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Fullerene and Boron Nitride Nanomaterials for Biomedical Applications

by

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ABSTRACT

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This thesis presents an evaluation of the potential of two new nanoparticles as biomedical agents, as well as an acid digestion method for exceptionally stable metal-doped nanoparticles, such as for the burgeoning field of boron nitride nanomaterials. With the birth of nanotechnology, researchers have explored functionalizing nanoparticles for various medical applications and have shown these nanoparticles to have many attractive properties. The present work strives to further understand the structure-function relationships for such biomedical nanomaterials.

This thesis examines two very different nanomaterials – C\textsubscript{60} fullerenes and boron nitride nanotubes (BNNTs) – and aims to answer key questions related to their biological potential. In the first part of the thesis, a biocompatible C\textsubscript{60} fullerene was functionalized to be radiolabeled in order to examine its biodistribution \textit{in vivo} using copper-64 positron emission tomography (PET). These studies showed that the resultant fullerene material was cleared rapidly and almost exclusively by the kidneys within three hours. This finding is in stark contrast to many other biocompatible fullerene materials in the literature with completely different
biodistribution profiles and extremely long clearance times (> 100 hours). These data clearly demonstrate that fullerene-based drug delivery agents must be approached rationally to design materials that have predictable and favorable biodistribution and pharmacokinetic profiles.

In the second part of the thesis, BNNTs were loaded with gadolinium chelates rendering the modified BNNT material a magnetic resonance imaging (MRI) contrast agent. However, the high stability of BNNTs prohibited accurate quantification of the metal content using inductively-coupled plasma (ICP) techniques because of incomplete digestion and subsequent release of Gd$^{3+}$ ions for quantification. Various acid digestion methods were explored and optimized. Complete digestion of the BNNT material was achieved only in the case of concentrated boiling perchloric acid at 203 °C. The gadolinium chelate loading capacity of the BNNT material was optimized and its MRI activity determined and studied. With the development of the perchloric acid preparation method, researchers now have a proven tool to quantify metal content of any new metal-doped BNNT material. This robust and time-dependent oxidation method also offers the potential of providing new BNNT materials with beneficial defect sites for further functionalization.
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Bassam Shakhshiri once said: “Science is fun, in the truest sense of the word. We’re not in it for cheap thrills. Science is fun because it is intellectually stimulating and emotionally rewarding.” My time in graduate school would not have been so fun without the people that I worked with over the years. First, I would like to thank my advisor, Prof. Lon Wilson, for many years of guidance, advice, and conversations about chemistry, travel, and much more. Thank you for being a kind and thoughtful mentor.

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<table>
<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>%ID/cc</td>
<td>Percent Injected Dose per Cubic Centimeter of Tissue</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
</tr>
<tr>
<td>BN</td>
<td>Boron Nitride</td>
</tr>
<tr>
<td>BNNT</td>
<td>Boron Nitride Nanotube</td>
</tr>
<tr>
<td>CNT</td>
<td>Carbon Nanotube</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared Spectroscopy</td>
</tr>
<tr>
<td>Gd@BNNT</td>
<td>BNNT loaded with Gd$^{3+}$ ions</td>
</tr>
<tr>
<td>Gd@BNNT$^2$</td>
<td>BNNT loaded with Gd(acac)$_3$·2H$_2$O</td>
</tr>
<tr>
<td>GNT</td>
<td>Gadonanotube (US-tube filled with Gd$^{3+}$ ions)</td>
</tr>
<tr>
<td>h-BN</td>
<td>Hexagonal Boron Nitride</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-Performance Liquid Chromatography</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively-Coupled Plasma Mass Spectrometry</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NOTA</td>
<td>2,2',2&quot;-(1,4,7-triazacyclononane-1,4,7-triyl)triacetic acid</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>PF</td>
<td>PromoFluor 633</td>
</tr>
<tr>
<td>RES</td>
<td>Reticuloendothelial System</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td></td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>Serinol</td>
<td>Serinolamide (water-solubilizing moiety)</td>
</tr>
<tr>
<td>T</td>
<td>Tesla</td>
</tr>
<tr>
<td>TAC</td>
<td>Time-Activity Curve</td>
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<td>Thermogravimetric Analysis</td>
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<tr>
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<td>Ultra-Short Carbon Nanotube</td>
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Chapter 1

Introduction

1.1. Overview

The field of nanomedicine has grown rapidly over many years.\textsuperscript{1,2} The new and attractive properties at the nanoscale that do not exist in bulk samples of the same material allow the potential for new avenues to diagnose and treat disease that traditional methods may have failed in the past. Many different materials have been extensively studied for diagnostic and therapeutic applications, including quantum dots,\textsuperscript{3} noble metal nanoparticles (mainly gold),\textsuperscript{4} silicon,\textsuperscript{5} boron nitride,\textsuperscript{6} and carbon-based nanomaterials.\textsuperscript{7–10} In particular, the molecule that founded the field of nanoscience, $C_{60}$ fullerene, has been of keen interest not only for its interesting, biologically-relevant properties, but also for its versatility as a chemical canvas that may be designed and functionalized for any biomedical application, as will be discussed subsequently in this thesis. $C_{60}$, though hydrophobic in nature, can
be easily derivatized for water solubility and hence, biocompatibility, while still retaining its inherent antioxidant properties and its ability to generate free radicals when subjected to light irradiation. Combined with the ability to release drugs and internalize into cells, C_{60} fullerene is poised to be a contender as a clinical nanomaterial.

A more recent addition to the biomedical nanomaterial community are boron nitride nanotubes (BNNTs), which are related rather distantly to fullerenes: whereas carbon nanotubes (CNTs) are cylindrical forms of fullerenes, BNNTs are structural counterparts to CNTs, with boron and nitrogen atoms replacing carbon atoms in the hexagonal lattice of the nanotube. BNNTs, while less developed for biomedical functions, have already been studied broadly for uses in drug delivery and boron neutron capture therapy. Because they are also hydrophobic in nature, research efforts have focused more on how to functionalize and suspend BNNTs in aqueous and biological media, as well as to determine biocompatibility and safety profiles, which preliminary studies show promising results that water-dispersible BNNTs are non-cytotoxic. BNNTs have also been investigated as magnetic resonance imaging (MRI) contrast agents, which has also been explored heavily for CNTs. Using CNTs as an example, insight on how to load BNNTs with MRI-active compounds and a new wet chemical method to digest metal-doped BNNTs for inductively-coupled plasma (ICP) analysis is presented in this thesis.

However, in the explosion of research to produce clinical nanomaterials, there have been many different materials studied with reports of chemical
composition, toxicity, and efficacy.\textsuperscript{11} Even if a specific nanomaterial reaches clinical trials and approval by the Food and Drug Administration (FDA), questions about scalability and advantages over other competing technologies will certainly be asked; questions that have different answers for various derivatives and formulations that may also vary in production batch-to-batch. In order to bring the concept of nanomedicine to fruition, attention must be paid to the rational design and reproducible synthesis of nanomaterials that have the best chance to achieve FDA approval and compete with current standards of care. This dissertation offers an evaluation of the biodistribution of C\textsubscript{60} fullerene for cancer drug delivery applications and an exploration of BNNTs for MRI contrast applications, in hopes that this work inspires future fundamental research in the design and functionalization of nanomaterials that will propel these materials from the lab into the clinic.

1.2. Organization

This thesis is divided into two main parts, each reviewing the relevant literature. Chapter 2 describes work done to synthesize a new radiolabeled C\textsubscript{60} fullerene derivative (C\textsubscript{60}-\textsuperscript{64}CuCu(NOTA)) based on the previous studied molecule, C\textsubscript{60}-serinol, which is covalently coated with water-solubilizing serinolamide moieties. The biodistribution and pharmacokinetic profile of C\textsubscript{60}-\textsuperscript{64}CuCu(NOTA) were determined using PET imaging in a live mouse model and a discussion on how functionalization affects tissue localization of C\textsubscript{60} fullerene is provided.
**Chapter 3** presents work on loading BNNTs with Gd$^{3+}$ chelates for potential use as MRI contrast agents. Also described is an improved wet chemical digestion method for ICP metal analysis of BNNTs and how this digestion method can be utilized as a means of oxidizing BN nanomaterials for further functionalization.

**Chapter 4** summarizes the important conclusions from each chapter and the main findings of this thesis as a whole, as well as potential directions for future studies.
Chapter 2

Development and Evaluation of Serinolamide-Derivatized Fullerene as a Drug Delivery Platform

2.1. Introduction and Background

2.1.1. Cancer and Drug Delivery

Cancer is the second-most lethal disease in the United States, and the American Cancer Society has estimated over 1.7 million new cancer cases and over 600,000 cancer deaths in 2018. An ideal cancer therapy should be both specific for its target (the tumor) as well as effective at killing malignant cells. Small-molecule chemotherapeutics are often used by oncologists to treat primary tumors that cannot be surgically removed, and to threat metastatic disease via systemic administration in concert with other therapies such as surgery, immunotherapy, and radiation. However, chemotherapies must be delivered more specifically such
that they interact more with tumor cells to increase the therapeutic payload delivered while reducing the delivery of the drugs to healthy tissues, which avoids debilitating side effects.\textsuperscript{13}

Drug delivery to solid tumors is impeded by a number of physical barriers in the body.\textsuperscript{14,15} First, the extracellular matrix the cancer cells excrete is quite dense, making diffusion of small molecules difficult. Another hurdle to cross is the chaotic vasculature network found in solid tumors. These blood vessels are not fully developed and are arranged in a haphazard manner as the tumor attempts to feed itself from the body’s blood supply and metastasize. \textbf{Figure 2.1} shows the difference between the structure of normal vasculature and tumor vasculature. Because the vessels lack their epithelial lining, this causes them to leak and raise the interstitial tumor pressure, which in turn also prevents extravasation of drugs to the tumor. Once the drug reaches the tumor microenvironment, it must then internalize into cells and later to its target. Because small-molecule drugs often cannot reach their target, they localize in healthy organs and cause debilitating side effects, which has led to research efforts to increase the therapeutic cargo to cancer cells.

![Normal blood vessels](image1.png) ![Tumor blood vessels](image2.png)
2.1.2. Fullerenes for Drug Delivery

Recently, nanoparticles have been studied for biomedical applications, including drug delivery for cancer therapy.\textsuperscript{17,18} For example, silicon nanoparticles,\textsuperscript{19} superparamagnetic iron oxide nanoparticles,\textsuperscript{20} and carbon nanotubes,\textsuperscript{21,22} among others, have been extensively studied for various imaging, therapeutic, and drug delivery applications. A particular nanostructure that shows promise for biomedical applications is the C\textsubscript{60} fullerene molecule, because this multi-functional medical platform has a variety of advantages.\textsuperscript{23} First, C\textsubscript{60} is chemically versatile, and the syntheses of many different adducts have already been developed,\textsuperscript{24–30} which provides great flexibility when designing a C\textsubscript{60} derivative for a particular application, such as drug delivery. Second, C\textsubscript{60} has shown strong antioxidant behavior through its ability to quench free radicals\textsuperscript{31,32} and has been observed to nearly double the lifespan of rodents, presumably because of this radical-scavenging property.\textsuperscript{33,34} Conversely, certain C\textsubscript{60} derivatives can generate reactive oxygen species under light irradiation, thus allowing them to be used for photodynamic therapy (PDT) against cancer.\textsuperscript{35,36} Furthermore, C\textsubscript{60} can be chemically decorated with molecular targeting agents,\textsuperscript{37,38} imaging agents,\textsuperscript{39–41} and anticancer drugs\textsuperscript{38,42–45} to be used as a drug delivery vector, a diagnostic agent, or a combination of the two.

In the pursuit of designing a platform that could be utilized for cancer drug delivery, Wilson and coworkers were inspired by the serinolamide moiety used to...
solubilize clinical iodobenzene X-ray contrast agents\textsuperscript{46} to functionalize C\textsubscript{60}. In a compound called C\textsubscript{60}-serinol, six malonate groups containing these serinolamide moieties surround the fullerene core in an octahedral arrangement (\textbf{Figure 2.2A}), which affords the material ultra-high water solubility and extremely low cytotoxicity.\textsuperscript{47,48} This C\textsubscript{60} derivative is a desirable material to use as a foundation for further functionalization because it is well-characterized, and like other hexakisadducts of C\textsubscript{60}, it is monoisomeric, which helps ensure a low polydispersity index necessary for clinical translation. C\textsubscript{60}-serinol materials have been shown to pass through restrictive biological barriers, such as the cellular and nuclear membranes of liver cancer cells,\textsuperscript{48} and can be used to deliver DNA to mouse fibroblasts and marrow stromal cells.\textsuperscript{49} These findings suggest C\textsubscript{60}-serinol may be able to penetrate the harsh tumor microenvironment described previously to reach individual tumor cells. In addition, when derivatized with paclitaxel, C\textsubscript{60}-serinol can kill cancer cells \textit{in vivo} without producing the weight loss associated with other formulations.\textsuperscript{50} These results demonstrate C\textsubscript{60}-serinol's potential to serve as a drug delivery platform, and also as a radical-quenching scaffold that may help meliorate the negative side effects of chemotherapy.
C₆₀ was discovered in 1985,⁵¹ has been available in bulk quantities since 1990,⁵² and has been extensively studied as evidenced by the thousands of peer-reviewed publications in the literature. However, no fullerene-based material in any form has yet to reach the clinic. An important characteristic of drug delivery platforms to study is the biodistribution and excretion profiles of the platform, in order to fine tune derivatization and treatment strategies of the formulation. Thus far, there have been numerous biodistribution studies of different C₆₀ derivatives, including C₆₀-serinol, showing vastly different uptake patterns with no consensus as to what kind of functionalization is optimum for C₆₀-based drug delivery, as will be discussed in this chapter. Therefore, a major gap of knowledge for C₆₀-serinol is the in vivo biodistribution profile and excretion mechanism of the compound with careful attention to functionalization. This work builds on known benefits for C₆₀-
serinol by providing a look into the unique in vivo behavior of this versatile nanomaterial using non-invasive positron emission tomography (PET).

PET is a clinically-used modality that can image whole organisms non-invasively with accurate quantifications and high sensitivity. A PET image is generated by an injected radionuclide that decays partly by positron (β+) emission. These positrons then annihilate with electrons in the vicinity, and two coincident gamma rays are generated at 180° apart with equal energies (511 keV). These gamma rays are identified by coincidence detectors that surround the subject and are reconstructed into an image that can be analyzed to determine the amount of radioactivity in a specific volume of interest (VOI), as shown in Figure 2.3. Of the many radionuclides currently used for PET imaging, copper-64 was elected because of its relatively low cost and long half-life (12.7 h), which permits imaging at later time points post-injection. In order to chelate copper-64 for radiolabeling C₆₀, a C₆₀-serinol derivative (Figure 2.2B) was synthesized with five malonates carrying serinolamide groups and a covalently linked chelate called NOTA because [⁶⁴Cu]Cu(NOTA) has been shown to be stable under biological conditions. Herein is presented a biodistribution study of the C₆₀-[⁶⁴Cu]Cu(NOTA) conjugate using dynamic and static PET imaging as a tool to assess the material’s pharmacokinetic profile. This work is viewed as a valuable first step toward developing C₆₀-serinol-derived materials as a new cancer drug delivery platform.
2.2. Experimental Methods

2.2.1. Synthesis and Characterization of C$_{60}$-Cu(NOTA)

All solvents and reagents were purchased from commercial sources and used as received. All synthetic reactions were performed under argon, unless otherwise stated. Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) was performed using a Bruker Autoflex MALDI ToF (time of flight) Mass Spectrometer (Bruker Corporation, Billerica, MA, USA). $^1$H nuclear magnetic resonance (NMR) was performed using a Bruker 400 MHz NMR Spectrometer (Bruker Corporation, Billerica, MA, USA). Fourier Transform Infrared (FTIR) spectroscopy was performed using a Nicolet 6700 FTIR spectrometer with an attenuated total reflectance (ATR) attachment (Thermo Fisher Scientific, Waltham, MA, USA). Purification of most intermediates was performed using an Agilent Technologies 971-FP Flash Purification System (Agilent Technologies, Inc., Santa...
Clara, CA, USA). All MS and $^1$H NMR spectra can be found in Appendix A. The hydrodynamic diameter and aggregate diameter of the C$_{60}$-NOTA conjugate were determined by dynamic light scattering (DLS) and atomic force microscopy (AFM), respectively. The zeta-potential ($\zeta$-potential) of the C$_{60}$-NOTA and C$_{60}$-Cu(NOTA) conjugates were determined as well. The DLS and $\zeta$-potential measurements were conducted using a Malvern Zetasizer Nano (Malvern Instruments, Malvern, UK). AFM images were taken using a Digital Instruments MultiMode AFM-2 (Digital Instruments, Santa Barbara, CA).

