Rice University

Imunoconjugates of Carbon Nanostructures

by

Jared Mark Ashcroft

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Abstract

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For the first time, carbon nanostructures have been designed and synthesized to form immunoconjugates with monoclonal antibodies (mAb) for use in cell-targeted cancer diagnosis and therapy. The immunoconjugates are derived from various nanoscale carbon-based building blocks, specifically fullerenes (C_{60}), gadofullerenes (M@C_{60}) and ultra-short carbon nanotubes (US-tubes). The exterior of each nanostructure has been derivatized with water-solubilizing addends using Bingel-type (nucleophilic cyclopropanation) addition chemistry to facilitate biocompatibility. Initially, conjugation to the murine anti-gp240 melanoma antibody (ZME-018 mAb) was completed with two different water-soluble C_{60} derivatives, only one of which had the potential to covalently attach to the ZME-018 mAb. After conjugation, this covalently linked C_{60}-SPDP conjugate incorporated 15 C_{60} moieties per antibody, while retaining 80% of the antibody's target specificity. In a second experiment a non-covalently linked C_{60}-Ser conjugate incorporated 38 fullerenes per antibody but retained only 4% of the antibody's target specificity. These findings suggest that covalent attachment of C_{60} derivatives to antibodies may not be essential for the development of fullerene immunotherapy (FIT), although the ratio of C_{60}:antibody may need to be minimized so as not to inhibit antibody targeting.

To study the cell internalization characteristics of the fullerene immunoconjugates, two water-soluble Gd@C_{60} derivatives, which allowed for Gd^{3+}
monitoring by inductively-coupled plasma mass spectrometry (ICP-MS) at concentrations <10 ppb, have been utilized. These studies have provided evidence that the fullerene-based immunoconjugates retain the ability to effectively internalize into target cells, with approximately 20% of the available Gd\(^{3+}\) internalizing into the A375m melanoma cells. These results suggest that immunoconjugates derived from C\(_{60}\)-based chemothepapeutics may become new-targeted therapies against cancer.

Of the carbon-based nanomaterials studied in this work, US-tubes are perhaps the most attractive candidates for nanomedicine platforms, due to the possibility of internally loading medically interesting materials, such as Gd\(^{3+}\) ions for magnetic imaging resonance (MRI) or iodine (I\(_2\)) for computed tomography (CT). Toward this end, single-molecule US-tubes have been isolated by chemical reduction of the US-tubes, followed by immediate functionalization using Bingel chemistry to produce debundled and derivatized US-tube materials. Three different malonate addends have been attached to the US-tubes, including serinol, polyethylene glycol (PEG) and amide malonates. Each of the US-tube derivatives exhibited varying degrees of solubilities in water ranging from 0.25 mg/mL to 1.00 mg/mL. An n-octanol/water partition coefficient has also been determined for each derivative, with values ranging from 0.25 to 1.20, which suggest that these derivatized nanocapsules might readily internalize into cells. Loaded internally with medically-useful materials such as Gd\(^{3+}\) ions, I\(_2\) or radionuclides for imaging and therapeutic applications, these biocompatible carbon nanocapsules may be engineered into a universal platform for the containment and delivery of an array of medical agents \textit{in vivo}. 
Acknowledgements

I wish to thank my thesis advisor Professor Lon J. Wilson for the opportunity of working with him in his laboratory over the last five years. His constant optimism and assistance has been invaluable to help foster my growth as a person and a scientist.

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Introduction

1. Serum Therapy: A Precursor to the Monoclonal Antibody

In 1890, Emil Behring and Shibasaburo Kitasato discovered that gradual doses of sterilized diphtheria and tetanus bacilli broth cultures caused animals to produce substances in their blood, known today as antitoxins, which could neutralize the toxins these bacilli generate. They also demonstrated that antitoxins from one animal had the ability to immunize and thus cure another animal showing analogous symptoms of diphtheria.\(^1\) Eventually, Behring concocted a toxin-antitoxin mixture designed to immunize mankind from the effects of toxins from diphtheria bacilli. This immunization led to the near eradication of diphtheria, which Behring regards as the crowning success of his life's work.

Ultimately, Behring's treatments became known as "serum therapy" since all preparations were derived from the serum of animal or human donors. Serum therapy was a common remedy against infectious diseases from 1890-1930.\(^2,3\) However, the introduction of sulphonamide antimicrobial therapy and other preferred classes of antimicrobial chemotherapy led to the abandonment of serum as an antibacterial agent. Still, one scientist, Paul Ehrlich, saw the potential of serum therapy and hypothesized that a "magic bullet" would one day selectively target specific diseases. That "magic bullet" became to be known as the monoclonal antibody (mAb), which are highly specific, purified antibodies derived from only one clone of cells that recognize a single antigen.
2. Hybridoma Technology: A New Era of Antibody Technology

The discovery of hybridoma technology (Figure 1) by Georges Köhler and César Milstein, in 1975, revolutionized antibody therapeutics. A hybridoma is created by fusing two cells, an antibody-producing plasma cell and a cancerous plasma cell, within a single membrane, resulting in a hybrid cell which can be cloned, producing several identical offspring. Over time these daughter clones will secrete the immune cell product. A B-cell hybridoma discharges a single specific antibody, known as the monoclonal antibody. As of 2004, twelve mAbs were licensed for various therapeutic uses, such as preventing organ rejection, treating lymphoma, asthma and Crohn's disease (Table 1). These mAb therapeutic agents have facilitated the urge to further develop new and improved mAb based therapies.

Figure 1. Schematic of hybridoma technology
Table 1. mAbs licensed for clinical use

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Use</th>
<th>Year Licensed</th>
</tr>
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<tbody>
<tr>
<td>Muromonab-CD3</td>
<td>Prevention of organ rejection</td>
<td>1986</td>
</tr>
<tr>
<td>Daclizumab</td>
<td>Prevention of organ rejection</td>
<td>1997</td>
</tr>
<tr>
<td>Rituximab</td>
<td>Treatment of non-Hodgkin's lymphoma</td>
<td>1997</td>
</tr>
<tr>
<td>Abciximab</td>
<td>During cardiac catheterization</td>
<td>1997</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td>Treatment of breast cancer</td>
<td>1998</td>
</tr>
<tr>
<td>Infliximab</td>
<td>Treatment of Crohn's disease</td>
<td>1998</td>
</tr>
<tr>
<td>Basiliximab</td>
<td>Prevention of organ rejection</td>
<td>1998</td>
</tr>
<tr>
<td>Palivizumab</td>
<td>Prophylaxis of RSV disease</td>
<td>1998</td>
</tr>
<tr>
<td>Alemtuzumab</td>
<td>Treatment of chronic lymphocytic leukemia</td>
<td>2001</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>Treatment of rheumatoid arthritis</td>
<td>2002</td>
</tr>
<tr>
<td>Ibritumomab-tiuxetan&lt;sup&gt;90&lt;/sup&gt;Y&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Treatment of lymphoma</td>
<td>2002</td>
</tr>
<tr>
<td>Tositumomab-&lt;sup&gt;131&lt;/sup&gt;I&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Treatment of lymphoma</td>
<td>2003</td>
</tr>
<tr>
<td>Omalizumab</td>
<td>Treatment of asthma</td>
<td>2003</td>
</tr>
<tr>
<td>Cetuximab</td>
<td>Treatment of colon cancer</td>
<td>2004</td>
</tr>
<tr>
<td>Bevacizumab</td>
<td>Treatment of colon cancer</td>
<td>2004</td>
</tr>
</tbody>
</table>

3. Passive Antibody Therapy

Passive antibody therapy has been used to combat many viral, bacterial, fungal and parasitic microorganisms that are responsible for disease (Table 2). This differs from mAb therapy, which is primarily used in treating malignancies and depends on discriminating between antigens expressed in normal and tumor cells. However, recent evidence revealed that mAbs can be generated which also protect against microorganisms, such as *Listeria monocytogenes*, *Histplasma capsulatum* and *Leishmania mexicana*. This revelation has augmented the interest for implementing mAbs for passive therapies. However, numerous challenges must be met for maximum effectiveness of mAb therapies against microorganisms to be attained.
Table 2. Microorganisms that have been targeted using antibody therapies

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Disease in humans</th>
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<tr>
<td><em>Bacillus anthracis</em></td>
<td>Anthrax</td>
</tr>
<tr>
<td><em>Bordetella pertussis</em></td>
<td>Whooping cough</td>
</tr>
<tr>
<td><em>Clostridium tetani</em></td>
<td>Tetanus</td>
</tr>
<tr>
<td><em>Clostridium botulinum</em></td>
<td>Botulism</td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em></td>
<td>Cryptococcosis</td>
</tr>
<tr>
<td><em>Cryptosporidium parvum</em></td>
<td>Cryptosporidiosis</td>
</tr>
<tr>
<td>Enterovirus</td>
<td>Gastrointestinal-tract infections</td>
</tr>
<tr>
<td>Group A streptococci</td>
<td>Necrotizing fascitis</td>
</tr>
<tr>
<td>Hepatitis B Virus</td>
<td>Hepatitis B</td>
</tr>
<tr>
<td>Measles virus</td>
<td>Measles</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>Tuberculosis</td>
</tr>
<tr>
<td><em>Neisseria meningitidis</em></td>
<td>Meningitis</td>
</tr>
<tr>
<td>Parvovirus</td>
<td>Aplastic anemia</td>
</tr>
<tr>
<td>Rabies virus</td>
<td>Rabies</td>
</tr>
<tr>
<td>Respiratory syncytial virus</td>
<td>RSV Infection</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>Pneumonia</td>
</tr>
<tr>
<td>Varicella-zoster virus</td>
<td>Shingles, chickenpox, pneumonia</td>
</tr>
<tr>
<td>Variola major</td>
<td>Smallpox</td>
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</table>

It is feasible to overcome the disadvantages evident in antibody therapies. Antibodies are natural products and thus produced in cell lines or other expression systems. This allows for the theoretical possibility of contamination with other infectious agents, such as prions or viruses. However, meticulous sterility during the antibodies preparation can nullify these possibilities. Another disadvantage is the tendency for the efficacy of antibody therapies to diminish over time. Therefore, for optimal results, antibody preparation must commence in order to coincide with need. Since potential markets for antibody reagents may be modest due to their high specificity, expensive production and laborious administrations, the need may be inadequate when juxtaposed with potential benefits. Fortunately, potential benefits of antibody therapy are immense and will be an impetus for further development of more advanced mAbs.
Antibody-based therapies have several distinct advantages. Since the majority of therapies use human or humanized antibodies, toxicity remains negligible, while maintaining high specificities. High specificity of an antibody is desirable, if not necessary, in order to target only the microorganisms or cells that cause a specific disease. However, this high specificity can be problematic if the microorganism has a high antigenic variation. More than one type of antibody preparation may be needed in order to consistently target specific microorganisms. Hence, high specificity can have deleterious effects on the therapy if there is an emergence of variants that lack the correct determinant recognized by the specific antibody. This could potentially be overcome by creating a "cocktail" of antibodies that exhibit specificity for several unique antigens.

Figure 2. The different biological effects of antibodies
Antibodies also possess the advantage in their versatility to mediate several biological effects after binding (Figure 2). Several modes of antibody function, such as toxin and virus neutralization, occur independent of other immune components. However, functions like antibody-dependent cellular cytotoxicity and opsonization depend on other cellular mediators to function. In these instances the antibody can act as an immunomodulator, assisting in the body's immune response to a foreign microorganism that would normally not cause a direct biological response. Therefore, an antibody can be an important regulator of cellular immunity and inflammatory response.

In addition, antibodies combined with other antimicrobial therapies have shown to increase the efficacies against fungal infections. Antibodies have also been instrumental in the advancement of new vaccines, similar to Behring's serum therapy designed to eradicate diphtheria. Passive antibody therapies have been utilized as vaccines against pneumonia. In fact, several antibody-based therapies have been shown to promote vaccine development. For all these reasons, it is obvious that the utilization of antibodies in medicine is and will continue to be of great interest, especially in the treatment of cancer.

4. "Magic Bullet" Therapies: mAbs as New Age Cancer Therapeutic Agents

Initially, mAb-based therapies for cancer treatments proved to be ineffective due to the murine origins of first generation mAbs. They showed low immunogenic responses and inferior ability to recruit immune effector mechanisms. This led to the development of chimeric and humanized mAbs that contain human Fc domains, but retain portions of
the murine variable region to maintain target specificity. Several of these customized mAbs have been approved for use in oncology therapies (Table 3).

**Table 3. Therapeutic mAbs approved for use in oncology**

<table>
<thead>
<tr>
<th>Generic name (trade name)</th>
<th>Indication</th>
<th>Year</th>
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<tbody>
<tr>
<td><strong>Unconjugated mAbs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trastuzumab (Herceptin)</td>
<td>Breast Cancer</td>
<td>1998</td>
</tr>
<tr>
<td>Rituximab (Rituxan)</td>
<td>Lymphoma</td>
<td>1997</td>
</tr>
<tr>
<td>Cetuximab (Erbitux)</td>
<td>Colorectal cancer</td>
<td>2004</td>
</tr>
<tr>
<td>Bevacizumab (Avastin)</td>
<td>Colorectal, lung cancers</td>
<td>2004</td>
</tr>
<tr>
<td>Alemtuzumab (Campath-1H)</td>
<td>Chronic lymphocytic leukemia</td>
<td>2004</td>
</tr>
<tr>
<td><strong>Immunocjugates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ibritumomab tiuxetan (Zevalin)</td>
<td>Lymphoma</td>
<td>2002</td>
</tr>
<tr>
<td>Tositumomab (Bexxar)</td>
<td>Lymphoma</td>
<td>2003</td>
</tr>
<tr>
<td>Gemtuzumab (MyloTarg)</td>
<td>Acute myelogenous leukemia</td>
<td>2000</td>
</tr>
</tbody>
</table>

Several other therapeutic agents have shown promising preliminary results and are in the late-stage trials for use as cancer therapeutics (Table 4). In fact, mAbs are now being studied that couple with toxic drugs or radionuclides for use in targeted therapies, known as immunocjugates. These immunocjugates are the catalyst behind cell specific drug delivery, known as immunotherapy.

