *et al.* used this approach (i.e., coatings with a siloxydithiocarbamate) to scavenge colloid gold from an aqueous solution for purposes of water purification (Scheme 2.2c, step 2). The reacted solution was then exposed to an external permanent magnet, and the water was purified of the reacted gold nanoparticles. In another example, Montazerabadi *et al.*¹⁸ used a gold seeding method (Scheme 2.2c, step 2) followed by Au (III) reduction to form Fe@Au NPs for MRI contrast. The following describes this procedure, which was followed for synthesis of Fe@Au NPs via a seeding method.

Following a modified procedure by Montazerabadi *et al.*,¹⁸ we used a short linker, 3-aminopropyl(triethoxysilane) (APTES), to coat the iron oxide NPs. Briefly, we reacted excess APTES with iron oxide NPs that were prepared according to the method of

Mikhaylova *et al.*²⁸ The resultant reaction mixture was sonicated for 30 minutes. The suspension was then rapidly stirred and heated at 60 °C in a sealed tube for 4 hours, followed by magnetic separation and washing with methanol to remove excess APTES. Comparison of the FT-IR spectrum of the starting “naked” iron oxide nanoparticles with that of the APTES-reacted nanoparticles (Figure 2.3) suggests the NPs were modified as desired, principally due to the appearance of C-H stretch (2929 cm^{-1} and 2840 cm^{-1}), N-H stretch (3358 cm^{-1}), and characteristic Si-O-R stretching (1043 cm^{-1}).

Figure 2.3. Comparison of FT-IR spectra of Fe₃O₄ NPs (top) and Fe₃O₄@APTES NPs (bottom).

With APTES-coated iron oxide nanoparticles in hand, we next examined how to attach the gold nanoseeds. In an effort to maximize attachment, we chose to follow a modified synthesis provided by Sharma *et al.*,²² utilizing the bidentate chelation afforded by reaction of the amine with CS₂. After reacting Fe₃O₄@APTES in borate buffer with saturated aqueous carbon disulfide, we added excess aqueous gold nanoseeds (2-3 nm). Gold nanoseeds were provided as a gift by Dr. Martin O'Toole's lab at the University of Louisville (see method of preparation by Yong *et al.*²⁹). The solution was stirred overnight, then purified via magnetic separation to give gold nanoseed-coated iron oxide NPs (Fe@nAu).

Figure 2.4. STEM Image of Fe@APTES NPs (light gray) decorated with gold nanoseeds (bright spots) to form Fe@nAu NPs.

High-resolution transmission electron microscopy (HR-TEM) and scanning transmission electron microscopy (STEM) imaging was performed on Fe@nAu NPs by

Dr. Jacek Jasinski. As seen in Figure 2.4, the Fe@APTES core is a lighter gray, and the gold nanoseeds are bright spots. Each image showed aggregation, but this is common when nanoparticles are dried and concentrated for imaging. Based on the HR-TEM images, the average size of the iron oxide NPs was 10 ± 3 nm. In addition to imaging, energy-dispersive X-ray spectroscopy (EDAX) was performed on a gold-seeded iron oxide sample. This allowed precise analysis of the chemical composition of the sample, which gave efficacy of coating. However, there was inconsistency in the coating. Dr. Jasinski provided EDAX readings for 3 different samples, and examined different regions within each sample. As seen in Figure 2.5, iron oxide is observed for regions 1 and 2, but only region 1 showed characteristic gold signals. The inconsistent coating is seen not only in multiple areas of the same sample, but also between different samples. This is troublesome because we expected gold nanoseeds to cover the iron oxide core in its entirety. Apparently only portions of the iron oxide core became coated with the gold nanoseeds. Despite this, we continued with the Fe@nAu, hoping the partial gold nanoseed coverage would still provide enough nucleation sites to form the gold shell.

Figure 2.5. EDAX of Fe@nAu, showing clear peaks for both iron oxide and gold in region 1 (left) but only iron oxide in region 2 (right). Cu seen in both samples is from the grid used for analysis.

Many researchers have reported gold shell formation after gold nanoseed attachment (i.e., nucleation). Procedures utilize chloroauric acid with various reducing agents to form the gold shell. Fe@nAu NPs were suspended in H₂O containing HAuCl₄, followed by addition of reducing agent. We examined several procedures that utilized sodium borohydride,³⁰ ascorbic acid,¹⁸ glucose,³¹ and sodium citrate.³² The product obtained by each method was characterized via UV-Vis, dynamic light scattering (DLS), and ζ -potential. UV-Vis allows the detection or absence of a surface plasmon resonance (SPR) that is commonly seen with GNPs or a gold shell. The SPR peak is due to the interaction of light with free surface electrons, which in turn oscillate. The electrons can

release their energy in the form of heat or light. Colloidal GNPs give a characteristic optical absorption peak around 520 nm.³³ DLS measures the hydrodynamic radius of the sample, and ζ -potential gives the charge of the NPs in a solution of water. Results are seen in Table 2.1.

Reduction method	SPR band	DLS (nm)	ζ -potential (mV)
Ascorbic acid	None observed	2500	-6.0
Sodium citrate	None observed	3000	-42.0
Glucose	None observed	700, 200	+32.2
Sodium borohydride	None observed	2400	-15.7

Table 2.1. Analysis for synthesis of Fe@Au NPs via hydrophilic methods. All samples were measured in 1 mL H₂O at a concentration of 0.2 mM.

Unfortunately, none showed a characteristic SPR band representative of gold coating. The lack of SPR peaks in any of the samples was disconcerting, and the hydrodynamic diameters were far too large to be considered useful. The ζ -potentials are relatively good. Because ζ -potential is a measure of the surface charge of the NPs, it is best of the observed number is largely negative or largely positive. This ensures repulsion between NPs, discouraging aggregation. Despite the good ζ -potential, the excessive hydrodynamic radii made it clear that this hydrophilic method approach was problematic. As mentioned previously, four common hydrophilic methods for gold coating were attempted, and each method used freshly made Fe@nAu NPs. Each method was performed only once, but the focus of this thesis work was to expand the use of a thermally labile linker to Fe@Au NPs for drug delivery. In order to perform the

necessary experiments, a reproducible method for Fe@Au NPs was necessary. Thus, instead of focusing on attempts to make the hydrophilic method work, we pursued a new method needed for Fe@Au NP synthesis.

While attempting to coat iron oxide with gold via gold nanoseed nucleation and reduction, we explored the possibility of capping the dithiocarbamate with methyl to produce stable xanthate functionality that could be analyzed prior to gold nanoseed capping. Mattingly *et al.*²⁶ previously attempted methyl capping of xanthate-coated iron oxide NPs with little success. Despite the failure to do so, a revised attempt to form dithiocarbamates followed by methyl capping yielded success. We followed the previous procedure for gold nanoseed capture, but added excess methyl iodide rather than gold nanoseeds. Characteristic stretching of the C–N bond of NCS₂Me at 1336 cm⁻¹ and asymmetric stretching of CS₂ at 960 cm⁻¹ provided evidence for methyl dithiocarbamate formation.³⁴ Whereas these results were promising, we did not pursue this approach further due to success in ongoing accompanying work that led to our goal.

2.3. CORE-SHELL IRON OXIDE-GOLD NANOPARTICLES

After failing to prepare Fe@Au NPs via a hydrophilic method, we chose to attempt synthesis via the thermal decomposition method to yield hydrophobic Fe@Au NPs. First, we synthesized oleyl amine-coated iron oxide NPs via a modified method developed by Park *et al.*³⁵ Briefly, we heated a biphasic water-ethanol/toluene solution of iron (II) and iron (III) salts, as well as sodium oleate, at 75 °C for 4 hours to give oleate-coated iron oxide NPs. The NPs were pelleted from excess reagents via centrifugation. HR-TEM, EDAX, and superconducting quantum interference device

(SQUID) were performed, as seen in Figure 2.6, and agreed with the literature characterizations.

Figure 2.6. Characterization of iron oxide NPs. a. HR-TEM image shows uniformly sized NPs. b. SQUID analysis with field cooling (blue) and zero-field cooling (red); right shows magnetic saturation and left shows blocking temperature determination. c. EDAX analysis to show characteristic iron and oxygen peaks; Cu seen in the sample is from the grid used for analysis.

HR-TEM revealed an average diameter of 5 ± 1 nm with good monodispersity. EDAX gave characteristic peaks for iron oxide. The Cu peaks are due to the copper grid used for analysis. SQUID measurements showed a magnetic saturation of 0.3 emu/g with a narrow hysteresis loop at room temperature. This indicated superparamagnetic NPs because a hysteresis loop should not be detected above the blocking temperature, in this case 27 °C.¹⁸

We then modified a synthesis procedure by Wang *et al.*²³ to coat our newly formed NPs with gold. Briefly, we combined iron oxide NPs with gold (III) acetate, oleic acid, oleyl amine, and 1,2-tetradecanediol in phenyl ether under nitrogen at room temperature. This solution was heated to 185 ± 5 °C for 90 minutes. After cooling to rt, the NPs were pelleted via centrifugation. This procedure allowed for removal of excess oleyl amine as well as any gold NPs formed during the synthesis. STEM, EDAX, UV-Vis and SQUID were performed on these NPs as seen in Figure 2.7.

Figure 2.7. Fe@Au NP Characterization. STEM image of Fe@Au NPs showing good monodispersity of Fe@Au NPs. Top right insert shows size of NP using STEM software. Bottom right insert shows size distribution based on diameter of NPs in image.

Figure 2.8. Fe@Au NP Characterization. a. UV-Vis reading that shows characteristic SPR absorption around 540 nm. b. EDAX analysis to show characteristic iron, oxygen, and gold peaks; C seen in the sample is from the grid used for analysis.

Figure 2.9. Fe@Au NP Characterization, SQUID analysis. a. Field loops up to 7 T at 1.9 K and 300 K. b. Field-cooled (blue) and zero-field-cooled (red) measurements showing temperature dependence of magnetization under 10 mT.

STEM in Figure 2.7 revealed an average NP diameter of 7 ± 3 nm with relatively good monodispersity, consistent with literature values.²³ EDAX showed characteristic peaks for both iron oxide and gold based on literature.²⁴ UV-Vis analysis (Figure 2.8) showed a characteristic SPR around 530 nm, consistent with literature values.^{23,24} SQUID measurements (Figure 2.9) showed a magnetic saturation of 6 emu/g with a narrow hysteresis loop at room temperature. These results confirm the presence of Fe@Au NPs, and we used this material in our subsequent experiments.

2.4. DUAL NANOPARTICLE SYSTEM

For reasons that will become clear in a later chapter, we also synthesized a dual NP system. As the name implies, the dual NP system consists of 2 different types of NPs: gold NPs for attachment of thiol based moieties, such as our linker, and iron oxide NPs for generation of local hyperthermia.

Because the thermal decomposition approach worked to prepare Fe@Au NPs, we chose a similar procedure sans the iron oxide core to give hydrophobic gold NPs (GNPs). de la Presa *et al.*³⁶ formed GNPs by reducing gold (III) acetate with oleylamine in phenyl ether, oleic acid, and 1,2-tetradecanediol at elevated temperatures, which is the synthesis we chose to follow. The resultant GNPs were characterized via DLS and SEM. DLS revealed a hydrodynamic diameter of 224.3 ± 11.2 nm, whereas SEM revealed 5 ± 3.7 nm (Figure 2.10).

Figure 2.10. GNP characterization. SEM image that shows monodispersed GNPs (bright spots) with minor aggregation (large bright spots). GNPs have an average diameter of 5 ± 3.7 nm.

The second portion of our dual NP system is the iron oxide NPs. As previously discussed, iron oxide NPs should be coated to avoid aggregation and prevent oxidation. Instead of using a metallic coating as done previously, we chose to make our iron oxide

NPs water-soluble by attaching polyethylene glycol (PEG). This procedure forms a ferrofluid, which is a colloidal suspension of surfactant coated magnetic nanoparticles. We followed a procedure from García-Jimeno and Estelrich³⁷ that combined PEG 2000 with iron (II) and iron (III) salts at slightly elevated temperatures. This was followed by addition of ammonium hydroxide to form water-soluble iron oxide NPs (MNPs), which were washed repeatedly with water to remove excess PEG 2000. Characterizations included ζ -potential, DLS and SEM. ζ -potential showed a surface charge of -22.4 ± 2.02 mV. DLS showed a hydrodynamic diameter of 716.2 ± 74.3 nm, whereas SEM gave a diameter of 50 ± 16 nm (Figure 2.11).

Figure 2.11. MNP characterization. SEM image that shows good monodispersity (average diameter 50 ± 16 nm), though aggregation was seen when sample was dried for analysis.

2.5. CONCLUSION

NP preparation is well described in the literature, yet the process of coating iron oxide NPs in a reproducible manner so as to exclude GNP formation is a major challenge. Despite following literature procedures, we did not enjoy success in making Fe@Au NPs via a gold seeding method, which led us to use a thermal decomposition method. The characterizations showed data that was consistent with literature for Fe@Au NP synthesis. HR-TEM images showed iron oxide cores around 5 nm, and STEM images

showed monodispersed, well-defined NPs around 7 nm. These diameters matched quite well with the diameters of the procedure we followed by Wang *et al.*²³ The Fe@Au NPs had a characteristic SPR peak for gold, and EDAX readings showing both iron oxide and gold consistently, something we had not achieved previously.

CHAPTER 3

LINKER SYNTHESSES

- 3.1. INTRODUCTION
- 3.2. AMINE LINKER
- 3.3. AMINE-FREE LINKER
- 3.4. SUMMARY

3.1. INTRODUCTION

We selected a linker for the Fe@Au NP delivery system based on previous work done in our lab. Knipp *et al.*¹ designed, synthesized, and attached a linker to iron oxide NPs for the same purpose, namely to respond to an external stimulus (AMF-generated local hyperthermia) so that intramolecular cyclization of the linker releases a payload (Scheme 3.1). The initial linker had 3 components: a terminal attachment point to load onto a NP surface, a reactive portion that consisted of a nucleophilic amine (blue) and an electrophilic carbonyl moiety (red), and a payload (green).

Scheme 3.1. Thermally responsive linker that undergoes intramolecular cyclization upon exposure to AMF to release a payload; blue = nucleophilic element (amine), red = carbonyl moiety, green = payload

Knipp *et al.*² first explored different parameters for optimal linker design prior to loading onto a surface (Figure 3.1). An allyl moiety was chosen as the surrogate for the attachment portion because it could easily be modified when needed yet remained inert during evaluation of the cyclization parameters. The reactive portion consisted of a nucleophilic amine and various electrophilic carbonyls, including ester (**1**), carbonate (**2**), carbamate (**3**), and amide (**4**). In addition, the carbon chain length was varied between the nucleophilic amine and the electrophilic carbonyl to assess the influence of ring size formation on exposure to heat. The payload used for the studies was anthracene, which is UV active and fluorescent and readily quantified using known methods.

Figure 3.1. Cyclization precursors ($R = 2$ -(9-anthracenyl)ethyl). Ring size after intramolecular cyclization given in parentheses. Adapted with permission from *Tetrahedron* **2014**, 70, 3422-3429. Copyright 2014 Elsevier.

After synthesis, each linker was incubated at 55 °C in methanol for 24 hours. Aliquots were taken at various times for analysis via HPLC to determine percent release. Ester **1.1** cyclized most readily, with nearly complete release of the anthracene alcohol group after incubation for 24 hours. Thus, this linker design was chosen for application in a carbonyl capture demonstration using a poly(dimethylsiloxane) (PDMS) microchannel. The attachment portion was modified to incorporate a triethoxysilane for covalent attachment to the microchannel. The anthracene payload was replaced with an aminooxy moiety capable of reacting with carbonyl analytes. To test the applicability of the system on a surface, the siloxane-linker was attached to the microchannel, and then incubated with an aqueous soluble fluorophore, fluorescein isothiocyanate (FITC), which was modified with a short chain containing an aldehyde.³ After rinsing to remove excess FITC, the

microchannel was incubated at 60 °C for 30 minutes and nearly complete release of the fluorophore was observed.

With this success, Knipp *et al.*¹ used the aforementioned linker design for attachment to iron oxide NPs once again using siloxane methodology, followed by AMF exposure to induce local hyperthermia and intramolecular cyclization. Ester **1.3** (Figure 3.1) was the linker of choice because it was resistant to cyclization until heated (i.e., exposure to local hyperthermia). As previously discovered by Riedinger *et al.*,⁴ iron oxide NPs exposed to an AMF provide enough thermal energy for azo bond cleavage, so Knipp hypothesized the same energy could drive the intramolecular cyclization. Because the temperature of the NP surface increases first, followed by the change in temperature of the bulk solution,⁵ the area immediately near the NP is much hotter than the bulk solution. Anthracene was used as the payload for its ease of detection using a fluorometer in a 2:1 water:acetonitrile solution. After 30 minutes of AMF exposure, a large amount of payload was detected in the supernatant. Unfortunately, release from the NP system without exposure to AMF was seen. The premature release was presumably caused by ester hydrolysis facilitated by iron oxide acting as a Lewis acid. To mask the oxide surface, the use of a silica shell, which reduced the Lewis acidity of the NPs, was examined. The *gem*-dimethyl carbonate **5** (Figure 3.1) was chosen as the linker for the silica-coated iron oxide NP studies because of its greater stability toward hydrolysis and relatively low payload release during previous studies.

Figure 3.2. Nucleophilic linker attached to silica-coated iron oxide NPs ($\text{Fe}@\text{SiO}_2$).

After the linker was attached to the silica-coated iron oxide NPs, both the Boc-protected amine **6** and free amine **7** (Figure 3.2) NPs were exposed to an AMF. Release was observed for **6**, which was unexpected because the Boc protecting group makes the amine less nucleophilic. More importantly, the Boc-protected amine is not able to cyclize because cyclization would result in a quaternary amine, which is highly unlikely. For this reason, a modified linker that lacked an amine and used a robust *N*-methyl carbamate moiety was explored. Stronger carbamate functionality would reduce the amount of undesired payload release but would release under forcing conditions when local hyperthermia was generated upon exposure to AMF. Instead of relying on intramolecular cyclization, the carbamate linker cleaved via pure hydrolysis. Almost 70% payload release was observed after 8 5-minute pulses at 500 A, while less than 10% payload release was observed at 37 °C. Knipp *et al.* concluded that the silica shell provided sufficient hydrogen bonding for accelerated hydrolysis and/or Boc-deprotection of the carbonate, as well as accelerated hydrolysis of the carbamate.

3.2. AMINE LINKER

This thesis work aims to apply the intramolecular cyclization strategy described above to NPs that are otherwise inert. Silica shells have numerous qualities appealing for

drug delivery, including controlled thickness of deposition onto an iron oxide core,⁶ covalent attachment of drugs or antibodies,⁷ and a variety of ways to coat the iron oxide core with silica.⁸ While the silica shell is important to increase functionalization of the iron oxide core, bare silica NPs are reactive both *in vitro* and *in vivo*, i.e. their silanol group is negatively charged at physiological pH (~7.4) and interacts with red blood cells, causing hemolysis.^{7,9} Even after coating the silica shell, it was still reactive, as demonstrated by Knipp *et al.* with ester and carbonate hydrolysis. We sought to use a gold shell because gold is bioinert¹⁰ and is functionalized easily to form covalent bonds with thiols.¹¹ We planned our studies to use a mono-thiol because we felt in this case a di- or tri-thiol was not necessary. The 7-membered ring formation was used because of its stability at physiological temperatures with response to thermal stimuli at relatively high temperatures. The payload portion of the linker utilized the aminooxy moiety for facile attachment of fluorescent carbonyl containing compounds seen in Figure 3.3.

	Knipp <i>et al.</i>			Biladeau
Core Shell	Fe ₃ O ₄ -----	Fe ₃ O ₄ SiO ₂		Fe ₃ O ₄ Au
Functionalization	Siloxane condensation			Thiol monolayer
Linker synthesis with FL prior to loading onto NPs	4 steps	7 steps	10 steps	11 steps
Carbonyl functionality	Ester	Carbonate	Carbamate	Carbonate
Ring size	5	7	Amine-free	7
Result from 37 °C incubation	Uncontrolled hydrolysis	Uncontrolled hydrolysis	Limited hydrolysis	No uncontrolled hydrolysis
Result from AMF exposure	Uncontrolled hydrolysis	AMF-promoted hydrolysis	AMF-promoted hydrolysis	Intramolecular cyclization

Table 3.1. Summary of improvements relative to Knipp *et al.*¹

Figure 3.3. Fluorophores used as payloads in the present study. a. Aminoxy of amine-based linker reacts quickly and irreversibly with carbonyl-containing compound. b. Fluorophores reacted with **15** to prepare LS1-FL.

Initially, boron-dipyrromethene (BODIPY) was the fluorophore used per recommendation of collaborators. BODIPY is often used in biochemistry for conjugation of nucleotides, amino acids, and other low molecular weight ligands.¹² It was used in early studies because of its superior fluorescence that spans the visible spectrum, depending on the modifications. BODIPY was modified with aminoacetaldehyde to yield a carbonyl containing BODIPY-CHO (Figure 3.3a). Ultimately, we found little success using this fluorophore because it was insoluble in water, despite use of 2:1 water:acetonitrile as well as derivatization of the fluorophore to aid in solubility. We also examined the anthracene fluorophore from the work of Knipp *et al.* in initial proof of concept studies. Finally, water-soluble molecules were used later in our studies, including lissamine rhodamine B (LRB), and fluorescein isothiocyanate (FITC). All fluorophores were attached to the protected linker system (LS) via the click oximation reaction depicted in Figure 3.3a. Lissamine rhodamine B combines the core of rhodamine with the sulfonyl groups of lissamine, which can be used for functionalization. In our case, a terminal sulfonyl chloride was used, and, following a procedure by Park and Yousaf,¹³ 1,4-dioxo-8-azaspiro[4.5]decane was attached followed by stirring with acid to yield LRB-CHO (Figure 3.3b). Unfortunately, we had little success with LRB, despite following the literature procedure carefully. Instead, a derivatized FITC was used, which was synthesized previously in our lab. FITC itself does not contain a carbonyl to react with our linker, so it was modified with a short chain that contained a terminal amine, which reacted with the isothiocyanate, as well as a terminal acetal, which is simply a masked carbonyl (Scheme 3.2).²

Scheme 3.2. FITC-CHO synthesis. Step 1: 4-aminobutylaldehyde diethyl acetal reacts at the carbon of the isothiocyanate to install masked carbonyl. Step 2: Acetal hydrolysis to give aldehyde.