High performance liquid chromatography (HPLC) was performed using 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). Preparative HPLC was performed on an Agilent 1260 Infinity II (Agilent Technologies, Inc., Santa Clara, CA, USA) with the following method: 5% solvent B (in solvent A) to 30% solvent B over 15 min, then 30% solvent B to 95% solvent B over the next 15 min with a 15 mL/min flow rate. A Luna® C18 5 µm column with 21.2 x 250 mm dimensions (Phenomenex, Torrance, CA, USA) was used. Analytical HPLC was performed on an Agilent 1100 Series with the following method: 5% solvent B to 95% solvent B over 15 min with a 1 mL/min flow rate. An XBridge® C18 3.5 µm column with 4.6 x 250 mm dimensions (Waters Corp., Milford, MA, USA) was used.

2.2.1.1. Synthesis of Compound 1

Benzyl N-[(4-aminophenyl)methyl]carbamate (2.00 g, 7.80 mmol), ethyl hydrogen malonate (1.03 g, 7.80 mmol), and N, N-diisopropylethylamine (Hünig’s base; 1.50 mL, 8.58 mmol) were dissolved in dry dichloromethane under argon at
0°C. Diisopropylcarbamide (DIC; 1.25 mL, 7.96 mmol) was added dropwise over 10 min. The reaction was stirred and allowed to reach room temperature over 24 h. The solution was then dried by rotary evaporation that yielded an orange oil. Toluene was added to the oil until a white precipitate formed that was removed by vacuum filtration and discarded. The filtrate was purified by column chromatography using a gradient of dichloromethane/methanol as the eluent. The second fraction was then recrystallized from dichloromethane using hexanes, forming compound 1 as white needle-like crystals (0.376 g, 13% yield). m.p.: 108-109 °C. IR νmax (neat): 3331, 3300, 2976, 1736, 1686, 1530, 1265, 1243, 1133, 1034, 971 cm⁻¹. ¹H NMR (400 MHz, CDCl₃): δ 9.23 (s, 1H), 7.48-7.44 (m, 2H), 7.35-7.27 (m, 5H), 7.22-7.17 (m, 2H), 5.34 (t, J=5.9 Hz, 1H), 5.11 (s, 2H), 4.30 (d, J=5.9 Hz, 2H), 4.22 (q, J=7.2 Hz, 2H), 3.42 (s, 2H), 1.30 (t, J=7.2 Hz, 3H). MALDI-MS: m/z calculated for C₂₀H₂₂N₂O₅, 370.41; found, 371.13 [M⁺], 393.15 [M+Na⁺], 409.20 [M+K⁺].

### 2.2.1.2. Synthesis of Compound 2

C₆₀ (1.00 g, 1.39 mmol) was dissolved in about 800 mL of dry toluene by bath sonication. Compound 1 (0.645 g, 1.74 mmol) and carbon tetrabromide (0.922 g, 2.78 mmol) were also added to the reaction mixture. A 1 M solution of 1,8-diazabicycloundec-7-ene (DBU) in toluene (1.94 mL, 1.94 mmol) was then added in two aliquots 10 min apart, and the reaction stirred for 4.5 h at room temperature. A black precipitate formed during the reaction that was removed by vacuum filtration and discarded before the reaction mixture was purified by column chromatography
using a gradient of toluene/ethyl acetate as the eluent. The second fraction was collected, and the solvent was removed by rotary evaporation to afford compound 2 as a dark red solid (0.121 mg, 8% yield). MALDI-MS: \( m/z \) calculated for \( \text{C}_{30}\text{H}_{20}\text{N}_{2}\text{O}_{5} \), 1089.05; found, 1089.63 [M⁺], 1112.69 [M+Na⁺], 1127.62 [M+K⁺].

### 2.2.1.3. Synthesis of Compound 3

Compound 2 (0.048 g, 44.5 \( \mu \)mol) was dissolved in a 1:1 mixture of dry toluene and dry dichloromethane. \( \text{N,N'}\text{-bis[2-(acetyloxy)-1-[acetyloxy)methyl]ethyl]-malonamide} \) (protected serinolamide malonate; 0.187 g, 0.448 mmol), which was previously synthesized according to the method reported by Wharton \textit{et al.}, was dissolved in dry dichloromethane and added to the reaction flask. Carbon tetrabromide (0.221 g, 0.667 mmol) was then added to the reaction mixture followed by DBU (0.245 mL, 0.245 mmol) in 6 mL of toluene dropwise over 6 hours. The reaction mixture was purified by column chromatography using a gradient of chloroform/methanol as the eluent. The first fraction was collected, and the solvent was removed by rotary evaporation giving compound 3 as a light red solid (0.128 g, 91% yield). IR \( \nu_{\text{max}} \) (neat): 3294, 3064, 2958, 1736, 1663, 1538, 1366, 1219, 1041 cm\(^{-1}\). MALDI-MS: \( m/z \) calculated for \( \text{C}_{165}\text{H}_{140}\text{N}_{12}\text{O}_{55} \), 3170.96; found, 3169.64 [M⁺], 3192.63 [M+Na⁺].

### 2.2.1.4. Synthesis of Compound 4

Trifluoroacetic acid (TFA; 3.10 \( \mu \)L, 40.5 \( \mu \)mol) was dissolved in isopropyl alcohol to give a 1.04 M solution. Compound 3 (0.128 g, 40.5 \( \mu \)mol) was then added
along with methanol until everything was dissolved. Pd/C (0.27 g) was added to the reaction mixture, and the vial was placed in a pressurized vessel with 10.2 atm of H₂. The reaction mixture was stirred at room temperature for about 12 h. The mixture was then filtered by vacuum filtration to remove the Pd/C, and the solvent was removed by rotary evaporation to give compound 4 as a red solid without further purification (0.091 g, 74% yield). MALDI-MS: m/z calculated for C₁₅₇H₁₃₄N₁₂O₅₃, 3036.83; found, 3038.59 [M+], 3058.43 [M+Na⁺].

2.2.1.5. Synthesis of Compound 5

Compound 4 (0.091 g, 29.9 µmol), 4-(4,7-bis(2-(tert-butoxy)-2-oxoethyl)-1,4,7-triazonan-1-yl)-5-(tert-butoxy)-5-oxopentanoic acid (NODAGA-t-Bu; 0.016 g, 29.9 µmol), and Hünig’s base (5.73 µL, 32.9 µmol) were dissolved in dry dichloromethane at 0°C. DIC (4.78 µL, 30.5 µmol) was then added dropwise over 10 min. The reaction was stirred for 12 h and warmed to room temperature as the reaction proceeded. The solvent was removed by rotary evaporation to afford compound 5 as a red solid without further purification. (0.093 g, 87% yield). IR νₘₚₙ (neat): 3323, 2979, 1739, 1680, 1538, 1368, 1224, 1150, 1046 cm⁻¹. MALDI-MS: m/z calculated for C₁₈₄H₁₈₁N₁₅O₆₀, 3562.52; found, 3562.83 [M⁺].

2.2.1.6. Synthesis of Compound 6 (C₆₀-NOTA conjugate)

Compound 5 (0.091 g, 25.5 µmol) was dissolved in about 10 mL of p-dioxane. 12 M HCl (0.833 mL) was added to the solution, and it was stirred for 5 d at room temperature under atmospheric conditions. The reaction mixture separated into
two phases for the first 24 h. Distilled water was added at 48 hours until the 
reaction mixture became homogeneous. After the fifth day, the mixture was dried 
using rotary evaporation, and the product was dissolved in distilled water and 
purified using dialysis centrifuge tubes with a 3500 molecular weight cut-off 
membrane in water (stirred at 3600 rpm for 60 min). The dialysate was collected 
and subjected to additional dialysis using a 2000 molecular weight cut-off 
membrane cartridge in distilled water for two weeks. After drying by lyophilization, 
compound 6 was afforded as an orange solid (7.16 mg, 11% yield). The identity and 
purity of the compound was confirmed by analytical HPLC (retention time = 7.6 
min) and \(^1\)H NMR through confirmation that the acetate protecting groups on the 
serinolamide moieties had been completely hydrolyzed (absence of peak at 2.08 
ppm). IR \(v_{\text{max}}\) (neat): 3268, 3063, 2943, 2880, 1652, 1531, 1460, 1283, 1040 cm\(^{-1}\).

2.2.1.7. Synthesis of C\(_{60}\)-Cu(NOTA)

\(\text{CuCl}_2\) (1.30 mg, 10.0 \(\mu\)mol) was dissolved in 1 mL of 0.1 M NaOAc buffer, 
\(pH=6\), and \(C_{60}\)-(NOTA) (6.40 mg, 2.50 \(\mu\)mol) was added in one portion under 
atmospheric conditions. The reaction mixture was heated to 45 °C, stirred 
overnight, and then purified using preparative HPLC to afford the title compound as 
an orange solid.

2.2.2. Radiochemistry Studies

All solvents and reagents were purchased from commercial sources and used 
as received. Water was deionized using a Milli-Q integral water purification system
(MilliporeSigma, Burlington, MA, USA). $[^{64}\text{Cu}]\text{CuCl}_2$ was produced from a 16 MeV proton/deuteron GE PET trace 10 cyclotron (GE Healthcare, Chicago, IL, USA) using an EDS/PTS solid target station (Comecer S.p.A., Castel Bolognese, Italy) in the Cyclotron Radiochemistry Facility at the MD Anderson Cancer Center. Radioactivity was detected during HPLC using a Bioscan Model 106 detector interfaced with the analytical HPLC through an Agilent Interface 35900E (same analytical and preparative instruments, methods, and columns as described previously).

2.2.2.1. Radiolabeling of $\text{C}_{60}-[^{64}\text{Cu}]\text{Cu}$(NOTA)

Cyclotron-produced $[^{64}\text{Cu}]\text{CuCl}_2$ in 0.1 M HCl (2.69 mCi, 3 µL) was added to 100 µL of 0.1 M NaOAc buffer, pH=5.6. $\text{C}_{60}$-NOTA (80 µg, 31 nmol) in 40 µL of water was added, and the reaction was heated to 37°C for 60 minutes. Reaction completion was confirmed with analytical HPLC. The reaction mixture was loaded onto a PD-10 column (GE Healthcare, Chicago, IL, USA) and eluted with 5 mL of 1x phosphate buffered saline (PBS) in 500 µL fractions. A decay-corrected radiochemical yield of 49 ± 5% was obtained. $\text{C}_{60}-[^{64}\text{Cu}]\text{Cu}$(NOTA) was obtained in 98% purity with a molar activity of 1.35-3.06 GBq/µmol.

2.2.2.2. Radiolabeling of $[^{64}\text{Cu}]\text{Cu}$(NOTA)

Cyclotron-produced $[^{64}\text{Cu}]\text{CuCl}_2$ in 0.1 M HCl (1.027 mCi, 1 µL) was added to 100 µL of 0.1M NaOAc buffer, pH=5.6. NOTA (40 µg, 130 nmol) in 40 µL of water was added, and the reaction was heated to 37°C for 45 minutes. Reaction completion was confirmed with analytical HPLC. The reaction mixture was buffered
with 300 µL of 1x PBS and injected into mice without further purification. [\(^{64}\text{Cu}\)]\text{Cu}(\text{NOTA}) was obtained in >99% purity with a molar activity of 0.268-0.601 GBq/µmol.

### 2.2.2.3. Shelf and Serum Stability Studies

\(C_{60}-[^{64}\text{Cu}]\text{Cu}(\text{NOTA})\) (120 µCi, 200 µL) was incubated with 200 µL of human serum at 37°C. After 20 and 48 hours, the sample was analyzed with analytical HPLC to determine integrity. A shelf stability test with \(C_{60}-[^{64}\text{Cu}]\text{Cu}(\text{NOTA})\) (529 µCi, 500 µL) and \([^{64}\text{Cu}]\text{Cu}(\text{NOTA})\) (235 µCi, 90 µL) in PBS was also conducted at room temperature and at 4 °C. After 24 and 48 hours, the samples were analyzed with analytical HPLC to determine integrity.

### 2.2.3. PET Imaging and Metabolism Studies

PET and CT scans were performed using a small animal Bruker Albira PET/SPECT/CT scanner (Bruker Biospin Corp., Billerica, MA, USA). PET images were analyzed using PMOD Version 3.505 (PMOD Technologies Ltd., Zürich, Switzerland). PET/CT maximum intensity projections were constructed using Inveon Research Workplace (Siemens Medical Solutions USA, Inc., Malvern, PA, USA). Statistical analysis was performed on GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).
2.2.3.1. PET Image Acquisition

All mice were manipulated in accordance to the MD Anderson Cancer Center's IACUC guidelines. Mice were anesthetized with 2% isoflurane using oxygen as a carrier. For both \( \text{C}_60-[^{64}\text{Cu}]\text{Cu(NOTA)} \) and \([^{64}\text{Cu}]\text{Cu(NOTA)}\), six male nude mice were injected on the bed with ~200 µCi of activity in 150 µL of saline through a tail vein catheter. At the start of injection, a 20-minute dynamic PET scan was acquired using the Bruker Albira PET/SPECT/CT scanner with a 15 cm field of view (FOV). The actual injected dose was calculated by measuring the pre- and post-injection activity in the syringe using a CRC-15R dose calibrator (Capintec, Inc., Florham Park, NJ, USA). The data were binned into 20-second time frames for the first 5 minutes, 60-second frames for the next 5 minutes, and 5-minute frames for the next 10 minutes. Static PET images were then recorded at 3 hours and 24 hours post-injection (p.i.) for 10 minutes. A static PET image at 48 hours p.i. was recorded for 20 minutes for \( \text{C}_60-[^{64}\text{Cu}]\text{Cu(NOTA)} \) only. Following all PET scans, two 3-minute CT scans were performed to cover the whole mouse body with a total FOV of 12.6 cm (400 µA, 45 kV, 250 projections).