**Table 4. Selected novel (unapproved) mAbs in late-stage trials for cancer**

<table>
<thead>
<tr>
<th>Monoclonal Antibodies</th>
<th>Indication</th>
<th>Sponsor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ch14.18</td>
<td>Neuroblastoma</td>
<td>NCI</td>
</tr>
<tr>
<td>Renarex (WX-9250; cG250)</td>
<td>Kidney cancer</td>
<td>Wilex</td>
</tr>
<tr>
<td>MDX-010</td>
<td>Melanoma</td>
<td>Medarex</td>
</tr>
<tr>
<td>Panitumumab (ABX-EGF)</td>
<td>Lung cancer</td>
<td>Abgenix/Immunex</td>
</tr>
<tr>
<td>Remitogen (Hu1D10)</td>
<td>Lymphoma</td>
<td>Protein Design Labs</td>
</tr>
</tbody>
</table>
5. Immunotherapy: Optimizing Cell-Specific Drug Delivery

Imunoconjugates are mAbs coupled to highly toxic agents that are incapable of administering medicinal needs when left alone. Unmodified mAbs may show therapeutic potency, but their effects are sporadic and thus not curative. Therefore, investigators have shifted attention to conjugating therapeutic agents, such as drugs, toxins or radionuclides to the mAbs. It is critical to select the correct therapeutic agent for this type of therapy to succeed. These therapeutic agents must exhibit three essential properties: 1) demonstrate enough stability to allow the agent to reach the target site before decomposition or decay yields the drug ineffective 2) conjugate in a manner that does not perturb the mAb binding characteristics or drug efficacies and 3) attach the mAb to therapeutics using cross-linkers which allow the immunoconjugates to arrive at the malignant cells still intact so maximum amount of drug delivery can occur. To date, three immunoconjugates have been approved for clinical use. Two implement murine radiolabeled mAbs to treat B-cell lymphomas: a CD20-specific IgG1κ radiolabeled with $^{90}\text{Y}$ (ibritumomab tiuxetan)$^{15}$ and a CD20-specific IgG2aλ radiolabeled with $^{131}\text{I}$ (tositumomab).$^{16}$ The third immunoconjugate is a humanized, CD33-specific IgG4κ mAb attached to a calicheamicin derivative (gemtuzumab ozogamicin) used in leukemia treatment.$^{17}$ Currently, a plethora of other radio-immunoconjugates ($^{131}\text{I}$, $^{90}\text{Y}$ and $^{177}\text{Lu}$ are most common) are undergoing clinical evaluation (Table 5).$^{18}$
Table 5. Selected immunoconjugates in clinical development for cancer therapy

<table>
<thead>
<tr>
<th>Immunoconjugates</th>
<th>Coupled To</th>
<th>Indication</th>
<th>Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1549</td>
<td>^90^Y</td>
<td>Ovarian cancer</td>
<td>Phase III</td>
</tr>
<tr>
<td>SGN-15</td>
<td>Doxorubicin</td>
<td>Lung cancer</td>
<td>Phase II</td>
</tr>
<tr>
<td>C242-DM1</td>
<td>Cytotoxic agent DM1</td>
<td>Pancreatic cancer</td>
<td>Phase II</td>
</tr>
<tr>
<td>CEA-Cide I-131</td>
<td>^131^I</td>
<td>Colorectal cancer</td>
<td>Phase II</td>
</tr>
<tr>
<td>RSB4 (dsFv)-PE38</td>
<td><em>Pseudomonas</em> exotoxin A</td>
<td>Leukemia</td>
<td>Phase II</td>
</tr>
<tr>
<td>SS1P (dsFv)-PE38</td>
<td><em>Pseudomonas</em> exotoxin A</td>
<td>Pancreatic cancer</td>
<td>Phase II</td>
</tr>
<tr>
<td>HuN901-DM1</td>
<td>Maytansinoid DM1</td>
<td>Lung cancer</td>
<td>Phase I/II</td>
</tr>
<tr>
<td>MLN2704</td>
<td>Maytansinoid DM1</td>
<td>Prostate cancer</td>
<td>Phase I/II</td>
</tr>
<tr>
<td>MLN591RL</td>
<td>^90^Y or ^177^Lu</td>
<td>Prostate cancer</td>
<td>Phase I/II</td>
</tr>
<tr>
<td>Lymphocide Y-90</td>
<td>^90^Y</td>
<td>Lymphoma</td>
<td>Phase I</td>
</tr>
<tr>
<td>CEA-Cide Y-90</td>
<td>^90^Y</td>
<td>Metastatic tumor</td>
<td>Phase I</td>
</tr>
</tbody>
</table>

Another area of burgeoning interest is the development of drug-based immunoconjugates in hopes of improving drug efficacy while limiting systemic toxicity by targeting specific malignant cells for drug delivery. For this method to be feasible, immunoconjugates must be produced that are non-immunogenic, display high affinities for tumor associated antigens, possess high drug potency, deliver the drug efficiently in its active state and efficiently internalize into tumor cells. In order to ensure these immunoconjugates' success, cross-linkers (compounds implemented in coupling antibodies to drugs) must be chosen that are relatively stable at neutral pH but undergo hydrolysis in mildly acidic conditions characteristic of lysosomes. Initial immunoconjugates (mAb-doxorubicin and mAb-calicheamicin) incorporated a hydrazone cross-linker as the coupling agent. Recent immunoconjugates have implemented disulfide linkages (mAb-DM1) and peptide linkages (mAb-auristatin) for coupling as seen in Figure 3. This technology is maturing rapidly and development of improved immunoconjugates has tremendous upside for use in cancer therapies.
Figure 3. Chemical structures of some advanced mAb-drug candidates (linkers in parentheses and the labile bonds leading to drug release are shaded).


Antibodies, also known as immunoglobulins play a significant role in the immune system. These targeting agents contain at least four subunits: two identical ~23 kDa light chains and two identical 53-75 kDa heavy chains as shown in Figure 4. These subunits form a Y-shaped symmetric molecule through disulfide bonds and non-covalent interactions. The antibody can be cleaved through proteolysis with the enzyme papain into three ~50 kDa fragments: two identical Fab fragments, which are the arms of the Y-shaped antibody that contain the antigen binding site and one Fc fragment, derived from the stem of the antibody. The Fab arms are connected to the Fc stem of the antibody by a
flexible hinge region, which may vary, causing the antibody molecule to not exhibit perfect symmetry. Antibodies can be developed which recognize only one antigen.

![Diagram of an antibody](image)

**Figure 4. Diagram of an antibody**

There is now a large body of literature regarding the development of cell-targeted delivery agents for imaging and therapeutic applications.\(^{22-24}\) Growth factors, cytokines and antibodies have all been extensively studied for their abilities to deliver payloads to the surface and the cytoplasm of target cells. The antibody designated ZME-018 targets the gp240 antigen (also known as the high molecular weight melanoma-associated antigen, HMWMAA) found on the surface of >80% of human melanoma cell lines and biopsy specimens.\(^{25}\) This antibody has been used extensively in clinical imaging trials\(^{26,27}\) and for the delivery of toxins, cytokines and other therapeutic agents to melanoma cells *in vitro* and *in vivo*.\(^{28}\) Immunoconjugates composed of ZME-018 have shown rapid
internalization into cultures of melanoma cells.\textsuperscript{29} Moreover, these conjugates effectively localize into melanoma xenografts after systemic administration and demonstrate impressive cytotoxic effects against established tumors in orthotopic models.\textsuperscript{30,31} For these reasons, the ZME-018 mAb is the ideal antibody for initial studies into nanotechnology-based immunoconjugation.

7. Small Things, Great Potential: Nanotechnology-based Immunoconjugates

An emerging field in nanotechnology is in biology, specifically medicinal applications. Current research in medical applications of fullerenes,\textsuperscript{32-34} metallofullerenes\textsuperscript{35} and nanotubes\textsuperscript{36,37} has helped foster interest in nanomedicine. Seven properties that make carbon nanostructures "special" for medical applications have been identified:

1. Properly derivatized, they are non-toxic in mammals (LD\textsubscript{50} = \sim 1.0 g/kg).

2. Properly derivatized, they clear efficiently from mammals.

3. Properly derivatized, they can be either intracellular (drug delivery) or extracellular (contrast agents) in mammals.

4. They are non-immunogenic in mammals.

5. They metabolize very slowly in mammals (weeks to months).

\[\text{\rightarrow} \quad \text{Bioinert}\]

6. They can be externally derivatized with medically interesting materials (peptides, drugs, antibiotics, radiopharmaceuticals, antibodies, etc.)

\[\text{\rightarrow} \quad \text{Bioinert Molecular Scaffolds}\]

7. They can be internally loaded with medically-interesting materials (radionuclides, magnetic materials, other small nanostructures, etc.)

\[\text{\rightarrow} \quad \text{Bioinert Molecular Capsules}\]
Fullerene (C$_{60}$) materials have been studied extensively for use in nanomedicine and show great promise. Water-soluble C$_{60}$ derivatives are now commonplace$^{38,39,40}$ and the discovery that water-soluble C$_{60}$ derivatives can cross cell membranes$^{41}$ and even produce transfection$^{42}$ has accelerated interest in utilizing C$_{60}$ for diagnostic and therapeutic medicine. Fullerene toxicity is of some concern, but several water-soluble C$_{60}$ derivatives have demonstrated acceptable cytotoxicity for drug-delivery applications.$^{43}$

A number of water-soluble C$_{60}$ derivatives have been suggested for various medical applications. These applications include neuroprotective agents,$^{44,45}$ HIV-1 protease inhibitors,$^{46}$ bone-disorder drugs,$^{47,48}$ transfection vectors,$^{42}$ x-ray contrast agents,$^{49}$ photodynamic therapy (PDT) agents,$^{50,51}$ and a C$_{60}$-paclitaxel chemotherapeutic.$^{52}$ In addition, endohedral metallofullerenes have demonstrated potential as radiopharmaceuticals$^{53}$ and MRI contrast agents.$^{54-58}$ Fullerene-based micelles have also been developed as a drug delivery system.$^{59}$ To date, however, only the bone-drug application has involved biological targeting of a C$_{60}$-based material,$^{47,48}$ although drug targeting is a desirable, if not essential, component of all drug-delivery strategies.

Interest in carbon nanotube medicinal potential has begun in earnest. Water-solubilizing nanotubes is essential for medical applications to become reality. Much effort has been put forth to attain sufficient water-solubility for implementation of nanotubes into biological systems.$^{60}$ Thus far, solubility properties of derivatized nanotubes have been inadequate for biological use, but several applications have been suggested for nanotube-based medicines including: a carbon nanotube-modified self referencing microelectrode specific to Auxin for use in cancer therapy,$^{61}$ a carbon
nanotube loaded with magnetic particles for magnetically guided drug delivery, a gadolinium filled ultra-short carbon nanotube has shown MRI efficacies 40 to 90 times larger than any other Gd\(^{3+}\)-based contrast agent in current use, and finally, a calcium-doped titanate nanotube has been developed that shows favorable bioactivity in regards to bone regeneration. Similar to fullerene, biological targeting has not been achieved for nanotube-based therapies, which would significantly increase the probability of producing a nanotube-based therapeutic or diagnostic agent.

This work details the conceptual and synthetic development of new water-soluble fullerenes (\(C_{60}\)), endohedral gadofullerenes (Gd@\(C_{60}\)) and ultra-short carbon nanotubes (US-tubes) designed to interact with the ZME-018 mAb as an initial step toward targeted fullerene immunotherapy (FIT) and nanotube immunotherapy (NIT). The nanostructures developed or used in this work to form immunoconjugates with the ZME-018 mAb are shown in Figure 5.
Figure 5. The nanostructures developed or used to form immunoconjugates with the ZME-018 monoclonal antibody
Experimental

1. Materials and Methods

All reagents used were reagent grade or better. Anhydrous material purification was performed under N₂ or Ar (Trigas, purified) atmosphere. Further purification of inert gases was performed in a schlenk line containing R3-11 catalyst (Chemical Dynamics Corp.) on vermiculite and Drierite (CaSO₄). For anaerobic reactions, the solvents were degassed with N₂ or Ar. Desiccators contained Drierite desiccant. Solvent purification procedures were performed according to literature precedent.⁶⁵

The following reagents were used as received: C₆₀ (99.5+% pure, MER Corp.), tetrahydro-1,3-thiazine-2-thione (Aldrich), CS₂ (Aldrich), tert-butanol (Aldrich), P₂O₅ (Fisher), NaHCO₃ (Fisher), ethyl malonyl chloride (Aldrich), NaCl (Fisher), MgSO₄ (Fisher), CBr₄ (Aldrich), DBU (Aldrich), tert-butyl N-(3-hydroxypropyl)-carbamate (Aldrich), TFA (Acros), NaOH (Fisher), conc. HCl (Fisher), SPDP (Pierce), 2-amino-1,3-propanediol (Aldrich), CuSO₄ (Baker), Na₂CO₃ (Fisher), diethyl malonate (Aldrich), 1% F₂ gas in He (Air Products), HiPco Single-walled carbon nanotubes (SWNTs) (Carbon Nanotechnologies Inc.), Na metal (Aldrich), K metal (Fisher), malonyl dichloride (Aldrich), NaH (Acros), oxalyl chloride (Aldrich), PEG (Aldrich) H₂EDTA (Aldrich), CaSO₄ (Drierite), CaH₂ (Acros), 2-iminothiolane (Pierce), iodoacetamide (Aldrich), Na₃PO₄ (Fisher), urea (Pierce).

The following reagents were purified as described: TEA (Acros) was refluxed and distilled from CaH₂ and Ac₂O (Fisher) was distilled.
The following solvents were used as received: petroleum ether (Fisher), acetone (Fisher) and DI water from the laboratory DI faucet.

The following solvents were purified as described: toluene (Fisher) was distilled over Na with a benzophenone indicator, methylene chloride (Fisher) was pre-dried with CaCl₂ and distilled over P₂O₅, EtOAc (Fisher) was distilled from MgSO₄, pyridine (Fisher) was dried with KOH with distillation over molecular sieves and solid KOH, chloroform (Fisher) was distilled over CaCl₂, MeOH (Fisher) was distilled, hexanes (Fisher) were dried with CaCl₂ and distilled over molecular sieves, EtOH (Fisher) was distilled over CaSO₄, THF (Fisher) was distilled from K/Na.

Column chromatography was performed with 70-230 mesh silica gel powder, which was slurry-packed using toluene as the solvent. ZME-018 immunoconjugates were purified with a G-25 sephadex size-exclusion chromatography, after which Bio-Rad protein assays determined the concentration of ZME-018 in the immunoconjugate solution. Enzyme-linked immunosorbent assay (ELISA) was utilized to establish the IC₅₀ value for each immunoconjugate. An ELX 800 UV-vis spectrometer from Bio-Tek Instrument was used to analyze the Bio-Rad assay and ELISA plates at 595 nm. Thin layer chromatography was carried out using silica gel 60, F-254 flexible TLC plates.

High-performance liquid chromatography (HPLC) purification was accomplished on a Hitachi L-6200A Intelligent Pump HPLC system with a Hitachi Model L-3000 UV-vis photodiode array detector using an econosil silica 10μ column (Alltech).