Figure 3.4. ^1H NMR spectrum of FITC-CHO in CD_3OD . The characteristic aldehyde proton is not evident around 9 ppm. Instead, FITC-CHO reacted with CD_3OD to form a hemiacetal (red arrow).

Scheme 3.3. Synthesis of protected LS1 **15** a. TBSCl, Et₃N, CH₂Cl₂, 0 °C; b. MsCl, Et₃N, CH₂Cl₂, 0 °C; c. Phthalimide, K₂CO₃, DMSO, 75 °C; d. Hydrazine monohydrate, CH₂Cl₂:EtOH, 0 °C; e. MeCN, 55 0 °C, 43% over 5 steps; f. Boc₂O, Et₃N, CH₂Cl₂, 0 °C; g. TBAF, THF, 0 °C, 98% over 2 steps; h. 1,1'-carbonyldiimidazole, (*i*-Pr)₂NEt, CH₂Cl₂, 0 °C; i. *N*-(2-hydroxyethyl)phthalimide, DBU, MeCN, rt, 51% over 2 steps; j. N₂H₄•H₂O, CH₂Cl₂, 0 °C, 99%; Tr = CPh₃, Boc = C(O)Ot-Bu, TBS = SiMe₂*t*-Bu

The synthesis of the amine linker, protected LS1, is outlined in Scheme 3.3. Each product was characterized by ¹H NMR, ¹³C NMR, and IR, with key intermediates characterized by HRMS. To install the amine moiety, commercially available 1,4-butanediol **10** was mono-TBS protected and followed by mesylation using standard protocols. Reaction with phthalimide under Gabriel reaction conditions followed by hydrazinolysis gave primary amine **11**. Initial syntheses were done on the milligram scale but after optimization, the reactions were scaled up and performed on the gram scale (e.g. Step a in Scheme 3.4 yielded 4.52 g of a monoprotected diol). Confirmation of formation of **11** can be seen in the ¹H NMR, ¹³C NMR, and IR. The phthalimide

(Scheme 3.4, step c) gives characteristic ^1H NMR peaks in the aromatic region, but these signals are no longer evident in amine **11** (Figure 3.5).^a ^{13}C NMR shows peaks in the aromatic region as well as the carbonyl carbon signal, but are no longer present after hydrazinolysis (Figure 3.6). IR analysis shows a strong characteristic C=O stretch at 1707 cm^{-1} from the phthalimide group but is absent in **11** (Figure 3.7).

Figure 3.5. ^1H NMR of TBS-protected phthalimide (top) and amine **11** (bottom). Aromatic peaks indicative of phthalimide (blue box). Phthalimide protons zoomed in (top) to show doublet of doublet. ^aSynthesis of amine **11** has been reported by Krivickas *et al.* (JOC, 72, 8280)

Figure 3.6. ^{13}C NMR comparison of TBS-protected phthalimide (top) and **11** (bottom). Aromatic peaks (120-140 ppm) and carbonyl peak at 168 ppm indicative of phthalimide.

Figure 3.7. IR spectrum of TBS-protected phthalimide (black) with characteristic $\text{C}=\text{O}$ stretch at 1707 cm^{-1} that is absent in amine **11** (red).

Commercially available 1,3-dibromopropane was reacted with triphenylmethanethiol to give the tritylprotected-thiol bromide **3**. Bromide **12** was reacted with **11** then purified via column chromatography to give the secondary amine **13**. After purification, the yield after 5 steps was 43%. Purified amine **13** was Boc protected, followed by deprotection of the TBS group using fluoride-mediated desilylation. The purified product was reacted with carbonyl diimidazole to give **14**. Characterization using ^1H NMR of crude **14** showed a downfield shift in alpha protons after transformation from a hydroxyl to an acyl imidazole (Figure 3.8, proton *a*, *c*). In addition, the hydroxyl proton disappears while the acyl imidazole protons are seen in the aromatic region (Figure 3.8, proton *b*, *d*). The crude acyl imidazole was reacted with a previously synthesized phthalimide alcohol,¹⁴ followed by hydrazinolysis to reveal aminooxy **15**. At this point, any molecule with a carbonyl could be attached for delivery using this system. Characterization using ^1H NMR of crude **15** showed protons of the aminooxy as a broad singlet around 5.5 ppm in addition to distinction of the ethylene protons *a* and *b* (Figure 3.9). This was used for attachment of either anthracene aldehyde or FITC-CHO, followed by trityl deprotection¹⁵ to reveal the thiol for attachment to Fe@Au NPs.

Figure 3.8. ^1H NMR spectra of alcohol (top) and acyl imidazole **14** (bottom). Each sample was dissolved in CDCl_3 with TMS and spectra were taken on a 400 MHz NMR machine.

Figure 3.9. ¹H NMR spectra of phthalimide (top) and aminoxy **15** (bottom). Each sample was dissolved in CDCl₃ with TMS and spectra were taken on a 400 MHz NMR machine.

3.3. AMINE-FREE LINKER

In anticipation of demonstrating that the mechanism of payload release will proceed via intramolecular cyclization, we pursued synthesis of a control linker system that lacked the amine moiety while retaining all other aspects. Without an amine moiety in the connecting chain, no release of payload can be expected based on an intramolecular cyclization.

Scheme 3.4. Synthesis of protected LS2 **18** a. Triphenylmethanethiol, K_2CO_3 , EtOH:H₂O, 90 °C; b. 1,1'-carbonyldiimidazole, $(i\text{-Pr})_2\text{NEt}$, CH_2Cl_2 , 0 °C; c. *N*-(2-hydroxyethyl)phthalimide, DBU, MeCN, rt, 54% over 3 steps; d. $N_2H_4 \cdot H_2O$, CH_2Cl_2 , 0 °C, 98%.

The synthesis for the amine-free linker is outlined in Scheme 3.4. Each product was characterized by 1H NMR, ^{13}C NMR, and IR, with key intermediates were characterized by HRMS. Commercially available 6-bromo-1-hexanol **16** was reacted with triphenylmethanethiol to give trityl protected thio-alcohol as described in the amine-based linker, followed by reaction with 1,1'-carbonyldiimidazole to give the acyl imidazole intermediate **17**. Characterization of both the alcohol and acyl imidazole products via 1H NMR is seen in Figure 3.10 and via IR is seen in Figure 3.11. As seen with LS1, the proton from the alcohol disappears and a downfield shift is seen in the alpha protons. In addition, the characteristic OH stretch is seen for the alcohol and the C=O stretch is seen with the acyl imidazole. **17** was reacted with a previously synthesized phthalimide alcohol¹⁴ and purified via column chromatography. The yield over 3 steps after purification was 54%. The phthalimide was cleaved via hydrazinolysis to give aminooxy **18**. Characterization using 1H NMR of crude **18** showed protons of the aminooxy as a broad singlet around 5.5 ppm in addition to distinction of the ethylene protons *a* and *b* (Figure 3.12). This was used for attachment of either the anthracene

aldehyde or FITC-CHO, followed by trityl deprotection¹² to reveal the thiol for attachment to Fe@Au NPs. Spectral comparison of protected LS1 and protected LS2 is seen in Figure 3.13.

Figure 3.10. ¹H NMR spectra of alcohol (top) and acyl imidazole **17** (bottom). Each sample was dissolved in CDCl₃ with TMS and spectra were taken on a 400 MHz NMR machine.

Figure 3.11. IR spectrum of alcohol (black) with characteristic OH stretch at 3362 cm⁻¹ that is absent in acyl imidazole **17** (red), but instead has a characteristic C=O stretch at 1768 cm⁻¹.

Figure 3.13. ^1H NMR spectral comparison of **15** and **18**.

3.4. SUMMARY

Our goal was to prepare a linker system that:

TASK 1: could be readily “loaded” with a carbonyl substrate (e.g, FL or drug)

TASK 2: could be readily attached to GNPs

TASK 3: is resistant to non-triggered hydrolysis

TASK 4: responds to local hyperthermia to release attached carbonyl substrate

The following features within the linker system address each task:

SOLUTION 1: -ONH₂ methodology allows for facile “click” attachment of carbonyl substrates.

SOLUTION 2: -SH functionality allows for strong binding to surface of GNPs as well as Au shells.

SOLUTION 3: Carbonate functionality is more resistant to hydrolysis than ester functionality. In addition, 7-membered ring formation (slow) was selected to discourage unassisted intramolecular cyclization.

SOLUTION 4: The pendant amine reacts only when local heat available to power a 7-ring intramolecular cyclization, which results in substrate cleavage.

As mentioned previously, researchers explored the idea of thermally responsive linkers with either azo or retro-DA functionality. These applications were attached to iron oxide NPs and were successful in externally triggered payload release. The use of linkers that clearly utilize organic reactions for payload release (i.e. azo decomposition, retro-DA

reactions) is limited, so we sought to extend AMF technology to a more readily applied release mechanism of intramolecular cyclization in an effort to expand this type of drug delivery system. Use of a gold shell allows a quick and facile covalent bond between the gold and the thiol functionality without the use of heat or vacuum as seen previously with a silica shell. In addition, gold is inert, meaning it would not have interaction with the linker other than initial attachment so undesired payload release would be minimal. For these reasons, modifications were made for attachment to a gold shell and for incorporation of a water-soluble fluorophore that would mimic release *in vitro* and *in vivo*.

CHAPTER 4

ASSEMBLY AND TESTING OF Fe@Au NP SYSTEMS

- 4.1. INTRODUCTION
- 4.2. INITIAL STUDIES – ANTHRACENE
- 4.3. WATER SOLUBLE STUDIES – FITC
- 4.4. DUAL NANOPARTICLE OPTION
- 4.5. CONCLUSION

4.1. INTRODUCTION

Our amine linker attached to Fe@Au NPs is designed to cyclize in response to local hyperthermia that can be generated upon exposure to an alternating magnetic field (AMF). AMF is an effective method to create local hyperthermia when using magnetic NPs, as described in Chapter 1. The amount of heat generated by the NPs in watts per gram of iron (W/g_{Fe}) is denoted as the specific absorption rate (SAR). This can vary when the machine is “on” because it is not only based on NP morphology, but is also determined by the settings of the instrument, mainly power (W), current frequency (Hz), and current amplitude (A). The current is the flow of electrons, and the electron flow acts as a wave with variances in frequency and amplitude, as illustrated in Figure 4.1.

Figure 4.1. Electrons behave as waves in a current. The frequency is the number of cycles completed per unit time (blue). The amplitude is the maximum height of the wave from its start point (red). An alternating current has positive voltage (above gray line) and negative voltage (below gray line).

An electrical current running through coils generates a magnetic field, as seen in Figure 4.2. In the case of an AMF, the current alternates direction based on the frequency, which in turn alternates the direction of the magnetic field.

Figure 4.2. Magnetic field generation. When current flows through the coil (black), a magnetic field (green) is generated in the direction. In this example, the current flows in through the bottom of the coil and out through the top of the coil, and the magnetic field direction is indicated by the arrows.

Heat generation, or SAR, is dependent partially on the magnetic field strength (kA m^{-1}) and partially on frequency (Hz). Guardia *et al.*¹ explored both magnetic field strength and frequency as it relates to SAR. For magnetic nanocubes, SAR varied linearly with the square of the strength of the magnetic field (Figure 4.3a), and SAR varied linearly with the frequency of the current (Figure 4.3b). In terms of AMF settings, this means a larger amplitude and/or frequency of current leads to larger heat dissipation, or local hyperthermia.

Figure 4.3. a. At constant magnetic field strength (solid: 14 kAm⁻¹, open: 18 kAm⁻¹), SAR (W/g_{Fe}) readings correlate in a direct linear fashion with frequency (kHz). b. At constant frequency (700 kHz), SAR (W/g_{Fe}) readings correlate in a direct linear fashion with the square of the magnetic field strength (kAm⁻¹). Adapted with permission from *ACS Nano* **2012**, 6, 3080-3091. Copyright 2012 American Chemical Society.

Generating local hyperthermia for cancer therapy is an area of great interest. However, setting AMF parameters too high can lead to thermal ablation rather than local

hyperthermia, so settings must be carefully regulated for *in vivo* treatment. In 1984, Atkinson *et al.*ⁱⁱ implanted thermoseeds in healthy volunteers and concluded tolerance up to $35.8 \text{ A} \cdot \text{turns m}^{-1}$ at a frequency of 13.56 MHz for 1 hour. Safety limits from this study led to the “Brezovich criterion”, an equation where the product of the amplitude and frequency should not exceed $4.5 \times 10^8 \text{ Am}^{-1}\text{s}^{-1}$.ⁱⁱⁱ Various *in vivo* testings have shown adherence to this criterion, as seen in Table 4.1.

Magnetic field strength (kA/m); Frequency (kHz)	Nanomaterial	NP concentration	Duration	Adjuvant therapy	Cancer type	Year
< 4; 50	CoPd rods	1 cm apart throughout tumor	60 min	Yes (rad)	Prostate	2003 ^{iva}
2-18; 100	SPIONs with aminosilane-type shell	112 mg/mL	120 min	Yes (rad)	Brain Tumor	2003-2005 ^{4b}
0-18; 100	SPIONs with aminosilane-type shell	120 mg/mL ;0.2 mL mag fluid/mL tumor	60 min	No	Prostate	2005 ^{4c}
2-15; 100	SPIONs with aminosilane-type shell	112 mg/mL; 0.28 mL/cm ³ tumor	60 min	Yes (rad)	Recurrent glioblastoma multiforme	2011 ^{4d}
7.2-13.5; 334.5	Iron oxide NPs	5 nM	60 min			2013 ^{4e}
11.3; 332.5	Iron oxide NPs	2.5 x 10 ⁻³ wt% and 2.5 x 10 ⁻² wt%	2-27.5 min			2013 ^{4f}
1.98; 203	Silica-coated iron oxide NPs	7 mg/0.75 mL	6 x 5 min bursts			2015 ^{4g}
15; 500	Iron oxide NPs	20-25 µg Fe/mL	10-60 sec			2016 ^{4h}
None given; 1950	Zn/Co/Mn doped Fe ₂ O ₄	None given	5 min			2017 ⁴ⁱ
1.98; 203	Gold-coated iron oxide NPs	10 mg/mL	4 x 15 min bursts			2017

Table 4.1. Examples of *in vivo* thermotherapy that adhere to the Brezovich criterion. Light gray portion represents NPs with thermally labile linkers, and settings used for the present thesis work are in blue. See Chapter 4, reference 4, for references to each work.

Recent payload release studies via a thermally labile linker show relatively similar AMF settings (Table 4.1, light gray). Adherence to the Brezovich criterion is crucial for drug delivery that will rely on AMF exposure. Thus, we chose our AMF settings to be similar to the recent given examples in literature (Table 4.1, blue).

4.2. INITIAL STUDIES – ANTHRACENE

The setup of AMF for the following studies is seen in Figure 4.4. The machine is an Ambrell[®] EasyHeat Induction Heating System 10.0 kW with a 5-turn coil for AMF exposure. The machine settings, such as current amperage and duration of AMF exposure, can be adjusted, but the frequency is set at 203 kHz (Figure 4.4a, red box). The bulk solution temperature is monitored using Neoptix NeoLink software, and the computer is connected to the temperature monitor (Figure 4.4a, green box) with a fiber optic probe (Figure 4.4a, yellow). The sample in an Eppendorf tube, surrounded by ceramic paper (Ambrell) to prevent heat from the coils to influence the bulk temperature, is placed on a plastic box at the center of the coils (Figure 4.4a, orange box; Figure 4.4b).

Figure 4.4. AMF machine setup. a. Overall view. In the red box, settings such as current amperage and duration could be adjusted. The frequency for this machine was set to 203 kHz and could not be adjusted. In the green box, a computer uses Neoptix NeoLink software and is connected to a temperature monitor (grey box) with a fiber optic probe (yellow). The orange box contains the sample with the probe to monitor bulk solution temperature placed in the middle of the coils. b. A closer view of the orange box from a shows the sample in an Eppendorf tube surrounded by ceramic paper (Ambrell®, 0.06" thick) to prevent heat transfer from coils. Inset shows aerial view of sample setup.

Prior to loading LS1 (Scheme 4.1, **20**) on Fe@Au NPs, we attached a fluorophore via the aminoxy functionality. For initial studies, an anthracene-aldehyde **8** was used, as previously studied in our lab.ⁱ Fluorophore **8** was loaded on protected LS1 **15** to give protected LS1-anthracene **19**, which was characterized via ¹H NMR, ¹³C NMR, and IR. Figure 4.5 shows **19** as both *E* and *Z* isomers, as indicative of signals from protons *a* and *b*.

Figure 4.5. ^1H NMR spectrum of protected LS1-anthracene **19**. The chemical shift differences between the *E* and *Z* isomer are seen in proton *a* and proton *b*.

Because the payload release weight is identical for both isomers, the geometry of the isomer is trivial. The trityl and Boc protecting groups were simultaneously removed following a literature procedure to give LS1-anthracene **20**.ⁱ Briefly, **19** was dissolved in 1:1 TFA: CH_2Cl_2 at 0 °C, followed by addition of Et_3SiH . The importance of Et_3SiH for the mechanism of trityl deprotection can be seen in Scheme 4.1.

Scheme 4.1. Mechanism of trityl deprotection.

After TFA protonates the sulfur (Step 1), S_N1 chemistry occurs to form a thiol and a tertiary carbocation (Step 2). The hydride of Et_3SiH reduces the carbocation to give triphenylmethane and triethylsilyl trifluoroacetate (Step 3). The solution was stirred for 30 minutes, and upon completion was concentrated *in vacuo* to give **20** and was used without further purification. **20** was not characterized via NMR to prevent dithiol formation prior to loading onto NPs, but a clear decrease in R_f value indicated completion. After concentration *in vacuo*, **20** was redispersed in CH_2Cl_2 , added dropwise to NPs in hexanes, and stirred rapidly for 4 hours. The ratio of linker to NPs was 0.04 mmol linker per 1 mg NPs. This initial loading method was determined our collaborators, who also work in the nanoparticle field, Dr. Ralf Schirmacher and Dr. Jun Zhu at McGill University, Montreal, Canada. After 4 hours, the NPs were precipitated

with EtOH, purified via centrifugation, and dried *in vacuo* to give Fe@Au@LS1-anthracene NPs (Scheme 4.1, **21**).

Scheme 4.2. Synthesis of Fe@Au-LS1-anthracene (**5**). a. CH₂Cl₂, rt, 16 h, 99%; b. Et₃SiH, 1:1 TFA:CH₂Cl₂, 0.5 h; c. CH₂Cl₂, rt, 16 h.

The loaded NPs then were dispersed in 1 mL of a 2:1 water:acetonitrile in a cuvette, followed by exposure to an AMF at 500 A for 15 minutes. After exposure, the NPs were separated via a strong magnet (N52 neodymium 1” magnetic cube, Applied Magnets, Texas) and the supernatant was analyzed via matrix assisted laser desorption ionization-time of flight (MALDI-TOF). When detecting a peak of interest in MALDI, it is possible for the molecule to be protonated (mass + 1), sodiated (mass + 23), or potassiated (mass

+ 39). Thus, masses for each of these versions were calculated and then inspected during analyses.

Figure 4.6. MALDI-TOF spectrum of supernatant obtained from Fe@Au@LS1-anthracene experiment after exposure to AMF at 500 A for 15 minutes. The theoretical m/z for the protonated “payload” fragment corresponds to the found m/z value of 279.22, confirming payload release. The signal at 242.47 corresponds to a peak from the matrix 2,5-DHB.

As seen in Figure 4.6, the signal for the protonated “payload” fragment $[22]^+$ is observed at a m/z of 279.22. This data strongly supports the notion that **22** is released as a result of the AMF exposure, but the actual mechanism of cleavage must still be verified. To investigate our intramolecular cyclization hypothesis, we loaded Fe@Au NPs with our amine-free linker, LS2-anthracene (**24**). The method in which the NPs were reacted with the linker thiol was kept the same. Click-chemistry oximation of anthracene-aldehyde **8** onto protected LS2 **18** delivered protected LS2-anthracene **23**. Protected LS2-anthracene **23** was characterized via ^1H NMR, ^{13}C NMR, and IR. Trityl deprotection gave LS2-anthracene **24**, which was used without further purification. **24** was not characterized via

NMR to prevent dithiol formation prior to loading onto NPs, but a clear decrease in R_f value indicated completion. After concentration *in vacuo*, **24** was redispersed in CH₂Cl₂, added dropwise to NPs in hexanes, and stirred rapidly for 4 hours. The ratio of linker to NPs was 0.04 mmol linker per 1 mg NPs. After 4 hours, the NPs were precipitated with EtOH, purified via centrifugation, and dried *in vacuo* to give Fe@Au@LS2-anthracene NPs (Scheme 4.2, **25**). The NPs were dispersed in 1 mL of a 2:1 water:acetonitrile cuvette, followed by exposure to an AMF at 500 A for 15 minutes. After exposure, the NPs were separated via a strong magnet and the supernatant was analyzed via MALDI-TOF. As seen in Figure 4.7, the m/z signal of 279.22 for the protonated “payload” fragment **[22]**⁺ expected in this case was not seen, indicating payload release had not occurred.

Scheme 4.3. Synthesis of Fe@Au-LS2-anthracene (**9**). a. CH₂Cl₂, rt, 16 h, 99%; b. Et₃SiH, 1:1 TFA:CH₂Cl₂, 0.5 h; c. CH₂Cl₂, rt, 16 h.

Figure 4.7. MALDI-TOF spectrum of supernatant obtained from Fe@Au@LS2-anthracene experiment after exposure to AMF at 500 A for 15 minutes. The theoretical m/z of 279.22 for protonated “payload” fragment is not seen in the spectrum, nor is the theoretical m/z for the sodiated or potassiated “payload” fragment. This led us to believe a payload was not released. The signal at 205.22 corresponds to a peak from the matrix 2,5-DHB.