The PET images were reconstructed using the Maximum Likelihood Expectation Maximization (MLEM) method with 12 iterations. The CT images were reconstructed using the Filtered Back Projection (FBP) method. Scatter, randoms, decay, and attenuation corrections were applied through the Bruker Albira software. Volumes-of-interest (VOIs) were drawn for the major organs on the decay-corrected PET images using PMOD, and injected doses were used to calculate
percent of injected dose per cubic centimeter of tissue (%ID/cc). Errors in the averaged %ID/cc were reported as standard error of the mean (SEM). A non-linear regression analysis of the dynamic PET data for both C$_{60}$-[$^{64}$Cu]Cu(NOTA) and [$^{64}$Cu]Cu(NOTA) was performed and fitted to a two-phase decay model to determine the blood half-life of each compound.

2.2.3.2. Metabolism Stability Studies

Radioactive doses of 179 µCi, 204 µCi, and 205 µCi of C$_{60}$-[$^{64}$Cu]Cu(NOTA) were injected through a tail vein catheter into three nude mice under anesthesia with 2% isoflurane using oxygen as a carrier. The mice remained anesthetized, and blood and urine were collected from each mouse after 20 minutes and put on dry ice to halt metabolism. The urine was injected directly onto the analytical HPLC. The blood was spun down via centrifugation with acetonitrile, and the serum/acetonitrile layer was injected onto the analytical HPLC. Additional urine was collected from anesthetized mice at 24 hours and injected directly onto the analytical HPLC.

2.3. Results and Discussion

2.3.1. Synthesis and Characterization of C$_{60}$-NOTA Conjugate

The structure of a C$_{60}$-serinol derivative amenable to PET imaging was constructed to include a chelate moiety that could be radiolabeled with copper-64 with high radiochemical purity and resistance to demetallation in vivo. The
bifunctional chelate derivative of NOTA (2-(4,7-bis(carboxymethyl)-1,4,7-triazonan-1-ylpentanedioic acid, or NODAGA) was chosen as NOTA has been shown to exhibit the most kinetic stability for copper in radiochemical studies when compared to other chelates.\textsuperscript{55,57,58} The C\textsubscript{60}-NOTA conjugate was synthesized according to the procedure outlined in Experimental Methods Section 2.2 of this chapter and the synthetic scheme is presented in Figure 2.4. Compound 1 was formed using a DIC-mediated amide bond formation reaction between benzyl N-[[4-aminophenyl)methyl]carbamate and ethyl hydrogen malonate in the presence of Hünig’s base. Formation of compound 2 utilized a Bingel-Hirsch reaction with the derivatized malonate to replace one 6-6 double bond on the fullerene with a cyclopropane ring after \textit{in situ} formation of the bromo-malonate. The monoadduct was easily separated from unreacted C\textsubscript{60} and the bisadduct using column chromatography. A subsequent Bingel-Hirsch reaction was then performed with a large excess of the protected serinolamide compared to compound 2 to control the number of groups added to the surface. Slow DBU addition and long reaction times also showed to lead to the formation of higher adduct products. The hexakisadduct (compound 3) showed a favorable formation using these conditions; however, a small amount of pentakisadduct sometimes remained in the final product. The benzyloxy carbamate protecting group was then removed from the malonate linker via hydrogenolysis, and the tert-butyl protected NOTA chelate was added through another DIC-mediated coupling in the presence of Hünig’s base to give compound 5. Finally, a global acid deprotection removed the tert-butyl and acetate protecting groups to give the final C\textsubscript{60}-NOTA conjugate (compound 6).
Figure 2.4 Complete synthesis of the $C_{60}$-NOTA conjugate (compound 6). (i): DIC, Hünig's base, $0 \, ^\circ \text{C} \to \text{RT}, 12 \, \text{h}$. (ii): $C_{60}$, CBr$_4$, DBU, RT, 4.5 h. (iii): N,N'-bis[2-(acetoxy)-1-(acetoxy)methyl]ethyl-malonamide (protected serinolamide malonate), CBr$_4$, DBU, RT, 6 h. (iv): H$_2$, Pd/C, RT, 12 h. (v): NODAGA-t-Bu$_3$, DIC, Hünig's base, $0 \, ^\circ \text{C} \to \text{RT}, 12 \, \text{h}$. (vi): 1 N HCl, dioxane, RT, 4-5 d.

The $C_{60}$-NOTA material (compound 6) was characterized by FTIR spectroscopy to confirm introduction of functional groups to the $C_{60}$ cage. The FTIR spectrum of $C_{60}$-NOTA was distinct from the naked $C_{60}$ and the serinolamide groups (Figure 2.5A). $C_{60}$-NOTA contained peaks at 1410, 1652, 2943, and 3268 cm$^{-1}$
representing the carboxylic acid O-H stretch (from NOTA), the C=C stretch of the C_{60} cage, the N-H stretch (from serinolamide and NOTA linker malonates), and the O-H stretch from the many hydroxyls on the serinolamide groups, respectively. Dynamic light scattering (DLS) was employed to measure the hydrodynamic diameter of the nanostructure in solution (Figure 2.5B). The DLS measurements showed that C_{60}-NOTA conjugate forms uniform aggregates in water with an average diameter of 182 nm and an average polydispersity index of 0.27. These results agreed with the aggregate size that C_{60}-serinol itself has shown to form in aqueous solution (median aggregate size 100-200 nm).\textsuperscript{59} These DLS results were also in good agreement with the aggregate size found in the solid state as determined by atomic force microscopy (AFM) images (Appendix A) which showed the average aggregate size to be 195 nm.
Figure 2.5 Chemical characterization of C$_{60}$-NOTA. (A) FTIR spectra of naked C$_{60}$ (orange), the deprotected serinolamide malonate compound that is conjugated to the C$_{60}$ cage (green), and the C$_{60}$-NOTA conjugate (black). (B) DLS spectrum showing the average hydrodynamic diameter of C$_{60}$-NOTA. (C) HPLC chromatograms of C$_{60}$-NOTA (orange), C$_{60}$-Cu(NOTA) (green), and NOTA alone (black). Peak at 3.1 min is an impurity in the commercial NOTA compound that did not affect radiochemistry.

The C$_{60}$-NOTA conjugate was then labeled with nonradioactive Cu$^{2+}$ to demonstrate the chelating ability of NOTA while appended to the C$_{60}$ cage. Following a literature procedure, the material was labeled with CuCl$_2$ dissolved in an sodium acetate buffer at pH 6, which was then stirred overnight at 45°C. The metallated conjugate, C$_{60}$-Cu(NOTA), the unmetallated conjugate, C$_{60}$-NOTA, and the unmetallated chelate, NOTA, were characterized by HPLC and were found to have...
different retention times (2.8 min for NOTA, 7.0 min for \( \text{C}_{60} \)-Cu(NOTA) and 7.6 min for \( \text{C}_{60} \)-NOTA; **Figure 2.5C**). These results demonstrated that conjugation of NOTA to \( \text{C}_{60} \) was successful and that the chelate successfully bound copper. Furthermore, the HPLC plots show there is no free NOTA within the \( \text{C}_{60} \)-NOTA material, which would have been problematic during the radiolabeling because NOTA and \( \text{C}_{60} \)-NOTA would compete for chelation with copper-64. The \( \zeta \)-potential of \( \text{C}_{60} \)-NOTA and \( \text{C}_{60} \)-Cu(NOTA) were also investigated to determine the surface charge on the conjugate, which can affect the biodistribution of the material.\(^{61}\) The \( \zeta \)-potential of \( \text{C}_{60} \)-NOTA and \( \text{C}_{60} \)-Cu(NOTA) were -22.5 mV and -11.4 mV, respectively, which is similar to \( \text{C}_{60} \)-serinol and other water soluble \( \text{C}_{60} \) materials.\(^{59,62,63}\)

### 2.3.2. Radiolabeling of \( \text{C}_{60} \)-NOTA

The \( \text{C}_{60} \)-NOTA conjugate was radiolabeled with copper-64, and its radiochemical purity and stability in biological media were evaluated. The conjugate was radiolabeled with copper-64 in a sodium acetate buffer (pH 5.6) at 37 °C for 1 h, and the reaction shown in **Figure 2.6A** was tracked by analytical radio-HPLC shown in **Figure 2.6B**. \( \text{C}_{60} \)-[\(^{64}\text{Cu}\)]Cu(NOTA) exhibited a different retention time than [\(^{64}\text{Cu}\)]Cu(NOTA) and free copper-64 on radio-HPLC. After purification, the radio-HPLC chromatograms show that \( \text{C}_{60} \)-[\(^{64}\text{Cu}\)]Cu(NOTA) had a radiochemical purity of 97%. Furthermore, the metal ion stability of \( \text{C}_{60} \)-[\(^{64}\text{Cu}\)]Cu(NOTA) was challenged in PBS for 24 and 48 h at 25 °C and human serum for 20 and 48 h at 37 °C (stability studies at 48 h shown in **Appendix A**). If the copper demetallated from \( \text{C}_{60} \)-[\(^{64}\text{Cu}\)]Cu(NOTA) in vivo, PET would not be able to distinguish between the two
radioactive species. In all cases, C$_{60}$-$[^{64}\text{Cu}]$Cu(NOTA) proved to be stable with 93% and 95% retention of copper after the shelf stability challenge in PBS for 24 h and 48 h, respectively. In addition, there was 91% and 89% retention of copper after the stability challenge in human serum for 20 h and 48 h, respectively. These results demonstrated that copper-64 is stable in the NOTA chelate C$_{60}$-$[^{64}\text{Cu}]$Cu(NOTA) under simulated biological conditions, which was important to establish prior to in vivo biodistribution experiments. It is important to note that the Cu(NOTA) complex is both thermodynamically stable (logK = 23.33)$^{64}$ and kinetically stable (i.e. the rate at which Cu$^{2+}$ ion disassociates from the NOTA ligand is very low). Typically, kinetic stability is more relevant for biological stability, as it is a measure that Cu(NOTA) does not readily undergo trans- or demetallation over time.$^{65}$ Furthermore, HPLC data confirmed the stability of the amide linkages found within the structure under these biological-challenge conditions.
Figure 2.6 Radiolabeling of C₆₀-NOTA. (A) Radiolabeling reaction scheme. Copper-64 was dissolved in an acetate buffer prior to reacting with C₆₀-NOTA. (B) Radio-HPLC chromatograms of [⁶⁴Cu]CuCl₂ (black), [⁶⁴Cu]Cu(NOTA) (blue), and C₆₀-[⁶⁴Cu]Cu(NOTA) (green), C₆₀-[⁶⁴Cu]Cu(NOTA) challenged against PBS at 25 °C for 24 h (orange), C₆₀-[⁶⁴Cu]Cu(NOTA) challenged against human serum for 20 h (gray).

2.3.3. PET Imaging of C₆₀-[⁶⁴Cu]Cu(NOTA)

C₆₀-[⁶⁴Cu]Cu(NOTA) was then administered by tail-vein injection to mice (~200 µCi in 150 µL of saline) and were imaged using PET/CT. First, a continuous, 20-min dynamic scan was acquired for each animal, followed by static scans that were taken at 3, 24, and 48 h p.i. (Figure 2.7). Representative PET images (Figure
2.7A) and the time activity curve (TAC) of the dynamic scan (Figure 2.7B) show that \( \text{C}_{60}-[^{64}\text{Cu}]\text{Cu(NOTA)} \) spiked in tissue accumulation as it traveled through the blood and then decreased rapidly in all organs except the kidneys. This suggests a renal clearance mechanism, which is most likely due to the very hydrophilic nature of the serinolamide groups of \( \text{C}_{60}-[^{64}\text{Cu}]\text{Cu(NOTA)} \). Specifically, it has been previously described in a review by Aggarwal, et al. that protein aggregation on the surface of nanoparticles tends to increase with the more hydrophobic surface exposed.\(^{66}\) This process, called opsonization, can lead to macrophage uptake and excretion through organs in the reticuloendothelial system (RES) such as the liver and spleen. Therefore, coating the hydrophobic surface of \( \text{C}_{60} \) with hydrophilic serinolamide groups could have prevented formation of this protein corona and nanoparticle aggregation and allowed for \( \text{C}_{60}-[^{64}\text{Cu}]\text{Cu(NOTA)} \) to be excreted through the kidneys as single particles.

The conjugate material cleared rapidly over the first 6 min p.i. from the heart, liver, and lungs after traveling through those organs, while showing negligible uptake in the brain and muscle. At 20 min p.i., the kidneys and bladder still had the highest uptake of \( \text{C}_{60}-[^{64}\text{Cu}]\text{Cu(NOTA)} \), as seen in the PET image for that time point. The amount of radioactivity significantly decreased at 3 and 24 h p.i. for all organs, and there was negligible uptake for any organ at 48 h, indicating the material had effectively cleared from the mice (Figure 2.7C). These biodistribution data resemble trends seen for X-ray contrast agents functionalized with serinolamide groups including the rapid renal clearance.\(^{46}\)
Figure 2.7 PET images and biodistribution of C₆₀⁻[⁶⁴Cu]Cu(NOTA). (A) Whole-body dynamic and static PET images acquired at various time points post-injection (p.i.). Organs labeled are brain (B), heart (H), kidney (K), and bladder (BL). (B) Time activity curve (TAC) of 20 min dynamic scan showing initial biodistribution and rapid drop-off in uptake in most organs. (C) Quantification of radioactivity in VOIs showing accumulation in various organs with a close-up view of the graph to highlight lower accumulation. Data represent mean ± SEM, n = 6.

Because PET detects signal from the radioactive copper-64 ion and not from the fullerene compound itself, it was important to determine whether or not the conjugate was releasing copper in vivo. To answer this question, blood and urine samples from mice were collected at 20 min p.i. and analyzed using radio-HPLC to determine the integrity of the conjugate material (Figure 2.8). These data indicated
that the conjugate is highly stable in vivo with 97% and 92% retention of copper for urine and blood, respectively. Furthermore, there was no evidence that any $[^{64}\text{Cu}]\text{Cu}(\text{NOTA})$ was cleaved from the fullerene conjugate, which suggested the material was excreted without significant modification.

![Graph showing metabolites](image)

**Figure 2.8** Metabolism analysis of $C_{60}[^{64}\text{Cu}]\text{Cu}(\text{NOTA})$ from urine and blood samples of mice. Radio-HPLC chromatograms of $[^{64}\text{Cu}]\text{CuCl}_2$ (black), $[^{64}\text{Cu}]\text{Cu}(\text{NOTA})$ (blue), and $C_{60}[^{64}\text{Cu}]\text{Cu}(\text{NOTA})$ (green), mouse urine sample at 20 min p.i. (orange), mouse blood sample at 20 min p.i. (gray).

This rapid, complete clearance of the $C_{60}[^{64}\text{Cu}]\text{Cu}(\text{NOTA})$ conjugate is unusual for a nanoparticle, as they typically collect in the liver, spleen, and lungs.\textsuperscript{14} However, exceptionally fast clearance might be advantageous when dealing with a targeted anticancer drug carrier. Since only a small fraction of injected dose of
traditional chemotherapeutic agents localizes in the tumor, having a drug delivery platform that can be targeted to the tumor yet be cleared rapidly from healthy organs could potentially mitigate some of the common side-effects of chemotherapy. For example, Wilson and colleagues, among others have shown that carbon nanotubes typically localize to the lungs, liver, and spleen. Silica or gold nanoparticles functionalized using PEG for water solubility tend to have longer blood circulation times, often showing retention in the blood for 24 h p.i., as well as uptake in RES organs. This is commonplace for PEGylated nanostructures, and more rapidly-clearing nanostructures may be beneficial, which would be less likely to cause harmful side effects due to long residence times in vivo. Unlike these examples, serinolamide-functionalized C_{60}-[^{64}\text{Cu}]\text{Cu}(\text{NOTA}) did not collect in the lungs or liver for any significant period of time, which helps mitigate any long-term toxicity concerns.