The cation-exchange resin (Bio-Rad) AG 50W-X2 (H⁺ form) removed cations from the serinol adducts of fullerene. Before use, the resin was washed extensively with DI water.
Nuclear magnetic resonance (NMR) solvents were used as received from Cambridge Isotope Laboratories. NMR spectra were obtained on a Bruker 400 MHz spectrometer. Solid-state $^{13}\text{C}$ NMR spectra were obtained on a Bruker AVANCE-200 NMR spectrometer (50.3 MHz $^{13}\text{C}$, 200.1 MHz $^1\text{H}$). A Perkin Elmer Paragon 1000 PC spectrometer collected FT-IR spectra. UV-Vis spectroscopy was performed on a Cary 4 spectrometer with a 1.0 mm quartz cell containing 500 μl of sample in water. The water solubilities and n-octanol/water partition coefficients of the $\text{C}_{60}$ and nanotube materials were determined by UV-vis spectroscopy at 25 °C by the method of Leo.\textsuperscript{66}

Mass Spectra were obtained on a Finnigan Mat 95 mass spectrometer or a Bruker Biflex III MALDI-TOF mass spectrometer. For the MALDI spectra, an elemental sulfur matrix was added to analyte and deposited on the sample plate.

Triplet-triplet absorption measurements and triplet-state decay kinetics were determined after excitation with a 532 nm pulse from a small Q-switched Nd:Yag laser. The samples were dissolved in water and freeze-pumped-thaw degassed three times to remove oxygen.

Thermal Gravimetric Analysis (TGA) was carried out using a SEIKO 1 TG/DTA 200 instrument with an Al pan under argon. The temperature was ramped 10 °C/min.

Transmission electron microscopy (TEM) images were captured with a single drop of nanomaterial deposited on a 300 mesh copper grid, Lacey Carbon Type-A support film, manufactured by Ted Pella, Inc. The sample was allowed to air-dry for 5 min under ambient conditions before imaging. A JEOL 2010 model TEM, operating at 100 keV imaged samples at 30,000X and 80,000X magnification.
Atomic force microscopy (AFM) was obtained using samples which were spin coated on a mica wafer after dispersion and sonication in THF, followed by AFM analysis using tapping mode on a DI Nanoscript 3A instrument.

The concentration of gadolinium in the Gd@C_{60}[C(COOH)_{2}]_{10} and Gd@C_{60}(OH)_{30} samples and immunoconjugates were determined using ICP-AE with a Varian Vista Pro Simultaneous Axial Inductively Coupled Atomic Emission Spectrometer with an atomic emission CCD detector. A calibration curve was obtained using 0.1, 1, 2, 4, 8 and 16 ppm Gd^{3+} standard and sample concentrations were collected three times in replicate with a standard deviation of <2%. The Gd^{3+} concentration for the cell internalization studies were acquired with a Perkin-Elmer Elan 900 inductively coupled plasma-mass spectrometer (ICP-MS). A calibration curve was produced from 0.1, 0.5, 1, 2, 4, 8 and 16 ppb Gd^{3+} standards and sample concentrations collected three times in replicate with standard deviation of <2%. The Gd@C_{60}[C(COOH)_{2}]_{10} and Gd@C_{60}(OH)_{30} samples were graciously donated by TDA Research Inc. of Wheat Ridge, Colorado.

Nanotube functionalization was characterized by elemental analysis using a PHI Quantera X-ray photoelectron spectrometer (XPS). A Monel flow apparatus using a gaseous mixture of 1% F_{2} in He was used to fluorinate the SWNTs, which were then pyrolyzed at 1000 °C in a tube furnace to produce US-tubes. The HiPco SWNTs were obtained from Carbon Nanotechnologies, Inc. of Houston, Texas.
2. Syntheses

All new fullerene compounds have been fully characterized by $^1$H and $^{13}$C NMR, FT-IR, UV-vis spectroscopy and mass spectrometry as appropriate. The data appear in Appendix II. Partial characterization was also performed on previously synthesized compounds and compared with literature reports. The characterization of the new carbon nanotube materials is discussed separately in the Results section.

A. Fullerene Chemistry

Tetrahydro-1,3-thiazine-2-thione (1)$^6$7

In a 100 mL rfb, 10.0 g (0.046 moles) of 3-bromopropylamine hydrobromide was chilled on ice. While stirring, 3 molar equivalents, 10.4 g (0.14 moles) of CS$_2$ was added. In a separate rfb, 4.0 g of NaOH (0.10 moles) was dissolved in 25 mL of ice cold DI H$_2$O. The two solutions were combined and stirred overnight at 0 °C. The solid product was collected by vacuum filtration and washed three times with DI H$_2$O. The crude solid was purified by recrystallization in EtOH to give 4.5 g (0.034 moles) of pure 1 as a white powder; yield 74%. mp 114-116 °C; $^1$H NMR (400 MHz, CDCl$_3$) δ (ppm) 2.20 (p, 2H, CH$_2$), 3.00 (t, 2H, CH$_2$), 3.48 (t, 2H, CH$_2$), 8.63 (bs, 1H, NH); $^{13}$C NMR (400 MHz, CDCl$_3$) δ (ppm) 20.91 (1C, CH$_2$), 30.41 (1C, CH$_2$), 44.80 (1C, CH$_2$), 195.28 (1C, C=O); FT-IR (KBr) ν (cm$^{-1}$) 3442, 1647 (N-H), 2361 (C-S), 1547 (N-C=S); EI-MS calculated for C$_4$H$_8$NS$_2$ (M$^+$) 134.0, found 134.0.
3-Amino-propane-1-thiol (2)\(^{68}\)

10.0 g of 1 (0.075 moles) was dissolved in 75 mL of 18\% HCl to generate a bright yellow solution, which was refluxed until the bright yellow color converted to a clear liquid. The solvent was then removed under reduced pressure to yield an impure white solid coated with a clear oily residue. The crude product was purified in a scintillation vial by placing it under high vacuum overnight. This caused the oily residue to evaporate, leaving 9.1 g (0.072 moles) of pure 2 as a white solid; yield 95\%. \(^1\)H NMR (400 MHz, D\(_2\)O) \(\delta\) (ppm) 1.98 (p, 2H, CH\(_2\)), 2.60 (t, 2H, CH\(_2\)), 3.10 (t, 2H, CH\(_2\)); \(^{13}\)C NMR (400 MHz, D\(_2\)O) \(\delta\) (ppm) 20.88 (1C, CH\(_2\)), 30.88 (1C, CH\(_2\)), 38.38 (1C, CH\(_2\)) ; El-MS calculated for C\(_3\)H\(_6\)NS (M\(^+\)) 91.0, found 91.0.

3-tert-Butylsulfanylpropylamine (3)

2.0 g (0.016 moles) of 2 was combined with 1.5 g (0.020 moles) of tert-butanol and refluxed in 7.0 mL (0.014 moles) of 2 N HCl for 12 hrs. The HCl was then removed under reduced pressure to leave a white solid which was then retreated to 3.5 mL of 2 N HCl and tert-butanol and condensed an additional 10 hr. After removal of HCl a crude solid remained that was purified by recrystallization from toluene to give a white solid. This solid was then placed in a scintillation vial and dried overnight with P\(_2\)O\(_5\) in a drying pistol, giving 2.3 g (0.015 moles) of pure 3; yield 96\%. \(^1\)H NMR (400 MHz, D\(_2\)O) \(\delta\) (ppm) 1.30 (s, 9H, CH\(_3\)), 1.95 (p, 2H, CH\(_2\)), 2.67 (t, 2H, CH\(_2\)), 3.10 (t, 2H, CH\(_2\)); \(^{13}\)C NMR (400 MHz, D\(_2\)O) \(\delta\) (ppm) 24.73 (1C, CH\(_2\)), 27.45 (1C, CH\(_2\)), 30.24 (3C, CH\(_3\)), 39.06 (1C, C-S), 43.15 (1C, CH\(_2\)) El-MS calculated for C\(_7\)H\(_{18}\)NS (M\(^+\)) 148.1, found 148.0.
N-(3-tert-butylsulfanyl-propyl)-malonamic acid ethyl ester (4)

0.9 g (0.006 moles) of 3 was dissolved in 30 mL of anhydrous CH₂Cl₂ on ice and combined with 30 mL of ice-cold saturated sodium bicarbonate. Drop-wise addition of 1.3 mL (0.01 moles) of ethyl malonyl chloride in 6 mL of CH₂Cl₂ initiated the nucleophilic substitution reaction. The solution was stirred on ice for 20 min, followed by stirring at room temperature overnight. DI H₂O was added to quench the reaction and the crude product was obtained by extraction with EtOAc. The aqueous portion was washed three times with EtOAc, which was added to the organic layer. Further washing of the organic layer was accomplished with water (three times) and brine (three times), with subsequent drying over MgSO₄. MgSO₄ was removed by gravity filtration and EtOAc removed under reduced pressure to give impure 4. The crude product was purified using column chromatography with chloroform as the eluant on silica gel, giving 1.2 g (0.0046 moles) of 4 as a golden viscous liquid; yield 92%. ¹H NMR (400 MHz, CDCl₃) δ (ppm) 1.25 (t, 3H, CH₃), 1.28 (s, 9H, CH₃), 1.72 (p, 2H, CH₂), 2.49 (t, 2H, CH₂), 3.23 (s, 2H, CH₂), 3.28 (q, 2H, CH₂), 4.14 (q, 2H, CH₂); ¹³C NMR (400 MHz, CDCl₃) δ (ppm) 14.43 (1C, CH₃), 26.00 (1 C, CH₂), 29.84 (1C, CH₂), 31.29 (3C, CH₃), 39.24 (1C, CH₂), 41.66 (1C, C-S), 42.46 (1C, CH₂), 61.90 (1C, CH₂), 165.48 (1C, C=O), 169.87 (1C, C=O); EI-MS calculated for C₁₂H₂₃O₃NS (M⁺) 261.0, found 261.1.
Asymmetric-protected thiol fullerene (C_{60}) derivative (5)

Using Bingel chemistry,^{69} 500 mg (0.69 mmol) of C_{60} was dissolved in 500 mL of anhydrous toluene in a 1000 L rbf. 45.3 mg (0.17 mmol) of 4 was then added to the reaction flask followed by 56.4 mg (0.17 mmol) of CBr_{4} and drop-wise addition of 52.4 mg (0.34 mmol) of DBU. Stirring continued for 1 hr, and the toluene was removed under reduced pressure. Unreacted C_{60} was removed on a silica gel column using toluene as the eluant. A 10:1 toluene/EtOAc eluant was then used to obtain the pure C_{60} monoadduct. The solvent was removed under reduced pressure to give 71.6 mg (0.073 mmol) of 5 as a red solid; yield 43%. The ^1H NMR spectrum could not be obtained due to broadening of the spectral signals. This is likely due to the product being somewhat paramagnetic. MALDI-MS calculated for C_{72}H_{21}O_{3}NS (M^+) 979.0, found 979.9. Removal of the tert-butyl protecting group to form the free thiol derivative of fullerene (5b) proved fruitless.

Malonic acid 3-tert-butoxycarbonylamino-propyl ester ethyl ester (6)^{52}

According to published procedure, 2.5 g (0.014 moles) of tert-butyl-N-(3-hydroxypropyl)carbamate and 1.5 mL (0.019 moles) of pyridine were combined in 100 mL of anhydrous CH_{2}Cl_{2}. The solution was cooled in an ice bath, during which 2.0 g (0.013 moles) of ethyl malonyl chloride was added drop-wise to the reaction flask under nitrogen. The mixture was stirred at room temperature for 12 hr, followed by quenching of the reaction with DI H_{2}O. Extraction was performed using CH_{2}Cl_{2} with subsequent washing of the organic layer three times with DI H_{2}O. The CH_{2}Cl_{2} was removed under reduced pressure, and the crude product purified on a silica gel column using a 1:1 hexanes/EtOAc eluant to give 2.9 g (0.0098 moles) of pure 6 as a pale yellow oil; yield 76%. ^1H NMR (400 MHz, CDCl_{3}) δ (ppm) 1.28 (t, 3H, CH_{3}), 1.43 (s, 9H, CH_{3}), 1.85 (p,
2H, CH₂), 3.20 (m, 2H, CH₂), 3.39 (s, 2H, CH₂), 4.18-4.28 (m, 4H, CH₂), 5.30 (bt, 1H, NH); ¹³C NMR (400 MHz, CDCl₃) δ (ppm) 14.06 (1C, CH₃), 28.70 (3C, CH₃), 28.94 (1C, CH₂), 37.23 (1C, CH₂), 41.50 (1C, CH₂), 61.42 (1C, CH₂), 62.88 (1C, CH₂), 78.88 (1C, O-C), 156.10 (1C, C=O), 166.61 (1C, C=O), 166.73 (1C, C=O); EI-MS calculated for C₁₃H₂₃O₆N (M⁺) 290.2, found 290.4.

Asymmetric-protected amine fullerene (C₆₀) derivative (7)⁵²

500 mg (0.69 mmol) of C₆₀ was dissolved in 700 mL of toluene, followed by sequential addition of 100 mg (0.34 mmol) 6, 120 mg (0.36 mmol) of CBr₄ and 105 mg (0.69 mmol) of DBU with stirring at room temperature for 1 hr. Toluene was removed under reduced pressure, and the C₆₀ monoadduct purified with column chromatography on a silica gel column using toluene as eluant to remove non-reacted C₆₀. This was followed by a 10:1 toluene/EtOAc eluant to give 170 mg (0.17 mmol) of pure 7 as a reddish-brown solid; yield 50%. ¹H NMR (400 MHz, CDCl₃) δ (ppm) 1.46-1.54 (m, 12H, CH₃), 2.06 (p, 2H, CH₂), 3.38 (m, 2H, CH₂), 4.52-4.60 (m, 4H, CH₂), 4.79 (bt, 1H, NH); ¹³C NMR (400 MHz, CDCl₃) δ (ppm) 14.30 (1C, CH₃), 28.43 (3C, CH₃), 29.18 (1C, CH₂), 37.31 (1C, CH₂), 52.10 (bridgehead C), 63.57 (1C, CH₂), 64.86 (1C, CH₂), 71.52 (C₆₀ sp³ C) 79.47 (1C, O-C), 138.84, 139.18, 140.99, 141.89 142.21, 143.04, 143.90, 144.64, 144.66, 144.70, 144.91, 145.14, 145.16, 145.20, 145.29 (C₆₀ sp² C), 155.94 (1C, C=O), 163.58 (1C, C=O), 163.79 (1C, C=O); MALDI-MS calculated for C₇₃H₂₁O₆N (M⁺) 1008, found 1007.
Asymmetric-amine fullerene (C₆₀) derivative (8)⁸²

170 mg (0.17 mmol) of 7 was dissolved in 100 mL of a 1:1 CH₂Cl₂/TFA solution and stirred for 30 min. The solvents were evaporated off under reduced pressure giving 155 mg (0.17 mmol) 8 as a reddish-brown solid, yield 100%. ¹H NMR (400 MHz, DMSO-d6) δ (ppm) 1.40 (t, 3H, CH₃), 2.09 (p, 2H, CH₂), 2.95 (m, 2H, CH₂), 4.51-4.57 (m, 4H, CH₂), 7.89 (s, 3H, NH₃⁺); ¹³C NMR (400 MHz, DMSO-d6) δ (ppm) 14.95 (1C, CH₃), 27.15 (1C, CH₂), 36.87 (1C, CH₂), 52.97 (bridgehead C), 64.55 (1C, CH₂), 65.45 (1C, CH₂), 72.17 (C₆₀ sp³ C), 139.24, 141.35, 142.22, 142.23 142.57, 143.37, 143.42, 144.24, 144.96, 145.04, 145.07, 145.22, 145.46, 145.50, 145.56 (C₆₀ sp² C), 163.48 (1C, C=O), 163.52 (1C, C=O); MALDI-MS calculated for C₆₈H₁₄O₄N (M⁺) 908, found 908.