While this result was what we expected, it is not conclusive (e.g., lack of a signal may be due to other factors). It is possible that, despite following the same preparation, LS2-anthracene **24** was not loaded onto the Fe@Au NPs as efficiently. We therefore sought a method to chemically cleave the payload from the Fe@Au-LS2-anthracene NPs **25** to demonstrate that the system, in fact, did load LS2-anthracene **24**, but simply did not release it upon exposure to an AMF (Figure 4.9). To cleave carbonate functionality, forcing conditions, such as strong base, are usually required. Concellón and del Solarⁱ used 50% w/w aqueous NaOH at room temperature to hydrolyze a chiral carbonate in MeOH to afford enantiopure diols. We tested protected LS2-anthracene **23** prior to trityl deprotection and loading onto NPs following this procedure. Briefly, we added 50% aqueous NaOH to protected LS2-anthracene **23** in methanol and monitored the reaction

via TLC. The starting material was not consumed, even after 4 hours. Despite this, the reaction was worked up, and analysis of the crude product via ^1H NMR revealed no carbonate cleavage. Subsequent experiments at an elevated temperature (i.e. 70 °C for 8 hours) did provide evidence for carbonate cleavage via TLC monitoring, but ^1H NMR analyses were not entirely clear. We next explored reducing the carbonate using lithium aluminum hydride (LAH), a different method used by Concellón and del Solar for carbonate cleavage (Figure 4.9).

Scheme 4.4. Protected LS2-anthracene cleavage using LAH. Ethylene protons that showed signal shift are indicated by *a* and *b*.

LAH is a strong reducing agent that reacts with carbonate functionality at room temperature (25 °C).⁷ ^1H NMR analyses of the reaction of **23** with LAH in THF at room temperature for 1 hour showed peak shifts in the signals from the ethylene protons between the carbonate and payload (Scheme 4.3), indicative of carbonate cleavage. This led us to testing the linker on Fe@Au@LS2-anthracene **25** to determine whether or not the NPs were initially loaded. After reaction of **25** with LAH at 25 °C for 1 hour, the supernatant was analyzed via MALDI. The protonated payload release *m/z* was present, which confirmed that **24** was indeed loaded onto the NPs. However, reaction of **25** with LAH resulted in numerous byproducts as seen in MALDI.

For cleaner analysis, instead of cleaving the carbonate to prove linker attachment, we sought a method to cleave the gold-thiol bond to give the linker system plus payload in its entirety (Figure 4.9). We chose the reducing agent, dithiothreitol (DTT), which is used in both gold-thiol bond cleavage as well as in dithiol reduction.ⁱⁱ Modifying a literature procedure initially disclosed by Storhoff *et al.*,ⁱⁱⁱ we incubated 10 mg of Fe@Au@LS2-anthracene **25** with 400 μ L of 0.1 M DTT for 2 hours, followed by magnetic separation and supernatant analysis.

Figure 4.8. MALDI-TOF spectrum of supernatant obtained from Fe@Au@LS2-anthracene experiment after incubation with DTT for 2 hours (no AMF application prior). The theoretical m/z of 478.15 for potassiated LS2-anthracene corresponds to the found m/z value of 478.14, confirming linker loading. The signal at 411.49 corresponds to a peak from the matrix 2,5-DHB.

This was done without exposure of the NPs to an AMF prior to DTT cleavage to determine if, in fact, **24** was loaded. MALDI-TOF analysis (Figure 4.8) showed a m/z

peak at 478.14, corresponding to the mass of the entire linker **20** plus potassium ($m/z = 478.15$). This data confirmed loading of LS2-anthracene onto the Fe@Au NPs. The previous negative results of payload release from AMF exposure of Fe@Au@LS2-anthracene, followed by these results confirming LS2-anthracene **24** loading, proved our hypothesis. The mechanism for release was not due to carbonate hydrolysis; rather, intramolecular attack of the amine on the carbonate, powered by the local hyperthermia after exposure to an AMF, is a reasonable action to expect for release of anthracene fragment **22** from LS1 versus LS2, where the attack is not possible.

Figure 4.9. Cleavage strategies to confirm retention of payload in **25**. The first and second strategies to cleave the carbonate bond using aq. NaOH and LAH, respectively, gave analytically complicated results. Cleavage of the gold-thiol bond using DTT, however, clearly showed the linker in its entirety.

4.3. WATER SOLUBLE STUDIES – FITC

Knowing that LS1 on Fe@Au NPs responded to AMF exposure, we sought to examine the system using aqueous conditions to determine its release profile in comparison to LS2. The amount of LS loaded onto NPs was modified slightly for these studies. Previously synthesized Fe@Au-LS1-anthracene underwent thermogravimetric analysis (TGA), an analytical method that monitors the mass of a substance as a function

of temperature. The TGA results are seen in Figure 4.10, with calculations below. An amount of 2.457 mg of Fe@Au-LS1-anthracene was heated from 25 °C to 800 °C over 30 minutes with a loss of 0.2015 mg (pink). Using the molecular weight of LS1-anthracene (468.2 g/mol), the amount of mmol LS1-anthracene lost for 2.457 mg of Fe@Au-LS1-anthracene was calculated as 4.3×10^{-4} mmol (Figure 4.10a). By subtracting the amount of LS1-anthracene lost from the total weight, the amount of Fe@Au NPs could be calculated (Figure 4.10b). The amount of mmol LS loaded on each mg NP was calculated as 1.9×10^{-4} mmol/mg (Figure 4.10c). Optimal loading amount of linker to NP was to be 100 times excess as determined by consultation with a collaborator, who works in the gold nanoparticle field, Mr. Kurtis James of the University of Louisville, Kentucky. A total of 0.02 mmol LS per 1 mg Fe@Au NP was used. This is half that of previous studies (0.04 mmol LS per 1 mg Fe@Au NP), meaning linker was not wasted during loading.

Figure 4.10. TGA analysis to determine weight of LS1-anthracene loaded onto Fe@Au NPs. An amount of 2.457 mg Fe@Au-LS1-anthracene lost 0.2015 mg LS1-anthracene. The total amount of LS1-anthracene loaded onto 1 mg Fe@Au NPs was calculated as 1.9×10^{-4} mmol.

Prior to NP loading, LS1 and LS2 required a water-soluble fluorophore, and, as mentioned previously, we chose FITC-CHO. Synthesis of FITC-CHO **9** was described in Scheme 3.4. The procedure for fluorophore loading and attachment to NPs is the same as with anthracene aldehyde **8** but with FITC-CHO **9** instead (Scheme 4.4, 4.5). Briefly, **15** or **18** was dissolved in 9:1 CH₂Cl₂:MeOH followed by addition of FITC-CHO to give crude protected LS1-FITC **26** or protected LS2-FITC **27**. Characterization of **26** via ¹H NMR in CDCl₃ (Figure 4.11, top) reveals a lack of –ONH₂ protons, but the FITC-CHO is not seen. This is because FITC-CHO is not soluble in CDCl₃, but when **26** was dissolved

in DMSO- d_6 (Figure 4.11, bottom), it not only showed the fluorophore attached but also showed *E* and *Z* isomers as indicated by the blue boxes. Protecting group removal with TFA and Et_3SiH revealed LS1-FITC **28** or LS2-FITC **29**. After concentration *in vacuo*, **28** or **29** was redispersed in 9:1 $CHCl_3$:MeOH and added to rapidly stirring Fe@Au NPs dissolved in $CHCl_3$. The mixture was allowed to stir overnight and concentrated *in vacuo* to give Fe@Au-LS1-FITC NPs **30** and Fe@Au-LS2-FITC NPs **31**. The NPs were washed sequentially with EtOH, saturated sodium bicarbonate, and water, pelleting each time via centrifugation before the next wash. Once loaded onto the NPs, Fe@Au-LS1-FITC NPs **30** were water soluble, but unexpectedly Fe@Au-LS2-FITC NPs **31** were not water-soluble, as seen in Figure 4.12. The key difference between the linkers is the secondary amine. We surmise that its ability to hydrogen bond with solvent aids in the water solubility of Fe@Au@LS1-FITC NPs **30**. Fortunately, a common ligand to increase NP water-solubility is polyethylene glycol (PEG) – the oxygen abundant polymer forms hydrogen bonds with water. For that reason, we pursued incorporating PEG to increase water solubility of our NPs.

Figure 4.11. ^1H NMR spectra of **26** in DMSO- d_6 . The protons from FITC-CHO are indicated in green boxes. Signals between 9.0 and 10.2 ppm indicate excess FITC-CHO in solution. As seen with the ethylene protons of protected LS1-anthracene **19**, *E* and *Z* isomers are seen (blue boxes).

Scheme 4.5. Synthesis of Fe@Au-LS1-FITC (**30**). a. 9:1 CHCl₃, rt, 16 h, 99%; b. Et₃SiH, 1:1 TFA:CH₂Cl₂, 0.5 h; c. CH₂Cl₂, rt, 16 h.

Scheme 4.6. Synthesis of Fe@Au-LS2-FITC **31**. a. 9:1 CHCl₃, rt, 16 h, 99%; b. Et₃SiH, 1:1 TFA:CH₂Cl₂, 0.5 h; c. CH₂Cl₂, rt, 16 h.

Figure 4.12. Dispersion of Fe@Au@LS1-FITC NPs (left) and Fe@Au@LS2-FITC NPs (right) in 1X PBS buffer (pH = 7.4) after sonication. Fe@Au@LS1-FITC NPs are well dispersed indicated by the homogenous color, but Fe@Au@LS2-FITC NPs sediment at bottom of the vial.

For our NPs in particular, we used a PEG (MW 5000) modified with a terminal thiol for attachment to our Fe@Au NPs.¹ We explored a variety of ratios of linker to PEG (Table 4.2) to maximize payload release while maintaining water solubility. That is, the greater amount of LS attached, the greater amount of payload carried for release. Nevertheless, an appropriate amount of PEG must be used to maintain water solubility.

Ratio (LS:PEG)	LS1-FITC	LS2-FITC
1:1	Soluble	Soluble
7:3	Soluble	Not Soluble
9:1	Soluble	Not Soluble
10:0	Soluble	Not Soluble

Table 4.2. Ratios of LS:PEG explored. Each ratio used with LS1-FITC was soluble in 1X PBS, whereas only 1:1 LS2-FITC:PEG was soluble in 1X PBS.

Following Carril *et al.*² in their coating of Fe@Au NPs, we began with a 1:1 mixture of mmol linker: mmol PEG-SH. Preparation of the linker system for NP coating remained the same, but prior to its addition, PEG-SH was added to the mixture. The solvent medium changed from CH₂Cl₂ to CHCl₃ to follow a literature prep.¹⁰ A 1:1 mixture of **28** or **29** and PEG-SH was dissolved in 9:1 CHCl₃:MeOH and added to rapidly stirring Fe@Au NPs dissolved in CHCl₃. The mixture was allowed to stir overnight, concentrated *in vacuo*, redissolved in CHCl₃, suspended in EtOH, and purified via centrifugation. After centrifugation, water solubility was evaluated by adding water to the NPs followed by sonication. Both Fe@Au-LS1-FITC-PEG NPs (Figure 4.14a) and Fe@Au-LS2-FITC-PEG NPs (Figure 4.14b) were water-soluble, as seen in Figure 4.12. We also explored linker:PEG ratios of 7:3, 9:1, and 10:0 for both linker systems (Table 4.2). Because Fe@Au@LS1-FITC NPs **30** were water-soluble without the addition of PEG, each of the ratios was water-soluble as well, as expected. Surprisingly, the only ratio that was water soluble for LS2-FITC:PEG was 1:1. Despite long sonication times to aid in dispersion, none of the ratios that increased the linker to above 1:1 were water-soluble. Thus, we continued our studies with this ratio.

Once the NP system was optimized, Fe@Au-LS1-FITC-PEG NPs **30** were characterized via UV-vis spectroscopy, ζ -potential, DLS, and SEM. UV-vis showed a characteristic surface plasmon resonance band at 531 nm, indicating the presence of gold. The ζ -potential was 12.6 ± 0.212 mV. This is not surprising because both LS1-FITC and PEG are neutral in water. The DLS reading was 229.5 ± 0 nm, but the particles show relatively good monodispersity and have an average diameter of 23 ± 7 nm based on SEM imaging (Figure 4.13).

Figure 4.13. Fe@Au-LS1-FITC-PEG NPs **30** characterization. SEM image shows moderate monodispersity of NPs (bright spots) with some salts from washing (light gray spots).

Figure 4.14. a. Fe@Au-LS1-FITC NPs **30** and b. Fe@Au-LS2-FITC NP **31**, with optimal ratio of PEG to LS-FITC as 1:1.

The release profiles were evaluated for both Fe@Au@LS1-FITC-PEG NPs **30** and Fe@Au@LS2-FITC-PEG NPs **31**. Prior to the studies, a calibration curve was created to determine the amount of payload release, as seen in Figure 4.15. Though the fit is not ideal, this calibration curve incorporates all concentration values seen in the following experiment. The excitation and emission of FITC was chosen as 475 nm and 525 nm, respectively, based on maximum excitation and emission studies. These were input into the software for single read mode. FITC has a relatively high fluorescence quantum yield, that is “the fraction of the number of quanta absorbed by a molecule that are emitted as fluorescence” is near 1.0.³ For this reason, FITC could be detected in the picomolar range on the fluorometer. The settings on the AMF were maintained as in our initial studies using the anthracene payload, 500 A and 203 kHz, but the exposure time was different.

Figure 4.15. FITC calibration curve to include concentration values 17 pM to 15.63 mM. Concentrations were established through serial dilutions of FITC dissolved in 1X PBS buffer followed by fluorescence readings. Though R2 value is only 0.977, the large concentration range included the smaller percent release from Fe@Au-LS2-FITC-PEG as well as the larger percent release from Fe@Au-LS1-FITC-PEG and “100%” after DTT incubation.

Our initial studies showed release after only 15 minutes, but we wanted use exposure times similar to that of current testing *in vivo*. In order to track how much payload was released over time, we chose to expose the NPs to four 15-minute AMF bursts with fluorescence readings after each burst. We also chose to pellet the NPs as a means to completely remove them from suspension so as not to interfere with the readings. For the studies, 10 mg of Fe@Au-LS-FITC-PEG were placed in an Eppendorf tube with 1 mL 1X PBS buffer (pH 7.4) and then sonicated to suspend. The Eppendorf tube was placed at the center of the AMF coil, and a fiber optic temperature sensor was placed in the solution to monitor bulk solution temperature. All data was recorded using Neoptix NeoLink software. The NPs were exposed to 15 minutes of AMF (500 A, 203

kHz), followed by centrifugation at 10K rpm for 5 minutes (Figure 4.16a). The supernatant was removed via pipette, added to a plastic cuvette with 2 mL fresh 1X PBS buffer, and analyzed via a fluorometer. The concentration of cleaved payload was calculated based on the calibration curve. After each reading, the solution that was analyzed was added back to the tube containing the NPs and resuspended via sonication so that a cumulative measurement could be made. The total amount of fluorophore loaded onto the NPs – 100% release – was measured after the 4th reading by addition of 400 μ L of a 0.1 M solution of DTT, incubation at rt for 2 hours, centrifugation at 10K rpm for 5 minutes, addition of the supernatant to the cuvette followed by a final reading (Figure 4.16b). Each percent release was determined relative to the total to establish percent released. The studies via AMF were performed on both Fe@Au-LS1-FITC-PEG NPs **32** and Fe@Au-LS2-FITC-PEG NPs **33**, and each linker system was done in triplicate.

Figure 4.16. a. Fe@Au-LS1-FITC-PEG NPs **32** were exposed to AMF for 15 minutes then pelleted via centrifugation for fluorescence readings. This process was repeated a total of four times. After the final reading, the NPs were incubated with DTT for 2 hours then pelleted via centrifugation to give “100%” fluorescence reading. This entire process was performed with Fe@Au-LS2-FITC-PEG NPs **33** as well. b. As exposure time to AMF increased, the percent of payload release increased, as indicated by the darker colors, with the darkest color (i.e. 100% release) seen after DTT incubation.

Figure 4.17. Release profile for Fe@Au-LS1-FITC-PEG NPs **30** (red) and Fe@Au-LS2-FITC-PEG NPs **31** (blue) with 10 mg NP system in 1 mL 1X PBS buffer (pH 7.4). Percent payload released after 4 15-minute bursts of AMF exposure (500A, 203 kHz). The bar gives average release, and the error bars display standard deviation (n = 3).

The release profile for both Fe@Au-LS1-FITC-PEG NPs **30** and Fe@Au-LS2-FITC-PEG NPs **31** is seen in Figure 4.17. After the first 15-minute burst, almost 15% of the payload for LS1 system was released into solution, followed by reaching a plateau of ~40% release after the next 3 bursts. As anticipated, after a total exposure time of 60 minutes, LS2 showed significantly less release than LS1 with only 10% release after 1 hour total exposure time. Starting at room temperature (average temperature of 25 °C), the temperature of the bulk solution reached a maximum temperature of 33 °C, 34.3 °C, 32.5 °C, and 33.3 °C for the 1st through 4th 15-minute exposures, respectively. The bulk temperature of solution with 1X PBS only had a maximum temperature of 32.1 °C after 15 minutes of exposure to AMF. Release profiles were also explored at physiological temperature (37 °C) as well as 50 °C. After incubation of 10 mg Fe@Au-LS1-FITC-PEG in 1X PBS buffer for 2 hours (no AMF) at either 37 °C or 50 °C, only $6.8 \pm 1.4\%$

and $8.4 \pm 6.4\%$ release was seen, respectively. These results suggest that bulk solution temperature does not play a pivotal role in payload release, rather it is the temperature generated at the surface of the NPs that contributes (i.e., actuates intramolecular cyclization) to cause payload release. The high temperature at the surface of the nanoparticle is accompanied by a very fast drop in temperature farther from the surface (i.e., 0-5 nm vs >5 nm), according to the findings by Riedinger *et al.*⁴

While these results were exciting and showed a new mechanism is operative for the first time, the plateau at 40% release perplexed us. A 100% payload release can be expected because the mechanism should not be hindered in some NPs versus others. We postulated that perhaps the addition of the PEG coating was trapping the payload after release from the linker. To explore this possibility, we performed the AMF studies with Fe@Au-LS1-FITC-PEG NPs **30** by washing the NPs with 1X PBS buffer. That is, we placed the first supernatant after AMF exposure into an empty cuvette, resuspended the NPs in fresh 1X PBS via sonication followed by centrifugation. The supernatant was removed and placed into the cuvette, and this process was repeated once more. This method was performed after each 15-minute exposure to AMF to determine the non-cumulative amount released after each burst. After the 4th reading, the NPs were incubated with 400 μ L 0.1 M DTT for 2 h and washed as just described. Each release profile was calculated based on the calibration curve. To make the results comparable to the previous cumulative results, each concentration after exposure was added to the previous results (e.g. concentration reading after 15 minutes was 2.72 pM and concentration reading after 30 minutes was 0.93 pM meaning the total release at 30 minutes was 3.65 pM). The reading after DTT that represented 100% release added all

readings together. Overall, the percent release was similar to the readings without washing. This disproved our hypothesis of the released fluorophore being trapped within the PEG layer. The plateau observation remains to be explained.

We next thought to increase the amount of local hyperthermia generated from the Fe@Au NPs. As stated previously, the maximum bulk temperature for each burst was no higher than 35 °C, and the bulk temperature of 1X PBS buffer without NPs was 32 °C. We thought it possible that the concentration of iron oxide might be too low to produce the necessary thermal energy for intramolecular cyclization and payload release. This could be overcome by either decreasing the amount of solvent with the same amount of iron oxide or increasing the amount of iron oxide with the same amount of solvent. We chose the latter and explored “spiking in” iron oxide NPs to cause the bulk solution to heat to a greater extent, thus triggering more payload release. Our first attempt involved combining 10 mg of Fe@Au-LS1-FITC-PEG NPs **30** in 1 mL 1X PBS buffer with 10 mg iron oxide NPs, previously made for the gold nanoseed project. The cumulative release method was reinstated, as the washing method was clearly no better. Analysis of the supernatant showed release percentages similar to what we observed before. However, we noted that in this experiment the Fe₃O₄ NPs were not water-soluble and thus were not adequately suspended in solution. Rather, the added iron oxide NPs sank to the bottom of the Eppendorf tube during AMF treatment, while Fe@Au-LS1-FITC-PEG NPs **30** remained suspended in solution. The bulk solution had an average temperature of 39 °C, which was higher than the temperature seen with Fe@Au NPs. Likely, the heat necessary to power the intramolecular cyclization at a reasonable rate was not concentrated near the linker system, which may explain why the release percentages were

no better. To determine whether suspension of the iron oxide is truly necessary for greater release, we added ~10 μ L of commercial ferrofluid, EMG 304, FerroTec, into a solution of Fe@Au-LS1-FITC-PEG NPs **30**. The release profile was higher than that of Fe@Au NPs alone, with a maximum of 57% release after 60 minutes total exposure time. In addition, the temperature was significantly higher, with an average maximum bulk temperature solution of 47 °C after 15 minutes, a temperature well above any previous study we performed. These promising results of increased percent payload release prompted us to improve the heating delivery as a means to increase the percent payload release. This ultimately led to the use of water-soluble iron oxide NPs suspended in solution with LS1-FITC coated gold nanoparticles (GNP@LS1-FITC NPs).

4.4. DUAL NANOPARTICLE OPTION

After finding more release and a higher bulk solution temperature with a greater concentration of iron oxide NPs in solution, we chose to explore how to optimize payload release with different concentrations of iron oxide added as part of a dual NP option. We chose not to use Fe@Au NPs to circumvent any potential issues of variance between linker on the gold coating versus the amount of iron oxide present at the core due to size variance in iron oxide core diameter, but instead opted to use a dual NP system consisting of gold NPs (GNPs) and water-soluble magnetic iron oxide NPs (MNPs), as seen in Figure 4.23b. This approach provided a platform for linker attachment (GNPs) with consistent variance in the amount of local hyperthermia generated (MNPs). The mechanism of substrate release was already established from previous studies, which meant only LS1 needed testing. Because Fe@Au-LS1-FITC **30** was water soluble, PEG-

SH was not necessary, and concentration of GNPs in solution could be decreased since the theoretical amount of linker on 10 mgs of Fe@Au-LS1-FITC-PEG NPs **30** was about the same amount on 5 mg of GNP@LS1-FITC NPs. In addition, we decreased the amount of LS1-FITC used for loading, and determined that only 0.01 mmol LS1-FITC per 1mg GNP was necessary. GNP@LS1-FITC NPs were characterized via ζ -potential, DLS, and SEM. ζ -potential showed a surface charge of -20.6 ± 1.63 mV. DLS readings gave an average hydrodynamic diameter of 226.8 ± 26.77 nm, and SEM also shows aggregation of that size (Figure 4.18a). Each individual particle, though, was much smaller, as seen in Figure 4.18b. The fluorescence release was at a lower concentration, so the calibration curve maximum concentration value was decreased. A new calibration curve is seen in Figure 4.19.