While PEG is usually employed to increase the circulation half-life of a material, Peng and coworkers recently reported a fast-clearing PEGylated C_{60} derivative. They found that a PEGylated, radiolabeled (by ^{64}\text{Cu} using NOTA as a chelator) C_{60} material of similar size (2-3 nm diameter) and surface charge as C_{60}-[^{64}\text{Cu}]\text{Cu}(\text{NOTA}) was cleared from all major organs by 24 h p.i. While this biodistribution data reported by these authors at later time points generally agree with the present results here, conducting a dynamic PET scan after administration of C_{60}-[^{64}\text{Cu}]\text{Cu}(\text{NOTA}) allowed for a more thorough study of the biodistribution profile, which was especially important when evaluating a material that clears so quickly. In addition, the authors examined the toxicity of their material and found
that the compound was not cytotoxic to U87MG cancer cells at 100 µg/mL exposure, and it was previously shown that C$_{60}$-serinol is likewise non-cytotoxic to cancer cells at the same concentration.$^{48}$

### 2.3.4. PET Imaging of $[^{64}\text{Cu}]\text{Cu}(\text{NOTA})$

With the biodistribution of the C$_{60}$-$[^{64}\text{Cu}]\text{Cu}(\text{NOTA})$ conjugate well characterized, it was of interest to further evaluate C$_{60}$-serinol’s potential as a drug delivery platform. To improve the efficiency of cancer drug delivery and to reduce side effects for healthy organs, a good drug delivery vehicle should have a predictable biodistribution that is relatively independent of the therapeutic cargo. Comparing present results for C$_{60}$-$[^{64}\text{Cu}]\text{Cu}(\text{NOTA})$ to previous work with a fluorescently-labeled PromoFluor 633 (PF) C$_{60}$-serinol conjugate (C$_{60}$-serinol-PF; Figure 2.9),$^{48}$ dramatically different profiles were found even though both nanomaterials are derivatives of C$_{60}$-serinol. C$_{60}$-serinol-PF was predominately retained in the tumor, kidneys, liver, and brain in a liver cancer mouse model (Hep3B) for longer than 100 h p.i. Although no tumor model was used in the present study, the C$_{60}$-$[^{64}\text{Cu}]\text{Cu}(\text{NOTA})$ conjugate showed very little uptake in the liver at 24 h p.i. and essentially no uptake in the brain. These strikingly different biodistribution profiles beg the question as to why these two C$_{60}$-serinol-based materials behave so differently $\textit{in vivo}$. Furthermore, controls for the effects of derivatization of various nanomaterials are often lacking in the literature,$^{72}$ and unfortunately, a control experiment examining the biodistribution of the PF fluorophore alone is also not available. However, a control experiment was included
in the present study by examining the biodistribution of $^{64}$CuCu(NOTA) under the same conditions and at the same time points used to characterize C$_{60}$-$^{64}$CuCu(NOTA) (Figure 2.10).

Figure 2.9 Structure of the fluorescently-labeled C$_{60}$-serinol-PF conjugate. Figure adapted with permission from Raoof, et al.$^{48}$
Figure 2.10 PET images and biodistribution of $[^{64}\text{Cu}]\text{Cu}($NOTA). (A) Whole-body dynamic and static PET images acquired at various time points post-injection (p.i.). Organs labeled are brain (B), heart (H), kidney (K), and bladder (BL). White arrows for 3 and 24 h p.i. images highlight a breathing pad that was placed under the mice during imaging. The radioactivity at 48 h p.i. was so low it could not be imaged and quantified accurately. (B) Time activity curve (TAC) of a 20 min dynamic scan showing initial biodistribution and rapid drop-off in uptake in most organs. (C) Quantification of radioactivity in VOIs showing accumulation in various organs with a close-up view of the graph to highlight lower accumulation. Data represent mean $\pm$ SEM, n = 6.

These studies showed that $[^{64}\text{Cu}]\text{Cu}($NOTA) has a very similar biodistribution profile to the C$_{60}$-$[^{64}\text{Cu}]\text{Cu}($NOTA) conjugate with significant uptake in the kidneys at 20 min p.i. compared to other major organs at the same time point. However, accumulation decreased rapidly at 3 and 24 h p.i. Although mice were imaged at 48
h p.i., the detected signal was negligible, so these data are not shown. This evidence indicated that most of the material was cleared by renal excretion between 20 min and 3 h p.i. These data are supported by previous biodistribution studies of other small-molecule, macrocyclic copper chelates of similar structure, such as DOTA and TETA, which were also found to be almost completely cleared within 24 h.\textsuperscript{73}

A non-linear regression analysis of the heart TAC data was also performed using a two-phase decay model, which gave a p value of $< 0.0001$ when compared to the one-phase model through an extra sum-of-squares F test. The two-phase decay model consists of a distribution phase and an elimination phase. The fast distribution phase describes the rapid circulation of the material from the plasma to highly-perfused tissues, and the slow elimination phase describes the material being cleared from the plasma and tissues through excretion. From this analysis, a blood half-life ($t_{1/2}$) and rate constant ($\alpha$, $\beta$) for each phase were determined along with the clearance (CL) and the area under the curve (AUC) (Table 2.1).
<table>
<thead>
<tr>
<th>Material</th>
<th>Distribution t_{1/2} [min]</th>
<th>Distribution rate constant ((\alpha)) [min^{-1}]</th>
<th>Elimination t_{1/2} [min]</th>
<th>Elimination rate constant ((\beta)) [min^{-1}]</th>
<th>Clearance [cc/min]</th>
<th>Area under curve [%ID-min/(\text{cc})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{64}\text{Cu})Cu(^{60}) (NOTA)</td>
<td>0.6436</td>
<td>1.077</td>
<td>7.078</td>
<td>0.09793</td>
<td>3.161 x 10^{-5}</td>
<td>316.4</td>
</tr>
<tr>
<td>(^{64}\text{Cu})Cu (NOTA)</td>
<td>0.4975</td>
<td>1.393</td>
<td>8.153</td>
<td>0.08501</td>
<td>3.152 x 10^{-5}</td>
<td>317.3</td>
</tr>
</tbody>
</table>

Two-phase decay model: \(r^2 = 0.996\) for both data sets

**Table 2.1 Pharmacokinetic Parameters for \(^{60}\text{C}_{60}\)-\(^{64}\text{Cu}\)Cu(NOTA) and \(^{64}\text{Cu}\)Cu(NOTA)**

From the blood half-life data, it is evident that clearance characteristics of \(^{60}\text{C}_{60}\)-\(^{64}\text{Cu}\)Cu(NOTA) and the \(^{64}\text{Cu}\)Cu(NOTA) imaging agent control are very similar. While the appended imaging agent may be slightly influencing the pharmacokinetics of \(^{60}\text{C}_{60}\)-\(^{64}\text{Cu}\)Cu(NOTA), both \(^{60}\text{C}_{60}\)-\(^{64}\text{Cu}\)Cu(NOTA) and \(^{64}\text{Cu}\)Cu(NOTA) behaved as small molecules *in vivo*. \(^{60}\text{C}_{60}\)-\(^{64}\text{Cu}\)Cu(NOTA) clears much more quickly from the blood, with t_{1/2} values of 0.6436 and 7.078 min for the respective distribution and elimination half-lives, compared to reported \(^{60}\text{C}_{60}\)-drug conjugates with similar hydrodynamic diameters and \(\xi\)-potentials with t_{1/2} values between 200-500 min.\(^{74,75}\) These \(^{60}\text{C}_{60}\)-drug conjugates bound to monomethyl fumarate and tamoxifen were both made water soluble with four units of PEG (tetraethylene glycol). Another
study reported an even longer $t_{1/2}$ (1.8 x $10^4$ min), which is likely due to the hydrophobic nature of the conjugate, as it was just derivatized with the hydrophobic drug docetaxel.\textsuperscript{76} This biodistribution pattern of [$^{64}$Cu]Cu(NOTA) is similar to that of the C$_{60}$-$[^{64}$Cu]Cu(NOTA) conjugate, yet it is quite distinguished from that of the fluorescently-labeled C$_{60}$-serinol-PF nanostructure, demonstrating that differences in an appended imaging agent can dramatically affect the biodistribution profile. The biodistribution data for C$_{60}$-serinol-PF also showed significant uptake in the heart at greater than 100 h p.i., indicating that the material was still circulating in the blood as a blood-pool agent. In fact, a recent study of C$_{60}$-serinol-PF biotransport kinetics reported that the material does not leak from normal vasculature as it does in tumor vasculature,\textsuperscript{59} leading to the conclusion that the PF fluorophore converts the C$_{60}$-serinol platform into a blood pool agent.

As is the case with any nanomaterial, surface chemistry is known to play an important role in the \textit{in vivo} behavior of the material.\textsuperscript{77} For example, Rajagopalan \textit{et al.} examined a monosubstituted, water-soluble derivative of C$_{60}$ fullerene and concluded that the material did not clear through the kidneys, because urine samples collected 24 h after administration did not contain detectable amounts of the material.\textsuperscript{78} They also found that the material possessed a greater affinity for tissue over plasma proteins. These results, while very different from the data presented here for C$_{60}$-$[^{64}$Cu]Cu(NOTA), are not surprising because the largely-exposed hydrophobic fullerene surface undoubtedly encouraged binding of hydrophobic regions of proteins or lipophilic tissue. Conversely, we have shown
here that a different highly hydrophilic C$_{60}$ material can be cleared by the kidneys in much less than 24 h.

Other studies have reported that C$_{60}$ fullerenol (hydroxylated fullerene) derivatives localize to the liver, lung, bone, muscle, and spleen at 30 h p.i. with slow clearance from the body in mice.$^{40,79,80}$ Such C$_{60}$ fullerenols with their slow clearance profiles and uptake by the liver and spleen provide evidence that certain surface functionalization can result in very long residence times for C$_{60}$ material in mice, which could possibly lead to negative long-term effects. In addition, C$_{60}$ fullerenol derivatization procedures are difficult to control and produce final products that are heterogeneous in nature. Conversely, the serinolamide malonate functionalization procedure used in this work to produce C$_{60}$-serinol and C$_{60}$-[$^{64}$Cu]Cu(NOTA) is predictable and easily controlled by varying the substituents on the malonate groups, as well as the number of malonates themselves.

Yet other C$_{60}$ materials decorated with a few carboxyl groups have been found to localize mainly in the liver, muscle, bone, and spleen at late time points following administration to mice, further demonstrating that a highly hydrophobic C$_{60}$ surface produces longer circulation times and retention in vivo.$^{81,82}$ While we have shown that hydrophilic fullerene derivatives encourage rapid renal clearance, leaving some lipophilic character on the C$_{60}$ surface can result in the penetration of certain restrictive membranes, such as the blood-brain-barrier, as was recently reported by Dugan and coworkers when administering $^{14}$C-labeled e,e,e-methanofullerene(60)-63-tris malonic acid (C$_3$).$^{83}$ C$_3$ showed significant liver and
kidney uptake at 12 and 24 h p.i., which resulted in fecal excretion as an additional and key route of clearance, unlike the $C_{60}^{[64}\text{Cu}]\text{Cu}(\text{NOTA})$ conjugate of this work.

2.4. Summary and Conclusion

Herein is presented the synthesis, characterization, radiolabeling, and PET imaging of a highly water-soluble $C_{60}$ derivative, based on a $C_{60}$-serinol platform. The $C_{60}$-NOTA conjugate was synthesized using Bingel-Hirsch chemistry with five malonate groups loaded with serinolamide moieties and was characterized by FTIR, DLS, $\xi$-potential, and HPLC. The $C_{60}^{[64}\text{Cu}]\text{Cu}(\text{NOTA})$ conjugate exhibited high radiochemical purity and survived challenge for thermodynamic and kinetic stability under biological conditions up to 48 h. The material was cleared rapidly from mice via the kidneys as evidenced by dynamic and static PET imaging and VOI analysis and exhibited almost total clearance by 48 h p.i. Furthermore, it was demonstrated by comparing the biodistribution and pharmacokinetic parameters of $C_{60}^{[64}\text{Cu}]\text{Cu}(\text{NOTA})$ and $^{[64}\text{Cu}]\text{Cu}(\text{NOTA})$ that $C_{60}^{[64}\text{Cu}]\text{Cu}(\text{NOTA})$ exhibits small-molecule behavior $\textit{in vivo}$. This work also presented a good control study for the imaging agent when conducting biodistribution studies on $C_{60}$ derivatives, which is important when designing treatment and functionalization strategies for fullerene-based drug formulations.
Chapter 3

Boron Nitride Nanotubes Loaded with Gd(III) Chelates for Magnetic Resonance Imaging Applications

3.1. Introduction and Background

3.1.1. Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) is one of the most common medical imaging modalities used in the clinic today, providing noninvasive anatomical information with ultra-high resolution. An MR image is obtained when water protons in the subject are aligned in parallel with an external magnetic field (along the z-axis), which is known as longitudinal magnetization. Radiofrequency (RF) pulses are emitted to disturb the alignment of the protons causing some protons to be no longer aligned with the external magnetic field, which decreases longitudinal magnetization and creates what is called transverse magnetization in the x-y plane.
The time constant for the relaxation time it takes for the longitudinal magnetization to return to its original value is defined as $T_1$, and the time constant for the time it takes for the net transverse magnetization to disappear is defined as $T_2$ (see Figure 3.1). The rates at which these respective relaxation phenomena occur are $R_1$ and $R_2$.\textsuperscript{85} Paramagnetic materials that can interact with nearby protons and cause them to relax faster, enhancing the contrast and sensitivity of the MR image are known as contrast agents (CAs) and have been used in standard clinical practice for the past 30 years. MRI CAs typically utilize iron or gadolinium ions and the CA performance is characterized by its relaxivity ($r$), which is the rate at which the CA relaxes the system and is defined per paramagnetic ion in units of mM$^{-1}$s$^{-1}$. 