Asymmetric-SPDP fullerene (C₆₀) derivative (9)

TEA was added drop-wise to 150 mg (0.165 mmol) of 8 in 20 mL of anhydrous CH₃Cl until the solid completely dissolved, followed by addition of 50 mg (0.160 mmol) of SPDP at room temperature with stirring overnight. The product was purified with column chromatography on silica gel using a 1:1 ratio of toluene/EtOAc as the eluant. Additional purification was performed using HPLC with a 15:1 ratio of toluene/MeOH eluant to give 40 mg (0.036 mmol) of 9 as a reddish-brown solid; yield 23%. ¹H NMR (400 MHz, CDCl₃) δ (ppm) 1.48 (s, 3H, CH₃), 2.10 (p, 2H, CH₂), 2.66 (t, 2H, CH₂), 3.10 (t, 2H, CH₂), 3.50 (q, 2H, CH₂), 4.54-4.61 (m, 4H, CH₂), 6.84 (bt, 1H, NH), 7.16 (m, 1H, ArH), 7.62 (m, 2H, ArH), 8.48 (d, 1H, ArH); ¹³C NMR (400 MHz, CDCl₃) δ (ppm) 14.52 (1C, CH₃), 29.01 (1C, CH₂), 35.33 (1C, CH₂), 36.08 (1C, CH₂), 36.68 (1C, CH₂), 52.30 (bridgehead C), 63.87 (1C, CH₂), 65.23 (1C, CH₂), 71.70 (C₆₀ sp³ C), 120.69 (1C, ArC), 121.37 (1C, ArC), 125.51 (1C, ArC), 137.31, 139.08, 139.33, 142.08, 142.10, 142.42,
143.23, 143.26, 144.10, 144.86, 144.91, 145.12, 145.33, 145.40, 145.41, 145.50 (C_{60} sp^2 C), 149.81 (1C, ArC), 163.88 (C=O), 163.89 (C=O), 171.35 (C=O); MALDI-TOF MS calculated for C_{76}H_{20}O_{3}N_{2}S_{2}: 1104; found: 1105.

\(N,N'-\text{bis}[2-\text{acetyloxy}-1-[(\text{acetyloxy})\text{methyl}]\text{ethyl}]-\text{malonamide (10)}^{39}\)

10 was prepared by slight modifications of a literature procedure. 10.0 g (0.11 moles) of serinol (2-amino-1,3-propanediol) was combined with 7.5 g (0.045 moles) of diethyl malonate and refluxed, using a sand bath, with vigorous stirring at 200-225 °C for 45 min in a 100 mL rbf. The rbf was then removed from the heat to evaporate off the EtOH. The solid, colorless residue was then treated with 40 mL of distilled Ac_2O and pyridine with continuous stirring for 18 hr at room temperature. Finally, 20 mL of chilled MeOH was added carefully to the reaction flask in an ice bath. Solvents were then removed under reduced pressure, and subsequently 75 mL of EtOAc was added. The organic solution was then washed three times with H_2O and saturated NaCl in a 500 mL separatory funnel. The organic layer was dried by contact with MgSO_4, followed by removal of the MgSO_4 by gravity filtration. The EtOAc was removed under reduced pressure to give a yellow solid. The product was further purified by recrystallization from a 2:1 ratio of EtOAc/hexanes to give 12.2 g (0.029 moles) of pure 10 as a white powder; yield 65%. ^1H NMR (400 MHz, CDCl_3) δ (ppm) 2.11 (s, 12H, CH_3), 3.21 (s, 2H, CH_2), 4.21 (m, 8H, CH_2), 4.43 (m, 2H, CH), 7.53 (d, 2H, NH). EI-MS calculated for C_{13}H_{23}O_{6}N (M^+) 290.2, found 418.1.
Asymmetric-protected amine + protected serinol fullerene (C_{60}) derivative (11)

100 mg (0.10 mmol) of 7 was dissolved in 100 mL of toluene, followed by sequential addition of 210 mg (0.50 mmol) of 10, 170 mg (0.51 mmol) of CBr₄ and 120 mg (0.78 mmol) of DBU. After stirring overnight, the solvent was removed under reduced pressure and the crude product purified with column chromatography using a 2:3 ratio of acetone/toluene eluant on silica gel to give 70 mg (0.031 mmol) of 11 as a reddish-orange solid; yield 38% based on the trisadduct of 11. MALDI-TOF MS calculated for C_{90}H_{45}O_{16}N_{3} (M⁺ bisadduct) 1424, found 1423, calculated for C_{107}H_{69}O_{26}N_{5} (M⁺ trisadduct) 1840, found 1840, calculated for C_{124}H_{93}O_{36}N_{7} (M⁺ tetraadduct) 2256, found 2257. For purposes of antibody conjugation, the various isomers of the derivative were not separated.

Asymmetric amine + protected serinol fullerene (C_{60}) derivative (12)

70 mg (0.031 mmol) of 11 was dissolved in 100 mL of a 1:1 CH₂Cl₂/TFA solution and stirred for 30 min. The solvent was removed under reduced pressure to give 52 mg (0.030 mmol) of 12 as a reddish-orange solid, yield 97%. MALDI-TOF MS calculated for C_{85}H_{38}O_{14}N_{3} (M⁺ bisadduct) 1324, found 1325, calculated for C_{102}H_{62}O_{24}N_{5} (M⁺ trisadduct) 1740, found 1741, calculated for C_{119}H_{86}O_{34}N_{7} (M⁺ tetraadduct) 2156, found 2158.
**SPDP + protected serinol fullerene (C_{60}) derivative (13)**

2 mL of TEA was added drop-wise to 100 mg (0.046 mmol) of 12 dissolved in 20 mL of CH\(_2\)Cl, followed by addition of 50 mg (0.160 mmol) of SPDP. The solution was then stirred at room temperature overnight. The crude product was purified by column chromatography on silica gel using a 1:1 ratio of toluene/EtOAc eluant. Further purification using HPLC and a 1:1 toluene/EtOAc solvent system was performed to ensure removal of all unreacted SPDP. The final product gave 28 mg (0.012 mmol) of 13 as a brown-red solid; yield 26%. MALDI-TOF MS calculated for C\(_{95}\)H\(_{44}\)O\(_{13}\)N\(_4\)S\(_2\) (M\(^+\) bisadduct) 1520, found 1523, calculated for C\(_{110}\)H\(_{68}\)O\(_{25}\)N\(_6\)S\(_2\) (M\(^+\) trisadduct) 1936, found 1940, calculated for C\(_{127}\)H\(_{92}\)O\(_{35}\)N\(_8\)S\(_2\) (M\(^+\) tetraadduct) 2352, found 2357.

**Water-soluble SPDP fullerene (C\(_{60}\)) derivative (14)**

Acetate protecting groups were removed from 13 by dissolving 25 mg (0.013 mmol) of 13 in 5 mL degassed methanol, with the subsequent addition of 15 mg (0.137 mmol) Na\(_2\)CO\(_3\) and 1.0 mL of degassed DI H\(_2\)O under argon. The reddish-orange solution was then stirred for 1.5 h, after which a cation exchange resin (H\(^+\) form) was added until the solution was pH 7. After an additional 1.0 h of stirring, the solid impurities were removed by gravity filtration and solvent removed under reduced pressure. The crude solid was dissolved in MeOH to perform column chromatography on silica gel with MeOH eluant. The MeOH was then removed under reduced pressure, to give 12 mg (0.006 mmol) of purified 14 as a reddish-orange solid; yield 49%. MALDI-TOF MS calculated for C\(_{94}\)H\(_{52}\)O\(_{17}\)N\(_6\)S\(_2\) (M\(^+\) trisadduct) 1600, found 1620 (M\(^+\) + 1 epoxide Os); \(^1\)H NMR (D\(_2\)O) was performed to verify that the pyridine moiety was still present on 14 after removal of acetate protecting groups: δ 7.26 (m, 1H), 7.79-7.85 (m 2H), 8.42 (m 1H).
Protected serinol fullerene (C_{60}) derivative (15)\textsuperscript{39}

The multi-adduct C_{60} serinol derivative was prepared by dissolving 50 mg (0.069 mmol) of C_{60} in 250 mL anhydrous toluene. To this solution, 145 mg (0.35 mmol) of 10, 120 mg (0.36 mmol) of CBr\textsubscript{4} and 85 mg (0.55 mmol) of DBU were added sequentially. The solution was stirred at room temperature overnight to help ensure the maximum degree of functionalization. Toluene was removed under reduced pressure to give a red solid, which was purified by column chromatography using a 4:6 ratio of acetone/toluene eluant on silica gel. After solvent removal, the red solid was dried over P_{2}O_{5} in a drying pistol. This gave 116 mg (0.041 mmol based on the pentaadduct) of purified 15 as a red solid; yield 60%. MALDI-TOF MS calculated for C_{145}H_{120}N_{10}O_{50} (M\textsuperscript{+}, pentaadduct) 2801, found 2804 (observe M\textsuperscript{+}- 35, 2769; M\textsuperscript{-} 80, 2724; M\textsuperscript{2-} 122, 2682; M\textsuperscript{3-} 167, 2637; M\textsuperscript{4-} 208, 2596; M\textsuperscript{5-} 248, 2556; M\textsuperscript{6-} 290, 2514; M\textsuperscript{7-} 335, 2469 loss off -OAc groups).

Deprotected (water-soluble) serinol fullerene (C_{60}) derivative (16)\textsuperscript{39}

The serinol functional groups were deprotected by first dissolving 116 mg (0.041 mmol) of 15 in 10 mL of degassed MeOH under Ar. To this solution, 70 mg (0.66 mmol) of Na\textsubscript{2}CO\textsubscript{3} and 2 mL degassed DI H\textsubscript{2}O were added. The red solution was stirred for 1.5 hr, after which a cation exchange resin (H\textsuperscript{+} form) was added until the solution was pH 7. The solution was then stirred an additional 1 hr and the solvent removed to give 75 mg (0.038 mmol) of 16 as a red solid; yield 93%. MALDI-TOF MS calculated for C_{87}H_{48}O_{18}N_{6} (M\textsuperscript{+} trisadduct) 1464, found 1467, calculated for C_{96}H_{64}O_{24}N_{6} (M\textsuperscript{+} tetraadduct) 1712, found 1716, calculated for C_{105}H_{80}O_{30}N_{10} (M\textsuperscript{+} pentaadduct) 1960, found 1963.
B. Ultra-short Carbon Nanotube Chemistry

Malonic acid bis-(3-tert-butoxycarbonylamino-propyl) ester (19)\textsuperscript{70}

According to literature procedure, 5.0 g (0.029 moles) of tert-butyl N-(3-hydroxypropyl) carbamate was dissolved in 250 mL anhydrous CH\textsubscript{2}Cl\textsubscript{2}, followed by addition of 2.0 g (0.014 moles) malonyl chloride. 2.2 g (0.028 moles) of anhydrous pyridine was then slowly added to the reaction vessel. After stirring overnight the reaction was quenched with DI H\textsubscript{2}O. The aqueous and organic layers were separated and the organic layer washed three times with DI H\textsubscript{2}O. CH\textsubscript{2}Cl\textsubscript{2} was then removed under reduced pressure to form a viscous yellow liquid. Further purification of the crude product with column chromatography using a 1:1 ratio of hexane/EtOAc eluant on silica gel gave 3.2 g (0.008 moles) of 19 as a viscous bright yellow liquid; yield 55%. \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) \delta (ppm) 1.38 (s, 18H, CH\textsubscript{3}), 1.86 (p, 4H, CH\textsubscript{2}), 3.19 (q, 4H, CH\textsubscript{2}), 3.39 (s, 2H, CH\textsubscript{2}), 4.20 (t, 4H, CH\textsubscript{2}), 5.30 (s, 2H, NH).

Reduced and fluorinated ultra-short single-walled carbon nanotubes (20)

The SWNTs were produced by the high pressure carbon monoxide (HiPco) process.\textsuperscript{71} Raw SWNTs were fluorinated in a custom-made flow apparatus using a gaseous mixture of 1% F\textsubscript{2} in He at 50 °C for 2 hr, a condition which gave F-SWNTs with a stoichiometry of CF\textsubscript{x} (x ≤ 0.2).\textsuperscript{72} Under an argon atmosphere, the F-SWNTs were pyrolyzed in a tube furnace at 1000°C, driving off volatile fluorocarbons to yield a chemically-cut ultra-short nanotube (US-tube). Upon cooling, the sample was bath sonicated in concentrated HCl for 1 hr to remove iron catalyst impurities. This process produced bundled US-tubes of average length ~30 nm, with ~90% of them shorter than 50 nm\textsuperscript{73} and residual iron of less
than 1.5% by mass. Reduction of the US-tubes was carried out as follows: 30 mg of US-tubes were added to a 250-mL oven-dried rbf, which was then purged with argon. After the addition of 200 mg potassium (or sodium) and 150 mL of anhydrous THF, the reaction mixture was refluxed for 2 hr, followed by 1 hr of sonication. The reduced US-tubes exhibited solubility in THF for 10 days with no visible bundling or precipitation. Excess potassium (or sodium) was removed from the reaction flask in preparation for the Bingel reaction.