Figure 4.18. SEM of GNP@LS1-FITC. a. Aggregation of NPs shows a diameter of 158.5 nm, consistent with DLS measurements. b. Individual NPs show a diameter of 23.57 nm.

Figure 4.19. FITC calibration curve for dual NP system (5 mg GNP@LS1-FITC with 5 mg, 10 mg, or 15 mg of MNP) to include concentration values 17 pM to 1.37 mM. Concentrations were established through serial dilutions of FITC dissolved in 1X PBS buffer followed by fluorescence readings.

The dual NP system was tested under the same conditions as in previous studies with AMF settings of 500 A (magnetic field strength of 1.98 kA/m) and 203 kHz, and exposure to an AMF for four 15-minute bursts. Fluorescent readings were taken between each burst followed by 100% release through DTT incubation. Each experiment contained a ratio of 5 mg of GNP@LS1-FITC NPs with either an equal, twice, or thrice amount of MNP by weight. Each experiment was performed in triplicate, and the results are seen in Figure 4.20.

Figure 4.20. Release profile at 500 A for GNP@LS1-FITC NPs:MNP at ratios of 1:1 (blue), 1:2 (red) and 1:3 (green), showing percent payload release after 4 15-minute bursts of exposure to an AMF (500A, 203 kHz). The 1:1 shows results similar to previous studies with Fe@Au. Significantly more release is seen with both 1:2 and 1:3, almost twice that of previous studies with Fe@Au. The bar gives average release, and the error bars display standard deviation (n = 3).

The data show that suspending iron oxide NPs in solution together with the GNP@LS1-FITC NPs provides the necessary heat on AMF pulsing for payload release to occur. A significant effect is noted when the amount of MNPs were twice or thrice that of GNPs.

We also explored the release profile of the same dual NP system ratios at a lower magnetic field strength (1.19 kA/m, or AMF setting of 300 A and 203 kHz). With the lower magnetic field strength, we hypothesized that payload release would be less than that at 500 A because not as much local hyperthermia would be generated. The dual NP system was tested under the same conditions as in previous studies with exposure to an AMF for 4 15-minute bursts with fluorescent readings taken between each burst followed by 100% release through DTT incubation, but with an AMF setting of 300 A (magnetic

field strength of 1.19 kA/m) and 203 kHz. Each experimental ratio was performed in triplicate, and the results are seen in Figure 4.21.

Figure 4.21. Release profile at 300 A for GNP@LS1-FITC NPs:MNP at ratios of 1:1 (blue), 1:2 (red) and 1:3 (green), showing percent payload release after 4 15-minute bursts of exposure to an AMF (300A, 203 kHz). Release is drastically reduced for each ratio in comparison to release at 500 A, but percent release increases with a greater concentration of MNP. The bar gives average release, and the error bars display standard deviation (n = 3).

As expected, the percent release at 300 A is lower than the percent release at 500 A. This data also shows that the AMF exposure is responsible for the release of the payload, and its decrease substantially effected substrate release. With a lower magnetic field strength, payload release to only 35% is seen. In addition, as seen with the experiments at 500 A, an increased ratio of MNP to GNP@LS1-FITC NPs shows greater percent payload release. Clearly, both the amount of MNP in solution, along with the AMF settings, contributes to the overall percent payload release.

Release profiles were also explored for each experimental ratio (i.e. GNP@LS1-FITC NPs with equal, twice, or thrice the amount of MNP) at physiological temperature (37 °C). Each ratio was incubated in 1X PBS buffer for 2 1-hour intervals at 37 °C followed by centrifugation and analysis. The release profiles after 1 hour for 1:1, 1:2, and 1:3 was 12%, 18%, and 24%, respectively. After the second hour of incubation, the percent release for 1:1, 1:2, and 1:3 increased to 24%, 30%, and 41%, respectively. These values are much larger than the values from Fe@Au NPs. The MNPs were coated in PEG following a literature procedure, but the release profiles suggest the PEG coverage is not complete. One possibility is that the increased hydrolysis found by Knipp *et al.* is happening with the dual NP system at 37 °C, i.e. the iron oxide core is exposed and, because of its Lewis acidity, increased hydrolysis of the carbonate bond. It is also possible that an exposed iron oxide core, which is anionic in nature, leads to local hyperbasicity upon exposure to an AMF for carbonate cleavage. This result suggests controlling the amount added and quality of coating must be controlled.

The Sun group first explored the synthesis of dumbbell-like Au-Fe₃O₄ NPs.⁵ Briefly, the GNPs are synthesized, followed by epitaxial growth (i.e., “single crystalline material grows on single-crystalline substrate”)⁶ of Fe₃O₄ onto GNPs, which allows for two distinct surfaces onto which attachment can be made. The Sun group later used the dumbbell-like NPs as a drug delivery system.⁷ A Her2-specific monoclonal antibody Herceptin was attached onto the iron oxide and a modified cisplatin was attached onto the gold with a short thiol terminated linker, as seen in Figure 4.22.

Figure 4.22. Dumbbell-like NPs with Herceptin attached to iron oxide NP via a catechol and cisplatin attached to gold via a thiol. Adapted figure with permission from *Nano Lett.* **2005**, 5, 379-382. Copyright 2005 American Chemical Society.

The functionalized NPs were incubated at 37 °C with Sk-Br3 cells (Her2-positive breast cancer cells) and MCF-7 cells (Her2-negative breast cancer cells). As expected, the Her2 receptor NPs better targeted the Sk-Br3 cells as compared to the MCF-7 cells. In addition, 70% modified cisplatin was released into solution at pH 6 after one hour incubation at 37 °C. By using a “dual NP” system that attaches the NPs together, distribution can be improved and both NPs arrive at the same intended destination. If the dumbbell-like NPs were not attached, the 2nm and 8nm GNPs would largely be found in the kidneys, whereas the larger iron oxide NPs would not.⁸

Figure 4.23. Different NP systems with exposure to AMF for payload release. a. Core-shell Fe@Au NPs with coating of PEG (~~~~) and linker in a ratio of 1:1. Upon exposure to an AMF, the core creates local hyperthermia to induce intramolecular cyclization (amine = •, carbonate = •) and payload release (•). b. Dual NP system with GNP coated with LS1-FITC and MNP. Upon exposure to an AMF, the MNPs create local hyperthermia to induce intramolecular cyclization and payload release from the GNPs.

4.5. CONCLUSION

This thesis work has established that an alternating magnetic field can be used to cause substrate release from NP carriers (Figure 4.23). We have shown for the first time that intramolecular cyclization of linkers attached to Fe@Au NPs can function as a triggerable mechanism for delivery. The challenge of creating monodispersed, evenly coated Fe@Au NPs with minimal contamination by pure GNPs is a substantial hurdle to

using this NP system as a carrier. The Fe@Au NPs we prepared and characterized were likely contaminated with substantial amounts of GNPs. Despite that, our Fe@Au NP system clearly released payload (~40%) upon exposure to an AMF. The dual NP system released payload at both a higher setting of 500 A – nearly complete release was seen – and a lower setting of 300 A. The concentration of iron oxide greatly improved local hyperthermia generation and can improve release efficiency. Because the premature payload release is a common problem when designing drug delivery systems containing Fe₃O₄, the carbonate moiety may not be ideal for further development. The carbonate functionality proved not to withstand mild heating without payload release, as seen in the dual NP system. If this remained the case with pure Fe@Au NPs, carbamate functionality would be the next option (Figure 4.24). The delivery system described in this work is flexible and can accommodate such structural changes.

Figure 4.24. Modification of (a) LS1 from carbonate to (b) carbamate as indicated by the rectangle. Upon exposure to AMF, the carbamate will cyclize, release the payload (right), and leave a cyclic urea on the NP (left).

CHAPTER 5

EXPERIMENTAL PROCEDURES

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5.1. GENERAL STATEMENT

Reagent grade solvents were used for extraction and flash chromatography. Acetonitrile was dried by distillation from CaH_2 . All other commercial reagents were used as received without additional purification. The progress of reactions was monitored by thin-layer chromatography (TLC) using pre-coated silica plates (EMD Silica Gel 60 F254). Visualization was accomplished by staining plates with PMA (3% phosphomolybdic acid/ethanol solution) or PAA stain (2.5% p-anisaldehyde acid/ethanol solution). UV active compounds were visualized by UV light (254 nm). Silica gel 60 (230-400 mesh) was used for flash column chromatography. ^1H NMR spectra were recorded at 400 or 500 MHz, and ^{13}C spectra were recorded at 100 or 125 MHz, respectively, in the indicated solvents. The chemical shifts are reported in ppm values relative to the solvent residual peak CDCl_3 (7.26 ppm for ^1H NMR and 77.23 ppm for ^{13}C NMR) or TMS (0.00 ppm for ^1H NMR). Coupling constants are reported in hertz (Hz). High-resolution mass spectra were obtained using a FT-ICR-MS system (LTQ FT, Thermo Electron Corp.) housed at the Center for Regulatory and Environmental Analytical Metabolomics (CREAM) Mass Spectrometry Facility, University of Louisville. Fluorescent measurements were taken on a Perkin Elmer LS 55 fluorescence spectrometer at an excitation wavelength of 475 nm and emission wavelength of 525 nm. Fluorescent measurements were taken using a plastic VWR two-sided spectrophotometer cell with a 10 mm light path. Scanning electron microscopy (SEM), transmission electron microscopy (TEM), and scanning transmission electron microscopy (STEM) was used to analyze size distribution and morphology of fabricated nanoparticles at the Conn Center for Renewable Energy Research, University of Louisville, Kentucky or at the

Nanotechnology Core Facility, University of Louisville, Kentucky. For SEM and STEM, hexane-based (Fe@Au and GNPs) or water-based (Fe@Au-LS1-FITC NPs, GNP@LS1-FITC NPs, and MNPs) nanoparticle dispersions were prepared and drop-casted on commercially available 200 mesh TEM support Cu grids coated with ultra thin carbon films. After solvent evaporation, the samples were imaged on a 200 carbon/copper mesh with a Zeiss Supra Scanning Electron Microscope at a voltage between 10 kV and 25 kV. For TEM, water-based nanoparticle dispersions were prepared and drop-casted on commercially available 300 mesh TEM support Cu grids coated with ultra thin carbon films. After water evaporation, samples were transferred to and analyzed using a field emission gun FEI Tecnai F20 transmission electron microscope operating at the accelerating voltage of 200 kV. Energy Dispersive A-ray Analysis (EDAX) was performed on the Zeiss SUPRA-FE-SEM (Peabody, MA) at 20 keV. Samples were prepared on a carbon coated copper mesh grid with a working distance of 8 mm. Thermogravimetric analysis (TGA) measurements were made on a TA Instruments Hi-Res TGA 2950 Thermogravimetric Analyzer using a Pt basket and maintaining a flow of N₂ gas through the oven. Each TGA experiment was run from 35 C to 800 C at a ramp rate of 20 °C/min. UV/Vis readings were taken using a Varian Cary 50 Bio. DLS and ζ -potential measurements were taken using a Malvern Zetasizer: Nano-Zs90. All measurements were taken using aqueous colloids of the nanoparticles in Millipore water. The alternating magnetic field (AMF) was generated with a Ambrell EasyHeat L1 set at either 499.6 amps or 300.2 A and 203 kHz using a 5-turn coil. MALDI-TOF analysis was done on a Voyager DE-Pro MALDI-TOF instrument (PE Biosystems). Sample preparation was provided by Bruker Guide to MALDI Sample Preparation. Briefly,

sample solvent (30:70 v/v acetonitrile:0.1% TFA in water) was added to an equal amount of sample in liquid medium after testing. The matrix solution was prepared by addition of 20 mg/mL 2,5-dihydroxybenzoic acid (2,5-DHB) to sample solvent. This was added to an equal amount of sample in sample solvent and mixed vigorously. 0.5 μ L sample + matrix was spotted onto a ground steel MALDI plate. For matrix reference, 0.5 μ L matrix solvent was spotted onto a ground steel MALDI plate as well. Spectra were acquired in positive reflectron mode and calibration was achieved by using known peaks from the 2,5-DHB matrix.

5.2. CHAPTER 2 EXPERIMENTAL PROCEDURES

5.2.1. Preparation of gold seeded iron oxide NPs

Preparation of Fe₃O₄@APTES

Modifying the literature procedure of Montazerabadi et al.,¹ 3-aminopropyl(triethoxysilane) (300 μ L, 1.28 mmol) was added to iron oxide NPs (157.5 mg, 0.68 mmol) and sonicated at rt for 30 minutes. This mixture was heated at 60 C for 4 h, followed by magnetic separation and washing with MeOH. The NPs were dried in vacuo and characterized by FT-IR, which showed characteristic C-H stretch at 2929 cm^{-1} and 2840 cm^{-1} , N-H stretch at 3358 cm^{-1} , and Si-O-R stretch at 1043 cm^{-1} (Chapter 2, Figure 2.3).

Preparation of Fe₃O₄@nAu

Modifying the literature procedure of Sharma et al.,² CS₂ (1.5 mL, 24.94 mmol) was added to a suspension of Fe₃O₄@APTES (157.5 mg) in borate buffer (1.5 mL) and stirred for 1 h at rt. To this solution was added gold nanoseeds (1.5 mL of 10mM soln) and stirred rapidly overnight. The NPs were magnetically separated and rinsed with DI water (5 x 1 mL) to give Fe₃O₄@nAu NPs. Fe₃O₄@nAu NPs were characterized by HR-TEM and EDAX (Chapter 2, Figure 2.4 and Figure 2.5), which showed inconsistent coating of gold nanoseeds onto the iron oxide core.

Preparation of Fe@Au by reduction with ascorbic acid

Modifying the literature procedure of Montazerabadi et al.,¹ HAuCl₄ (0.5 mL of 10 mM soln) was added to Fe₃O₄@nAu NPs (18 mg) in water (2 mL) and sonicated for

30 mins at rt. To the suspension was added ascorbic acid (100 mg, 0.57 mmol) and stirred vigorously for 2 h, followed by magnetic separation and washing with water until supernatant was clear (2 x 5 mL) then resuspended in water (10 mL) to give Fe@Au NPs. These NPs were characterized via UV-Vis, DLS, and ζ -potential. UV-Vis did not show a SPR band, DLS revealed an average hydrodynamic diameter of 2500 nm, and ζ -potential showed an average surface charge of -6.0 mV.

Preparation of Fe@Au by reduction with sodium citrate

Modifying the literature procedure of Hu et al.,³ HAuCl₄ (0.5 mL of 10 mM soln) was added to Fe₃O₄@nAu NPs (18 mg,) in water (200 mL) while stirring vigorously then immediately heated to 100 °C and held there for 10 minutes. To this solution was added sodium citrate (10 mL of 5 wt % soln) and stirred for another 20 minutes at 100 °C. Upon cooling, the NPs were magnetically separated and washed with DI water (2 x 10 mL) then resuspended in water (10 mL) to give Fe@Au NPs. These NPs were characterized via UV-Vis, DLS, and ζ -potential. UV-Vis did not show a SPR band, DLS revealed an average hydrodynamic diameter of 3000 nm, and ζ -potential showed an average surface charge of -42.0 mV.

Preparation of Fe@Au by reduction with glucose

Modifying the literature procedure of Mandal et al.,⁴ glucose (0.5 g, 2.77 mmol), Fe₃O₄@nAu NPs (18 mg), and HAuCl₄ (0.5 mL of 10 mM soln) were added together and sonicated for 15 mins., then heated at 35-40 °C for 1 h. The NPs were magnetically separated and washed with DI water (3 x 5 mL) then resuspended in water (10 mL) to

give Fe@Au NPs. These NPs were characterized via UV-Vis, DLS, and ζ -potential. UV-Vis did not show a SPR band, DLS revealed a cluster of NPs with hydrodynamic diameter of 700 and 200 nm, and ζ -potential showed an average surface charge of +32.2 mV.

Preparation of Fe@Au by reduction with sodium borohydride

Modifying the literature procedure of Rivas,⁵ sodium borohydride (10 mL of 0.6M soln) was added to Fe₃O₄@nAu NPs (18 mg), followed by addition of HAuCl₄ (1 mL of 10mM soln) and stirred vigorously at rt for 5 h. The NPs were magnetically separated and washed with DI water (5 mL) then resuspended in water (10 mL) to give Fe@Au NPs. These NPs were characterized via UV-Vis, DLS, and ζ -potential. UV-Vis did not show a SPR band, DLS revealed an average hydrodynamic diameter of 2400 nm, and ζ -potential showed an average surface charge of -15.7 mV.

5.2.2. Fe@Au NPs

Modifying the literature procedure of Park *et al.*,⁶ FeCl₃·6H₂O (540.6 mg, 2 mmol), FeCl₂·6H₂O (198.8 mg, 1 mmol), and C₁₈H₃₃ONa (2.4 g, 8 mmol) were dissolved in ethanol (6 mL), deoxygenated water (4.5 mL), and toluene (10.5 mL). This mixture was heated at 74 °C for 4 h under nitrogen. The resulting black mixture was cooled to room temperature and ethanol (50 mL) was added to precipitate the particles. The mixture was centrifuged at 4000 rpm for 10 min at 4 °C to pellet. A clear, light orange supernatant was decanted off and the residual black pellet was re-dispersed in hexane (5 mL) by vortexing. Redispersed particles were precipitated with ethanol (40 mL) then

centrifuged at 4000 rpm for 10 min at 4 °C to pellet. Redispersal, precipitation, and centrifugation were repeated two more times. The pelleted particles were redispersed in hexane (40 mL) and undispersed residues removed by centrifugation at 4000 rpm for 5 min at 4 °C. The resulting black supernatant was decanted, and hexane removed *in vacuo* to obtain nanoparticles in solid form. Nanoparticles were stored under nitrogen at rt until further use. NPs were analyzed via HR-TEM, EDAX, and SQUID (Chapter 2, Figure 2.6). HR-TEM revealed an average diameter of 5 ± 1 nm with good monodispersity. EDAX gave characteristic peaks for iron oxide. The Cu peaks are due to the copper grid used for analysis. SQUID measurements showed a magnetic saturation of 0.3 emu/g with no hysteresis loop at room temperature. This indicated superparamagnetic NPs because a hysteresis loop should not be detected above the blocking temperature, in this case 27 °C.

Modifying the literature procedure of Wang *et al.*,⁷ a solution of as prepared iron oxide NPs (16 mg, 0.07 mmol), gold (III) acetate (80 mg, 0.2 mmol), oleic acid (50 μ L, 0.15 mmol), oleylamine (300 μ L, 0.911 mmol), and 1,2-tetradecandiol (0.3 g, 1.3 mmol) in phenyl ether (10 mL) under nitrogen was heated at 185 °C for 1.5 h. After cooling to rt, EtOH (40 mL) was added to precipitate the core/shell NP. The mixture was centrifuged at 3000 rpm for 5 min and the supernatant was discarded. The precipitate was redissolved in hexane (5 mL), followed by addition of EtOH 40 mL). The mixture was centrifuged at 3000 rpm for 10 min and the upper purple GNP solution was discarded. This process was repeated 5 times. The core/shell NP was collected after centrifuge and NP were stored in solid form under nitrogen at rt until further use. NPs were characterized via STEM, EDAX, UV-Vis, and SQUID (Chapter 2, Figure 2.7, Figure 2.8, Figure 2.9). STEM revealed an average NP diameter of 7 ± 3 nm with

relatively good monodispersity, consistent with literature values.² EDAX showed characteristic peaks for both iron oxide and gold based on literature.² UV-Vis analysis showed a characteristic surface plasmon resonance around 530 nm, consistent with literature values.² SQUID measurements showed a magnetic saturation of 6 emu/g with no detectable hysteresis loop above the blocking temperature of 27 °C.

5.2.3. Gold NPs

Modifying the literature procedure of de la Presa *et al.*,⁸ to a rb flask a solution of gold (III) acetate (100 mg, 0.27 mmol) and 1,2-tetradecanediol (500 mg, 2.17 mmol) in phenylether (30 mL) were added at rt. The solution was heated up to 80 °C under nitrogen, then oleic acid (0.32 mL, 1.01 mmol) and oleylamine (0.34 mL, 1.03 mmol) were added and heated to reflux (260 °C) for 30 minutes. After cooling to room temperature, the GNPs were precipitated by adding EtOH (40 mL) and pelleted via centrifugation at 3000 rpm for 5 min and the supernatant was discarded. The precipitate was redissolved in hexane (5 mL), followed by addition of EtOH (40 mL). The mixture was centrifuged at 3000 rpm for 10 min and the supernatant was discarded. This was done once more, and after the final removal of supernatant, the NPs were dried *in vacuo* and stored in solid form under nitrogen at room temperature until further use. NPs were characterized via DLS and SEM (Chapter 2, Figure 2.10). DLS revealed a hydrodynamic diameter of 224.3 ± 11.2 nm, whereas SEM revealed 5 ± 3.7 nm.

5.2.4. Water-soluble iron oxide NPs

Modifying the literature procedure of García-Jimeno and Estelrich,⁹ PEG 2K (6.0 g, 3 mmol) was dissolved in a rb flask containing water (5 mL) while stirring at 45 °C. To this was added FeCl₂•4H₂O (0.16 g, 0.8 mmol) and FeCl₃•6H₂O (0.435 g, 1.6 mmol). After dissolution, NH₄OH (10 mL of a 0.75M solution) was added under vigorous stirring and stirred a further 30 min. Upon cooling to rt, the MNPs were poured into a beaker and placed on a neodymium magnet (N52 neodymium 1” magnetic cube, Applied Magnets, Texas). The supernatant was decanted and the MNPs were washed four more times with water. Water was added until the desired concentration was achieved and sonicated for 12 minutes. NPs were characterized by ζ-potential, DLS and SEM (Chapter 2, Figure 2.11). ζ-potential showed a surface charge of -22.4 ± 2.02 mV. DLS showed a hydrodynamic diameter of 716.2 ± 74.3 nm, whereas SEM gave a diameter of 50 ± 16 nm.