![Diagram](https://example.com/diagram.png)
Figure 3.1 MRI proton relaxation process. (A) Protons are aligned with the magnetic field (longitudinal magnetization). (B) Protons move into the xy-plane after an RF pulse (transverse magnetization). (C) Longitudinal relaxation begins after the RF pulse is switched off (T₁ relaxation). (D) Protons from the xy-plane realign with the magnetic field along the z-axis. Adapted with permission from Currie et al.\textsuperscript{85}

In the medical imaging field, T₁ image-brightening is employed more often than T₂ image-darkening, making T₁-weighted imaging the preferred method for intravenously (i.v.)-injected contrast studies.\textsuperscript{85} The T₁ relaxation rate (R₁) depends on the tumbling rate of the CA; this generates a fluctuating magnetic field that surrounding protons experience, which reduces T₁. Gd(III) and Fe(III) ions are often used in CAs, with Gd typically used for T₁-weighted images and Fe typically used for T₂-weighted images.\textsuperscript{86,87} The more paramagnetic ions in a CA, the faster the agent can relax protons, yielding higher relaxivities per agent and more efficient CAs.\textsuperscript{85,88,89} Relaxivities are highly field dependent and efficacy of T₁-weighted CAs decreases with higher field strength, whereas the efficacy of T₂-weighted CAs increases with higher field strength. Clinical MRI CAs currently used are various Gd\textsuperscript{3+} ion polyaminocarboxylic acid chelates, with r₁ values around 3-4 mM\textsuperscript{-1}s\textsuperscript{-1} at 1.5 T magnetic field strength.\textsuperscript{90}

3.1.2. Gadonanotubes as High-Performance MRI CAs

For many years, carbon nanotubes (CNTs) have been studied extensively for biomedical imaging and therapeutic applications.\textsuperscript{21,22} In 2005, Wilson and colleagues discovered a material that dramatically enhances MRI using a CNT
platform. They found that ultra-short single-walled carbon nanotubes (20-80 nm in length, ~ 1 nm in diameter; dubbed US-tubes) loaded Gd$^{3+}$-ion clusters exhibited higher relaxivity compared to commercial CAs by almost two orders of magnitude (150 mM$^{-1}$s$^{-1}$), rendering the material the world’s most effective MRI CA.\textsuperscript{91,92} Called Gadonanotubes (GNTs), this material can be wrapped with a nonionic surfactant, used as a scaffold for \textit{in situ} polymerization,\textsuperscript{93} or covalently functionalized\textsuperscript{94} to render the material dispersible in aqueous and biological media and is extremely well tolerated by cells and mice alike (Figure 3.2).\textsuperscript{68,95} The unique GNT properties – particularly the defect sites in the US-tubes where the ion clusters form – stem from a culmination of many beneficial factors within the particle to create a system that has yet to be improved on a per Gd$^{3+}$ ion basis.\textsuperscript{96,97}

Figure 3.2 Gadonanotubes (GNTs) as MRI CAs. (A) Skematic of GNTs showing Gd$^{3+}$-ion clusters within defect sites on CNTs. (B) High-resolution TEM image of Gd$^{3+}$-ion clusters within US-tubes. (C) TEM image of a GNT-labeled stem cell.
Red arrows point to GNT aggregates in the cell cytoplasm. (D) T₁-weighted MR images at 1.5 T and 25 °C of (left to right) unlabeled stem cells, Gd-DTPA (diethylenetriaminepentaacetic dianhydride)-labeled stem cells, and GNT-labeled stem cells. Figure reproduced with permission from Hernandez-Rivera et al.²¹

Using the GNTs as a model nanomaterial for enhancing MRI contrast, it was of interest to learn how the GNT properties could be applied to another nanotube system of recent interest, boron nitride nanotubes (BNNTs). In order to generate shorter BNNTs with defect sites, a chemical cutting process reported for CNTs was attempted.⁹⁸ However, that procedure was not feasible, because BNNTs are too structurally different from CNTs for this to be accomplished under the same conditions that US-tubes were produced. Therefore, full-length, multi-walled BNNTs were employed to create a new counterpart to the high-performing GNTs to explore the possibilities of BNNT-based MRI CAs.

3.1.3. Boron Nitride Morphologies

Boron nitride (BN) is an inorganic material with different amorphous and crystalline motifs. Various forms of BN include cubic BN (c-BN), hexagonal BN (h-BN), and boron nitride nanotubes (BNNTs). Hexagonal-BN is the structural equivalent to graphite and it consists of flat, monoatomic sheets of alternating, sp²-hybridized boron and nitrogen atoms arranged in a hexagonal lattice.⁹⁹,¹⁰⁰ Because B-N bonds are more polar than C-C bonds, h-BN sheets are held together tightly by dipole-dipole and pi-pi stacking interactions, where the B and N atoms from adjacent sheets directly line up in what is known as “lip-lip” interactions; this is in
contrast with graphene sheets in graphite that are held together by pi-pi stacking alone.\textsuperscript{101,102} These intermolecular forces combined with the high surface area make h-BN the most stable of the BN morphologies. This causes exfoliation of single h-BN sheets to be much more difficult to achieve than exfoliation of graphene sheets.\textsuperscript{101}

Like CNTs, BNNTs can be considered to be rolled-up cylindrical sheets of h-BN. Although they were first predicted in 1994 and synthesized in 1995,\textsuperscript{103,104} BNNTs are not as well-studied as the other BN motifs. BNNTs can be produced by arc-discharge (usually with the use of a metal catalyst),\textsuperscript{104} laser ablation,\textsuperscript{105} chemical vapor deposition,\textsuperscript{106} and ball-milling/annealing methods,\textsuperscript{107,108} among others.\textsuperscript{109} BNNTs can also be made using a CNT substitution reaction, using the carbon nanotube as a template, where CNTs and boron oxide react under a nitrogen atmosphere at high temperatures.\textsuperscript{110} The BNNT material studied in this work was produced using a pressurized vapor/condenser (PVC) method, which involves condensation of seed boron particles in a plume of pure boron vapor at high temperature (\ (> 4000 °C) combined with nitrogen gas at elevated pressures.\textsuperscript{111}

Although some studies on single-walled BNNTs exist,\textsuperscript{112–115} multi-walled BNNTs are the much more common morphology, due to stronger interactions between concentric nanotubes. The axis about which the h-BN sheet is rolled determines the chirality of the BNNT. The three most common morphologies observed in CNTs are also observed in BNNTs: the armchair morphology, in which the B-N bonds are perpendicular to the tube axis; the zig-zag morphology, in which the B-N bonds are parallel to the tube axis; and the chiral morphology, in which the
B-N bonds lie at an angle to the tube axis (Figure 3.3). Unlike CNTs, however, the zig-zag conformation is the most common for BNNTs, whereas all CNT helicities are equally probable.\textsuperscript{116,117} Furthermore, BNNT properties are far less dependent on their chirality than are CNT properties.

![Figure 3.3 BNNT nanotube chiralities. From top to bottom: Armchair, Zig-zag, and Chiral morphologies. Figure adapted with permission from Zhi et al.\textsuperscript{118}](image)

### 3.1.4. Properties of BNNTs

Multi-walled BNNTs are light weight and usually range in diameter from 10-50 nm, with lengths ranging from 100-200 \( \mu \text{m} \).\textsuperscript{111,119} BNNTs produced by the PVC method contain only a few walls, with two to five walls being most common, resulting in smaller outer diameters for the tubes (5-8 nm). A comparison of some
general properties of CNTs and BNNTs are summarized in **Table 3.1.** While the mechanical properties of CNTs and BNNTs are quite similar,\textsuperscript{120-122} BNNTs have a number of interesting properties that are very different from those of CNTs. CNTs can be metallic or semiconducting, whereas all BNNTs are electrical insulating, with a uniform wide band gap.\textsuperscript{123} This band gap can also be tuned by doping with various elements, such as magnesium and molybdenum,\textsuperscript{124} fluorine,\textsuperscript{125,126} and carbon\textsuperscript{127} to produce n-type or p-type semiconducting BNNTs. Additionally, BNNTs have much higher resistance to thermal oxidation: CNTs oxidize around 450-500 °C, while BNNTs do not oxidize until 950-1000 °C.\textsuperscript{128,129} BNNTs are also very chemically inert, making chemical oxidation and covalent functionalization difficult.

<table>
<thead>
<tr>
<th>Property</th>
<th>CNTs</th>
<th>BNNTs</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common morphologies (diameters)</td>
<td>Single-walled (1-2 nm)</td>
<td>Multi-walled (10-50 nm)</td>
<td>Odom\textsuperscript{130} Smith\textsuperscript{111}</td>
</tr>
<tr>
<td>Tensile strength</td>
<td>11-63 GPa</td>
<td>Up to 33 GPa</td>
<td>Yu\textsuperscript{122} Chen\textsuperscript{120}</td>
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<tr>
<td>Electrical conductivity</td>
<td>Metallic/Semiconducting</td>
<td>Insulating</td>
<td>Odom\textsuperscript{130} Blase\textsuperscript{123}</td>
</tr>
<tr>
<td>Electronic band gap</td>
<td>0-2 eV</td>
<td>5.5-6 eV</td>
<td>Popov\textsuperscript{131} Blase\textsuperscript{123}</td>
</tr>
<tr>
<td>Oxidation temperatures</td>
<td>450-500 °C</td>
<td>950-1000 °C</td>
<td>Datsyuk\textsuperscript{132} Chen\textsuperscript{129}</td>
</tr>
<tr>
<td>------------------------</td>
<td>------------</td>
<td>-------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Thermal conductivity</td>
<td>$\sim 3500 \text{ Wm}^{-1}\text{K}^{-1}$</td>
<td>$\sim 360 \text{ Wm}^{-1}\text{K}^{-1}$</td>
<td>Popov\textsuperscript{131} Chang\textsuperscript{133}</td>
</tr>
</tbody>
</table>

**Table 3.1 Properties of Carbon and Boron Nitride Nanotubes**

### 3.1.5. Functionalization and Applications of BNNTs

The methods to functionalize BNNTs are similar to those of CNTs,\textsuperscript{134–137} including non-covalent modification, where the nanotubes are wrapped with an amphiphilic polymer or surfactant that coats the hydrophobic nanotube surface, and chemical functionalization, where different moieties are covalently attached to the nanotube surface.\textsuperscript{138} Both techniques can be employed to tailor the material to a specific application. For non-covalent modification, the van der Waals forces that hold BNNTs and coatings together are most often pi-pi stacking, ion/dipole, or hydrophobic interactions. Such modification has been reported using different polymers,\textsuperscript{139} such as DNA,\textsuperscript{140} PEGylated phospholipids,\textsuperscript{141} and many others.\textsuperscript{142} For covalent functionalization, BNNTs have been modified using radical polymerization,\textsuperscript{143} oxygen and nitrogen plasmas,\textsuperscript{144} diazonium salt hydrolysis,\textsuperscript{145} and Billups-Birch reaction conditions\textsuperscript{146} to attach various substituents to the nanotube surface. Other groups have achieved BNNT oxidation with hydrogen peroxide at high temperatures and pressures\textsuperscript{147} and by sonication with nitric acid followed by silanization.\textsuperscript{148}
Whether or not modification was performed, BNNTs have been used for a variety of applications. For example, BNNTs have been used as polymer and metal matrix composites,\textsuperscript{149,150} as superhydrophobic surfaces,\textsuperscript{151} as components in water purification from oil and salt,\textsuperscript{152,153} and as hydrogen storage materials.\textsuperscript{154,155} Researchers have also been exploring how to load BNNTs with different metals to produce BNNTs for electronic\textsuperscript{156-159} and luminescent\textsuperscript{160} applications on both theoretical and experimental grounds. In one instance, Golberg and coworkers extensively studied metal-filled BNNTs by first loading multi-walled CNTs with a metal, followed by the C to BN conversion process previously described.\textsuperscript{161} However, each of these studies rely on loading metals into the BNNTs at the tube-growth stage in which the nanotubes were produced in the presence of the dopant metal. There are few reports of loading metal ions or metal complexes directly into BNNTs, which the work detailed in this chapter explores by analyzing the metal chelate loading capacity of BNNTs, specifically for biomedical applications.

In addition to the above-mentioned applications, BNNTs have also been studied for applications in biomedicine,\textsuperscript{162} specifically as drug and gene delivery agents,\textsuperscript{163,164} boron neutron capture agents,\textsuperscript{165,166} and as imaging contrast agents.\textsuperscript{167-170} Biocompatible coatings for BNNTs include non-covalent suspensions using amphipathic dendrimers,\textsuperscript{171} glycol-chitosan (GC),\textsuperscript{172} poly-L-lysine (PLL),\textsuperscript{173} and many others.\textsuperscript{174} The PLL-BNNT material was found to internalize into cells and accumulate in vesicles after 12-19 h of incubation, and no signs of toxicity were observed up to 20 \( \mu \)g/mL.\textsuperscript{173} The GC-BNNT material was shown to be cytocompatible at concentrations up to 50 \( \mu \)g/mL, and found signs of early
apoptosis and production of reactive oxygen species only at the 100 µg/mL in the case of glycol-chitosan. BNNT materials have also been investigated in vivo, for preliminary toxicological and biodistribution studies, showing no adverse effects, although follow-up studies are required to confirm these findings. However, it is important to note that the BNNT material stability, cellular internalization, localization, and toxicity are often coating-dependent, and the biocompatibility of the whole material must be considered. While this area of BNNT research is still developing, there is a possibility of translating BNNT-based nanomaterials into the clinic. However, it is important to evaluate this potential on all fronts in order to determine a niche for BNNTs in imaging, therapeutic, or other biomedical applications.

### 3.1.6. MRI Applications of BNNTs

Returning to the discussion on MRI CA nanomaterials, there have been relatively few reports on BNNTs for MRI applications. Calucci and coworkers investigated the relaxivity of BNNTs made from a ball milling and annealing procedure that contained ~1.5 wt% metal impurities, specifically iron and chromium. These nanotubes were wrapped in PLL and when the relaxivities of the materials were measured at 1.5 T, the authors found $r_1$ and $r_2$ to be 31 and 270 mM$^{-1}$s$^{-1}$, respectively. They also measured the relaxivities at 7.05 T and, later, at 3 T, observing a decrease in $r_1$ and an increase in $r_2$, as is typical for CAs under increasing magnetic field strengths. Ciofani et al. have also loaded Gd$^{3+}$ ions into oxidized BNNTs to use as MRI CAs, and demonstrated that hydroxyl groups on the
oxidized nanotubes bind free Gd$^{3+}$ ions to a loading capacity of 0.4 wt%.$^{168}$ They measured the MR contrast ability of their material at 7 T, and confirmed its biocompatibility \textit{in vitro} with human SH-SY5Y neuroblastoma cells 72 h after treatment with up to 100 µg/mL of material.

Metal chelates can also be used to load Gd$^{3+}$ ions into nanotubes, as was demonstrated with carbon US-tubes by Law \textit{et al.}$^{176}$ The authors of this recent study found that using Gd(acac)$_3$:2H$_2$O afforded the material much higher relaxivity (103 mM$^{-1}$s$^{-1}$) than commercial products and Gd(acac)$_3$:2H$_2$O alone. It was hypothesized that Gd chelates could similarly be loaded into BNNTs and hold more Gd than what has been reported above for oxidized BNNTs. Therefore, this work presents the loading, characterization, and T$_1$-weighted MRI studies at 1.5 T of Gd(acac)$_3$:2H$_2$O within BNNTs. Reported also is a new technique to digest extremely chemically inert BNNTs, employing an antique wet chemical method, for accurate metal quantification using inductively-coupled plasma mass spectrometry (ICP-MS).

\textbf{3.2. Experimental Methods}

\textbf{3.2.1. Instrumentation}

Fourier-transform infrared (FTIR) spectroscopy was performed using a Nicolet 6700 FTIR spectrometer with an attenuated total reflectance (ATR) attachment (Thermo Fisher Scientific, Waltham, MA, USA). Thermogravimetric analysis (TGA) was achieved using a Q-600 Simultaneous TGA/DSC (TA Instruments, New Castle, DE, USA). Elemental analysis was performed with X-ray
photoelectron spectroscopy (XPS) and inductively-coupled plasma mass spectrometry (ICP-MS) with a PHI Quantera XPS spectrometer (Physical Electronics, Chanhassen, MN, USA) and a Perkin Elmer Nexion 300 instrument (Waltham, MA, USA), respectively. High-resolution transmission electron microscopy (HR-TEM) was performed using a JEOL 2100 Field Emission Gun Transmission Electron Microscope (Akishima, Tokyo, Japan). Energy dispersive X-ray spectroscopy (EDS) was performed using a FEI Quanta 400 high resolution field emission scanning electron microscope with an EDS detector (FEI Company, Hillsboro, OR, USA). Elemental map images were taken using a Hitachi SU-8230 SEM with an EDS detector (Tokyo, Japan). Bath sonication was used in all preparations using a Cole-Parmer 8891-21 sonicator (Vernon Hills, IL, USA) unless otherwise stated.