Fluorinated US-tubes were prepared using a gaseous mixture of helium-diluted F₂, as described above, at 100 °C. The increased temperature was to help ensure maximum fluorination of US-tubes.


2 g (0.005 moles) of 19 was added to the reduced US-tube solution from 20 or to 25 mg of fluorinated US-tubes suspended in 150 mL of dry THF in a 500 mL rbf. After sequential addition of 2.5 g (0.008 moles) CBr₄ and 1.5 g (0.010 moles) of DBU the reaction was sonicated for 2 hr and then stirred overnight. The solid was then washed extensively with THF and ether (until a clear wash solution was obtained) on a Pyrex Buchner funnel with a fritted disc to avoid US-tube material affixing to the filter. Finally, the solid was placed in a 35 °C oven and dried overnight to give 15 mg of 21 as a black powder; yield 50%.
Protected-serinol functionalized ultra-short carbon nanotube (22)

Reduced US-tubes were functionalized with 10 using the same methodology as for compound 21. 2 g (0.005 moles) of 10 was added to the reduced US-tube solution from 20 in anhydrous THF. While sonicating, 2.5 g (0.008 moles) of CBr₄ and 1.5 g (0.010 moles) of DBU were added sequentially to the reaction flask, sonicated an additional 1 hr and then stirred overnight. The solid was washed with THF and ether similar to 21 and dried overnight in a 35 °C oven. The total amount of 22 recovered was 15 mg; yield 50%.

Deprotected (water-soluble) serinol functionalized ultra-short carbon nanotube: US-tube(Ser) (23)

Acetate protecting groups were removed by sonicating 25 mg of 22 in 50 mL of degassed MeOH for 1 hr, followed by addition of 500 mg Na₂CO₃ and 5 mL of degassed DI H₂O. The solution was then sonicated for 1.5 hr after which cation exchange resin (H⁺ form) was added until the solution was pH 7. The solution was then sonicated for an additional 1 hr. The Na₂CO₃ was removed by washing the US-tube(Ser) three times with DI H₂O, with subsequent centrifugation in a 3200-rpm centrifuge and removing the supernatant, which contained the Na₂CO₃. The total amount of 23 recovered was 6 mg; yield 24%.

Reduced US-tubes were functionalized with diethyl malonate using the same methodology as compound 21 with slight modifications. 50 mg (0.21 mmol) of diethyl bromomalonate was added to 20 mg of reduced US-tubes in a 1:1 ratio of anhydrous toluene/THF solvent system under argon. While stirring, 50 mg (2.0 mmol) of NaH was added to the reaction flask and allowed to stir overnight. The solid was then collected and washed with EtOH and H2O on a PTFE filter to remove excess NaH. After washing the solid was placed in a 35 °C oven to dry overnight. The total amount of US-tube(Ester) recovered was 12 mg; yield 60%.


Hydrolysis of 24 was accomplished by suspending 20 mg of 24 in 5 mL MeOH, followed by the addition of 5 mL 1 M NaOH. The solution was then stirred at room temperature (avoid decarboxylation) for 24 hr; yield 100%.

PEG functionalized ultra-short nanotubes: US-tube(PEG) (26)

1.0 mL (0.011 moles) of oxalyl chloride was added directly to the solution in 25 and sonicated for 24 hrs under Ar. 1.0 mL of PEG, which had been dried over P2O5, was then added to the reaction flask and condensed at 120 °C for 5 days. The solid was collected and washed with EtOH on a PTFE filter to give 9 mg of US-tube(PEG); yield 20%.
C. Antibody Chemistry

**C_{60}-SPDP and C_{60}-serinol ZME-018 Immunoconjugates (17,18)**

2.0 mg of ZME-018 mAb was added to 3.4 mL of phosphate/saline buffer. TEA was then added until pH 8.0, followed by the addition of 1 mM H_{4}EDTA. Free thiol functionalities were then attached to the antibody with addition of 7.8 μL 2-iminothiolane to the above solution with constant stirring under nitrogen at 4°C for 90 min. Non-reacted 2-iminothiolane was removed with a G-25 sephadex size-exclusion column using an eluant consisting of 5 mM bis/tris, 50 mM NaCl and 1 mM H_{4}EDTA at pH = 5.8. Fractions containing the thiol-derivatized antibody were determined using a Bio-Rad protein assay. The antibody fractions were then combined and pH brought to 7.0 with TEA. The antibody solution was halved to allow for immunoconjugation with both the C_{60}-SPDP and C_{60}-Ser samples. 123.7 μL C_{60}-SPDP and 130.8 μL C_{60}-Ser were each added to one of the resulting antibody solutions (10:1 C_{60}:antibody) and stirred overnight at 4°C. A white solid of unreacted antibody precipitated out of the solution during the night. This solid was removed by centrifugation. The immunoconjugates were then purified with a G-25 sephadex size-exclusion column using a buffer of 10 mM Na_{3}PO_{4} and 140 mM NaCl at pH = 7.2 to remove any non-conjugated C_{60} material from the sample. A Bio-Rad protein assay was utilized to determine which fractions contained the immunoconjugate. Aliquots of the purified immunoconjugates were taken and dialyzed overnight in 6 M Urea to ascertain whether any covalent linkages formed between the C_{60}-SPDP and antibody. Several analytical techniques were implemented in the characterization of the immunoconjugates, including triplet-triplet absorption, UV-vis,
transmission electron microscopy (TEM), and Bio-Rad protein assays. These are discussed in full in the Results section.

Enzyme-linked immunosorbent assay (ELISA) was performed to determine if the C₆₀-immunoconjugates retain specificity to the A375m melanoma cells. ELISA plates were prepared by versene-stripping 50,000 gp240-antigen-positive A375m melanoma cells from tissue culture flasks, which were washed 2 times with DPBS. The cells are then rehydrated in DPBS in the individual wells of a Falcon 3912 96-well µl-plates, leaving 2 empty wells for blanks. The plates were dried overnight at 37 °C and stored at 4 °C until used. The ELISA was initiated by adding 200 µl of blocking buffer to each well with incubation for 1 hr at room temperature. The blocking buffer was removed by decanting, followed by immediate addition of 100 µl/well of various antibody standards and unknowns. The plate was incubated for 3 hr at room temperature and solution removed. Each well was washed three times with a washing buffer for preparation of IgG component detection. Concurrently, anti-mouse IgG-HRP was diluted 1:1000 in a dilution buffer, making 11 ml/plate. A 100 µl/well aliquot was added to the cells and incubated for 15 min at room temperature. The wells were then washed three times with a washing buffer. Simultaneously, 11 µl of H₂O₂ was added to 11 ml of ABTS, which was added 100 µl/well to the cells and incubated for 10 min at room temperature. The plate was then read at 405 nm to plot the ELISA binding curve in order to calculate the IC(50) values.
Results and Discussion

1. $C_{60}$-SPDP: The ZME-018 mAb Cross-linking Agent

The goal of this work was to design and develop a new-targeted drug delivery system using carbon-based nanomaterials, including fullerenes ($C_{60}$), gadofullerenes (Gd@$C_{60}$) and ultra-short carbon nanotubes (US-tubes). Derivatives of each nanomaterial have been developed to allow for coupling with the murine anti-gp240 antibody, ZME-018, which has been utilized in clinical trials for imaging in patients with metastatic melanoma.\textsuperscript{29} These derivatives were designed to make optimal use of the special physical and chemical properties exhibited by each nanomaterial, while maintaining the melanoma specificity inherent in the ZME-018 mAb.

Initial focus for antibody coupling was the development of a $C_{60}$-based cross-linking agent. $C_{60}$ is an ideal starting material due to its facile functionalization and nontoxic nature after proper derivatization.\textsuperscript{33,74,75} Also advantageous is the possibility of combining a cell-specific $C_{60}$-antibody conjugate in concert with either a $C_{60}$-drug derivative, such as a $C_{60}$-paclitaxel conjugate\textsuperscript{52} or an endohedral $C_{60}$-radionuclide, such as $^{225}\text{Ac}@C_{60}$,\textsuperscript{53} to serve as the foundation for fullerene immunotherapy (FIT). The practicality of this approach depends on the ability to form a strong interaction, which remains intact under biological conditions, between $C_{60}$ and ZME-018 without disrupting the binding affinity exhibited by the antibody for its antigen (gp240). A conjugation method that had demonstrated the ability to retain binding affinities of antibodies previously was the cross-linking agent, N-succinimidyl-3-(2-pyridyldithio)-propionate
Due to its previous success, SPDP was also chosen as the cross-linking agent for coupling C₆₀ to ZME-018.³⁷

SPDP is a heterobifunctional cross-linking agent, which can undergo aminolysis with its N-hydroxysuccinimide ester (a in Scheme 1) or disulfide exchange with 2-pyridyl disulfide (b in Scheme 1). Recently, SPDP has linked human IgM mAb 16-88 to cobra venom factor,³⁸ mAb 138H11 to the DNA-cleaving enediyne, calicheamicin³⁹ and a fifth generation polyamidoamine (starburst) dendrimer to oligosaccharide moieties away from the antigen binding site of the chimeric mAb, cetuximab.⁴⁰ It is feasible to derivatize C₆₀ with either a thiol (-SH) or an amine (-NH₂), with subsequent coupling with a SPDP derivatized antibody for the thiol derivatized C₆₀ or direct attachment of SPDP to the amine derivatized C₆₀. The advantages and disadvantages of each C₆₀-derivative were compared in order to ascertain which derivative was more conducive for conjugation to the ZME-018 mAb.

Scheme 1. Possible modifications of proteins by N-succinimidyl-3-(2-pyridyl-dithio) propionate (SPDP) (a) introduction of a 2-pyridyl disulfide group into a non-thiol protein by aminolysis and (b) introduction of N-hydroxy succinimide ester structure into a thiol protein by thiol-disulfide exchange
Initial coupling of SPDP to proteins normally occurs via aminolysis (a in Scheme 1), followed by attachment of a thiol-containing compound through new disulfide bond formation with the SPDP-conjugated protein (b in Scheme 1). Therefore, attempts to synthesize a C\textsubscript{60}-thiol derivative (6b in Figure 6) for conjugation to the ZME-018 mAb were first explored.

![Chemical structures](image)

**Figure 6.** Two possible C\textsubscript{60} derivatives designed for conjugation to ZME-018 mAb

Bingel chemistry is a common functionalization method for C\textsubscript{60},\textsuperscript{69} employing cyclopropanation of C\textsubscript{60} with a bromonated malonate in the presence of base. This chemistry is advantageous for three reasons: (1) mild reaction conditions give high yields, (2) exclusive adduct formation at [6,6] double bonds, and (3) the ability to synthesize higher adducts (bis up to hexakis) in one-step with defined stereochemistry using activated 9,10-dimethylanthracene (DMA).\textsuperscript{81} In the infancy of this method, adducts of C\textsubscript{60} were prepared from brominated malonates and NaH.\textsuperscript{69} Recently a more efficient synthesis was developed incorporating an *in situ* bromination of malonates using CBr\textsubscript{4} and DBU.\textsuperscript{82} The second method eliminates the tedious synthesis of bromomalonates,
which have characteristically been difficult to purify due to the formation of dibromomalonate. Therefore, *in situ* Bingel derivatization was chosen for the initial attachment of malonates to C₆₀, followed by generation of a thiol-C₆₀ and subsequent antibody coupling with the SPDP derivatized ZME-018 mAb.

First, N-(3-tert-butylsulfanyl-propyl) malonamic acid ethyl ester (4 in **Scheme 2**) was prepared for attachment to C₆₀. The synthesis began by reacting 3-bromopropylamine with carbon disulfide to form cyclic tetrahydro-1,3-thiazine-2-thione, 1. Acid hydrolysis of 1 with 18% hydrochloric acid and heat produced 3-aminopropane-1-thiol, 2. The thiol functionality was then protected with tert-butyl to form 3. Finally, nucleophilic substitution of ethyl malonyl chloride with the primary amine from 3 gave the desired malonate, 4, for use in Bingel addition to C₆₀.

**Scheme 2.** Synthesis of N-(3-tert-butylsulfanyl-propyl) malonamic acid ethyl ester for Bingel addition to C₆₀
Scheme 3. Synthesis of the thiol-derivatized C$_{60}$

Preparation of the C$_{60}$-thiol derivative 5b (Scheme 3) proved problematic. After Bingel reaction of 4 to C$_{60}$, MALDI TOF-MS of 5 (Appendix A-14) contained the desired molecular weight of the protected thiol malonate adduct (5a, $M^+ = 980$). However, the free thiol molecular ion peak (5b, $M^+ = 925$) was also evident. Unfortunately, it was not possible to determine if this was an ionization fragment or an actual molecular ion peak of 5b. Several attempts to remove the tert-butyl protecting group on 5a using various reaction conditions were unsuccessful. Another difficulty was the inability to obtain a $^1$H NMR spectrum due to peak broadening, which is characteristic of paramagnetic behavior that had been demonstrated by several amide Bingel products.$^8$ Therefore, an alternative C$_{60}$-amine derivative, 8, was prepared for coupling to the ZME-018 mAb. The C$_{60}$ derivative, 8, contains an amine arm that is capable of attaching to SPDP for coupling to thiol-derivatized antibodies. A drawback to
this method is that the antibody must first be derivatized with 2-iminothiolane, forming a free-thiol that can then displace the disulfide on the SPDP attached to C₆₀. This increased the steps in antibody preparation, but allowed for a more useful functionalization of C₆₀.

Synthesis of 8 was accomplished as shown in Scheme 4. First, nucleophilic substitution of ethyl malonyl chloride with tert-butyl-N-(3-hydroxypropyl)carbamate formed the asymmetric malonate, 6. For the C₆₀-antibody coupling an asymmetric malonate was desired for several reasons: (1) to allow for future fluorescent tagging through the non-conjugated malonate arm, (2) to allow for a single SPDP molecule attachment to the antibody per C₆₀ moiety, and (3) to allow for future drug attachment to the non-conjugated malonate arm in order to facilitate targeted drug delivery. The asymmetric malonate, 6, was attached to C₆₀ using in situ Bingel conditions, giving the protected amine C₆₀, 7. Deprotection of the tert-butoxy protecting group with TFA liberated the primary amine, forming the desired C₆₀ derivative, 8, which was characterized using NMR and MALDI-TOF MS (Appendix A-19, A-20).

Scheme 4. Synthesis of asymmetric amine C₆₀ monoadduct
A C\textsubscript{60}-SPDP monoadduct (9 in Scheme 5) was then prepared via aminolysis to test the feasibility of attaching the cross-linker, SPDP to C\textsubscript{60}. TEA was added slowly to 8, followed by the addition of SPDP to form the amide linkage to C\textsubscript{60}, 9, with release of N-hydroxysuccinimide. The NMR and MALDI-TOF MS characterization of 9 is given in (Appendix A21-A23).