5.3. CHAPTER 3 EXPERIMENTAL PROCEDURES

5.3.1. Amine Linker

4-((*tert*-Butyldimethylsilyl)oxy)butan-1-ol. A solution of TBSCl (6.00 g, 39.8 mmol) in dry CH₂Cl₂ (100 mL) was added dropwise over 1 h to a solution of 1,4-butanediol (16.9 mL, 191 mmol) and Et₃N (7.77 mL, 55.7 mmol) in dry CH₂Cl₂ (100 mL) at 0 °C. The reaction mixture was stirred overnight at rt. The solvent was removed *in vacuo* and the remaining oil was extracted with hexanes (4x) and the combined extractions were washed twice with sat. NH₄Cl, once with brine, and then dried over Na₂SO₄, filtered and concentrated *in vacuo* to afford crude 4-((*tert*-butyldimethylsilyl)oxy)butan-1-ol (7.28 g, 89%) as a colorless oil. This material was used in the next step without further purification. Spectral characteristics agreed with published data.¹⁰ IR ν (cm⁻¹) 3349; ¹H NMR (400 MHz, CDCl₃) δ 0.07 (s, 6H), 0.90 (s, 9H), 1.60-1.69 (m, 2H), 2.86 (br s, 1H), 3.62-3.68 (m, 4H); ¹³C NMR (100 MHz, CDCl₃) δ -5.4, 18.3, 25.9, 29.8, 30.1, 62.7, 63.4. HRMS m/z calcd [C₁₀H₂₅O₂Si]⁺ 205.1618, observed 205.1618.

4-((*tert*-Butyldimethylsilyl)oxy)butyl methanesulfonate. Methanesulfonyl chloride (1.09 mL, 14.1 mmol) was added to a solution of crude 4-((*tert*-butyldimethylsilyl)oxy)butan-1-ol (2.51 g, 12.3 mmol) and Et₃N (2.59 mL, 18.4 mmol) in dry CH₂Cl₂ (50 mL) at 0 °C and the reaction mixture was stirred for 2 h at 0 °C and washed twice with sat. NH₄Cl. The combined aqueous layers were extracted twice with

CH₂Cl₂. The combined organic phases were washed once with brine and then dried over Na₂SO₄, filtered and concentrated *in vacuo* to afford 4-((*tert*-butyldimethylsilyl)oxy)butyl methanesulfonate (3.22 g, 93%) as an orange oil. This material was used without further purification. IR ν (cm⁻¹) 1173, 1426; ¹H NMR (400 MHz, CDCl₃) δ 0.05 (s, 6H), 0.89 (s, 9H), 1.63 (m, 2H), 1.84 (m, 2H), 3.00 (s, 3H), 3.65 (t, *J* = 6.0 Hz, 2H), 4.27 (t, *J* = 6.8 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃) δ -5.4, 18.3, 25.9, 28.6, 37.3, 62.2, 70.2.

2-(4-((*tert*-Butyldimethylsilyl)oxy)butyl)-2,3-dihydro-1*H*-isoindole-1,3-dione.

Phthalimide (3.19 g, 21.7 mmol) was added to a solution of 4-((*tert*-butyldimethylsilyl)oxy)butyl methanesulfonate (3.22 g, 11.4 mmol) and K₂CO₃ (1.89 g, 13.7 mmol) in DMSO (40 mL). The solution was heated to 75 °C for 17 h. The reaction was then cooled to rt and quenched with water. The aqueous solution was extracted with EtOAc (4x) and the combined organic phases were washed with water (3x). The organic phase was then washed with brine, dried over Na₂SO₄, filtered and concentrated *in vacuo* to afford 2-(4-((*tert*-butyldimethylsilyl)oxy)butyl)-2,3-dihydro-1*H*- isoindole-1,3-dione as white crystals, mp 188-190 °C, and was used without further purification. IR ν (cm⁻¹) 1707; ¹H NMR (400 MHz, CDCl₃) δ 0.04 (s, 6H), 0.88 (s, 9H), 1.53-1.60 (m, 2H), 1.71-1.79 (m, 2H), 3.64 (t, *J* = 6.2 Hz, 2H), 3.72 (t, *J* = 7.2 Hz, 2H), 7.71 (dd, *J* = 3.0, 5.4 Hz, 2H), 7.84 (dd, *J* = 3.2, 5.2 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃) δ -5.2, 18.5, 25.3, 26.2, 30.2, 38.0, 62.7, 123.3, 132.4, 134.0, 168.6.

(4-Aminobutoxy)(*tert*-butyl)dimethylsilane (11). Hydrazine monohydrate (2.95 mL, 60.9 mmol) was added to a solution of crude 2-(4-((*tert*-butyldimethylsilyl)oxy)butyl)-2,3-dihydro-1*H*-isoindole-1,3-dione (4.06 g, 12.2 mmol) in 2:1 CH₂Cl₂:EtOH (60 mL) at 0 °C. The reaction mixture was stirred overnight, allowing the reaction to come to rt. The solution was diluted with CH₂Cl₂, the white precipitate was filtered, and the filter cake was washed with ample CH₂Cl₂. The crude solution was concentrated *in vacuo* to afford **11** (2.37 g, 98% over 2 steps) as a light yellow oil, having spectral characteristics in agreement with published data,¹¹ and was used without further purification. IR ν (cm⁻¹) 3024, 3058; ¹H NMR (400 MHz, CDCl₃) δ 0.06 (s, 6H), 0.89 (s, 9H), 1.60-1.67 (m, 2H), 1.79-1.87 (m, 2H), 3.01 (t, *J* = 7.2 Hz, 2H), 3.65 (t, *J* = 6.0 Hz); ¹³C NMR (100 MHz, CDCl₃) δ -5.3, 18.2, 24.6, 25.9, 29.7, 39.9, 62.3. HRMS *m/z* calcd [C₁₀H₂₆NOSi]⁺ 204.1778, observed 204.1779.

(((3-Bromopropyl)sulfanyl)diphenylmethyl)benzene (12). K₂CO₃ (0.80 g, 5.8 mmol) followed by 1,3-dibromopropane (2.67 mL, 26.6 mmol) was added to a solution of triphenylmethanethiol (1.5 g, 5.4 mmol) in dry THF (27 mL) under N₂. The reaction was refluxed for 24 h before cooling to rt. The reaction solution was washed twice with water, extracted twice with Et₂O, washed with brine, dried over Na₂SO₄, filtered and concentrated *in vacuo*. The excess 1,3-dibromopropane was removed by distillation to

afford **12** (1.99 g, 92%) as white crystals, mp 85-86 °C, (lit 90-92 °C) having spectral characteristics in agreement with published data.¹² IR ν (cm⁻¹) 660, 1254; ¹H NMR (400 MHz, CDCl₃) δ 1.78-1.85 (m, 2H), 2.33 (t, J = 7.0 Hz, 2H), 3.33 (t, J = 6.6 Hz, 2H), 7.20-7.31 (m, 9H), 7.41-7.43 (m, 6H); ¹³C NMR (100 MHz, CDCl₃) δ 30.5, 31.8, 32.5, 66.9, 126.9, 128.1, 129.8, 144.9.

12,12,13,13-Tetramethyl-1,1,1-triphenyl-11-oxa-2-thia-6-aza-12-silatetradecane (13).

(((3-Bromopropyl)sulfanyl)diphenylmethyl)benzene (742 mg, 1.87 mmol) was added to a solution of (4-aminobutoxy)(*tert*-butyl)dimethylsilane (950 g, 4.68 mmol) in MeCN (20 mL) and the reaction was heated to 55 °C for 24 h. After cooling to rt, the reaction was quenched with sat. NaHCO₃ and extracted three times with EtOAc. The combined organic phases were washed with brine, dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude material was purified by column chromatography (SiO₂, 0:1 to 1:4, MeOH:CH₂Cl₂ with 1% NH₄OH gradient) to give **13** (692 mg, 71%) as an orange oil. IR ν (cm⁻¹) 697, 3300; ¹H NMR (400 MHz, CDCl₃) δ 0.04 (s, 6H), 0.89 (s, 9H), 1.45-1.52 (m, 4H), 1.53-1.60 (m, 2H), 2.19 (t, J = 7.2 Hz, 2H), 2.52 (q, J = 7.2 Hz, 4H), 3.60 (t, J = 5.8 Hz, 2H), 7.17-7.28 (m, 9H), 7.40-7.42 (m, 6H); ¹³C NMR (100 MHz, CDCl₃) δ -5.1, 18.5, 26.1, 26.7, 29.1, 29.9, 30.8, 49.0, 49.8, 63.2, 66.7, 126.7, 128.0, 129.7, 145.1. HRMS m/z calcd [C₃₂H₄₆NOSSi]⁺ 520.3064, observed 520.3060.

tert-Butyl N-(4((tert-butyldimethylsilyl)oxy)butyl)-N-(3-((triphenylmethyl)sufanyl)propyl)carbamate. Boc₂O (327 mg, 11.5 mmol) was added to a solution of 12,12,13,13-tetramethyl-1,1,1-triphenyl-11-oxa-2-thia-6-aza-12-silatetradecane (710 mg, 1.37 mmol) and Et₃N (211 μ L, 1.5 mmol) in CH₂Cl₂ (14 mL) at 0 °C. After 3 h, the reaction solution was washed twice with sat. NH₄Cl and the aqueous phase was extracted twice with CH₂Cl₂. The combined organic phases were washed with brine, dried over Na₂SO₄, filtered and concentrated *in vacuo* to afford crude *tert*-butyl *N*-(4((*tert*-butyldimethylsilyl)oxy)butyl)-*N*-(3-((triphenylmethyl)sufanyl)propyl)carbamate as an orange oil and was used without further purification. IR ν (cm⁻¹) 1693; ¹H NMR (400 MHz, CDCl₃) δ 0.04 (s, 6H), 0.88 (s, 9H), 1.39 (br s, 4H), 1.45 (br s, 2H), 1.53 (s, 9H), 2.13 (t, *J* = 7.6 Hz, 2H), 3.05 (br s, 4H), 3.58 (t, *J* = 5.6 Hz, 2H), 7.18-7.29 (m, 9H), 7.39-7.41 (m, 6H); ¹³C NMR (100 MHz, CDCl₃) δ -5.1, 18.5, 25.4, 26.2, 27.6, 28.6, 29.6, 30.3, 46.5, 47.0, 63.0, 66.8, 79.3, 126.8, 128.1, 129.8, 145.1, 155.6.

tert-Butyl N-(4-hydroxybutyl)-N-(3-((triphenylmethyl)sulfanyl)propyl)carbamate. TBAF (1.5 mL of 1 *M* solution in THF, 1.5 mmol) was added to a solution of crude *tert*-butyl *N*-(4((*tert*-butyldimethylsilyl)oxy)butyl)-*N*-(3-((triphenylmethyl)sufanyl)propyl)carbamate (1.4 mmol) in dry THF (5 mL) at 0 °C. The reaction mixture was stirred overnight, allowing the reaction to warm to rt. Upon completion as determined by

TLC analysis, the reaction was washed twice with sat. NaHCO₃ and the combined aqueous phases were extracted three times with Et₂O. The combined organic layers were washed with brine, dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude material was purified by column chromatography (SiO₂, 3:7, EtOAc:CH₂Cl₂) to give *tert*-butyl *N*-(4-hydroxybutyl)-*N*-(3-((triphenylmethyl)sulfanyl)propyl)carbamate (673 mg, 98% over 2 steps) as a light yellow oil. IR ν (cm⁻¹) 1669, 3426; ¹H NMR (400 MHz, CDCl₃) δ 1.39 (s, 9H), 1.45-1.51 (m, 4H), 1.54-1.58 (m, 2H), 2.14 (t, *J* = 7.2 Hz, 2H), 2.29 (br s, 1H), 3.05 (br s, 4H), 3.60 (t, *J* = 5.6 Hz, 2H), 7.17-7.28 (m, 9H), 7.39-7.41 (m, 6H); ¹³C NMR (100 MHz, CDCl₃) δ 24.9, 28.0, 28.6, 29.6, 29.8, 46.6, 46.9, 62.4, 66.8, 79.6, 126.8, 128.1, 129.8, 145.0, 155.7. HRMS *m/z* calcd [C₆₂H₇₉N₂O₆S₂]⁺³ 1011.5363, observed 1011.5385.

4-(((*tert*-Butoxy)carbonyl)((3-((triphenylmethyl)sulfanyl)propyl))amino) butyl 1H-imidazole-1-carboxylate (14). 1,1'-Carbonyldiimidazole (154 mg, 0.95 mmol) was added to a solution of *tert*-butyl *N*-(4-hydroxybutyl)-*N*-(3-((triphenylmethyl)sulfanyl)propyl) carbamate (320 mg, 0.63 mmol) and (*i*-Pr)₂NEt (166 μ L, 0.95 mmol) in dry CH₂Cl₂ (2 mL) at 0 °C. The mixture was stirred overnight, allowing the reaction to warm to rt. The reaction solution was washed twice with water and extracted once with CH₂Cl₂. The combined organic layers were washed twice with sat. NH₄Cl, once with brine, were dried over Na₂SO₄, filtered and concentrated *in vacuo*

to afford crude **5** (350 mg, 92%) as a light yellow oil and was used without further purification. IR ν (cm⁻¹) 1686, 1760; ¹H NMR (400 MHz, CDCl₃) δ 1.39 (s, 9H), 1.52-1.61 (m, 4H), 1.70-1.77 (m, 2H), 2.15 (t, J = 7.2 Hz, 2H), 3.09 (br s, 4H), 4.40 (t, J = 6.6 Hz, 2H), 7.06 (s, 1H), 7.18-7.29 (m, 9H), 7.39-7.41 (m, 7H), 8.13 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 24.6, 26.0, 28.2, 28.5, 29.5, 46.5 (2 C's), 66.8, 68.1, 79.6, 117.2, 126.7, 128.0, 129.7, 130.8, 137.2, 144.9, 148.8, 155.4. HRMS m/z calcd [C₃₅H₄₂N₃O₄S]⁺ 600.2891, observed 600.2892.

2-(2-(((4-(((*tert*-Butoxy)carbonyl)((3-((triphenylmethyl)sulfanyl)propyl))amino)butoxy)carbonyl)oxy)ethoxy)-2,3-dihydro-1*H*-isoindole-1,3-dione. DBU (434 μ L, 2.80 mmol) was added to a solution of crude *tert*-butyl *N*-(4-hydroxybutyl)-*N*-(3-((triphenylmethyl)sulfanyl)propyl)carbamate (1.64 g, 2.80 mmol) in dry MeCN (12 mL). After stirring for 10 min, 2-(2-hydroxyethoxy)-2,3-dihydro-1*H*-isoindole-1,3-dione (579 mg, 2.80 mmol) was added and the reaction was stirred overnight. Upon completion, the reaction was washed twice with sat. NH₄Cl and the combined aqueous layers were extracted three times with EtOAc. The combined organic layers were washed with brine, dried over Na₂SO₄, filtered and concentrated *in vacuo*. The crude material was purified by column chromatography (SiO₂, 3:17, EtOAc:CH₂Cl₂) to give 2-(2-(((4-(((*tert*-butoxy)carbonyl)((3-((triphenylmethyl)sulfanyl)propyl))amino)butoxy)carbonyl)oxy)ethoxy)-2,3-dihydro-1*H*-isoindole-1,3-dione (350 mg, 60%) as a colorless oil. IR ν (cm⁻¹

¹) 1685, 1733, 1793; ¹H NMR (400 MHz, CDCl₃) δ 1.40 (s, 9H), 1.48-1.60 (m, 6H), 2.16 (t, *J* = 7.0 Hz, 2H), 3.06 (br s, 4H), 4.14 (t, *J* = 5.2 Hz, 2H), 4.43-4.44 (m, 4H), 7.18-7.28 (m, 9H), 7.39-7.41 (m, 6H), 7.69-7.72 (m, 2H), 7.91-7.81 (m, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 24.6, 26.1, 28.2, 28.6, 29.5, 46.5, 46.6, 65.2, 66.8, 68.1, 75.7, 79.5, 123.8, 126.8, 128.0, 129.0, 129.7, 134.8, 145.0, 155.1, 155.5, 163.5. HRMS *m/z* calcd [C₄₂H₄₇N₂O₈S]⁺ 739.3048, observed 739.3058.

***N*-(4-(((2-(aminooxy)ethoxy)carbonyl)oxy)butyl)-*N*-((*tert*-butoxy)carbonyl)-3-((triphenylmethyl)sulfanyl)propan-1-amine (15).** Hydrazine monohydrate (356 μL, 7.3 mmol) was added to a solution of 2-(2-(((4-(((*tert*-butoxy)carbonyl)((3-((triphenylmethyl)sulfanyl)propyl))amino)butoxy)carbonyl) oxy)ethoxy)-2,3-dihydro-1*H*-isoindole-1,3-dione (1.08 g, 1.5 mmol) in CH₂Cl₂ at 0 °C and stirred for 2 h. When complete, the white precipitate was removed by filtration and the filter cake was washed with ample CH₂Cl₂. The filtrate was concentrated *in vacuo* to afford **6** (899 mg, 99%) as a colorless oil and was used without further purification. IR ν (cm⁻¹) 1687, 1744, 3323; ¹H NMR (400 MHz, CDCl₃) δ 1.39 (s, 9H), 1.50-1.62 (m, 6H), 2.14 (t, *J* = 7.2 Hz, 2H), 3.05 (br s, 4H), 3.85 (t, *J* = 4.2 Hz, 2H), 4.13 (t, *J* = 6.2 Hz, 2H), 4.32 (t, *J* = 4.4 Hz, 2H), 5.52 (br s, 2H), 7.18-7.29 (m, 9H), 7.39-7.41 (m, 6H); ¹³C NMR (100 MHz, CDCl₃) δ 24.7, 26.2, 27.5, 28.6, 29.5, 46.7 (2 C's), 65.5, 66.8, 68.0, 73.4, 79.5, 126.8, 128.0, 129.8, 145.0, 155.5. HRMS *m/z* calcd [C₆₈H₈₉N₄O₁₂S₂]⁺ 1217.5918, observed 1217.5917.

5.3.2. Amine-free Linker

6-(Tritylthio)hexan-1-ol. 6-bromohexanol (0.526 g, 2.90 mmol), triphenylmethanethiol (0.802 g, 2.90 mmol), and K_2CO_3 (0.802 g, 5.80 mmol) were added to a 1:1 mixture of ethanol:water (15.0 mL) and the reaction was heated to 90 °C for 18 h. After cooling to rt, the reaction was quenched with 1M HCl and extracted three times with CH_2Cl_2 . The combined organic layers were washed with water, brine, dried over anhydrous Na_2SO_4 , filtered, and concentrated *in vacuo* to give 6-(tritylthio)hexan-1-ol (0.88 g, 82%) as a yellow oil and was used without further purification. IR ν (cm^{-1}) 3362; 1H NMR (500 MHz, $CDCl_3$) δ 1.19-1.30 (m, 4H), 1.37-1.43 (m, 2H), 1.45-1.50 (m, 2H), 2.14 (t, $J = 7.5$ Hz, 2H), 3.58 (t, $J = 6.5$ Hz, 2H), 7.19-7.29 (m, 9H), 7.40-7.42 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.5, 28.8, 32.1, 32.7, 63.0, 66.6, 126.7, 128.0, 129.8, 145.2.

6-(Tritylthio)hexyl 1H-imidazole-1-carboxylate (17). 1,1'-carbonyldiimidazole (1.27 g, 7.86 mmol) was added to a solution of *N,N*-diisopropylethylamine (1.02 g, 7.86 mmol) and 6-(tritylthio)hexan-1-ol (1.97 g, 5.24 mmol) in CH_2Cl_2 (25 mL) at 0 °C and was stirred overnight. The reaction mixture was washed twice with water and extracted once with CH_2Cl_2 . The combined organic layers were washed twice with sat. NH_4Cl , once with brine, were dried over anhydrous Na_2SO_4 , and concentrated *in vacuo* to give **8** as a yellow oil and was used without further purification. IR ν (cm^{-1}) 1768; 1H NMR (500 MHz, $CDCl_3$) δ 1.26-1.34 (m, 4H), 1.38-1.44 (m, 2H), 1.68-1.73 (m, 2H), 2.16 (t, $J =$

7.0 Hz, 2H), 4.35 (t, $J = 6.5$ Hz, 2H), 7.11 (s, 1H), 7.19-7.29 (m, 9H), 7.40-7.42 (m, 6H), 8.11 (s, 1H); ^{13}C NMR (100 MHz, CDCl_3) δ 25.4, 28.4, 28.6, 31.7, 66.6, 68.4, 117.2, 126.7, 128.1, 129.7, 130.8, 137.2, 145.1, 148.9; HRMS m/z calcd $[\text{C}_{29}\text{H}_{32}\text{N}_2\text{O}_2\text{S}]^+$ 471.2101, observed 471.2096.

2-((6-(Tritylthio)hexyl)oxy)isoindoline-1,3-dione. DBU (1.65 mL, 11.1 mmol) was added to a solution of crude 6-(tritylthio)hexyl 1*H*-imidazole-1-carboxylate (5.21 g, 11.1 mmol) in dry MeCN (50 mL). After stirring for 10 min, *N*-(2-Hydroxyethyl)phthalimide (2.29 g, 11.1 mmol) was added and the reaction was stirred overnight. Upon completion, the reaction was washed twice with sat. NH_4Cl and the combined aqueous layers were extracted three times with EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4 , filtered and concentrated *in vacuo*. The crude material was purified by column chromatography (SiO_2 , 3:7 Hex:EtOAc) to give 2-((6-(tritylthio)hexyl)oxy)isoindoline-1,3-dione (4.48 g, 66% over 2 steps) as a clear oil. IR ν (cm^{-1}) 1735, 1793; ^1H NMR (500 MHz, CDCl_3) δ 1.19-1.27 (m, 4H), 1.36-1.41 (m, 2H), 1.54-1.59 (m, 2H), 2.13 (t, $J = 7.5$ Hz, 2H), 4.08 (t, $J = 7.0$ Hz, 2H), 4.43-4.47 (m, 4H), 7.19-7.29 (m, 9H), 7.40-7.42 (m, 6H), 7.73-7.75 (m, 2H), 7.83-7.84 (m, 2H); ^{13}C NMR (100 MHz, CDCl_3) δ 25.4, 28.4, 28.6, 32.0, 65.2, 66.6, 68.4, 75.7, 123.8, 126.7, 128.0, 129.0, 129.8, 134.8, 145.2, 155.1, 163.5.