3.2.2. Purification of raw BNNTs

BNNTs (P2 Beta type; BNNT, LLC. Newport News, VA, USA) were purified following a modified literature procedure. BNNTs (gray in color) were first exfoliated in ethanol via bath sonication and then oxidized in air at 800 °C for 1 h to oxidize elemental boron impurities. The material was subsequently washed three times with boiling water, 3 M HCl, and finally 18 MΩ water to yield a white solid (~55% recovery). The integrity of the material was confirmed by XPS, FTIR, HR-TEM, and TGA.
3.2.3. Gd Loading of BNNTs

Different methods were used to produce two kinds of Gd-loaded BNNT materials. First, Gd$^{3+}$ ions were loaded into BNNTs by combining GdCl$_3$ dissolved in 18 MΩ water, with purified BNNTs suspended in water, in a 1.5:1 w/w ratio of Gd:BNNTs and 750 µL of 5% HCl. The mixture was then sonicated for 1 h to produce Gd@BNNT. Finally, the Gd@BNNT material was filtered through a 0.2 µm PTFE filter and washed with 18 MΩ water to remove excess Gd$^{3+}$ not bound to the BNNTs, until ICP-MS analysis showed < 0.5 ppb of Gd in the filtrate.

The second material was produced by loading Gd(acac)$_3$·2H$_2$O into BNNTs following a procedure outlined previously for Gd(acac)$_3$-loaded US-tubes, to produce Gdac@BNNT.$^{178}$ The Gd(acac)$_3$·2H$_2$O complex was first synthesized by the following method: 6.359 mmol (1 eq.) of gadolinium (III) chloride hexahydrate was dissolved in a minimal amount of water; separately, 19.077 mmol (3 eq.) each of acetylacetone (acac) and ammonium hydroxide were dissolved in a minimal amount of methanol. The solution of GdCl$_3$·6H$_2$O was added slowly to the deprotonated acetylacetone. Excess water was added to the reaction mixture to ensure the resulting chelate precipitated completely. The white solid product was washed with water, collected by vacuum filtration, and air-dried. Gd(acac)$_3$·2H$_2$O and purified BNNTs were suspended in methanol (~ 2 mg/mL each) separately via bath sonication. The two mixtures were then combined in various ratios (1:1, 10:1, and 100:1 w/w Gd(acac)$_3$·2H$_2$O:BNNT) and sonicated for 1 h (2 h for 100:1 Gd(acac)$_3$·2H$_2$O:BNNT). The obtained Gdac@BNNT materials were then filtered
through a 0.2 μm PTFE filter and washed with methanol until the filtrate showed < 0.7 ppb Gd by ICP-MS to remove excess Gd(acac)$_3$·2H$_2$O not bound to the BNNTs. The loaded BNNT samples were collected and dried at 80 °C overnight.

3.2.4. Digestion of Gd-loaded BNNTs for ICP-MS Analysis

Trace metal grade 70% nitric acid (HNO$_3$) was used to prepare all samples for ICP-MS, unless otherwise stated. Gd$^{3+}$ analysis was performed on samples of the filtrate when washing the Gd@BNNT and Gdac@BNNT materials to ensure all excess Gd$^{3+}$ ions and Gd$^{3+}$ chelates were removed from the overall material. For the Gd@BNNT material, 200 μL of each wash filtrate was diluted to 10 mL with 2% HNO$_3$, and the Gd$^{3+}$ content was determined by ICP-MS. For the Gdac@BNNT material, 200 μL of each wash filtrate was treated with ~ 4-5 mL of concentrated HNO$_3$ repeatedly over 2-3 d and heated to dryness in order to digest the acac ligands. The dried residue was dissolved in a minimal amount of 2% HNO$_3$, passed through a 0.22 μm syringe filter, and diluted to 10 mL with 2% HNO$_3$ before the Gd$^{3+}$ content was determined by ICP-MS. Because of the exceptional stability of BNNTs, it was hypothesized that conventional nitric acid digestion methods were not sufficiently digesting the material and releasing all of the loaded Gd$^{3+}$ for quantification by ICP-MS. Therefore, different digestion techniques were attempted to optimize the release of Gd$^{3+}$ from the BNNT material, using the following methods.
3.2.4.1. Nitric Acid Digestion

Samples of each material were weighed (0.1-0.3 mg) on a Cahn C-31 microbalance (Cahn Scientific, LLC. Irvine, CA, USA) and digested by sonicating the material in concentrated HNO₃ and heating them to dryness. The dried residue was dissolved in a minimal amount of 2% HNO₃, passed through a 0.22 µm syringe filter, and diluted to 10 mL with 2% HNO₃ in a centrifuge tube.

3.2.4.2. Nitric Acid/Aqua Regia Digestion

Samples were sonicated for 1 h in 5 mL of concentrated HNO₃ and heated to dryness (repeated five times). To the dried residue about 4-5 mL of aqua regia (3:1 v/v HCl:HNO₃) was added, and the samples were again sonicated for 20 min and heated to dryness. The remaining residue was dissolved in a minimal amount of 2% HNO₃ and passed through a 0.22 µm syringe filter before adjusting the volume to 10 mL with 2% HNO₃ in a centrifuge tube.

3.2.4.3. Oxidation Digestion

Because BNNTs begin to oxidize at 800 °C, digestion of the material was attempted using oxidation at high temperatures in air. Samples were weighed in alumina TGA pans using a TGA instrument balance, and placed in a ceramic boat that was loaded into a tube furnace at 1000 °C for 2.5 h. The TGA pans were then rinsed with 2% HNO₃, and the wash was passed through a 0.22 µm syringe filter before the volume was adjusted to 10 mL with 2% HNO₃ in a centrifuge tube.
3.2.4.4. Liquid Fire Reaction Digestion

Samples were first sonicated in 4 mL of concentrated HNO₃ in a round-bottom flask to suspend BNNTs. The samples were then boiled until dry, in order for the HNO₃ to oxidize any material that is easily oxidized. Once no HNO₃ remained in the flask, 4 mL of concentrated trace metal grade perchloric acid (HClO₄) was added to the flask, sonicated briefly to suspend the material, and refluxed for ~ 4 h (set up shown in Figure 1). **Warning:** perchloric acid at high temperatures (203 °C) and organic perchlorate salts are extremely corrosive and reactive. All organic material that is not first oxidized with nitric acid may lead to an extremely violent and explosive reaction. All glass joints were sealed with grease and no stir bar was used, as perchloric acid dissolves Teflon at high temperatures. White vapors were produced but were contained to the reaction flask and the reflux remained well-controlled with no violent reactions observed. The reaction mixture changed from cloudy and gray in color to clear, indicating that the BNNTs were completely digested (Figure 3.4). Appropriate precautions were taken to ensure that no perchloric acid vapors escaped the experimental set-up and it was assumed that the volume of liquid in the reaction vessel remained constant for the duration of the reflux. A 10 µL aliquot of the HClO₄ reaction mixture was diluted to 10 mL with 2% HNO₃, as the concentration of HClO₄ must remain at ≤ 0.1% or below for ICP-MS analysis (instrument requirement).
Figure 3.4 Liquid Fire reaction as a digestion method for Gd-loaded BNNTs. (A) Experimental set-up of perchloric acid reflux. A vacuum distillation condenser was placed as precaution on top of the reflux condenser, in case HClO₄ vapors escaped the reflux condenser, the vapors would collect as liquid in the collection flask. In the unlikely event that the liquid HClO₄ evaporated from the collection flask, those vapors would simply flow into a flask of water (not shown) to form dilute HClO₄. (B) Photographs of reaction mixtures before boiling in HNO₃ (top), after boiling in nitric acid and before HClO₄ reflux (middle), and after HClO₄ reflux (bottom).

3.2.5. Characterization of the Liquid Fire Reaction

In order to understand the effects of the Liquid Fire reaction on BNNTs and to explore this process as a possible means of functionalization, the digestion procedure was repeated using purified BNNTs and commercially-available h-BN (purchased from Sigma Aldrich) for comparison. Both materials were digested as
described above, except now by varying the perchloric acid reflux time (1, 2, 3, and 4 h). The reaction mixtures were then centrifuged at 7000 rpm for 2 h. FTIR and XPS analyses were performed for all samples that yielded pellets after centrifugation (only nitric acid-treated BNNTs and all h-BN samples but the 4 h perchloric acid reflux time yielded pellets). For samples that did not yield a pellet after centrifugation, the HClO₄ reaction mixture was neutralized with 1 N NaOH until basic to pH paper and then lyophilized until dry.

3.2.6. Relaxivity Measurements on Gdac@BNNTs

Samples for relaxivity measurements were prepared by suspending the Gdac@BNNT material in a 0.17% w/v Pluronic F-108 via tip sonication (1 s pulse, 2 s pause for 15 min). Measurement of T₁ relaxation times for the samples were carried out using MR imaging at room temperature (RT, 25 °C) on a 1.5 T MR Imager (Achieva, Philips Health Care, the Netherlands). A transmit body coil and a 16 channel receiver head coil were used for radio-frequency signal transmission and reception, respectively. An inversion recovery prepared turbo-spin echo pulse sequence was used to measure the T₁ relaxation times of the samples. The 2D images were acquired over a field-of-view of 100 × 120 mm with a slice thickness of 8 mm and an acquisition voxel size of 0.20 × 0.20 × 8.00 mm³. Inversion times (TI) of 50, 55, 60, 65, 70, 75, 80, 85, 90, 100, 150, 200, 500, 1000, 2000, 3000, 4000 and 5000 ms were used for the inversion recovery preparation sequence, and the T₁ values were calculated using the standard inversion recovery equation.
3.3. Results and Discussion

3.3.1. Purification of Raw BNNTs

The as-received raw BNNT material is a mixture of multi-walled BNNTs (~3-4 concentric walls), some h-BN, and elemental boron. The material was first purified using a known method to remove mainly elemental boron impurities and to exfoliate the BNNTs. Although there is some h-BN remaining from the manufacturing process, there is no known method to effectively separate the h-BN from BNNTs. The procedure for BNNT purification is outlined in Experimental Methods Section 3.2.2 and was modified from a previously reported method. The raw BNNT material (gray in color) was sonicated in ethanol to exfoliate the nanotubes, and was then oxidized in air at 800 °C for 1 h. Subsequently, the BNNT material (now white in color) was washed with boiling water, 3 M HCl, and RT water to remove boron oxide formed during oxidation.

The integrity of the material was verified and the spectroscopic characterization of the purified BNNTs is shown in Figure 3.5. In addition to the B-N longitudinal and out-of-plane vibrations around 1350 and 788 cm\(^{-1}\) in the raw material, FTIR data show a peak around 3200 cm\(^{-1}\), corresponding to O-H moieties on oxidized boron in the raw material, which disappears after purification (Figure 3.5A). In addition, a shoulder on the B-N longitudinal vibration centered around 1190 cm\(^{-1}\) found in the spectrum of the raw material corresponds to B-N-O vibration modes, which further indicate the presence of oxygen-containing moieties in the BN starting material that are removed with the purification method. The TGA plot of
the raw material shows the weight percent increases around 275 °C, which corresponds to the oxidation of elemental boron impurities (see Figure 3.5B). After purification, the TGA of BNNTs is stable with negligible weight loss until 900 °C, where the BNNTs begin to oxidize. The small weight loss (about 10 wt%) is likely caused by desorption of moisture or gases.\textsuperscript{180,181} XPS spectroscopy was performed, which confirmed the expected elemental composition of the BNNTs both before and after purification, with only boron, nitrogen, and adventitious carbon and oxygen impurities in the survey spectrum (Figure 3.5C,D). High-resolution elemental analysis shows the calculated B:N ratios to be near 50:50 (atomic percentages shown as insets on the survey spectra, high resolution data not shown). High-resolution TEM images were also obtained, which showed less amorphous material and that the BNNTs were reasonably pristine after purification with little to no defects, as displayed in Figure 3.6. These purified BNNTs were used for all subsequent experiments without further oxidation or functionalization.
Figure 3.5 Characterization of raw and purified BNNT material. (A) FTIR of raw (blue) and purified (black) BNNTs. (B) TGA of raw (blue) and purified (black) BNNTs. (C) XPS showing elemental analysis of raw BNNTs. (D) XPS showing elemental analysis of purified BNNTs. Insets on panels (C) and (D) show atomic percentages calculated by high resolution XPS elemental analysis.
Figure 3.6 Representative high-resolution TEM image of the purified BNNT material. Inset: an individual BNNT.

3.3.2. Gd Loading of BNNTs

To make BNNTs MRI-active, the as-purified BNNTs were loaded with Gd$^{3+}$ from two different sources. Gd$^{3+}$-ion loading was accomplished using either free Gd$^{3+}$ ions or Gd$^{3+}$ chelates (Gd(acac)$_3$·2H$_2$O specifically) following the same procedures as previous studies loading Gd$^{3+}$ into carbon US-tubes.$^{91,176,178}$ BNNTs were sonicated with either free Gd$^{3+}$ ions or the Gd(acac)$_3$·2H$_2$O chelates, followed by filtration with water or methanol until the Gd$^{3+}$ level in the filtrate was negligible as confirmed by ICP-MS (Figure 3.7).
Figure 3.7 Gadolinium loading of BNNTs. (A) Loading scheme of BNNTs with free Gd\(^{3+}\) ions under acidic conditions. The plot shows the concentration of Gd\(^{3+}\) ions as measured by ICP-MS in each filtrate after the material was washed with water. Data represent mean ± standard deviation (SD), n = 3. (B) Loading scheme of BNNTs with Gd(acac\(_3\))\(\cdot\)2H\(_2\)O in methanol. The plot shows the concentration of Gd\(^{3+}\) ions also measured by ICP-MS in each filtrate sample after the material was washed with methanol. Data represent mean ± SD, n = 2. Some error bars in both plots are smaller than symbols.
To quantify the amount of Gd\(^{3+}\) associated with the BNNTs, samples of the Gd@BNNT and Gdac@BNNT materials were digested and prepared for ICP-MS analysis. Samples were sonicated in concentrated nitric acid, boiled until dry, dissolved in a minimal amount of 2\% nitric acid, and passed through 0.22 µm syringe filter to insure no insoluble material was injected into the ICP-MS instrument. As shown in Table 3.2, the amount of Gd loaded is 0.03 wt\%, which is considerably lower than that reported in the literature (0.4 wt\% by Ciofani et al.).\(^{168}\) The authors reported that the BNNTs were previously oxidized, which helped to bind the Gd\(^{3+}\) ions to their material, which explains why their material would contain more Gd\(^{3+}\) than that in the present study. Furthermore, they digested their material in a microwave system using nitric acid at high temperatures and pressures (200 °C, 400 psi), which led to the question of whether all Gd\(^{3+}\) in the present BNNT samples was being detected following digestion in boiling nitric acid with no microwave or pressurized systems. It is well known that BN materials are very stable towards chemical oxidation;\(^{101,128,129}\) therefore, the Gd\(^{3+}\) ions may be bound to insoluble, undigested BNNT fragments that were removed when the samples were filtered. This led to further investigation and optimization of the digestion method to assure complete digestion of the nanotubes and thus, accurate measurements of Gd\(^{3+}\) concentration.
<table>
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<tr>
<th>Material</th>
<th>Digestion Method</th>
<th>Wt% of Gd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gd@BNNT</td>
<td>HNO$_3$</td>
<td>0.03</td>
</tr>
<tr>
<td>Gd@BNNT</td>
<td>HNO$_3$/aqua regia</td>
<td>0.01</td>
</tr>
<tr>
<td>Gdac@BNNT</td>
<td>HNO$_3$/aqua regia</td>
<td>1.00</td>
</tr>
</tbody>
</table>

**Table 3.2 Comparison of BNNT loading and digestion methods.**

A second method to digest the BNNTs was employed with a combination of nitric acid and *aqua regia*. Gd@BNNT and Gdac@BNNT samples were first, sonicated and boiled in nitric acid five times followed by three similar treatments with *aqua regia*. The dried residue samples were then dissolved in a minimal amount of 2% nitric acid and filtered through a 0.22 μm syringe filter. As shown in **Table 3.2**, the Gd@BNNT digested with nitric acid/*aqua regia* released slightly less Gd$^{3+}$ ions from the material, while still on the same order of magnitude as the amount of Gd$^{3+}$ ions detected with nitric acid alone. For the case of Gdac@BNNT, the nitric acid/*aqua regia* digestion method demonstrated that the loading capacity of Gd$^{3+}$ in BNNTs was two orders of magnitude greater with Gd$^{3+}$ chelates than with naked Gd$^{3+}$ ion alone. Because the Gd(acac)$_3$·2H$_2$O complex allowed for greater retention of Gd$^{3+}$ in the BNNTs, this material (Gdac@BNNT) was selected for all follow-up experiments. However, because BN materials are so stable towards
oxidation, it was still unclear whether the optimum digestion procedure was accomplished; therefore, two other methods were investigated.