Scheme 5. Synthesis of C\textsubscript{60}-SPDP Monoadduct

Preparation of the ZME-018 mAb for coupling to 9 was achieved by attachment of a free-thiol arm to the ZME-018 mAb using 2-iminothiolane (Scheme 6). Nucleophilic attack on the electropositive carbon atom adjacent to the iminium ion allowed for the primary amines from the antibody to sever the C-S bond, thus liberating the alkyl thiol, which is necessary for covalent coupling to C\textsubscript{60}-SPDP derivative. On average, five thiol functionalities are attached to the antibody using this method.\textsuperscript{84} Non-reacted 2-iminothiolane is removed from the thiol-containing antibody by size exclusion chromatography.
Scheme 6. 2-iminothiolane conjugation to the ZME-018 mAb

The coupling of 9 with the ZME-018 mAb occurred by reacting 9 with the ZME-018 solution at pH 7.0. The conjugation reaction was stirred overnight to allow for the new disulfide linkage between C₆₀-SPDP and the ZME-018 mAb to form, with concurrent release of 1H-pyridine-2-thione (Scheme 7). Unfortunately, after stirring overnight, a precipitate was observed consisting of unreacted 9 and denatured ZME-018 mAb, with no indication of conjugate formation. This suggested that 9 was not sufficiently water soluble for coupling to ZME-018.

Scheme 7. Monoadduct C₆₀-SPDP coupling with the ZME-018 mAb
2. Biocompatible C₆₀ Derivatives

For successful coupling of C₆₀ to ZME-018 to occur, the C₆₀-SPDP derivative must display sufficient water solubility. Previously, attachment of serinol malonates, which consist of four hydroxyl water-solubilizing groups, have shown astounding C₆₀ water-solubilizing abilities. In fact, these malonates are the most efficient C₆₀ water-solubilizing adducts to date.⁴⁹ Thus, attaching multiple serinol moieties to the exterior of C₆₀ was used to obtain high water solubility for the C₆₀-SPDP derivative, while retaining the ability to functionalize so that coupling to ZME-018 occurred in a facile manner.

C₆₀-SPDP was made biocompatible by derivatization with 10 (synthesis shown in Scheme 8), followed by subsequent removal of the acetate protecting groups. First, diethyl malonate was condensed with serinol, with concomitant protection of the hydroxyl functional groups with acetate to give 10. As before, in situ Bingel addition was utilized to attach an average of three adducts of 10 to 7 (using a 5:1 ratio of 10:7) to form 11 (Scheme 9).

\[
\begin{align*}
\text{H₂N} & \quad \text{OH} \\
\text{OH} + \text{EtO} & \quad \text{O} \quad \text{Et} \\
& \quad \text{OEt} \quad \xrightarrow{1. \text{ 200-225 } \text{C}} \quad \text{AcO} \quad \text{AcO} \\
& \quad \text{2. Ac₂O, pyridine} \quad \text{AcO} \\
& \quad \text{AcO} \quad \text{O} \quad \text{O} \quad \text{OAc} \\
\text{10} & \quad \text{11}
\end{align*}
\]

Scheme 8. Synthesis of acetate-protected malonodiserinolamide
Biocompatible C\textsubscript{60}-SPDP 14 was obtained in three steps from 11. The tert-butoxy protecting group was removed with TFA to give the primary amine. Then aminolysis of SPDP with the primary amine of 11 was accomplished, yielding 13. Finally, the acetate protecting groups are cleaved, liberating the water-solubilizing hydroxyl functionalities to give biocompatible C\textsubscript{60}-SPDP, 14. Attachment of SPDP to C\textsubscript{60}, before the removal of acetate protecting groups, is vital for the successful preparation of 14.

Scheme 9. Synthesis of water-soluble C\textsubscript{60}-SPDP
A second water-soluble C₆₀ derivative, 16, without the ability to covalently couple with ZME-018 was prepared for use as a control in the conjugation reaction. This compound was previously reported, with attachment of five addends of 10 to C₆₀ (16 in Scheme 10). The reaction proceeds with addition of 10 to C₆₀ in a 10:1 ratio via *in situ* Bingel conditions to yield 15. As before, the acetate protecting groups were then removed, leaving water-solubilizing hydroxyl functional groups to obtain 16 (C₆₀-Ser). The antibody coupling reaction was then performed for both C₆₀-SPDP, 14 and C₆₀-Ser, 16.

![Scheme 10. Synthesis of water-soluble C₆₀-Ser](image-url)
3. Characterization Obstacles

The characterization of the C\textsubscript{60}-antibody conjugates had obstacles to overcome. Mainly, the size differential between the ZME-018 mAb (\textasciitilde 150 kDa) compared to the C\textsubscript{60} derivatives (\textasciitilde 2 kDa) impeded the use of conventional chemical characterization tools, such as NMR, MS, IR, etc. Alternative characterization methods were utilized that exploited the unique physical properties of the C\textsubscript{60} molecule that were not present in the ZME-018 mAb. Spectroscopic techniques, such as UV-vis and triplet-triplet absorption, as well as imaging methods like TEM can distinguish signals and show images from the C\textsubscript{60} molecule without interference from the ZME-018 mAb. Initial design of the C\textsubscript{60} derivatives took into consideration the fact that the C\textsubscript{60} molecule had to be derivatized with an acceptable amount of water-solubilizing addends, while avoiding any deleterious consequences to the unique spectroscopic signature of intact C\textsubscript{60}. It was observed that over-functionalization of C\textsubscript{60} could diminish the spectroscopic signatures. Therefore, it was established that for optimal functionalization, a maximum of four addends were selected for attachment to the C\textsubscript{60}-SPDP derivative (1 addend for SPDP linkage and 3 addends for water-solubilization) to allow for sufficient water solubility for the antibody coupling, while best preserving the C\textsubscript{60} molecule's spectroscopic properties.
4. Conjugation of Biocompatible C_{60} to the ZME-018 mAb

Several reports have been published regarding C_{60} interactions with large biomolecules. C_{60} derivatives have been developed to bind myoglobin,\textsuperscript{86} form electrostatic interactions with cytochrome c,\textsuperscript{87,88} induce protein clusters and complexes in human serum albumin,\textsuperscript{89,90} and enhance catalytic activity via conjugation with the serine protease, subtilisin.\textsuperscript{91} Finally, one study has reported the x-ray crystal structure of a C_{60}-specific monoclonal antibody.\textsuperscript{92} Together, these studies suggested the possibility of creating a C_{60}-antibody conjugate as a proof-of-principle step towards FIT.

Fluorescence spectroscopy and transient absorption spectroscopy have previously been used to detect dendritic C_{60} interactions with cytochrome c.\textsuperscript{88} These spectroscopic probes have the advantage of monitoring C_{60} without interference from the biomolecule. In particular, triplet $\rightarrow$ triplet absorption provides a method to sensitively and selectively monitor C_{60} derivatives through their known spectral and kinetic signatures.\textsuperscript{22} We therefore used transient and ground state absorption measurements to track the fullerene components in synthesized immunoconjugates.

The two C_{60} derivatives, C_{60}-SPDP 14 and C_{60}-Ser 16 were successfully conjugated to the ZME-018 mAb. Coupling of C_{60}-SPDP to the antibody (for ratios of 1:1, 5:1 and 10:1) was accomplished by reacting the thiol derivatized ZME-018 mAb with the SPDP sidearm of C_{60}-SPDP (Scheme 11). The coupling was performed in a salt solution to minimize fullerene aggregation.\textsuperscript{89} Products were purified by size-exclusion chromatography and then examined by transient absorption spectroscopy (Appendix A-33). As shown in Figure 7a, the C_{60} core's 690 nm triplet-triplet spectral signature was clearly present with intensities reflecting the reactant ratio. This technique was utilized as
proof that C\textsubscript{60} material did in fact interact with the ZME-018 mAb, but not to quantify the amount of C\textsubscript{60} enclosed within the immunoconjugate. Unfortunately, it was unclear whether covalent bonds had formed between C\textsubscript{60}-SPDP and the ZME-018 mAb. Therefore, the related water-soluble C\textsubscript{60}-Ser derivative (16), was substituted for C\textsubscript{60}-SPDP in the reaction schemes with ZME-018 mAb (10:1 C\textsubscript{60}-Ser:ZME-018). To our surprise, results for the C\textsubscript{60}-Ser derivative mirrored those of C\textsubscript{60}-SPDP. This implies that C\textsubscript{60}-antibody conjugate formation may not require covalent bond formation.

\begin{center}
\textbf{Scheme 11.} Schematic representation showing the formation of the C\textsubscript{60}-immunoconjugate from C\textsubscript{60}-SPDP (C\textsubscript{60} and antibody figures not to scale)
\end{center}
Figure 7. a) Triplet-Triplet spectrum of C₆₀-SPDP-(ZME-018) immunoconjugate prepared with three different ratios of fullerene to antibody, after chromatographic purification and b) UV absorption spectra of 0.40 μM ZME-018, the C₆₀-SPDP-(ZME-018) immunoconjugate (chromatographically purified), and an unreacted mixture of the two components.

Our quantitative characterization began with Bio-Rad protein assays, which use UV-vis spectroscopy at 595 nm (no C₆₀ interference as shown in Appendix A-34) that showed the concentration of ZME-018 in the chromatographically purified samples as 0.40 μM for C₆₀-SPDP-(ZME-018) and 0.36 μM for C₆₀-Ser-(ZME-018) (calculated from the calibration curve in Appendix A-35). To find the corresponding fullerene concentrations in these conjugates, we used UV-vis spectroscopy. At 440 nm, the molar absorptivity of C₆₀-Ser far exceeds that of ZME-018. The conjugate’s measured 440 nm absorbance (spectra and calibration curve shown in Appendix A-36) directly showed a C₆₀-Ser concentration of 15 μM, implying that the ratio of C₆₀-Ser:ZME-018 was 38:1. Spectral analysis of the C₆₀-SPDP-(ZME-018) conjugation was more complex because absorption bands of C₆₀-SPDP at 440 nm (Appendix A-37) are not intense enough for determining concentrations of <20 μM and at lower wavelengths (<350 nm) there is an overlap from absorption bands from the ZME-018 mAb. To account for this, we first
prepared a reference solution containing only 0.40 μM ZME-018. As shown in Figure 7b, this solution has significant absorption at 282 nm (this is an absorption maxima of the C₆₀-SPDP derivative as shown in Appendix A-37). We then added C₆₀-SPDP until the absorbance of the mixture near 282 nm matched that of the C₆₀-SPDP-(ZME-18) immunoconjugate known to contain a 0.40 μM concentration of antibody. The upper traces in Figure 7b show spectra of this mixture and the conjugate. From the amount of C₆₀-SPDP used to prepare the matching mixture, we deduced a C₆₀-SPDP concentration of 6 μM in the conjugate, corresponding to a C₆₀-SPDP:ZME-018 molar ratio of 15:1. Urea dialysis was performed on both the C₆₀-SPDP-(ZME-018) and C₆₀-Ser-(ZME-018) immunoconjugates in an attempt to determine if any C₆₀-SPDP was covalently attached to the ZME-018 mAb. Urea denatures proteins, which would theoretically cause release of any non-covalently linked C₆₀ material from the ZME-018 mAb, while retaining covalently attached C₆₀. However, after dialysis, both the C₆₀-SPDP (50% loss) and C₆₀-Ser (60% loss) immunoconjugates displayed a reduction in C₆₀ concentration. Even though C₆₀-Ser displayed a slightly greater reduction compared to C₆₀-SPDP, it was inconclusive whether C₆₀-SPDP-(ZME-018) contained any covalent attachment.

ELISA binding curves using antigen-positive cells as targets gave mid-points of 1.2 nM for the C₆₀-SPDP-(ZME-018) immunoconjugate, 26 nM for the C₆₀-Ser-(ZME-018) immunoconjugate (these values were adjusted by a factor of 2 after determining a more accurate ZME-018 concentration using the standard curve in Appendix A-34), and 724 nM for a non-specific, isotype-matched murine IgG antibody used as a control (Figure 8). Amazingly, the C₆₀-SPDP-(ZME-018) conjugate demonstrated binding midpoints nearly identical to the non-conjugated ZME-018 antibody (mid-point of 0.46
nm), even though 15% (by weight) of the immunoconjugate is fullerene. However, the non-covalently bound C$_{60}$-Ser-(ZME-018) conjugate, consisting of 26% (by weight) fullerene, exhibited a much lower affinity than C$_{60}$-SPDP-(ZME-018). Regardless, the C$_{60}$-Ser-(ZME-018) conjugate was still a factor of 30 more effective in binding the target than was the control.

![Graph showing antibody binding affinity](image)

**Figure 8.** ELISA: A375m Dead Cell: Testing of C$_{60}$-ZME-018 immunoconjugates

To visualize the two C$_{60}$-immunoconjugates, TEM images of both were obtained on a lacy carbon grid. Comparative images of the ZME-018 antibody and the immunoconjugates are shown in **Figure 9.** The figure shows that the free antibody appears to have a clear globular structure ~60 nm in diameter, whereas the image of the C$_{60}$-Ser and C$_{60}$-SPDP immunoconjugates are also globular, but 4-5 times larger in diameter. In addition, these immunoconjugate images reveal numerous dark spots scattered throughout the structure that can be attributed to small aggregates of C$_{60}$-Ser, ~2-5 nm in diameter. The larger immunoconjugate sizes may reflect disruption of hydrogen bonding networks inside the antibody or some aggregation effect.
Figure 9. TEM images of a) ZME-018 monoclonal antibody b) C₆₀-Ser-(ZME-018) immunoconjugate and c) C₆₀-SPDP-(ZME-018) immunoconjugate. The scale is the same for each frame; scale bar length is 20 nm. The solid curved feature in the image is the lacy carbon grid material.