***O*-(6-(Tritylthio)hexyl)hydroxylamine (18).** Hydrazine monohydrate (2.16 g, 43.11 mmol) was added to a solution of 2-((6-(tritylthio)hexyl)oxy)isoindoline-1,3-dione (5.25 g, 8.62 mmol) in CH₂Cl₂ (100 mL) at 0 °C and stirred for 2 h. When complete, the white precipitate was removed by filtration and the filter cake was washed with ample CH₂Cl₂. The filtrate was concentrated *in vacuo* to afford **9** (4.06 g, 98%) as a colorless oil and was used without further purification. IR ν (cm⁻¹) 1749, 3334; ¹H NMR (500 MHz, CDCl₃) δ 1.21-1.29 (m, 4H), 1.36-1.41 (m, 2H), 1.54-1.61 (m, 2H), 2.14 (t, *J* = 7.0 Hz, 2H), 3.86 (t, *J* = 4.0 Hz, 2H), 4.07 (t, *J* = 6.5 Hz, 2H), 4.33 (t, *J* = 4.5 Hz, 2H), 5.52 (s, 1H), 7.19-7.29 (m, 9H), 7.40-7.41 (m, 6H); ¹³C NMR (100 MHz, CDCl₃) δ 25.4, 28.5, 28.7, 31.9, 65.4, 66.6, 68.2, 73.4, 126.7, 127.9, 129.8, 145.2, 155.6; HRMS *m/z* calcd [C₂₈H₃₄NO₄S]⁺ 480.2203, observed 480.2206.

5.4. CHAPTER 4 EXPERIMENTAL PROCEDURES

5.4.1. Preparation of Fe@Au@LS-anthracene NPs

TGA calculation for LS loading

The amount of linker per milligram of NP was calculated based off TGA data seen in Chapter 4. Briefly:

$$\begin{array}{lcl} \text{a} & \frac{0.2015 \text{ mg lost}}{468.2 \text{ mg}} \times \frac{1 \text{ mmol LS1-anthracene}}{468.2 \text{ mg}} & = 4.3 \times 10^{-4} \text{ mmol LS1-anthracene} \\ \text{b} & 2.457 \text{ mg Fe@Au-LS1-anthracene} - 0.2015 \text{ mg LS1-anthracene lost} & = 2.2555 \text{ mg Fe@Au NPs} \\ \text{c} & \frac{4.3 \times 10^{-4} \text{ mmol LS1-anthracene}}{2.2555 \text{ mg Fe@Au NPs}} & = \frac{1.9 \times 10^{-4} \text{ mmol LS1-anthracene}}{1 \text{ mg Fe@Au NPs}} \end{array}$$

a. Total amount of LS1-anthracene lost is equivalent to total amount loaded onto NPs.

This amount is converted to mmol using molecular weight (465.2 g/mol).

b. Total amount of Fe@Au NPs is calculated by subtracting the amount of LS1-anthracene lost from the total weight.

c. Conversion: if 4.3×10^{-4} mmol LS1-anthracene loaded for 2.2555 mg Fe@Au NPs, then 1.9×10^{-4} would be loaded onto 1 mg Fe@Au NPs.

Calculations determined the amount of LS1-anthracene loaded onto 1 mg of NP to be 0.0002 mmol. In our preparation, we used 100 times excess linker, or 0.02 mmol, per milligram NP.

Preparation of protected LS1-anthracene **19**

Anthracene-CHO (296.4 mg, 1.35 mmol) was added to **15** (820.6 mg, 1.35 mmol) in CH₂Cl₂ (5 mL) at room temperature and stirred overnight. Upon completion as determined by TLC analysis, the reaction concentrated *in vacuo* to afford **19** (1.08 g,

99%) as an orange oil and was used without further purification. IR ν (cm^{-1}) 1684, 1724, 1741; ^1H NMR (500 MHz, CDCl_3) major isomer δ 1.38 (s, 9H), 1.45-1.65 (m, 6H), 2.13 (t, $J = 7.5$ Hz, 2H), 3.04 (br s, 4H), 3.72 (t, $J = 4.5$ Hz, 2H), 4.13 (t, $J = 6.5$ Hz, 2H), 4.33 (t, $J = 5.0$ Hz, 2H), 4.50 (d, $J = 6.5$ Hz, 2H), 7.18-7.27 (m, 9H), 7.39-7.40 (m, 6H), 7.46-7.52 (m, 4H), 8.02 (d, $J = 8.5$ Hz, 2H), 8.17 (d, $J = 8.5$ Hz, 2H), 8.42 (s, 1H); ^{13}C NMR (100 MHz, CDCl_3) major isomer δ 24.7, 25.4, 26.2, 28.6, 29.5, 43.3, 46.7 (2 C's), 66.2, 66.5, 67.8, 71.3, 79.5, 124.0, 125.3, 126.4, 126.8, 127.4, 128.0, 129.5, 129.8, 130.3, 131.7, 134.3, 145.0, 149.6, 155.5.

Preparation of protected LS2-anthracene **23**

Anthracene-CHO (495.4 mg, 2.25 mmol) was added to **18** (1.08 g, 2.25 mmol) in CH_2Cl_2 (10 mL) at room temperature and stirred overnight. Upon completion as determined by TLC analysis, the reaction concentrated *in vacuo* to afford **23** (1.53 g, 99%) as an orange oil and was used without further purification. IR ν (cm^{-1}) 1669, 1744; ^1H NMR (400 MHz, CDCl_3) major isomer δ 1.17-1.28 (m, 4H), 1.32-1.40 (m, 2H), 1.50-1.66 (m, 2H), 2.11 (t, $J = 9.0$ Hz, 2H), 4.03 (t, $J = 8.5$ Hz, 2H), 4.32 (t, $J = 6.5$ Hz, 2H), 4.51 (d, $J = 7.0$ Hz, 2H), 7.19-7.29 (m, 9H), 7.39-7.41 (m, 6H), 7.46-7.50 (m, 4H), 8.02 (d, $J = 10.0$ Hz, 2H), 8.30 (d, $J = 10.5$ Hz, 2H), 8.42 (s, 1H); ^{13}C NMR (100 MHz, CDCl_3): δ 25.2, 25.3, 28.5, 31.9, 66.0, 66.5, 68.0, 71.2, 124.0, 125.1, 126.2, 126.6, 127.2, 127.9, 128.4, 129.3, 129.7, 130.2, 131.5, 145.1, 149.4, 155.2.

Preparation of LS-anthracene (**20** and **24**)

To prepare LS1-anthracene for loading onto Fe@Au NPs, protected LS1-anthracene (364.8 mg, 0.45 mmol) was dissolved in CH₂Cl₂ (5 mL) at 0 °C. TFA (5 mL, 65 mmol) was added dropwise, followed by Et₃SiH (215.5 µL, 1.35 mmol) added dropwise. The mixture was stirred at 0 °C for 30 mins. Upon completion as determined by TLC analysis, the reaction was warmed to rt, concentrated *in vacuo*, and used crude immediately for addition to NPs. Both **20** and **24** followed this procedure.

Preparation of Fe@Au-LS-anthracene NPs (**21** and **25**)

LS1-anthracene **20** (112.32 mg, 0.24 mmol) was redissolved in CH₂Cl₂ (6.4 mL), then added to a solution of rapidly stirring Fe@Au NPs (6 mg) in hexane (15 mL) over 4 hours, followed by precipitation with EtOH (40 mL). NPs were pelleted via centrifugation at 10K rpm for 15 minutes. The supernatant was discarded, the NPs were redissolved in CH₂Cl₂ (5 mL) and precipitated with EtOH (45 mL). NPs were pelleted via centrifugation at 10K rpm for 15 minutes, and the supernatant was discarded. This washing process was performed 4 more times. After the final discard of supernatant, the NPs were dried *in vacuo* and stored in solid form under nitrogen at rt until further use. Both **21** and **25** were prepared in this manner.

5.4.2. AMF studies of Fe@Au@LS-anthracene (21** and **25**)**

Fe@Au@LS1-anthracene NPs **21** and Fe@Au@LS2-anthracene NPs **25** were separately subjected to one 15-minute burst of AMF exposure at 500 A. After the

exposure, the NPs were pelleted via centrifugation at 10K rpm for 5 min, and the supernatant was analyzed using MALDI-TOF.

To confirm initial loading of LS2-anthracene, freshly loaded Fe@Au@LS2-anthracene NPs **25** were incubated in 400 μ L 0.1M DTT for 2 h at rt, pelleted via centrifugation at 10K rpm for 5 min, and the supernatant was analyzed using MALDI-TOF.

5.4.3. Preparation of Fe@Au@LS-PEG NPs

Preparation of protected LS1-FITC **26**

FITC-CHO (112.2 mg, 0.24 mmol) was added to a **15** (143.3 mg, 0.24 mmol) in 9:1 CHCl₃:MeOH (3 mL) at room temperature and stirred overnight. Upon completion as determined by TLC analysis, the reaction concentrated *in vacuo* to afford **26** (226.2 mg, 90%) and was used without further purification. ¹H NMR (500 MHz, DMSO-d₆) δ 1.29 (s, 9H), 1.31- 1.52 (m, 6H), 1.57-1.76 (m, 2H), 2.06 (br s, 2H), 2.17-2.22 (m, 3H), 2.96 (br s, 4H), 3.48-3.56 (m 2H), 3.80-3.84 (m, 2H), 4.02-4.06 (m, 2H), 4.35-4.26 (m, 2H), 6.55-6.61 (m, 4H), 6.67 (s, 2H), 7.15-7.31 (m, 17H), 7.82-7.84 (m, 1H), 8.22 (s, 1H), 10.12 (brs, 2H).

Preparation of protected LS2-FITC **27**

FITC-CHO (522.7 mg, 1.1 mmol) was added to a **18** (526 mg, 1.1 mmol) in 9:1 CHCl₃:MeOH (5 mL) at room temperature and stirred overnight. Upon completion as determined by TLC analysis, the reaction concentrated *in vacuo* to afford **27** (915.4 mg, 89%) and was used without further purification. ¹H NMR (500 MHz, DMSO-d₆) δ 1.07-

1.23 (m, 4H), 1.27-1.29 (m, 2H), 1.47-1.49 (m, 2H), 1.90 (br s, 2H), 2.01-2.09 (m, 4H), 2.51 (s, 1H), 3.58-3.62 (m, 2H), 3.69-3.71 (m, 2H), 3.82-3.86 (m, 1H), 3.98-4.01 (m, 2H), 4.21-4.22 (m, 2H), 5.79 (br s, 1H), 6.57-6.65 (m, 4H), 6.70 (s, 2H), 7.16-7.24 (m, 11H), 7.28-7.39 (m, 6H), 7.85-7.87 (m, 1H), 8.10 (s, 1H), 10.12 (br s, 2H).

Preparation of LS-FITC (**28** and **29**)

To prepare LS1-FITC for loading onto Fe@Au NPs, protected LS1-FITC (95.7 mg, 0.09 mmol) was dissolved in CH₂Cl₂ (1 mL) at 0 °C. TFA (250 µL, 13 mmol) was added dropwise, followed by Et₃SiH (43 µL, .27 mmol) added dropwise. The mixture was stirred at 0 °C for 30 mins. Upon completion as determined by TLC analysis, the reaction was warmed to rt, concentrated *in vacuo*, and used crude immediately for addition to NPs. Both **28** and **29** followed this procedure.

Preparation of Fe@Au-LS-FITC-PEG NPs (**30** and **31**)

Following a modified procedure by Menichette *et al.*,¹³ PEG-SH (82.6 mg, 0.09 mmol) was added to LS1-FITC **28** (18 mg, 0.09 mmol) in 9:1 CHCl₃:MeOH (10 mL). This solution was added to rapidly stirring NPs (5 mg) in CHCl₃ (10 mL), and the solution was stirred rapidly overnight. The NPs were concentrated *in vacuo* and redissolved in EtOH (50 mL). NPs were pelleted via centrifugation at 10K rpm for 15 minutes. The supernatant was discarded, the NPs were redissolved in H₂O (50 mL) under sonication, pelleted via centrifugation at 10K rpm for 15 minutes, and the supernatant was discarded. The NPs were resissolved in saturated sodium bicarbonate (50 mL) under sonication, pelleted via centrifugation at 10K rpm for 15 minutes, and the supernatant

was discarded. The NPs were resissolved in 1X PBS buffer (50 mL, pH = 7.4) under sonication, pelleted via centrifugation at 10K rpm for 15 minutes, and the supernatant was discarded. The NPs were resissolved in DI water (50 mL) under sonication, pelleted via centrifugation at 10K rpm for 15 minutes, and the supernatant was discarded. This washing process with water was performed twice. After the final discard of supernatant, the NPs were lyophilized and stored in solid form under nitrogen at rt until further use. Both **30** and **31** were prepared in this manner. Fe@Au-LS1-FITC-PEG NPs **30** were characterized via UV-vis spectroscopy, ζ -potential, DLS, and SEM. UV-vis showed a characteristic surface plasmon resonance band at 531 nm, indicating the presence of gold. The ζ -potential was 12.6 ± 0.212 mV. This is not surprising because both LS1-FITC and PEG are neutral in water. The DLS reading was 229.5 ± 0 nm, but the particles have an average diameter of 23 ± 7 nm based on SEM imaging (Figure 4.13).

5.4.4. AMF studies of Fe@Au@LS-FITC-PEG NPs

A fresh batch of Fe@Au@LS1-FITC-PEG NPs **30** and Fe@Au@LS2-FITC-PEG NPs **31** were separately subjected to four sequential 15-minute bursts of AMF exposure at 500 A in 1 mL 1X PBS buffer (pH 7.4). After each exposure, the NPs were pelleted via centrifugation at 10K rpm, 5 min, the supernatant was added to 2 mL fresh 1X PBS buffer in a cuvette, and then analyzed using a fluorometer. Before each AMF exposure, 1 mL of the liquid in the cuvette was added to the NP pellet and resuspended. After the last reading, an aliquot of the supernatant was analyzed via MALDI-TOF (Figure 3e and 3f), then the NPs were incubated with 400 μ L 0.1M DTT for 2 h at rt, pelleted via

centrifugation at 10K rpm, 5 min, and then analyzed using a fluorometer. The DTT fluorescence reading was deemed “100% release”, and each reading prior gave % release based on this reading. Each linker system testing was done in triplicate.

A fresh batch of Fe@Au@LS1-PEG NPs **30** also was incubated for 2 hours, at 37 °C and at 50 °C, then incubated with 400 μ L 0.1M DTT for 2 h at rt followed by analysis using a fluorometer. The DTT fluorescence reading was deemed “100% release”, and each reading prior gave % release based on this reading. Each temperature study was performed in triplicate.

5.4.5. Preparation of GNP@LS1-FITC NPs

LS1-FITC **28** (118.7 mg, 0.16 mmol) in 9:1 CHCl₃:MeOH (20 mL) was added to rapidly stirring NPs (163.9 mg) in CHCl₃ (50 mL), and the solution was stirred rapidly overnight. The NPs were concentrated *in vacuo* and redissolved in EtOH (50 mL). NPs were pelleted via centrifugation at 10K rpm for 15 minutes. The supernatant was discarded, the NPs were redissolved in H₂O (50 mL) under sonication, pelleted via centrifugation at 10K rpm for 15 minutes, and the supernatant was discarded. The NPs were resissolved in saturated sodium bicarbonate (50 mL) under sonication, pelleted via centrifugation at 10K rpm for 15 minutes, and the supernatant was discarded. The NPs were resissolved in 1X PBS buffer (50 mL, pH = 7.4) under sonication, pelleted via centrifugation at 10K rpm for 15 minutes, and the supernatant was discarded. The NPs were resissolved in DI water (50 mL) under sonication, pelleted via centrifugation at 10K rpm for 15 minutes, and the supernatant was discarded. This washing process with water was performed twice. After the final discard of supernatant, the NPs were lyophilized

and stored in solid form under nitrogen at rt until further use. GNP@LS1-FITC NPs were characterized via ζ -potential, DLS, and SEM. ζ -potential showed a surface charge of -20.6 ± 1.63 mV. DLS readings gave an average hydrodynamic diameter of 226.8 ± 26.77 nm, and SEM also shows aggregation of that size (Chapter 4, Figure 4.18a). Each individual particle, though, was much smaller, as seen in Figure 4.18b.

5.4.6. AMF Studies of Dual NP system

For the dual NP system, 5 mg GNP@LS1-FITC was combined with an equal amount of MNP by weight (1:1), twice the amount of MNP by weight (1:2), and three times the amount of MNP by weight (1:3). Each freshly prepared ratio was subjected to four sequential 15-minute bursts of AMF exposure at 500 A in 1 mL 1X PBS buffer (pH 7.4). After each exposure, the NPs were pelleted via centrifugation at 10K rpm for 5 min, the supernatant was added to 2 mL fresh 1X PBS buffer in a cuvette, and then analyzed using a fluorometer. Before each AMF exposure, 1 mL of the liquid in the cuvette was added to the NP pellet and resuspended. After each exposure, the NPs were pelleted via centrifugation at 10K rpm for 5 min, and the supernatant was analyzed using a fluorometer. After the last reading, the NPs were incubated with 400 μ L 0.1M DTT for 2 h at rt, pelleted via centrifugation at 10K rpm for 5 min, and the supernatant was analyzed using a fluorometer. The DTT fluorescence reading was deemed “100% release”, and each reading prior gave % release based on this reading. Each dual NP system testing was done in triplicate.

A freshly prepared batch of each ratio of the dual NP system was also subjected to

AMF exposure at 300 A. The method remained the same as previously described, but the amperage was lowered to 300 A.

A freshly prepared batch of each ratio was incubated at 37 °C for 2 hours, pelleted via centrifugation at 10K rpm for 5 min, and the supernatant was analyzed using a fluorometer. The NPs were then incubated with 400 μ L 0.1M DTT for 2 h at rt, pelleted via centrifugation at 10K rpm for 5 min, and the supernatant was analyzed using a fluorometer. The DTT fluorescence reading was deemed “100% release”, and each reading prior gave % release based on this reading.

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- R.3. CHAPTER 3 REFERENCES
- R.4. CHAPTER 4 REFERENCES
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Appendix

- A.1.** SK Biladeau, WR Richmond, S Laulhé, S.; MH Nantz. Isotopically coded *N*-methoxy amide reagents for GC-MS profiling of carbonyl compounds *via* mass spectral tag generation. *Anal. Methods*, **2016**, 8, 3704-3710.

A.2. Spectral data of key intermediates

¹H NMR Spectrum of **11**
¹³C NMR Spectrum of **11**
IR Spectrum of **11**

¹H NMR Spectrum of **13**
¹³C NMR Spectrum of **13**
IR Spectrum of **13**

¹H NMR Spectrum of **14**
¹³C NMR Spectrum of **14**
IR Spectrum of **14**

¹H NMR Spectrum of **15**
¹³C NMR Spectrum of **15**
IR Spectrum of **15**

¹H NMR Spectrum of **17**
¹³C NMR Spectrum of **17**
IR Spectrum of **17**

¹H NMR Spectrum of **18**
¹³C NMR Spectrum of **18**
IR Spectrum of **18**

A.3. List of abbreviations

- A.1.** SK Biladeau, WR Richmond, S Laulhé, S.; MH Nantz. Isotopically coded *N*-methoxy amide reagents for GC-MS profiling of carbonyl compounds *via* mass spectral tag generation. *Anal. Methods*, **2016**, 8, 3704-3710.

My first two years of graduate school were devoted to a project unrelated to the present thesis. The focus during this time was synthesis and application of an isotopically labeled reagent for chemoselective capture of carbonyls for multiplex analysis. This research served as a platform for me to experience multistep synthesis, methods of compound purification, spectral analyses, and aminooxy chemistry, which came in handy once I began my thesis work. I have included the publication that resulted from the efforts during this time. The work shows how we designed a reagent *N*-methoxy-*N*-(2-aminooxyethyl)-propionate (MAP) to increase stability toward hydrolysis and acyl transfer. Because amides do not readily undergo α -cleavage as easily as esters, we used a methoxy amine moiety so that, upon α -cleavage, the nitrogen radical formed would be more stable, thus promoting α -cleavage (Scheme 2). In this manuscript, we describe the synthesis and application of MAP using a variety of carbonyl compounds to show its superiority.

Cite this: DOI: 10.1039/c5ay02253c

Isotopically coded *N*-methoxy amide reagents for GC-MS profiling of carbonyl compounds via mass spectral tag generation†

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Synthesis and application of isotopically labeled *N*-methoxy-*N*-(2-aminooxyethyl)-propionate (MAP), a chemoselective carbonyl derivatization reagent, is reported. To exploit the ready measurement of fragments serving as reporter ions in the m/z 32–34 range, MAP is designed to undergo electron ionization (EI)-induced fragmentation to expel labeled ethyl carbenium ions for relative quantifications in multiplexed analyses. A study of the EI-MS fragmentation behavior of a panel of MAP–carbonyl adducts revealed that the *N*-methoxy amide motif of MAP is highly predisposed to undergo carbonyl alpha cleavage to produce corresponding labeled carbenium ions in the targeted m/z range. Use of the *N*-methoxy amide functionality decreased undesired (e.g., uninformative) mass spectral fragmentations as well as provided good resistance to cleavage by amines or base relative to ester functionality. These properties should facilitate the use of MAP in multiplexed GC-MS analyses of complex mixtures containing aldehyde and ketone analytes. A representative multiplexed experiment using MAP isotopologues illustrates this approach for quantification of a carbonyl analyte in pooled sample mixtures.

Received 26th August 2015
Accepted 13th April 2016DOI: 10.1039/c5ay02253c
www.rsc.org/methods

Introduction

Liquid chromatography-mass spectrometry (LC-MS) is widely used for analysis of complex mixtures since analyte derivatization generally is not required.¹ Gas chromatography-mass spectrometry (GC-MS), on the other hand, often requires analyte derivatization to increase volatility and/or alter ionization character for suitable analysis.² The strategy of chemoselective derivatization enabled the practice of analyzing multiple samples at a time by LC-MS, or multiplexing (*i.e.*, with a single injection).⁴ Consequently, many derivatized metabolites, including carboxylic acids,⁵ fatty acids,⁶ steroids,⁷ amino⁸ and non-amino⁹ acids, have since been analyzed in multiplexed experiments by LC-MS.