The third digestion method that was examined took advantage of the fact that BNNTs oxidize at temperatures > 800 °C. Gd@BNNT samples were oxidized in air using a tube furnace at 1000 °C for 2.5 h. The residue was dissolved, filtered through a 0.22 μm syringe filter, and prepared for ICP-MS analysis with the rationale being that if the BN material is completely converted to boric oxide, the Gd$^{3+}$ ions would be released and detected. The final digestion method investigated is known as the “Liquid Fire Reaction,” which is digestion by refluxing concentrated perchloric acid at 203 °C.$^{182}$ Samples were first treated with boiling concentrated nitric acid to oxidize any readily-oxidizable material, thus preventing possible violent oxidation reactions (explosions).$^{182}$ The nitric acid was evaporated before adding concentrated perchloric acid and the mixture was refluxed for ~ 4 h, yielding a clear, transparent solution (Figure 3.4). These samples were not filtered because the syringe filters may leach unwanted impurities into perchloric acid. Additionally, an extremely small sample was taken from the reaction mixture (10 μL) for ICP analysis to remain compliant with the ICP-MS instrument requirements ( < 0.1% HClO$_4$). A comparison of the nitric acid/aqua regia, oxidation, and Liquid Fire digestion methods is shown in Figure 3.8.
As shown in Figure 3.8, the Liquid Fire reaction digested the BNNTs sufficiently well to release more Gd$^{3+}$ from the BNNTs than the other two methods tested, with 1.6 wt% Gd$^{3+}$ bound to the BNNTs. On the contrary, the oxidation digestion released less Gd$^{3+}$ (0.02 wt%) than the nitric acid/aqua regia digestion (1.0 wt%). While it seemed like the oxidation method was plausible, the small amount of Gd$^{3+}$ recovered from the nanotubes is likely a result of an inability to recover the oxidized material from the alumina TGA pans used to hold the Gdac@BNNT sample. The change in solution after reflux in perchloric acid from cloudy to clear indicated almost, if not complete, digestion of the BNNTs. The present findings clearly indicate that the Liquid Fire digestion method is superior to the other digestion methods investigated.
Using the Liquid Fire reaction as the digestion method, the loading capacity of Gdac@BNNTs was optimized by varying the ratios of Gd chelate to BNNT used when preparing the material. Batches of Gdac@BNNTs were prepared with 10:1 and 100:1 w/w Gd(acac)₃·2H₂O:BNNT ratios in order to determine if the BNNT material could absorb and retain more Gd³⁺ chelate, as this would increase the efficacy of the Gdac@BNNT as an MRI CA. It was found that the loading capacity of BNNTs increased with greater amounts of Gd(acac)₃·2H₂O, as shown in Figure 3.9, where it was observed that Gdac@BNNTs prepared with a 100:1 weight ratio contained 37 wt% Gd.

Figure 3.9 Loading capacity optimization of Gdac@BNNTs using different weight ratios of Gd(acac)₃·2H₂O to BNNT. Data represent mean ± SD, n = 3.
While the exact interaction by which the Gd(acac)$_3$·2H$_2$O complex is being retained by the BNNTs is unknown, it is hypothesized that the intermolecular forces between the BN material and the acac ligands are the primary driving forces for attachment. Because BNNTs are multi-walled (3-4 walls), the Gd chelate may also intercalate in between the concentric nanotube walls. Zhi et al. reported loading shortened BNNTs with DNA and polyvinylpyrrolidone (PVP), but did not suggest a retention mechanism other than that defect sites on the shortened BNNTs could allow for such molecules to enter and attach to the BNNTs. However, given the similarity in size between DNA, PVP, and BNNTs (shortened BNNTs were around 1 µm in length), it seems likely that such polymers could easily wrap around the outside of BNNTs instead of attaching to interior surfaces. Other reports demonstrate the loading metals into BNNTs via capillary action with seed particles that are then reduced to form a metal nanorod inside a BNNT. Goldberg and colleagues also reported a capillary-action loading mechanism when loading BNNTs with fluorescent dyes. A similar capillary-action process may play a role in the loading of Gd$^{3+}$ and Gd(acac)$_3$·2H$_2$O in the present study as well. To effectively wet BNNTs with a solution, solvents with lower surface tension, such as methanol (22.7 mN/m) should be used over solvents, such as water, since water forms a large contact angle with the hydrophobic BNNTs and has a much higher surface tension (72.8 mN/m). Since the BNNTs used in this study were not functionalized or oxidized, the material retained its hydrophobic character and would have wetted better with the methanol in the case of the Gdac@BNNT loading procedure than with the water in the Gd@BNNT loading procedure. This likely explains the large
difference in loading capacity between the Gd@BNNT and Gdac@BNNT materials. While capillary action may move the respective Gd species into or onto the BNNTs, nothing has been done in the present study to chemically alter the Gd species or the nanotubes to seal the metal ions or chelates inside the BNNTs. Therefore, it is plausible that strong dipole-dipole and hydrophobic interactions between the Gd(acac)_3·2H_2O chelate and the BNNT (whether on the external surface or in between concentric nanotubes) are primarily responsible for the attachment of the Gd^{3+} chelate to the BN material. Because the 100:1 loading ratio sample contained the greatest amount of Gd^{3+}, and therefore have the highest likelihood of performing the best as an MRI CA, this Gdac@BNNT material was selected for follow-up studies.

3.3.3. Characterization of Gdac@BNNTs

To further characterize the Gd-loaded BNNTs and to investigate the degree of functionalization, FTIR and TGA analyses were performed on Gdac@BNNTs. Again, the FTIR spectrum of the purified BNNT shows the longitudinal and out-of-plane vibration modes of BN at 1350 and 788 cm\(^{-1}\), respectively (Figure 3.10A). The FTIR spectrum of the Gd(acac)_3·2H_2O chelate shows peaks at 1257, 1376, 1577, and 3243 cm\(^{-1}\), which correspond to the C-O, C-H, C=C, and O-H vibration modes of the acac ligand and coordinated water molecules, respectively. The Gdac@BNNT FTIR spectrum shows peaks that overlap with both the Gd(acac)_3·2H_2O and BNNT components of the sample; however, because of the intense broad B-N peak around 1350 cm\(^{-1}\), many peaks corresponding to Gd(acac)_3·2H_2O adhered to the BNNTs are overshadowed. In Figure 3.10B, the TGA plot of the Gdac@BNNT material shows a
weight loss pattern similar to that of Gd(acac)$_3$·2H$_2$O, but the weight loss stabilized at a higher weight percent, indicating that the chelate is associated with the BNNTs. The difference in weight between the purified BNNT and the Gdac@BNNT is ~ 39%, which corresponds to the amount of Gd$^{3+}$ attached to the BNNTs, consistent with the ICP-MS results described above (37 wt%). The first derivative of the weight% loss pattern is shown in Figure 3.10C in order to highlight the weight loss events more clearly. There are three weight loss events with Gd(acac)$_3$·2H$_2$O: loss of water bound to the Gd ion between 40-110 °C and decomposition of the acac ligands, which occurs over multiple steps up to about 550 °C, consistent with previous studies of lanthanide acac chelates.$^{187}$ These weight-loss events also occur with Gdac@BNNTs, although they occur at higher temperatures, again indicating an interaction between the chelate and the thermally-stable BNNTs. There is an additional weight loss event around 47 °C with Gdac@BNNTs, which is likely the release of adsorbed gas or solvent from the BNNTs, as was seen previously in the pure BNNT material.$^{180,181}$
Figure 3.10 Characterization of Gdac@BNNT. (A) FTIR spectra of purified BNNT (black), Gd(acac)$_3$·2H$_2$O (orange), and Gdac@BNNT (green). (B) TGA plots of purified BNNT (black), Gd(acac)$_3$·2H$_2$O (orange), and Gdac@BNNT (green). (C) First derivative of TGA plots in panel (B) for Gdac@BNNT (green) and Gd(acac)$_3$·2H$_2$O (orange).

Elemental analysis was performed using EDS/SEM, which showed nearly complete overlap of B, N, and Gd elemental localization (Figure 3.11A). Because BN are insulating materials, it is difficult to obtain clear images on SEM. However, these images still demonstrate that the gadolinium is closely associated with the BNNTs. The EDS spectrum also confirmed the presence of Gd in the Gdac@BNNT material with peaks at 1.2 and 6.05 keV, corresponding to the M and L shell electron binding
energy, respectively, as shown in Figure 3.11B. A peak for silicon appears in the EDS spectrum at a binding energy of 1.75 keV as a result from the substrate. Sodium also appears in the spectrum at 1.05 keV, which is a ubiquitous, yet harmless, impurity. The acac ligand contains oxygen and carbon, which can be seen in the oxygen peak at 0.53 keV and the broad shoulder of the nitrogen peak centered around 0.29 keV, which agrees with the expected binding energy of carbon of 0.28 keV.188
Figure 3.11 Qualitative elemental analysis of Gdac@BNNT. (A) EDS/SEM elemental maps of boron (top left), nitrogen (top right), gadolinium (bottom left), and merged (bottom right). Scale bar = 30 μm. (B) EDS spectrum of Gdac@BNNT. Inset: close-up view of binding energies 0-2.5 keV.
To evaluate the properties of Gdac@BNNT as an MRI CA, aqueous suspensions were made at different concentrations and MR phantom images were acquired at various inversion times (TI). Again, because the BNNTs are inherently hydrophobic, it was necessary to first suspend the Gdac@BNNT material in a surfactant. The Gdac@BNNTs were dispersed via tip sonication in a 0.17% w/v solution of Pluronic® F-108 in order to form stable aqueous suspensions. The coronal MR phantom images and T₁ relaxation times of Gdac@BNNT are presented in Figure 3.12. As seen by the phantom images, the material generates clear visual contrast faster than controls with no Gd³⁺ present (Figure 3.12A). Gdac@BNNTs at a concentration of 0.1 mg/mL generate bright contrast beginning around 1.0-1.5 s TI, and produce contrast faster with increasing concentration, as shown by the images at 100 and 70 ms TI for the 0.25 and 0.5 mg/mL samples, respectively (see Figure 3.12B). The T₁ relaxation times also showed a concentration-dependent trend similar to that observed with the phantom images. Furthermore, the sagittal phantom images were also acquired and the T₁ values were also calculated. The values for the sagittal images agree well with the coronal images, which confirm that the samples are homogenous suspensions of Gdac@BNNTs (see Appendix B).
Table 3.12 MRI CA performance of Gdac@BNNTs at various inversion times (TI) and concentrations. (A) MR phantom images and T₁ relaxation times of Gdac@BNNT at 0.1, 0.25, and 0.5 mg/mL. T₁ values represent mean ± SD. (B) MR phantoms for 0.25 and 0.50 mg/mL Gdac@BNNT are shown in the red box at smaller TI increments to show relaxation effect more clearly. Black circles around the phantom images are shown to provide contrast between the image object and the background.

By plotting the Gd concentration against the inverse of T₁, the slope of the linear regression is the proton relaxivity of the CA, as shown in Figure 3.13. The proton relaxivity value of the Gdac@BNNT at 25 °C under 1.5 T magnetic field strength is 17 mM⁻¹s⁻¹. The relaxivity is greater than those reported for commercially-available MRI CAs such as Magnevist (gadopentetate dimeglumine), Multihance (gadobenate dimeglumine) and Prohance (gadoteridol), as well as the relaxivity previously reported for Gd(acac)₃·2H₂O; however, it is less than what was achieved with Fe impurities in ball milled and annealed BNNTs (31 mM⁻¹s⁻¹ at 1.5
The Fe-BNNT reported that the impurities formed an Fe nanoparticle at the end of the nanotubes, which may be the cause of the higher relaxivity compared to seemingly individualized Gd chelates associated with the BNNTs of the present study. The ultra-high relaxivity of the GNTs can also be explained due to a clustering effect. These results demonstrate that not only can BNNTs be loaded with Gd chelates, but also that the relaxivity of the Gd chelates is enhanced when sequestered with the nanotube material, as was shown previously. While the development of BNNTs as biomedical agents may be in the distant future, more analyses into the efficacy of these materials as medical imaging CAs, as well as closer investigations into biological toxicity, biodistribution, and clearance profiles will be necessary to realize the potential of the BNNT material. The present study serves first, as a proof-of-principle showing that metal-chelate loading into BNNTs, accurate quantification by ICP-MS using wet chemical methods, and potential use as MRI CAs are possible, and second, as a stepping-stone for further studies on BNNT-based biomedical materials. In addition, because of the exciting potential for the Liquid Fire reaction as a functionalization tool for BNNTs, the effects of refluxing perchloric acid on BN materials were studied in closer detail.
3.3.5. Oxidation of BN materials with the Liquid Fire Reaction

Because the oxidation and functionalization of BN nanomaterials is also of increased scientific interest, a preliminary investigation was performed into how the Liquid Fire reaction digested the BNNTs and whether step-wise oxidation of the BNNTs could be observed before ultimate digestion. None of the solid BNNT material remained after the 4 h perchloric acid reflux and could not be recovered after the reaction mixture was centrifuged for 2 h at 7000 rpm (see Figure 3.14). The photographs in Figure 3.14 show that the pellet of BNNTs grows smaller after each step of the liquid fire reaction until it disappears altogether after 2 h of reflux, suggesting that the BNNTs had been either suspended homogenously or completely dissolved by that time. In the samples where a solid material was recoverable, the

Figure 3.13 Plot of Gd concentration vs $1/T_1$. The slope of the linear regression is the relaxivity of Gdac@BNNT (17 mM$^{-1}$s$^{-1}$).