The above experiments confirm that covalent bond formation was not necessary to form immunoconjugates of water-soluble C₆₀ derivatives with an antibody, and that antibody to antigen binding was not significantly reduced for high C₆₀:antibody molar ratios (15:1). Further studies explored the cancer cell biology of these new C₆₀-immunoconjugates, as well as immunoconjugates derived from other fullerene-based nanostructures that have the potential for targeted imaging and therapy in medicine.⁵²,⁶³,⁹⁴
5. Cell Internalization of Gd@C$_{60}$-immunoconjugates

Cell internalization studies were performed to determine the efficiency with which the cell-specific C$_{60}$-immunoconjugates internalize into melanoma cells. Antigen positive (A375m) cells were prepared in a 96-well plate (5000 cells/well) using Dulbecco's modified eagle medium. The cells were incubated overnight at 37 °C, followed by addition of 100 µL/well of the C$_{60}$-immunoconjugates over various time frames. Incubation for 1, 4, 8, and 48 hr at 37 °C allowed for cell internalization to occur. At the zero point, the media was removed and each cell sample washed with DPBS to strip off any non-internalized C$_{60}$ immunoconjugate. Cells were then detached from the bottom of the plate and lysed in order to determine if the C$_{60}$-immunoconjugates internalized into the cells. Triplet-triplet absorption was once again implemented as a qualitative measure of C$_{60}$ cell internalization. Unfortunately, attempts at observing C$_{60}$ in the lysed cell solution by triplet-triplet absorption proved unproductive, showing no characteristic C$_{60}$ triplet-triplet bands. It was concluded that the sensitivity of triplet-triplet absorption spectrum was insufficient to detect the C$_{60}$-materials at concentrations <20 nM, which was the approximate amount of C$_{60}$ expected to internalize into the melanoma cells.

An alternative method that has shown sensitivity in the nM range is inductively-coupled plasma mass spectrometry (ICP-MS), with previous concentration determinations of several elements in water or waste extracts of digests <20 nM range, which was within the concentration range of C$_{60}$ expected to internalize into the cells. However, this method required an element other than carbon to detect. Fortunately, great strides have been made in the preparation and purification of water-
soluble gadofullerenes, Gd@C_{60}[C(COOH)_2]_{10} (Gd-COOH, Figure 5c) and Gd@C_{60}(OH)_{30} (Gd-OH, Figure 5d),\textsuperscript{46} which were implemented to monitor the amount of C_{60} internalized into the A375m melanoma cells.

Immonoconjugates of Gd@C_{60}(OH)_{30} and Gd@C_{60}[C(COOH)_2]_{10} were prepared in similar fashion as the C_{60}-based immunoconjugates. Using ICP-atomic emission spectroscopy (ICP-AE), the Gd-OH and Gd-COOH concentrations in the immunoconjugates were determined to be 180 nM and 47 nM. Bio-Rad protein assays then determined the antibody concentration in the Gd-OH-(ZME-018) to be 875 nM (for a 1:5 molar ratio of Gd-OH:antibody) and 624 nM for the Gd-COOH-(ZME-018) (for a 1:13 molar ratio of Gd-COOH:antibody). The amount of Gd@C_{60} was, therefore, significantly less than for empty C_{60} in the C_{60}-immunoconjugates prepared above. This may be attributed to greater aggregation of the Gd@C_{60}-derivatives, when compared to the empty C_{60} derivatives. This greater degree of aggregation stems from the inability of Gd@C_{60} aggregates to thoroughly separate at the salt concentration and low temperature utilized in the immunoconjugation.\textsuperscript{89} It appears that the C_{60}-Ser and C_{60}-SPDP are able to disaggregate to greater extents under the conditions used for immunoconjugation. This implies that the Gd@C_{60} immunoconjugates reported here actually contain mostly empty C_{60} derivatives, C_{60}(OH)_x and C_{60}(COOH)_x, rather than Gd@C_{60} materials. In fact, the initial Gd@C_{60}(OH)_{30} and Gd@C_{60}(COOH)_{10} samples used to prepare the immunoconjugates only contain 70% and 50% gadofullerene, respectively, with the remainder of the sample being empty C_{60} derivatives. In order to test this hypothesis, UV-vis spectra (Figure 10) of the Gd-OH-(ZME-018) immunoconjugate was obtained and compared with the Gd@C_{60}(OH)_{30} spectra at 180 nM (value of Gd^{3+} in immunoconjugate
as determined by ICP-AE). It was observed that the Gd-OH immunoconjugate spectrum exhibited an absorbance from 280-600 nm. In contrast, Gd@C_{60}(OH)_{30} diluted to 184 nM (concentration of Gd^{3+} in the immunoconjugate) displayed no absorbance over the same range. The most reasonable explanation for this observance was that the absorbance from the Gd-OH immunoconjugate is due to empty C_{60} derivatives within the ZME-018 mAb. For comparison, a 1 μM Gd@C_{60}(OH)_{30} solution was prepared, which shows an absorbance spectra similar to the immunoconjugate. This suggests that the Gd-OH immunoconjugate consisted of an abundance of empty C_{60} material compared to Gd@C_{60}(OH)_{30}. However, attempts to quantify the amount of empty C_{60} using UV-vis is not possible using a standard curve from Gd@C_{60}(OH)_{30} samples.

![Absorbance spectra](image)

*Figure 10. UV-vis spectrum of Gd@C_{60}(OH)_{30} and its immunoconjugate*

TEM images of the Gd@C_{60} immunoconjugates were acquired in order to visualize the Gd-OH and Gd-COOH interaction with the ZME-018 mAb (Figure 11). Similar to the C_{60}-based immunoconjugates, the ZME-018 mAb has increased in size and contains aggregates of the C_{60}-based nanomaterials as seen by the uniform black spots.
These results show that the Gd@C₆₀ materials display similar interactions (to a smaller extent) as the C₆₀-SPDP and C₆₀-Ser derivatives with ZME-018, but that conjugation conditions must be optimized in order to increase the amount of Gd@C₆₀-derivatives in any of the ZME-018 mAb conjugates.

![Figure 11. TEM images of a) Gd-OH-(ZME-018) and b) Gd-COOH-(ZME-018)](image)

Cell binding affinity was once again evaluated by calculating IC(50) values from ELISA plots. Similarly to the C₆₀-immunoconjugates, dry cell A375m (antigen +) cells were utilized. However, to better understand binding efficiencies, SK-BR-3 (antigen -) cells were also used for comparison with the antigen positive cells. The Gd-OH-(ZME-018) and Gd-COOH-(ZME-018) immunoconjugates ELISA biding curves and IC(50) were each analyzed with both cell lines (Figure 12). The IC(50) values and hence binding efficiencies to the A375m cells for the Gd-COOH immunoconjugate was 2.1 nM and Gd-OH immunoconjugate was 1.5 nM. This is practically identical to non-conjugated ZME-018, which demonstrated a IC(50) value of 3.6 nM (plot not shown). When juxtaposed with the SK-BR-3 antigen negative cell line, which showed the IC(50) values as 14 nM for the Gd-OH immunoconjugate (nine times less efficient) and 49 nM for the Gd-COOH-immunoconjugate (23 times less efficient), it is evident that the retained cell
specificity of the Gd@C$_{60}$-based immunoconjugates is a major step forward for the future development of FIT.

![Graph](image)

**Figure 12.** ELISA A375m and SK-BR-3 dead cell tests of the Gd@C$_{60}$-immunoconjugates

Cell internalization studies for the Gd@C$_{60}$-immunoconjugates were performed in a manner similar to that for the C$_{60}$-immunoconjugates. Deviations from the previous method occurred when lysing the cell. Instead of using a lysis buffer, cells were removed from the plate and placed in a scintillation vial. Approximately 1.5 mL of 25% chloric acid was added to the vial and heated to 90 °C for 30 min in order to consume the cells and destroy the C$_{60}$ cage around gadolinium. After cooling, 10 mL of 2% nitric acid was then added as the matrix utilized for ICP-MS.

The Gd$^{3+}$ concentration was determined in triplicate using ICP-MS for cell internalization studies using both Gd-OH-(ZME-018) and Gd-COOH-(ZME-018) (Figure 13). Standard deviations were determined for the three aliquots of one cell
internalization sample at each time point. This deviation only shows the accuracy of the ICP-MS. In order to obtain more accurate Gd\(^{3+}\) internalization data, a greater number of separate cell internalizations must be performed and analyzed. Regardless, for the Gd-COOH-(ZME-018) conjugate, it is clear that the amount of Gd-COOH immunoconjugate that internalizes remains relatively constant over time, between 10-13 nM. However, the Gd-OH immunoconjugate appears to exhibit a slight increase in delivery of Gd-OH, with the concentration increasing from 15 to 23 nM over time from 1 to 48 hr. This contrast could be attributed to much greater Gd-OH concentration found in the Gd-OH-(ZME-018) immunoconjugate (180 nM vs. 47 nM). It is reasonable for the Gd-OH-(ZME-018) to internalize Gd\(^{3+}\) ion to a greater extent due to its higher Gd\(^{3+}\) concentration in the immunoconjugate.

![Graph showing concentration over time for Gd-COOH and Gd-OH immunoconjugates](image)

**Figure 13.** Cell internalization of the Gd@C\(_{60}\)\([\text{C(COOH)}\]\)\(_{10}\) and Gd@C\(_{60}\)(OH)\(_{30}\) immunoconjugates over time
These initial internalization experiments demonstrate the feasibility of utilizing ICP-MS for determining $[\text{Gd}^{3+}]$ at very low concentrations after cell internalization of Gd-OH and Gd-COOH immunoconjugates into A375m cells. For comparison, attempts to internalize the Gd@$\text{C}_{60}$-immunoconjugates into TXM-1 antigen negative cells were performed. These internalizations showed no internalization of the Gd$^{3+}$ into the TXM-1 cells, demonstrating that the Gd@$\text{C}_{60}$-immunoconjugates retained their cell specific properties, as well as verifying that cell internalization into the A375m cells was successful. A second study, which analyzed both the cells and the exo-cellular wash solution, revealed that approximately 20% of Gd-OH immunoconjugate are internalized into cells, while the other 80% eluted with the wash solution. These results suggest that Gd@$\text{C}_{60}$-based immunoconjugates do internalize into cells and that optimization of this internalization will be needed for the future development of FIT.
6. Future Directions: Towards C$_{60}$-based Cancer Therapies

Fullerene (C$_{60}$) and Gd@C$_{60}$ immunoconjugates have been prepared using 10:1 ratios of C$_{60}$:antibody, yielding immunoconjugates that are loaded with C$_{60}$ material equal to 15:1 C$_{60}$:antibody, without negatively effecting the cell binding properties characteristic of the ZME-018 mAb. Determining optimal ratios of C$_{60}$:antibody to allow for efficient internalization into the A375m cells is imperative for further development of a cell-specific C$_{60}$-based cancer therapy. A major challenge in utilizing the Gd@C$_{60}$ derivatives for cell internalization is that these materials consist of 30-50% empty C$_{60}$. Obtaining 100% encapsulated Gd@C$_{60}$ material would allow for internalized C$_{60}$ materials to contain a greater amount of gadolinium, in effect increasing the amount of Gd@C$_{60}$ immunoconjugate available for internalization into the melanoma cells.

After determining the optimal C$_{60}$:antibody ratios for cell internalization, studies should progress towards the creation of a "drug cocktail" that is specific for cancer cells. Combining the immunoconjugates with a C$_{60}$ derivative externally loaded with drugs, such as the paclitaxel conjugate shown in Figure 14, could provide for time-released drug delivery to cancer cells. In time, it may be possible to attach several different drugs to the external cage of the same C$_{60}$ moiety, which could prove to be a significant advantage over current approaches in targeted chemotherapy.

![Figure 14. A C$_{60}$-paclitaxel conjugate](image)

Recent advances in Gd\textsuperscript{3+}-ion\textsuperscript{63} and iron oxide\textsuperscript{97} filled carbon nanotubes as contrast agents (CAs) for magnetic resonance imaging (MRI) has stimulated interest in developing diagnostic imaging agents from nanotube-based materials. Properly-engineered, shortened carbon nanotube "capsules" derived from full-length single-walled carbon nanotubes (SWNTs) and filled with medical imaging agents offer the opportunity to develop these diagnostic agents, specifically contrast agents for intracellular molecular imaging.\textsuperscript{98,99} Such bio-inert carbon nanocapsules, with their imaging-agent cargos safely sequestered within, are lipophilic and intrinsically intracellular agents, even when decorated externally with biocompatible coatings. New CAs were designed to be biocompatible over an extended period of time in vivo, for computed tomography (CT) x-ray imaging. In contrast most other CT agents in the clinic today are highly hydrophilic and intrinsically extracellular agents designed to clear within hours from the body, largely because of toxicity concerns. With the exception of orally-ingested BaSO\textsubscript{4} slurries for gastrointestinal imaging, the commonly-employed clinical CAs are based on the 2,4,6-triiodinated-5-aminoisophthalic acid structure (with Iohexol being a common example),\textsuperscript{100} where iodine (Z = 127) serves as the x-ray opaque element. It is feasible for these new CAs to form similar interactions with antibodies, as does C\textsubscript{60} for use in targeted single-molecule x-ray imaging.

Interest in these materials for diagnostic imaging is largely due to five reasons: 1) the exterior of nanotubes can be derivatized for biocompatibility and cellular targeting,\textsuperscript{101} 2) properly-derivatized nanotubes have demonstrated acceptable cytotoxicities for drug delivery,\textsuperscript{102,103} 3) nanotubes are relatively bio-inert and have been shown to be excreted
intact from mammals,\textsuperscript{104} 4) derivatized nanotubes readily translocate into mammalian cells,\textsuperscript{105} and 5) nanotubes are hollow and can easily be loaded with metal ions (Gd\textsuperscript{3+} for MRI or Mn\textsuperscript{2+} radionuclides for nuclear imaging)\textsuperscript{63} or small molecules (I\textsubscript{2} for computed tomography (CT) x-ray imaging)\textsuperscript{106} of medical interest. Collectively, these properties promise the potential for developing carbon nanotube materials into intracellular agents for molecular imaging, and because of their small size [especially for ultra-short carbon nanotubes (US-tubes) of \(\leq 50\) nm in length],\textsuperscript{72,73} a large number of such agents could accumulate within each cell to enhance image intensity. The US-tube material derived from the fluorine (F\textsubscript{2}) cutting of full-length SWNTs is ideal because the procedure creates uniform length nanocapsules (\(\sim 95\% \leq 50\) nm) with small defects in the side-walls which serendipitously facilitate a uniform internal loading of ions and small molecules.\textsuperscript{63} In order to maximize the potential of these loaded US-tubes they must be conjugated with antibodies for specific cell targeting. For this conjugation to be attainable the US-tubes must be made biocompatible and hence, water-solubilizing functionalities must be attached to the sidewalls of the US-tubes. We prefer HiPco SWNTs as the starting material of choice because purity and uniformity, two characteristics of HiPco SWNTs, are important for drug development.
8. Bingel Functionalization of US-tubes

One major hurdle, which plagues both SWNTs\textsuperscript{107} and US-tubes\textsuperscript{73} is the tendency to form bundles, impeding solubility, and thus implementation into biology and medicine. The large $\pi$-electron system, which contributes to SWNTs unique electronic properties, has deleterious consequences on solubility. The $\pi-\pi$ interaction results in aggregated bundles which have a van der Waals binding energy of $\sim$0.5 eV per nanometer of tube-tube contact.\textsuperscript{108} Debundling and subsequent water suspension of SWNTs has been achieved by wrapping them in polymers, such as polyvinyl pyrrolidone (PVP) and polystyrene sulfonate (PSS),\textsuperscript{109} and surfactants, like sodium dodecyl sulfate (SDS).\textsuperscript{110,111} Unfortunately, these methods are not capable of solubilizing US-tubes, as they flocculate from suspension with moderate centrifugation.