Whereas the use of isotopic labeling for LC-MS analyses has become prevalent in recent years, its application in GC-MS analyses is far less.¹⁰ A recent review by Bruheim *et al.*¹⁰ described only three cases of binary derivatization strategies for

analysis by GC-MS. The reagents *N*-methyl-trimethylsilyltrifluoroacetamide (MSTFA),¹¹ *N*-methyl-*N*-(*t*-butyldimethyl-silyl) trifluoroacetamide (MTBSTFA)¹² and methyl chloroformate (MCF)¹³ have been used in conjunction with the isotopologue reagents d_9 -MSTFA,¹¹ d_6 -MTBSTFA,¹² and d_3 -MCF,¹³ respectively, for simultaneous analyses of sample mixtures. To date, these limited examples of matched derivatizing agents allowed only for the GC-MS analyses of amino acids, organic acids, fatty acids and metabolites amenable to silyl derivatization. Furthermore, these approaches are binary, allowing only two samples to be analyzed at a time.

The ability to analyze three or more samples at a time has only recently been established and utilized.¹⁴ Progress in developing multiplexed approaches, however, has largely been confined to the LC-MS platform. For example, recent advances in multiplexed proteomic studies include the use of isobaric tags for relative and absolute quantification (iTRAQ)¹⁵ and mass differential tags for relative and absolute quantification (mTRAQ).¹⁶ Recently, groups have utilized iTRAQ¹⁷ and mTRAQ,¹⁸ and the methodology has since been expanded to analyze up to eight samples simultaneously (OxiTRAQ).¹⁹ A limited number of papers have described LC-MS multiplexing for analysis of lower molecular weight compounds, such as metabolites from biological extracts. In one example, Torde *et al.*²⁰ described analyses of carboxylic acids and other fatty acids in eggs from caged *versus* cage-free chickens using choline-amine- d_0 , $-d_3$, or $-d_9$ reagents. Given the benefits of more rapid sample analysis with enhanced analyte detection illustrated by the above-cited works, there is a surprising dearth of

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multiplexed GC-MS analyses. Extrapolation of multiplexing strategies to this platform may provide a means for rapid and efficient metabolite or biomarker detection. We report herein our efforts to develop and optimize chemoselective derivatization reagents for this purpose.

We recently described an isotope coding strategy to enable multiplexed analysis of carbonyl compounds from multiple samples using GC-MS.²¹ Our approach relies on the electron ionization-induced expulsion of isotopically labeled and quantifiable mass spectral reporter ions (Scheme 1). Specifically, treatment of a metabolite mixture with the aminooxy reagent AEP results in the chemoselective derivatization (oximation) of all aldehydes and ketones. The use of aminooxy reagents to label aldehydes and ketones is among the most efficient of chemoselective derivatizations.²² This click chemistry approach²³ has been widely studied²⁴ and is more robust than analogous derivatizations that utilize amine²⁵ or hydrazine-based²⁶ reagents. Subsequent GC-MS analysis of the AEP adducts 1 (Scheme 1) results in their ionization and accompanying fragmentation. One of the principal fragmentation modes for adducts 1 is an ester α -cleavage that gives rise to acylium ion [2], which spontaneously loses CO to yield the labeled mass spectral tag [3]. The ethyl carbenium ion [3] is purposely substituted with deuterium so that its $^{12}\text{C}_2$ -isotopologue is observed at m/z 32. An analysis of the mass range m/z 31–37 revealed sparse fragment ion population (unless specifically targeted) and low ion intensities for the few fragment ions that register within this range.²¹ Thus, fragments within this mass range, a zone of minimal interference (ZMI), can be cleanly quantified using ion count measurements. In a multiplexing scenario, the $^{12}\text{C}_2$ -isotopologue adducts 1 can be pooled with the adducts obtained from derivatization of a second and third metabolite mixture, using the $^{13}\text{C}_1$ - and $^{13}\text{C}_2$ -isotopologues of AEP, respectively, and then simultaneously analyzed by GC-MS. Importantly, for a given carbonyl analyte, the isotopic AEP-derivatives are chromatographically indistinguishable. Comparison of the unobstructed ZMI mass spectral tag ion counts at m/z 32, 33, and 34 then reveals the relative abundance of a given carbonyl metabolite in the three mixtures. In our

Scheme 1 Formation and EI-induced fragmentation of AEP-derived oxime ether adducts.

Scheme 2 Incorporation of an *N*-methoxy amide group to impart reagent and adduct stability and to promote EI-induced generation of the ZMI reporter ion.

initial disclosure,²¹ we demonstrated the average accuracy of the method to be 95%. One concern, however, for applicability under more forcing conditions is the susceptibility of the ester linkage toward hydrolytic cleavage as well as possible transacylation reactions with nucleophiles, either by the reagent AEP or its adducts 1. These undesired reactions could have the effect of cleaving or transferring the mass spectral tag onto metabolites not targeted by the aminooxy moiety. Also of interest in a redesign of the AEP reagent would be to enhance the α -cleavage process so as to boost mass spectral tag ([3]) detection in the ZMI while minimizing uninformative fragmentations that arise from EI-induced cleavage along the reagent backbone. With these goals in mind, we pursued synthesis and evaluation of an *N*-methoxy amide analog of AEP based on the rationale presented below.

Whereas amides are considerably more stable toward hydrolysis than esters, their α -cleavage fragmentations in EI-MS occur at a significantly lower rate, thus suggesting that conversion of AEP to an amide analog is likely to be counterproductive. However, we postulated that substitution of the amide nitrogen with alkoxy (e.g., amide 4, Scheme 2) would maintain the stability associated with amides yet possibly promote α -cleavage (5 $\rightarrow$ 6) due to neighboring oxygen stabilization of the resultant *N*-centered radical species.²⁷ Consequently, we set out to synthesize *N*-methoxy-*N*-(2-aminooxyethyl)-propionate (MAP; 5, Y = CH₂CH₂ONH₂) and examine the propensity for formation of mass spectral tag [3] from derived oxime ether adducts (Y = CH₂CH₂ON = CR(R')). Although syntheses and applications of *N*-methoxy amides are abundant in the literature (e.g., Weinreb amides²⁸), there are few reports on the EI-induced fragmentation of this functionality.

Experimental

MAP synthesis

We prepared the MAP isotopologues from Boc-protected methoxyamine²⁹ (7, Scheme 3). *N*-Alkylation using silyl-protected 2-bromoethanol³⁰ followed by mild acid work-up delivered amide-alcohol 8. Alcohol to alkoxy phthalimide transformation according to the method of Grochowski and

Scheme 3 Synthesis of MAP reagents (Phth = phthaloyl, *C = ¹²C or ¹³C). Conditions: (a) (i) NaH (1.05 eq.), DMF, 0 °C, (ii) Me₃SiOCH₂CH₂Br (1.0 eq.), rt, 12 h, (iii) H₂O, citric acid, 50%; (b) *N*-hydroxy-phthalimide (1.15 eq.), PPh₃ (1.15 eq.), DIAD (1.2 eq.), THF, rt, 16 h, 90%; (c) CH₂-Cl₂:CF₃CO₂H (1 : 1), 0 °C, 1 h, 66%; (d) RCO₂H (1.1 eq.), DIC (1.5 eq.), DMAP (cat.), CH₂Cl₂, 0 °C – rt, 16 h, 14–91%; (e) N₂H₄·H₂O (1.05 eq.), CH₂Cl₂, 0 °C – rt, 1 h, 20–53%.

Jurczak³¹ followed by Boc deprotection gave platform amine **9** in good yield. At this stage, *N*-acylations of **9** with ²H₃-labeled propionic acid and corresponding ¹³C₁- and ¹³C₂-isomers using standard carbodiimide methodology afforded entry to the propionamide isotopologues **10a–c**, respectively. Hydrazinolysis of **10a–c** furnished the labeled MAP panel. Detailed synthetic procedures and spectral characterizations are provided in the (ESI).†

Sample derivatization

MAP and AEP adducts were prepared by adding an equimolar mixture of MAP-32 (2 mg, 0.01 mmol) and AEP-32 (1.4 mg, 0.01 mmol) to a slight excess of select aldehydes and ketones (0.02 mmol) in CH₂Cl₂ (1 mL) at room temperature. After stirring 48 h, the solvent and unreacted carbonyls were removed by rotary evaporation. The isolated oxime ether adducts then were redissolved in CH₂Cl₂ (1 mL) for analysis by GC-MS.

MAP multiplexing study

Each MAP isotopologue was dissolved in acetone (5 mL) at room temperature. After stirring 24 h, the acetone was removed by rotary evaporation. Quantitative transformation to the corresponding oxime ether adducts was confirmed by GC using a reference sample (MAP-32-acetone adduct, ¹H NMR (CDCl₃, 400 MHz) δ 4.18 (t, *J* = 8.0 Hz, 2H), 3.86 (t, *J* = 8.0 Hz, 2H), 2.43 (br. s, 2H), 1.87 (s, 3H), 1.83 (s, 3H) ppm; ¹³C NMR (CDCl₃, 100 MHz) δ 69.3, 61.7, 45.4, 26.1, 25.2, 21.8 ppm). The adducts then were redissolved in CH₂Cl₂ (1 mL) for the multiplexing experiment. Multiplexed samples were prepared by combining

the isotopic adducts MAP-32-acetone, MAP-33-acetone, and MAP-34-acetone in the following respective proportions: 1 : 2 : 5, 2 : 5 : 1, 5 : 1 : 2. Details for analysis using GC-MS and accompanying spectra are provided in the ESI.†

Stability tests

A solution of MAP-32-acetone adduct (2.0 mg, 0.01 mmol) dissolved in CH₃CN (1 mL) was added to benzyl amine (2.18 mL, 0.02 mmol). The reaction mixture was heated to 85 °C and stirred overnight. Upon cooling, the solution was transferred to a vial for analysis using GC-MS. This procedure also was conducted using *N*-methyl-benzyl amine (2.58 mL, 0.02 mmol).

In a separate experiment, MAP-32-acetone (25.0 mg, 0.12 mmol) and 1,4-dimethoxy-benzene (5.0 mg, 0.04 mmol), serving as internal standard, were added to 1× PBS buffer (1 mL, pH 8.8). The reaction mixture was heated to 65 °C for 16 h. Upon cooling, the aqueous layer was extracted with CDCl₃ (0.5 mL) and the organic extract was analyzed using ¹H-NMR.

Results and discussion

A 1 : 1 mixture of MAP-32 and AEP-32 was reacted in turn with select aldehydes and ketones to form the corresponding 1 : 1 mixture of oxime ether adducts for subsequent comparisons of EI-induced fragmentations. For structural diversity, the panel of carbonyl compounds included saturated (acetone, 2-heptanone, 4-heptanone, hexanal), unsaturated ((*E*)-2-butenal) and aromatic examples (benzaldehyde, acetophenone) as well as an α-alkoxy substituted compound (2-(*n*-propoxy)ethanol). The 1 : 1 mixtures of MAP- and AEP-derived adducts obtained from reactions of the carbonyl compounds then were analyzed by GC-MS (Table 1). In addition to the formation of acylium [2] and mass spectral tag [3], the reagent-carbonyl adducts exhibited several other characteristic fragmentations (Scheme 4). Of interest to this study is the formation of the substrate-derived nitrilium ion [11] that accompanies oxime ether *N*-O cleavage.^{32,33} This mode of fragmentation provides useful substrate-dependent information (*i.e.*, nitrilium *m/z* + 1.992 provides carbonyl parent MW). In contrast, fragmentations leading to dioxolium (from AEP adducts) or oxazolium (from MAP) ions ([12], Scheme 4) provide no useful information in that these fragment ions are reagent-derived. The adducts also underwent classic McLafferty-type fragmentations. McLafferty fragmentations involving the reagent carbonyl (ester in AEP, amide in MAP) furnish radical cations [13] that yield no substrate information. When structurally possible, McLafferty fragmentations of the oxime ethers,³² however, furnish radical cations [14] that provide structural information specific to the adducts. An ideal 'next-generation' AEP reagent should improve the prevalence of [3] for quantifications and/or [11] and [14] to assist with analyte identifications while minimizing the formation of uninformative fragmentations, such as formation of [12] and/or [13]. The GC-MS data (Table 1) show that the MAP modification provides these benefits.

Examination of the MAP-adduct fragmentations reveals that α-cleavage of the *N*-methoxy amide construct to generate

Table 1 Relative abundance of fragment ions observed on EI-induced fragmentation of AEP- and MAP-derived oxime ether adducts (Scheme 4)^a

Entry	Carbonyl	Adduct (isomer) ^b	[2]	[3]	[11]	[12]	McLafferty ^c	
							[13]	[14]
1		AEP	83.5	21.0	100	53.1	0.2	
		MAP	100	17.5	37.7	12.5	0.1	Na ^d
2		AEP (Z)	100	30.2	2.4	61.2	7.6	
		MAP (Z)	100	20.9	17.3	1.2	0.4	
		AEP (E)	100	56.0	0.3	49.3	33.4	Na
		MAP (E)	100	25.5	0.1	21.3	0.8	
3		AEP (Z)	72.8	17.5	100	83.3	57.7	
		MAP (Z)	100	14.7	32.8	16.3	3.5	
		AEP (E)	74.3	20.5	100	77.3	71.7	Na
		MAP (E)	100	17.3	26.2	9.9	3.6	
4		AEP (Z)	100	35.9	49.9	68.4	12.0	
		MAP (Z)	100	18.2	21.0	14.5	0.0	
		AEP (E)	100	31.4	0.0	59.4	4.7	Na
		MAP (E)	100	14.9	9.9	8.9	0.1	
5		AEP (Z)	37.4	8.6	25.4	100	0.2	3.6
		MAP (Z)	100	17.8	55.8	36.8	0.2	0.1
		AEP (E)	35.0	8.3	3.4	100	0.2	17.2
		MAP (E)	100	18.7	7.7	49.5	0.2	7.5
6		AEP	51.7	17.3	1.7	100	1.7	2.1
		MAP	100	19.4	8.8	56.6	0.1	0.3
7		AEP (Z)	60.6	11.7	13.4	100	0.3	0.5
		MAP (Z)	100	14.8	4.8	8.9	0.1	0.2
		AEP (E)	50.4	10.5	2.5	100	0.2	8.9
		MAP (E)	100	13.1	2.1	6.5	0.1	0.3
		AEP (Z)	51.2	11.7	8.8	100	1.9	0.3
8		MAP (Z)	100	13.7	0.5	17.2	0.2	0.1
		AEP (E)	30.7	7.2	0.9	100	17.3	12.6
		MAP (E)	100	21.9	1.6	2.0	0.0	0.2

^a Parent *m/z* peak assigned a value of 100. ^b Oxime ether stereochemistry. ^c Fragments resulting from γ -H abstraction leading to α,β -scission. ^d Not applicable (no carbonyl γ -H).

Scheme 4 EI-MS induced fragmentations of oxime ether adducts.

acylium ion [2] is very facile. In every case examined this fragment ion was the predominant ion measured. In agreement with this observation, formation of the mass spectral tag [3]

from the MAP-adducts also was robust, although there was neither substantial increase nor decrease in the productions relative to the corresponding AEP-adducts. Substitution of amide functionality with methoxy, as anticipated, thus greatly enhanced EI-induced α -cleavage to furnish a mass spectral tag in the ZMI for quantification purposes. We also were gratified to observe that formation of the structurally informative nitrilium ion [11] from MAP-adducts was well populated and occurred to a greater extent in a few cases than the corresponding AEP-adducts (e.g., entries 5, 6). A notable difference between MAP and AEP behavior is fragmentation along the linking carbon chain to form the uninformative ions [12]. AEP-adducts are prone to give uninformative dioxolium ions [12] — these are often the predominant fragment ions (entries 5–8). In comparison, analogous formation of the oxazolonium ion [12] from MAP-adducts was suppressed in every case examined. Incidence of McLafferty fragmentation along the linking carbon chain also was greatly reduced in MAP-adducts relative to AEP-adducts. Radical cation [13] was formed to a lesser extent from MAP-adducts than AEP-adducts in all cases except with 2-heptanone (entry 5). McLafferty fragmentations involving the

Fig. 1 Multiplexed GC-MS analyses of three sample mixtures containing the isotopic MAP-32-, MAP-33- and MAP-34-acetone adducts as follows: mixture (A) 1 : 2 : 5; mixture (B) 2 : 5 : 1; mixture (C) 5 : 1 : 2. Insets depict the relative abundance of mass spectral tags [3] in the ZMI.

oxime ether moieties of either AEP or MAP adducts to form [14] was minimal relative to the aforementioned modes of fragmentation.

In modeling a multiplexed experiment, we pooled acetone adducts of the three isotopic reagents MAP-32, MAP-33 and MAP-34 at different ratios. Three sample mixtures with

respective adduct ratios of 1:2:5, 2:5:1, 5:1:2 were prepared and analyzed by GC-MS. Importantly, since no chromatographic separations based on differences in ^{13}C incorporation occurred, the relative quantification of adducts in each mixture is achieved by direct comparison of the isotopic carbenium ion mass spectral tags in the ZMI at m/z 32–34, as shown in Fig. 1.

To test the resistance of the *N*-methoxy amide functionality of MAP toward biological nucleophiles, we reacted the MAP-32-acetone adduct, as a representative carbonyl metabolite adduct, with excess benzyl amine and *N*-methyl-benzyl amine under forcing conditions (acetonitrile, 85 °C, 16 h). No observable adduct degradation or label transfer was noted in either reaction. The MAP-32-acetone adduct also exhibited robust stability when incubated in 1× PBS buffer (pH 8.8) at 65 °C for 16 h — no amide or oxime ether cleavage was observed by ^1H NMR analysis.

Conclusion

Substitution of the ester moiety of the previously reported²¹ AEP reagent with an *N*-methoxy amide affords MAP, a robust and chemoselective carbonyl derivatization reagent. Examination of the electron ionization-induced fragmentations of a representative carbonyl-MAP adduct panel reveals that *N*-methoxy amide functionality is highly predisposed to produce an acylium ion fragment and corresponding isotopically labeled carbenium reporter ion fragment in the m/z range 32–34. A representative multiplexed experiment using the MAP isotopologues demonstrated how relative quantification is achieved through reporter ion comparison in this range. The substitution to *N*-methoxy amide also decreased the prevalence of undesired (e.g., uninformative) fragmentations, such as oxazolium ion formation or McLafferty fragmentation associated with the reagent carbonyl. Finally, the *N*-methoxy amide functionality is suitably resistant to reaction with amines or base at elevated temperatures. These properties should facilitate the use of the MAP reagent panel in multiplexed GC-MS analyses of complex mixtures containing aldehyde and ketone analytes.

Acknowledgements

The authors thank Drs Xiang Zhang for helpful discussions and guidance with GC-MS instrumentation. SKB thanks the Department of Chemistry, University of Louisville for the award of a Graduate Research Fellowship.

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Electronic Supplementary Information

for the article entitled

**Isotopically Coded *N*-Methoxy Amide Reagents for GC-MS Profiling
of Carbonyl Compounds via Mass Spectral Tag Generation**

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I. General Synthesis Procedures

A. Reagents and Methods

Methoxyamine hydrochloride (98%), sodium hydroxide ($\geq 98\%$), 2-bromoethanol (95%), imidazole ($>99\%$), chlorotrimethylsilane (99%), triphenyl phosphine (99%), diisopropyl azodicarboxylate (98%), trifluoroacetic acid (99%), *N,N'*-diisopropylcarbodiimide (99%), 4-dimethylaminopyridine (DMAP) ($\geq 98\%$), and all solvents were purchased from Sigma-Aldrich and used without further purification. Sodium hydride (60% dispersion in mineral oil) was purchased from Acros Organics. Di-*tert*-butyl dicarbonate (99%) was purchased from Oakwood Chemical. *N*-Hydroxyphthalimide (98%), *N,N'*-diisopropylcarbodiimide (99%), and hydrazine monohydrate (99%) were purchased from Alfa Aesar. 3,3,3- $^2\text{H}_3$ -Propionic acid was purchased from Cambridge Isotope Laboratories, Inc. 3- $^{13}\text{C}_1$ -3,3,3- $^2\text{H}_3$ -Propionic acid and 2,3- $^{13}\text{C}_2$ -3,3,3- $^2\text{H}_3$ -propionic acid were synthesized using the published procedures.^{1,2} The progress of reactions was monitored by thin-layer chromatography (TLC, silica gel 60 Å F-254 plates). The plates were visualized first with UV illumination followed by staining either with phosphomolybdic acid, ninhydrin, or *p*-anisaldehyde solution. Column chromatography was performed using silica gel (230-400 mesh). NMR spectra were obtained using a Varian/Agilent 400-MR NMR spectrometer equipped with a 5 mm z-axis gradient AutoX probe operating at the nominal ^1H frequency of 399.66 MHz and ^{13}C frequency of 100.49 MHz. All spectra are reported in parts per million (ppm) relative to the residual solvent peak in ^1H NMR and the deuterated solvent peak in ^{13}C NMR.

B. MAP Synthesis

tert-Butyl N-methoxycarbamate (7). To a solution of methoxyamine hydrochloride (1.50 g, 179.60 mmol) and sodium hydroxide (7.18 g, 179.60 mmol) in a solution of dioxane/water (2:1, 210 mL) at 0 °C was added di-*tert*-butyl dicarbonate (43.1 g, 197.55 mmol). The solution was stirred for 5 minutes at 0 °C and then at room temperature overnight. Dioxane was removed via rotary evaporation and the aqueous layer was extracted with ethyl acetate (3 x 20 mL). The combined organic layer was washed with brine (20 mL), dried over sodium sulfate and concentrated by rotary evaporation. The crude liquid was purified via column chromatography (SiO₂, hexane:ethyl acetate, 1:1 v/v, *R_f* = 0.70) to afford amide **7** (25.4 g, 96% yield) as a clear liquid, having spectral characteristics in agreement with published data;³ IR ν (cm⁻¹) 1714, 3276; ¹H NMR (400 MHz, CDCl₃) δ 3.71 (s, 3H), 1.49 (s, 9H); ¹³C NMR (100 MHz, CDCl₃) δ 156.9, 81.2, 64.0, 28.1; FT-ICR-MS (ESI⁺, *m/z*) calcd for C₇H₁₅NO₃, [M + Na]⁺ 170.0788, found 170.0789.