![Figure 3.13](image.png)
supernatant was decanted and the solid was washed several times with water, dried, and analyzed by FTIR and XPS. The liquid samples were neutralized with sodium hydroxide until basic and dried until solid. However, because this step yielded a high sodium perchlorate content within the sample, relevant peaks in the FTIR and XPS were not observed above the background. Although a pellet of BNNTs remained after 1 h reflux, the solid was too small to be recovered after the washes with water and could not be analyzed. Additionally, because the FTIR and XPS spectra of purified BNNTs and nitric acid-treated BNNTs looked fairly identical (see Appendix B), a new system was required to study the effects of the Liquid Fire reaction.
Hexagonal BN nanosheets were studied next as a model for BNNT digestion, due to their similar properties and structure. Because h-BN sheets are known to be more stable than BNNTs,\textsuperscript{129}, which would allow the possibility of observing a step-wise oxidation of the material before complete digestion. Commercially-available h-BN was digested with the Liquid Fire reaction in the same manner as the BNNTs. The material was sonicated in nitric or perchloric acid to suspend the material prior to boiling or reflux, respectively. This time after each step of the liquid fire reaction, a solid remained after centrifugation and was analyzed by FTIR and XPS. As shown in Figure 3.15, the FTIR spectra of the pristine and nitric acid-treated material show a shift in the longitudinal BN stretch from around 1310 to 1380 cm\textsuperscript{-1}, while the transverse BN stretch remained at the same position, suggesting a change in bonding structure. The FTIR spectra of the h-BN samples refluxed in perchloric acid for 1, 2, and 3 h (after treatment with nitric acid) showed further changes to the line-shape of the longitudinal BN stretch. These extra peaks and shoulders suggest the formation of new vibrational modes in the BN material, such as what appears to be a B-N-O stretch at 925 cm\textsuperscript{-1} (highlighted in red), which is comparable to what was reported elsewhere for functionalized h-BN (959 cm\textsuperscript{-1}).\textsuperscript{179} Additionally, as shown in the XPS spectra (Figure 3.16, shown below), there appears to be oxidation in the pristine material, which can be seen in the FTIR spectrum as a shoulder of the longitudinal BN stretch. Unfortunately, as discussed previously, due to the broad,
intense FTIR peaks of pristine material, it is difficult to discern different functional
groups that usually overlap in that region from 650-1620 cm\(^{-1}\).\(^{179,189}\)

Figure 3.15 FTIR spectroscopy of h-BN digested using the Liquid Fire reaction
with various reflux times. FTIR spectra of pristine h-BN (black), h-BN treated
with boiling nitric acid (green), refluxing perchloric acid for 1 h (gold), 2 h
(gray), and 3 h (blue). The red box highlights possible B-N-O stretch around
925 cm\(^{-1}\). Red dotted line highlights center of pristine h-BN longitudinal peak
around 1310 cm\(^{-1}\).

XPS spectra were also obtained and are shown in Figure 3.16. The high-
resolution peaks for B 1s and N 1s electron binding energies grew more
asymmetrical with each step of the Liquid Fire digestion and were deconvoluted
into two peaks, showing the B-N peak centered at 190.8/398.4 eV for B/N and B-
O/N-O peak centered around 192.0/399.2 eV for B/N, which is consistent with other
reports.\textsuperscript{189,190} As shown in \textbf{Figure 3.16}, the area percentages of the B-O peaks increase with each step of the Liquid Fire reaction, which suggests greater oxidation of the material. After 3 h of perchloric acid reflux, the B-O peak area percentage decreases, while a third peak appears centered at 190.2 eV that suggests the formation of defects in the h-BN material, as Kidambi \textit{et al.} also suggested in their findings.\textsuperscript{190} The N-O peak in the N 1s spectrum does not appear to grow much until 2-3 h of perchloric acid reflux, indicating that the nitrogen in h-BN is much less reactive. However, the N-O peak area percentage grows to around 13\% at 2 and 3 h of perchloric acid reflux, suggesting that oxygen is binding to B and N sites on the h-BN sheets. The FTIR and XPS evidence presented here represent a promising start toward understanding how the Liquid Fire reaction oxidizes and ultimately digests BN nanomaterials.
Pristine h-BN

binding energy [eV]  

B-O 5%

B-N 95%

N-O 6%

N-O 13%

N-O 12%

Liquid Fire 1h

binding energy [eV]

B-O 10%

B-N 90%

B-N 87%

B-N 89%

Liquid Fire 2h

binding energy [eV]

B-O 13%

B-N 87%

B-N 87%

B-N 89%

Liquid Fire 3h

binding energy [eV]

B-O 7%

B-N 94%

B-N 95%

B-N 95%

Nitric Acid

binding energy [eV]

B-O 7%

B-N 93%

N-O 6%

Liquid Fire 1h

binding energy [eV]

B-O 10%

B-N 90%

B-N 87%

Liquid Fire 2h

binding energy [eV]

B-O 13%

B-N 87%

B-N 87%

B-N 89%

B-N 95%

B-N 94%

B-N 93%

B-N 93%

B-N 95%

Liquid Fire 3h

binding energy [eV]

B-O 7%

defects 4%

N-O 12%
Figure 3.16 XPS analysis of h-BN nanosheets digested using the liquid fire reaction at different reflux times. B 1s (left) and N 1s (right) deconvoluted peaks are shown for each step of the Liquid Fire reaction.

3.4. Summary and Conclusion

Herein is reported the Gd loading, digestion, and characterization of a new Gd-based BNNT MRI CA. This is the first known report of Gd chelates used in BNNT MRI CAs and the first known use of the Liquid Fire reaction to digest BNNTs for ICP analysis. BNNTs were loaded with both free Gd$^{3+}$ ions and with Gd(acac)$_3$$\cdot$2H$_2$O and found that the Gdac@BNNT material retained significantly more Gd than the Gd@BNNT material, most likely due to enhanced intermolecular interactions between the BNNTs and the chelates. The loading capacity of Gdac@BNNTs was also optimized by varying the loading ratios and it was observed that a 100:1 Gd(acac)$_3$$\cdot$2H$_2$O:BNNT w/w ratio yielded 37 wt% of Gd. This Gdac@BNNT material was analyzed for its MRI properties, and found that a relaxivity of 17 mM$^{-1}$s$^{-1}$ can be achieved at 1.5 T magnetic field strength. This enhancement effect agrees well with other reports of relaxivity enhancement due to confinement of Gd in nanotubes.

Furthermore, from the various digestion methods studied, the Liquid Fire reaction released the most Gd$^{3+}$ ions and sufficiently digested BNNTs; however, more studies into the structural remnants of the Liquid Fire reaction are necessary. From the preliminary studies into structural defects caused by the Liquid Fire reaction, it can be observed that oxidation occurred, and the bonding structure appeared to change after perchloric acid reflux. This study sheds light on how BNNT
materials can be manipulated for further functionalization for biomedical and other applications.
Chapter 4

Conclusions and Future Directions

4.1. $\text{C}_6\text{O}_2$-Serinol as a Drug Delivery Agent

$\text{C}_6\text{O}_2$-serinol may be further enhanced as a drug delivery platform with functionalization of targeting agents such as tumor-specific peptides or small antibody fragments that direct the material to the tumor. While these modifications would require a reevaluation of the biodistribution and pharmacokinetics of the nanomaterial, more studies of these modified $\text{C}_6\text{O}_2$-serinol materials would lead to a better understanding of how chemistry at the surface of $\text{C}_6\text{O}_2$-serinol affects in vivo character. In addition, using radioisotopes as an imaging tag for these biodistribution studies allows for tracking in vivo with high sensitivity and accuracy using non-invasive PET imaging over time. However, using a low molecular weight ligand or a drug with similar polarity and charge as $\text{C}_6\text{O}_2$-serinol would allow for an easier prediction of the biodistribution of that agent. Theoretically, there are 30
sites available on C₆₀ for Bingel-Hirsch chemistry,¹⁹¹ and in general, only two of those sites are required (based on past experience) for serinolamide moieties to achieve water solubility. In addition to water solubility, the advantage of the serinolamide groups is that they are non-toxic, compact, and uniform. Compared to PEGylation, addition of serinolamide groups gives shorter residence times because of their compact nature and allow for renal clearance of small nanomaterials.

Future directions for this work could include targeting the C₆₀-NOTA conjugate with a ligand specific to a tumor cell marker or receptor. For example, C₆₀-serinol could be functionalized to target the specific membrane antigen (PSMA) receptors overly expressed in prostate cancer using a relatively small urea-based ligand.¹⁹² Furthermore, curtailing the hydrophilic character of C₆₀-serinol is likely to increase residence time in vivo. This could lead to a biodistribution closer to that of the previously discussed examples, such as C₃, and perhaps a more favorable tissue localization for drug delivery. Moreover, using a C₆₀-based delivery vehicle allows for the potential for multimodal therapy. The attractive therapeutic properties of the C₆₀ core, such as potent antioxidant activity and PDT capabilities, create the opportunity for a therapeutic drug delivery platform, which is not possible with simply targeting small-molecule drugs or sequestering them within liposomes. The work presented here sheds light on rational design of future biomedical materials that offers a viable route toward C₆₀-based clinical materials for cancer therapy.
4.2. BNNTs as MRI CAs

The work presented in this thesis discusses how BNNTs can be loaded with Gd chelates to render the material MRI active at clinically-relevant magnetic field strengths, with a $r_1$ value of 17 mM$^{-1}$s$^{-1}$. In this work, it was discovered that free Gd$^{3+}$ ions do not bind to unfunctionalized BNNTs in the manner used in this study, like Gd$^{3+}$ ions bind to oxidized BNNTs or to US-tubes (GNTs), and therefore Gd chelates were necessary to load BNNTs with Gd$^{3+}$ ions. In pursuit of quantifying the Gd chelate loading capacity of BNNTs, investigation into how to effectively digest the BNNTs for ICP-MS metal analysis was performed, where it was found that the Liquid Fire reaction was successful over other acid and thermal digestion methods. Furthermore, it was found that some step-wise oxidation of h-BN nanosheets can be observed with XPS and FTIR, however more structural studies of how the h-BN lattice changes under Liquid Fire conditions are necessary to fully understand how to oxidize and digest BN nanomaterials.

This work accomplishes significant steps toward evaluating the potential for BNNT-based medical imaging CAs. First, the novel use of the Liquid Fire reaction as a wet chemical digestion method completely dissolves BNNTs at ambient pressures without the use of microwave or autoclave reactors. This is important in the development of not only imaging agents, but any metal-doped BNNT material where it is essential to quantify the metal in the nanotubes. Because MRI CA relaxivity is highly concentration dependent, it was vital to know the weight% of Gd within the BNNTs. Second, the Liquid Fire reaction could serve another useful function of
oxidizing, but not completely destroying the BNNTs in order to use the oxygen moieties as site of attachment for other molecules of interest. By controlling the Liquid Fire reaction times, the degree of oxidation and defects in the BN material could be controlled and could be proved experimentally, as shown in the present study. Furthermore, by adding defects and oxygen moieties, the material may be able to hold more Gd$^{3+}$ ions or chelates, thereby becoming more like the GNTs and perform better as MRI CAs. The closer comparison between BNNTs and GNTs will become clearer as shorter BNNTs (on the order of 20-80 nm) and single-walled BNNTs become more available.

Third, it has been shown here and previously that BNNTs and CNTs can absorb and retain Gd$^{3+}$ chelates.$^{176}$ Because the BNNTs are multi-walled, it is likely that the Gd(acac)$_3$·2H$_2$O likely intercalates in between the concentric nanotubes, as well as on the surface of BNNTs. Furthermore, the relaxivity per ion of the Gd(acac)$_3$·2H$_2$O was enhanced when the material was sequestered in BNNTs, consistent with the trend in previous studies with Gd chelates with US-tubes (< 5 mM$^{-1}$s$^{-1}$ alone; 17 mM$^{-1}$s$^{-1}$ in Gdac@BNNTs). However, the degree of enhancement with BNNTs was not nearly as significant as with US-tubes (~103 mM$^{-1}$s$^{-1}$ in Gd(acac)$_3$@US-tubes), but this is attributed to the specific nature of the US-tubes, which also contain paramagnetic free radical centers that have been shown to add to the relaxivity per ion, whereas the BNNTs do not affect the relaxivity. With the available data, it appears that US-tubes loaded with both free Gd$^{3+}$ ions and chelates are the best material by far as nanoparticle-based MRI CAs. Finally, using the GNT
material as a standard for nanotube-based MRI CAs, future studies with Gd-loaded BNNTs should include creating defects on multi- or single-walled BNNTs in order to possibly load more Gd$^{3+}$ ion or chelates to enhance the relaxivity.

Because BNNTs have been studied for therapeutic applications such as drug delivery and boron neutron capture therapy as well, BN-based materials have the potential to serve as multimodal agents, combining multiple diagnostic and anticancer capabilities in one particle, similar to C$_{60}$ fullerenes. In order to move BNNT-based materials toward clinical applications, first, a standard method to produce a homogenous material batch-to-batch is vital for clinical translation. Currently, BNNTs vary in number of walls, impurity content, diameters, and lengths across different methods of production, and while the variation in these properties did not affect the present results, reproducible production methods must be explored to minimize any side effects from these variables. Once this is accomplished, more detailed studies into the biological performance of BNNTs are necessary to understand the behavior of this nanomaterial as it interacts with cells and tissues \textit{in vivo}. 
References


63. Cheney, M. A. Radiofrequency-induced Cellular Hyperthermia: Water-soluble Fullerene as a New Cancer Therapeutic Agent. (Rice University, 2014).


Appendix A

Figure A.1. FTIR spectrum of Compound 1.

Figure A.2. $^1$H-NMR spectrum of Compound 1.
Figure A.3. MALDI-MS of Compound 1.

Figure A.4. MALDI-MS of Compound 2.
Figure A.5. FTIR spectrum of Compound 3.

Figure A.6. MALDI-MS of Compound 3.
Figure A.7. MALDI-MS of Compound 4.

M+Na⁺ 3058.346

M⁺ 3038.590

Figure A.8. FTIR spectrum of Compound 5.
Figure A.9. MALDI-MS of Compound 5.

Figure A.10. $^1$H-NMR of Compound 6.
Figure A.11. AFM image of aggregates of Compound 6.

Figure A.12. Radio-HPLC of PBS shelf stability challenge at RT at 48 h. The integration of the C$_{60}$-$[^{64}$Cu]Cu(NOTA) peak showed 95% retention of Cu$^{2+}$ in NOTA.
Figure A.13. Radio-HPLC of human serum stability challenge at 37 °C at 48 h. The integration of the C$_{60}$-[$^{64}$Cu]Cu(NOTA) peak showed 89% retention of Cu$^{2+}$ in NOTA.
## Appendix B

<table>
<thead>
<tr>
<th>Material</th>
<th>$T_1$ [ms] (Coronal)</th>
<th>$T_1$ [ms] (Sagittal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pluronic alone</td>
<td>2299.717 ± 25.499</td>
<td>2304.183 ± 25.875</td>
</tr>
<tr>
<td>BNNT alone</td>
<td>2513.428 ± 5.490</td>
<td>2506.390 ± 5.689</td>
</tr>
<tr>
<td>0.10 mg/mL Gdac@BNNT</td>
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<td>1542.649 ± 4.502</td>
</tr>
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<td>0.25 mg/mL Gdac@BNNT</td>
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<td>93.772 ± 1.107</td>
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<tr>
<td>0.50 mg/mL Gdac@BNNT</td>
<td>50.006 ± 0.551</td>
<td>45.67 ± 0.1287</td>
</tr>
</tbody>
</table>

Table B.1. $T_1$ values for varying concentrations of Gdac@BNNT wrapped in Pluronic F-108 surfactant using coronal and sagittal projections. Data represent mean ± SD.
Figure B.1. FTIR spectrum of purified BNNT (black) and HNO$_3$-treated BNNTs (green).