(2-Bromoethoxy)trimethylsilane. To a solution of 2-bromoethanol (10.0 g, 80.0 mmol), imidazole (13.62 g, 200.05 mmol), and DMAP (cat.) in dry CH₂Cl₂ (150 mL) at 0 °C was added chlorotrimethylsilane (10.43 g, 96.03 mmol). The solution was allowed to come to room temperature and stirred overnight. The reaction solution was washed with

water (2 x 50 mL) and brine (1 x 50 mL), dried over sodium sulfate, and purified by short path distillation (20 mbar, collect bp 149.5 °C) with the aid of an acetone-dry ice bath to cool the collection vessel. The product was isolated (14.3 g, 90% yield) as a clear liquid; IR ν (cm⁻¹) 1093, 1255; ¹H NMR (400 MHz, CDCl₃) δ 3.80 (t, J = 8.0 Hz, 2H), 3.34 (t, J = 8.0 Hz, 2H), 0.08 (s, 9H); ¹³C NMR (100 MHz, CDCl₃) δ 62.7, 35.2, 28.2.

tert-Butyl N-(2-hydroxyethyl)-N-methoxycarbamate (8). To a slurry of sodium hydride (2.46 g of 60% in mineral oil, 61.43 mmol) in dry DMF (80 mL) at 0 °C was added carbamate 7 (8.59 g, 58.5 mmol). The suspension was stirred until hydrogen evolution ceased whereupon (2-bromoethoxy)trimethylsilane (12.7 g, 64.4 mmol) was added dropwise. The reaction mixture was allowed to warm to room temperature and stirred overnight. The reaction mixture then was diluted with water (80 mL) and extracted with ethyl acetate (3 x 50 mL). The combined organic extract was washed successively with 1M citric acid solution (80 mL) and brine (50 mL), and then dried over sodium sulfate. The solvents were removed by rotary evaporation, and the crude liquid was purified by column chromatography (SiO₂, hexane:ethyl acetate, 7:3 v/v, R_f = 0.43) to afford carbamate 8 (5.13 g, 50% yield) as a clear liquid; IR ν (cm⁻¹) 1053, 1699, 3428; ¹H NMR (400 MHz, CDCl₃) δ 3.81 (t, J = 4.0 Hz, 2H), 3.71 (s, 3H), 3.64 (t, J = 4.0 Hz, 2H), 1.51 (s, 9H); ¹³C NMR (100 MHz, CDCl₃) δ 157.2, 82.1, 62.6, 60.9, 52.0, 28.5; FT-ICR-MS (ESI⁺, m/z) calcd for C₈H₁₇NO₄, [M + H]⁺ 192.1230, found 192.1232.

2-(2-(Methoxyamino)ethoxy)isoindoline-1,3-dione (9). To a mixture of carbamate **8** (0.813 g, 4.65 mmol), *N*-hydroxyphthalimide (0.985 g, 6.04 mmol) and triphenylphosphine (1.58 g, 6.04 mmol) in dry THF (20 mL) at 0 °C was added diisopropyl azodicarboxylate (1.2 mL, 6.04 mmol). The reaction solution was stirred at room temperature overnight whereupon the solvent was removed via rotary evaporation. The residue was diluted with ethyl acetate (20 mL) and the resultant solution was washed successively with sodium bicarbonate (3 x 20 mL) and brine (20 mL). The organic layer was dried over sodium sulfate and then concentrated by rotary evaporation. The crude product was purified via column chromatography (SiO₂, CH₂Cl₂:ethyl acetate, 9:1 v/v, *R_f* = 0.65) to afford intermediate *tert*-butyl *N*-(2-((1,3-dioxoisindolin-2-yl)oxy)ethyl)-*N*-(methoxy)carbamate (1.4 g, 90% yield) as a clear oil; IR ν (cm⁻¹) 1702, 1730; ¹H NMR (400 MHz, CDCl₃) δ 7.84 (m, 2H), 7.76 (m, 2H), 4.39 (t, *J* = 4.0 Hz, 2H), 3.86 (t, *J* = 4.0 Hz, 2H), 3.81 (s, 3H), 1.50 (s, 9H); ¹³C NMR (100 MHz, CDCl₃) δ 163.6, 156.5, 134.7, 129.1, 123.8, 82.1, 63.2, 48.3, 28.4; FT-ICR-MS (ESI⁺, *m/z*) calcd for C₁₆H₂₀N₂O₆, [M + Na]⁺ 359.1214, found 359.1213.

To a stirred solution of the *N*-methoxycarbamate intermediate (5.56 g, 16.5 mmol) in CH₂Cl₂ (57 mL) at 0 °C was added trifluoroacetic acid (3.8 mL, 49.5 mmol). After stirring 1 h, the reaction mixture was concentrated by rotary evaporation and then diluted by addition of ethyl acetate (20 mL). The resultant solution was washed with sodium bicarbonate (3 x 20 mL) and brine (20 mL), dried over sodium sulfate, and concentrated

via rotary evaporation. The crude product was purified via column chromatography (SiO₂, hexane:ethyl acetate, 1:1 v/v, *R_f* = 0.53) to afford amine **9** (2.57 g, 66% yield) as a white solid, mp 65.6-65.9 °C; IR ν (cm⁻¹) 1711, 3265; ¹H NMR (400 MHz, CDCl₃) δ 7.84 (m, 2H), 7.76 (m, 2H), 4.39 (t, *J* = 8.0 Hz, 2H), 3.61 (s, 3H), 3.25 (t, *J* = 4.0 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 163.8, 134.6, 128.8, 123.6, 74.8, 61.6, 49.6; FT-ICR-MS (ESI⁺, *m/z*) calcd for C₁₁H₁₂N₂O₄, [M + H]⁺ 237.0870, found 237.0872.

10a: R = CD₃CH₂
10b: R = ¹³CD₃CH₂
10c: R = ¹³CD₃¹³CH₂

***N*-(2-((1,3-Dioxoisindolin-2-yl)oxy)ethyl)-*N*-methoxy-3,3,3-³H₃-propionamide (**10a**).**

To a solution of amine **9** (0.405 g, 1.72 mmol) and DMAP (cat.) in CH₂Cl₂ (6 mL) at 0 °C was added *N,N'*-diisopropylcarbodiimide (0.7 mL, 4.44 mmol). After 5 minutes of stirring, 3,3,3-³H₃-propionic acid (0.116 mL, 1.56 mmol) was added and the reaction mixture was allowed to warm to room temperature and stirred overnight. The reaction mixture was filtered through a fritted glass funnel and then concentrated by rotary evaporation. The crude residue was purified by column chromatography (SiO₂, hexane:ethyl acetate, 3:7 v/v, *R_f* = 0.65) to afford amide **10a** (0.399 g, 91% yield) as a colorless oil; IR ν (cm⁻¹) 1728; ¹H NMR (400 MHz, CDCl₃) δ 7.83 (m, 2H), 7.75 (m, 2H), 4.39 (t, *J* = 4 Hz, 2H), 4.02 (t, *J* = 4 Hz, 2H), 3.81 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 176.3, 163.5, 134.7, 129.3, 123.8, 75.4, 62.7, 45.6, 25.4, 14.4; FT-ICR-MS (ESI⁺, *m/z*) calcd for C₁₄H₁₃D₃N₂O₅, [M + H]⁺ 296.1320, found 296.13226.

***N*-(2-((1,3-Dioxoisindolin-2-yl)oxy)ethyl)-*N*-methoxy-3-¹³C₁-3,3,3-²H₃-propionamide (10b).** Using the procedure outlined above for the synthesis of amide 10a, amine 9 (0.330 g, 1.40 mmol) was reacted with *N,N'*-diisopropylcarbodiimide (0.3 mL, 1.91 mmol), 3-¹³C₁-3,3,3-²H₃-propionic acid (0.1 mL, 1.27 mmol) and DMAP (cat.) to afford amide 10b (0.265 g, 79% yield); ¹H NMR (400 MHz, CDCl₃) δ 7.83 (m, 2H), 7.75 (m, 2H), 4.39 (t, *J* = 4 Hz, 2H), 4.00 (t, *J* = 4 Hz, 2H), 3.80 (s, 3H), 2.50 (s, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 163.5, 134.8, 129.1, 123.8, 75.3, 62.6, 45.4, 25.2, 8.6-7.4 (m); FT-ICR-MS (ESI⁺, *m/z*) calcd for C₁₃¹³CH₁₃D₃N₂O₅, [M + H]⁺ 297.1354, found 297.1356.

***N*-(2-((1,3-Dioxoisindolin-2-yl)oxy)ethyl)-*N*-methoxy-2,3-¹³C₂-3,3,3-²H₃-propionamide (10c).** Using the procedure outlined above for the synthesis of amide 10a, amine 9 (0.359 g, 1.52 mmol) was reacted with *N,N'*-diisopropylcarbodiimide (0.7 mL, 4.34 mmol), 2,3-¹³C₂-3,3,3-²H₃-propionic acid (0.115 mL, 1.45 mmol) and DMAP (cat.) to afford amide 10c (0.062 g, 14% yield); ¹H NMR (400 MHz, CDCl₃) δ 7.83 (m, 2H), 7.75 (m, 2H), 4.38 (t, *J* = 4 Hz, 2H), 4.01 (t, *J* = 4 Hz, 2H), 3.80 (s, 3H), 2.50 (dd, *J* = 4, 128 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 163.3, 134.5, 128.8, 123.6, 75.0, 62.4, 45.2, 25.3-24.8 (m), 8.5-7.0 (m); FT-ICR-MS (ESI⁺, *m/z*) calcd for C₁₂¹³C₂H₁₁D₃N₂O₅, [M + H]⁺ 298.1384, found 298.1389.

N-(2-(Aminoethoxy)ethyl)-*N*-methoxy-3,3,3-²H₃-propionamide (**MAP-32**). To a solution of **10a** (0.399 g, 1.35 mmol) in CH₂Cl₂ (5 mL) at 0 °C was added hydrazine monohydrate (0.069 mL, 1.42 mmol) dropwise. The reaction mixture was stirred at 0 °C for 10 minutes before warming to room temperature and then stirring 45 minutes. The reaction was filtered through a fritted glass funnel, and the filtrate was concentrated by rotary evaporation. The crude product was purified by column chromatography (SiO₂, CH₂Cl₂:MeOH, 9:1 v/v, *R_f* = 0.41) to afford **MAP-32** (0.118 g, 53% yield) as a clear liquid; IR ν (cm⁻¹) 1728, 2942, 2983; ¹H NMR (400 MHz, CDCl₃) δ 4.66 (br. s, 2H) 4.04 (t, *J* = 4 Hz, 2H), 3.56 (s, 3H), 3.15 (t, *J* = 4 Hz, 2H), 2.11 (s, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 70.9, 61.6, 50.0, 25.2, 14.0; FT-ICR-MS (ESI⁺, *m/z*) calcd for C₆H₁₁D₃N₂O₃, [M + H]⁺ 166.1265, found 166.1268.

N-(2-(Aminoethoxy)ethyl)-*N*-methoxy-3-¹³C₁-3,3,3-²H₃-propionamide (**MAP-33**). Using the procedure outlined above for the synthesis of **MAP-32**, amide **10b** (0.265 g, 1.00 mmol) was reacted with hydrazine monohydrate (0.058 mL, 1.05 mmol) to afford **MAP-33** (0.034 g, 20% yield); ¹H NMR (400 MHz, CDCl₃) δ 4.03 (t, *J* = 4 Hz, 2H), 3.55 (s, 3H), 3.14 (t, *J* = 4 Hz, 2H), 2.48 (s, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 172.5, 80.0, 61.6, 50.0, 25.0, 8.9-7.3 (m); FT-ICR-MS (ESI⁺, *m/z*) calcd for C₅¹³CH₁₁D₃N₂O₃, [M + H]⁺ 167.1299, found 167.1301.

***N*-(2-(Aminoxy)ethyl)-*N*-methoxy-2,3-¹³C₂-3,3,3-²H₃-propionamide (MAP-34).**

Using the procedure outlined above for the synthesis of MAP-32, amide 10c (0.062 g, 0.21 mmol) was reacted with hydrazine monohydrate (0.011 mL, 0.22 mmol) to afford MAP-33 (0.018 g, 51% yield); ¹H NMR (400 MHz, CDCl₃) δ 4.79 (br. s, 2H), 4.03 (t, *J* = 4.0 Hz, 2H), 3.55 (s, 3H), 3.14 (t, *J* = 4.0 Hz, 2H), 2.12 (d, *J* = 128, 2H); ¹³C NMR (100 MHz, CDCl₃) δ 73.1, 61.6, 49.8, 26.5-25.1 (m), 7.54-9.27 (m); FT-ICR-MS (ESI⁺, *m/z*) calcd for C₄¹³C₂H₉D₃N₂O₃, [M + H]⁺ 168.1330, found 168.1335.

II. GC-MS Analyses

A. General Procedure

All GC-MS analyses were performed using an Agilent Technologies GC-MS instrument (Agilent Technologies, Palo Alto, CA); GC consisting of an Agilent 7820A gas chromatograph, and an Agilent 5975 MSD. The GC was fitted with an HP-5MS column 30 m long with an internal diameter of 250 μm and a stationary-phase film thickness of 0.25 μm. The carrier was high-purity helium at a flow rate of 1.0 mL/min. Sample size was 1 μL with a 10:1 split ratio. The injector temperature was 275 °C; the column temperature was programmed to start at 60 °C and increase at 20 °C/min, to a maximum of 315 °C, giving a 12.5 minute run, although the run was usually stopped at about 10 minutes. A 120 second solvent delay was allowed. The transfer line temperature was 250 °C. The MS ion source was held at 230 °C and the MS Quad at 150 °C. The electron ionization energy was 70 eV. Mass spectra were collected from 25 to 400 *m/z*.

B. Sample Preparation for Study of GC Retention Time and MST Generation

Representative sample mixtures were prepared and derivatized by treatment with an excess (~2 equiv) of reagent, either MAP-32 or MAP-33, to afford the derivatized mixtures A1-32, A2-32, B1-33 and B2-33. Aliquots (0.5 mL) of the A1 and B1 derivatized mixtures, and the A2 and B2 mixtures, were combined, respectively, and then directly injected into the GC-MS instrument for analysis. The two sample mixtures were analyzed by GC-MS a total of three times.

Sample Mixture A1-32. To generate sample mixture A1-32, solutions of benzaldehyde (50 μ L of a 0.01 M solution), hexanal (50 μ L of a 0.01 M solution), and 2-propoxyacetaldehyde (50 μ L of a 0.01 M solution) were added to acetonitrile (5 mL). To this mixture at room temperature was added MAP-32 (250 μ L of a 0.015 M solution). After the solution was heated at 60 $^{\circ}$ C for 16 hours, the solution was allowed to cool to room temperature and the acetonitrile was removed via rotary evaporation. The mixture then was reconstituted by dissolving in acetonitrile (2 mL) to generate mixture A1-32.

Sample Mixture B1-33. To generate sample mixture B1-33, solutions of benzaldehyde (50 μ L of a 0.01 M solution), hexanal (50 μ L of a 0.01 M solution), and 2-propoxyacetaldehyde (50 μ L of a 0.01 M solution) were added to acetonitrile (5 mL). To this mixture at room temperature was added MAP-33 (200 μ L of a 0.019 M solution). After the solution was heated at 60 $^{\circ}$ C for 16 hours, the solution was allowed to cool to room temperature and the acetonitrile was removed via rotary evaporation. The mixture then was reconstituted by dissolving in acetonitrile (2 mL) to generate mixture B1-33.

Sample Mixture A2-32. To generate sample mixture A2-32, solutions of benzaldehyde

(50 μ L of a 0.01 M solution), hexanal (150 μ L of a 0.01 M solution), and 2-propoxyacetaldehyde (50 μ L of a 0.01 M solution) were added to acetonitrile (5 mL). To this mixture at room temperature was added MAP-32 (500 μ L of a 0.015 M solution). After the solution was heated at 60 °C for 16 hours, the solution was allowed to cool to room temperature and the acetonitrile was removed via rotary evaporation. The mixture then was reconstituted by dissolving in acetonitrile (2 mL) to generate mixture A2-32.

Sample Mixture B2-33. To generate sample mixture B2-33, solutions of benzaldehyde (50 μ L of a 0.01 M solution), hexanal (50 μ L of a 0.01 M solution), and 2-propoxyacetaldehyde (150 μ L of a 0.01 M solution) were added to acetonitrile (5 mL). To this mixture at room temperature was added MAP-33 (400 μ L of a 0.019 M solution). After the solution was heated at 60 °C for 16 hours, the solution was allowed to cool to room temperature and the acetonitrile was removed via rotary evaporation. The mixture then was reconstituted by dissolving in acetonitrile (2 mL) to generate mixture B2-33.

C. GC-MS Study of Pooled Sample Mixtures A1-B1 and A2-B2

Tables 1 and 2 summarize the MST ion counts measured for each injection of the multiplexed sample mixtures.

Table 1. Summary of measured ion counts for MSTs generated on analysis of multiplexed A1-B1 (*E*-isomer comparison).

Carbonyl Substrate	MST (<i>m/z</i>)	Actual Substrate Ratio (source)	Measured ion counts (<i>E</i> -isomer) over three injections	Total ion counts	Ratio
Benzaldehyde	32	1 (fr. A1)	398, 722, 608	1,728	0.94
	33	1 (fr. B1)	604, 628, 599	1,831	
Hexanal	32	1 (fr. A1)	6982, 6584, 7283	20,849	0.94
	33	1 (fr. B1)	6528, 6926, 6076	19,530	
<i>n</i> -Propoxy-acetaldehyde	32	1 (fr. A1)	6819, 7009, 7409	21,237	0.88
	33	1 (fr. B1)	8115, 7668, 8433	24,216	

Carbonyl Substrate	MST (<i>m/z</i>)	Actual Substrate Ratio (source)	Measured ion counts (<i>E</i> -isomer) over three injections	Total ion counts	Ratio
Benzaldehyde	32	1 (fr. A2)	6549, 6435, 6617	19,601	0.92
	33	1 (fr. B2)	6510, 7172, 7566	21,248	
Hexanal	32	3 (fr. A2)	55800, 66744, 60656	183,200	0.34
	33	1 (fr. B2)	18360, 23064, 20904	62,328	
<i>n</i> -Propoxy-acetaldehyde	32	1 (fr. A2)	11742, 14967, 13662	40,371	0.42

	33	3 (fr. B2)	27259, 35464, 32664	95,387	
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Table 2. Summary of measured ion counts for MSTs generated on analysis of multiplexed A2-B2 (*E*-isomer comparison).

Figure S1. GC trace obtained on injection of pooled sample mixture A2-B2 showing the retention times of MAP32- and MAP33-carbonyl adducts. The *E*-isomer adducts are indicated by colored arrows. The *Z*-isomer adducts of *n*-propoxyacetaldehyde and benzaldehyde are present in trace amounts, so all calculations were performed using the *E*-isomers.

III. Comparisons of EI-Induced Fragmentations of MAP- and AEP-Adducts

Shown below are the MS spectra obtained for the MAP-carbonyl adducts. To correlate signals of specific fragment ions derived from the MAP adducts to the corresponding ions derived from the AEP adducts, arrows are added to reflect relative changes: (1) an increase (up arrow) in signal intensity or decrease (down arrow) in signal intensity relative to the corresponding AEP-derived fragment ion; (2) ion resulting from

an informative (green) or uninformative (red) fragmentation. The fragment ion labels correspond to those identified in Scheme 5.

S14

S15

S16

S18

MAP-4-heptanone

40 70
[3]

(E)-MAP-2-hexenal

50 60

Relative Abundance

13

S19

(E)-MAF-2-hexanal

(Z)-MAF-a-propocycethanal

S20

IV. ^1H and ^{13}C NMR Spectra of MAP Reagent Panel

S21

S22

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A.2. NMR spectra of key intermediates

TMS

^1H NMR of **11**, 400 MHz, CDCl_3

11

^{13}C NMR of **11**, 100 MHz, CDCl_3

FT-IR with ATR of **11**

^1H NMR of **13**, 400 MHz, CDCl_3

13

197

NMR of 13, 100 MHz, CDCl₃

FT-IR with ATR of **13**

^{13}C NMR of **14**, 100 MHz, CDCl_3

FT-IR with ATR of **14**

15

¹H NMR of **15**, 400 MHz, CDCl₃

15

203

^{13}C NMR of **15**, 100 MHz, CDCl_3

15

FT-IR with ATR of **15**

NMR of **17**, 100 MHz, CDCl₃

FT-IR with ATR of **17**

18

TMS $\rightarrow$

CH_2Cl_2

ppm

^1H NMR of **18**, 500 MHz, CDCl_3

18

^{13}C NMR of **18**, 100 MHz, CDCl_3

18

FT-IR with ATR of **18**

A.3. List of abbreviations

h = hour

rt = room temperature

mmol = millimole

mg = milligram

TLC = thin layer chromatography

rb = round bottom

rpm = revolutions per minute

EtOH = ethanol

CH₂Cl₂ = dichloromethane

CHCl₃ = chloroform

TFA = trifluoroacetic acid

MeOH = methanol

nm = nanometer

MRI = magnetic resonance imaging

NMR = nuclear magnetic resonance

FTIR = fourier transform infrared radiation

HRMS = high resolution mass spectrometry

ATR = attenuated total reflectance

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Education

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Sci-Mix Poster Presentation	SERMACS 2014
Negishi-Brown Poster Award	2014
Red and Black Scholarship Reception	Spring 2015
College for a Day	May 2015, 2016
Speaker at Lassiter Middle School Career Fair	October 9, 2015
Speaker at Berea College	July 9, 2015
STEM Day at UofL Chemistry Tour Leader	April 18, 2016
Cardinal Preview Day	October 15, 2016
Graduate Teaching Assistant Award	April 19, 2017

Experience

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SK Biladeau, S Laulhé, S.; MH Nantz. *Isotopically coded Weinreb amide-inspired reagents for GC-MS profiling of aldehydes and ketones*. **Poster** 31; presented at the 7th Negishi-Brown Lectures, Lafayette, IN; October 6, 2014.

Poster 346; presented at the 66th Southeastern Regional Meeting of the American Chemical Society, Nashville, TN; October 17, 2014. **Poster** Graduate Research Symposium; November 14, 2014.

Publications

SK Biladeau, WR Richmond, S Laulhé, S.; MH Nantz. Isotopically coded *N*-methoxy amide reagents for GC-MS profiling of carbonyl compounds *via* mass spectral tag generation. *Anal. Methods*, **2016**, 8, 3704-3710.

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