The current model of SWNT dispersion postulates that sonication separates SWNTs at tube ends because they contain large length:width aspect ratios, imparting relative flexibility. Once the surfactant or polymer wraps around a tube end, it can propagate along the bundle length, eventually separating into an individual surfactant-coated nanotube.\textsuperscript{112} Current belief is that since US-tubes are so small ($<$50 nm), they act as rigid rods, making this method of debundling futile because there is insufficient torque to peel the tubes off from one another. Therefore an alternative method must be designed to create and isolate individual US-tubes if they ever expect to realize their potential for bioapplications.

Similar to fullerenes the Bingel reaction can be employed to functionalize US-tubes.\textsuperscript{69,82} This allows for further side chain chemistry off the ester or amide Bingel malonate addend, which can be used as a scaffold for various water-solubilizing
functional groups, such as amines and hydroxyls.\textsuperscript{39,70} Previously, Bingel addition has been performed on SWNTs using diethyl bromomalonate, making it an ideal candidate to functionalize US-tubes for use in bioapplications.\textsuperscript{113,114} However, for Bingel addition to be effective, both in regards to the degree of functionality and the attainment of single US-tubes, the US-tubes must first be debundled, followed by immediate functionalization, to prevent bundle reformation.

Two strategies to individualize US-tubes, which both allow for subsequent functionalization, were examined, fluorination and alkali reduction. Fluorinated SWNTs have been shown to exfoliate and allow for subsequent nucleophilic substitution (S\textsubscript{n,1}) reactions to occur.\textsuperscript{115} A second paradigm, the Birch reduction, uses alkali metals in liquid ammonia to form SWNT salt complexes that are soluble in organic solvents without using sonication, surfactants or functionalization.\textsuperscript{116,117} Attachment of malonate addends to US-tubes was accomplished using \textit{in situ} Bingel conditions, which enable US-tube functionalization without the complex preparation of bromomalonate, allowing for a variety of malonate addends to be affixed to the US-tubes.

A two-fold strategy was employed to develop Bingel US-tubes. First, the US-tubes are individualized to obtain single US-tubes by either fluorination or reduction, followed by immediate derivatization to prevent rebundling. Here, both methods were compared to determine the extent of exfoliation and the degree of subsequent functionalization.
Scheme 12. Synthesis of US-tube(amide), n=4-5 per nanometer

Protected amine functionalized US-tube derivatives, designated as US-tube(amide), were initially prepared to demonstrate successful implementation of our two-fold US-tube derivatization strategy. First, the malonate addend 19 was synthesized from tert-butyl N-(3-hydroxypropyl) carbamate and malonyl chloride via nucleophilic substitution as shown in Scheme 12. Attachment to individual US-tubes were then accomplished by in situ Bingel addition of 19 to reduced or fluorinated US-tubes to yield the US-tube(amide) 21. The deprotection of 21 to form the primary amine has yet to be explored. ATR-IR, TGA, XPS and NMR were used to investigate the extent of derivatization on the sidewall of the US-tube(amide).

The extent of exfoliation by reduction and fluorination was determined by AFM (Figure 15 and Appendix A-39). Measuring the z-heights of US-tubes and derivatives divulged substantial insight into the relative debundling of each species. A single HiPco tube is on average 1.0 nm diameter, though may vary from 0.5-2.0 nm. Initial AFM
analyses of purified US-tubes measured z-resolution heights in excess of 7.0 nm (Appendix A-39c)—clearly suggesting a heavily bundled US-tube sample. Subsequent AFM analysis on fluorinated-US-tubes showed z-resolution heights ranging from single US-tubes of 1.4 nm to significantly bundled US-tubes of over 5.5 nm (Appendix A-40d). In comparison, reduced US-tubes showed z-resolution heights ranging from 0.5-1.5 nm, corresponding to single US-tubes (Appendix A-40c). Clearly, reduction produced the most efficient debundling of US-tubes. The US-tube(Amide) derivatives mirrored debundling data with reduced US-tubes manifesting z-heights ranging from 1.1-2.1 nm (Figure 15), while US-tube(Amide) derivatives from fluorinated US-tubes revealed z-height ranges from 0.9-4.0 nm (Appendix A-39d).

![Figure 15. a) AFM height image of US-tube(Amide) after reduction and b) Z-scan resolution height analysis of US-tube(Amide) after reduction](image-url)
It would be expected that reduced US-tubes would functionalize to a greater extent due to their greater exposed surface area. However, elemental XPS data indicates the contrary. XPS analysis shows the presence of Bingel functionalization as measured by increased nitrogen-content resulting from Bingel addition of 19 to US-tubes (Table 6). For comparison, the Bingel reaction was performed on purified US-tubes, demonstrated moderate functionalization with an increase in nitrogen atomic percent of 1.5%. Both the fluorinated US-tube(Amide) (increase 4.5%) and reduced US-tube(Amide) (increase 3.1%) derivatives comprised a greater degree of functionalization relative to pure US-tubes, due to the inherent debundling of the reduced and fluorinated US-tubes. Subsequent reactions on US-tube(Amide) found that the nitrogen percent plateaus at about 5%. Assuming that 1 nm of US-tube consists of 120 carbons, it is calculated that approximately 4-5 Bingel malonate groups are attached per nm of US-tube.

Table 6: XPS analysis of US-tubes and derivatives (± 0.5%, numbers in atomic %)

<table>
<thead>
<tr>
<th>Sample</th>
<th>C %</th>
<th>F %</th>
<th>N %</th>
<th>K %</th>
<th>O %</th>
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<tr>
<td>US-tubes</td>
<td>90.4</td>
<td>0.9</td>
<td>0.5</td>
<td>0.0</td>
<td>8.0</td>
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<tr>
<td>Reduced US-tubes</td>
<td>75.3</td>
<td>0.6</td>
<td>0.4</td>
<td>12.5</td>
<td>10.5</td>
</tr>
<tr>
<td>Fluorinated US-tubes</td>
<td>71.2</td>
<td>18.7</td>
<td>0.5</td>
<td>0.0</td>
<td>9.6</td>
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<tr>
<td>US-tube(Amide)</td>
<td>91.8</td>
<td>0.3</td>
<td>2.0</td>
<td>0.0</td>
<td>5.9</td>
</tr>
<tr>
<td>Fluorinated US-tube(Amide)</td>
<td>80.7</td>
<td>5.3</td>
<td>5.0</td>
<td>0.0</td>
<td>9.3</td>
</tr>
<tr>
<td>Reduced US-tube(Amide)</td>
<td>86.4</td>
<td>0.3</td>
<td>3.5</td>
<td>1.2</td>
<td>8.1</td>
</tr>
</tbody>
</table>

The greater functionalization of fluorinated US-tubes over reduced US-tubes can be attributed to the electron-withdrawing character of fluorine. The Bingel malonate addend behaves as a nucleophile, reacting favorably with electron deficient carbons. The reduced US-tubes are coated with ~10 e⁻/nm, which causes an electrostatic repulsion to
exfoliate US-tubes, but impedes the nucleophilic Bingel addition of malonate addends to the US-tubes. Conversely, the fluorinated US-tube incorporates no additional negative charge, while possessing an abundance of electron-withdrawing fluorine atoms. The fluorine attached to the US-tube acts as an electron sink, causing an increase in electropositive character at the reaction site, creating an environment conducive for $S_n1$-reactions. A second hindrance exhibited by reduced US-tube is the tendency for negatively charged species to undergo hydrogenation, promoting C-H bond formation, which has previously been observed with reduced SWNTs.$^{119}$ Hydrogen ions are produced during the in situ bromination of 19, which can compete for reaction sites on the reduced US-tube, in effect diminishing the reaction sites available for attachment of 19, accounting for the lesser degree of functionalization exhibited by the US-tube(amide) from the reduced US-tubes.

The reduced US-tube(amide) was characterized by ATR-IR, TGA and NMR. The ATR-IR spectrum (Appendix A-41) confirms the presence of carbonyl functionality due to the strong C=O stretch at 1736 cm$^{-1}$. This corresponds to the carbonyl ester groups from attached malonate 19. Degradation of the US-tube(amide) is evident in the TGA plot as the temperature is ramped to 350 °C (Appendix A-42). This is characteristic of side-chain cleavage of the malonate from the US-tube(amide). TGA was performed on a US-tube/malonate mixture (not covalently attached) for comparison, in which the malonate volatilized at 200 °C, confirming that the loss of mass in the US-tube(amide) is indeed from covalently-attached malonates of the US-tubes. After cleavage, approximately 40% of the US-tube mass remains, indicating that approximately 60% of US-tube(amide) mass is contributed from the Bingel malonate addends. This is
consistent with XPS data that calculated the attachment of approximately 4-5 Bingel malonates per nm of the US-tubes.

The basic $^1$H-$^{13}$C cross-polarization/magic-angle spin (CP-MAS) spectra was acquired with 7 kHz MAS, a 1-ms contact time, 29.3-ms free induction decay (FID), and 5-s relaxation delay.$^{120}$ The FID after 48,400 scans was processed with 50 Hz (1 ppm) of line broadening. The dipolar-dephasing spectrum differed only in that after CP; two 25-μs dephasing periods with a 180° $^{13}$C refocusing pulse in the middle were used before FID acquisition in order to eliminate the methylene signals. The FID obtained after 67,600 scans was processed with 50 Hz of line broadening. Chemical shifts are reported relative to the carbonyl carbon of glycine defined as 176.46 ppm.$^{121}$

The basic $^1$H-$^{13}$C CP-MAS spectra (Figure 16) indicate sp$^3$ and sp$^2$ functionality. The upfield portion of the aliphatic signal results from overlapping signals from the tert-butyl methyl carbons and two of the three different types of methylene carbons. A peak maximum of 26 ppm is upfield of what would be expected for such carbons$^{70}$ and indicates that the US-tube is exerting a shielding effect on the addend.$^{120}$ The downfield tail of the aliphatic signal is consistent with overlapping signals from the different quaternary carbons of the cyclopropane ring, the methylene carbon adjacent to oxygen, and the tert-butyl quaternary carbon (also adjacent to oxygen). The carbons of the cyclopropane ring can be expected to give relatively weak signals in light of their distance from the nearest protons, while the tert-butyl quaternary carbon can be expected to give a relatively weak signal resulting from weak $^1$H-$^{13}$C dipole-dipole interactions with the highly mobile methyl protons.$^{122}$ The prominent sp$^2$ signal at about δ120 clearly results from unfunctionalized sp$^2$ carbons of the US-tube, while its downfield tail is
consistent with overlapping signals from the carbamate and ester carbonyl carbons. The signal at about 8120 can reasonably arise from cross polarization from methylene protons of the addend lying along the US-tube, a particularly clear example of the through-space nature of cross polarization.

The CP-MAS spectrum with a pair of 25-μs dephasing periods (Figure 17) displays only attenuated signals from methyl and quaternary carbons. The tert-butyl methyl signals are clearly weak after only 50 μs of dephasing;122 this may reflect only partial cross polarization with just a 1-ms contact time before the dephasing process. Lengthening the contact time to 3 ms did not result in a detectable aliphatic signal after 17,400 scans, which suggests that $T_{1p}(H)$ is no more than a few milliseconds. Regardless, peak maxima at about 15-20 ppm are clearly upfield of what would be expected for tert-butyl methyl carbons, as these signals are at 829 in the precursor malonate or correspondingly functionalized C₆₀.70 The other types of quaternary aliphatic carbon would definitely give signals further downfield. Therefore, the US-tube is obviously exerting a shielding effect on the addend. It can be speculated that the malonate functional group is tightly wrapped around the nanotube, which contains a small residual negative charge from the reduction reaction. This could account for the shielding of the methyl signals. This NMR data strongly suggests that the formation of covalently functionalized US-tube(Amide) was accomplished.
Figure 16. $^1$H-$^{13}$C CP-MAS NMR of US-tube(Amide)

Figure 17. Dipolar dephasing NMR of US-tube(Amide)

The successful preparation of US-tube(Amide) via Bingel conditions make it expedient to design US-tube malonate addends, which can tailor solubility to a desired solvent necessary for biological applications.

Currently, the methods of water-solubilizing SWNTs take several approaches including: surface modification using acid oxidation,\textsuperscript{123} covalent modification with organic functionalities,\textsuperscript{124,125} synthesis of water-soluble graft copolymers using poly(aminobenzene sulfonic acid) PABS and polyethylene glycol (PEG),\textsuperscript{126} non-covalent association with linear polymers\textsuperscript{111} and non-covalent sidewall functionalization.\textsuperscript{127,128} Each of these methods water-solubilize SWNTs to a certain extent. However for use in medicines, such as drug transport and diagnostic agents, water-solubilized tubes must be single non-toxic molecules that are non-immunogenic and clear efficiently from the body. The ability to cross cell membranes is also a beneficial property, which has been observed in SWNTs functionalized with small molecules and proteins.\textsuperscript{105} Characteristically; these medicines are small (<50 nm) and contain non-ionic character.

Biologically compatible, empty US-tube materials (\textbf{Figure 18}) were developed. The US-tube nanocapsules have been individualized using the same Na\textsuperscript{0}/THF reduction procedure\textsuperscript{117} and Bingel derivatization used in synthesizing the individual US-tube(Amide).
The US-tubes were prepared, purified and reduced as discussed in experimental section, then functionalized (R groups in Figure 18) with carboxylic acid, serinolamide (Scheme 13) and PEG (Scheme 14) moieties using in situ Bingel reaction conditions. The Bingel conditions produce protons, which undoubtedly protonate, and thus competes for reaction sites on the reduced US-tubes, in a similar manner to when reduced SWNTs are quenched with MeOH or water.
Scheme 13. Synthesis of US-tube(Ser)

Biocompatible serinol functionalized US-tubes, designated as US-tube(Ser) were prepared by *in situ* Bingel addition of 10 to form 22. Subsequent cleavage of the acetate protecting groups gave the US-tube(Ser) US-tube derivative 23. PEG US-tubes, designated as US-tube(PEG) were prepared using a modified Bingel procedure. Diethyl malonates were attached to the US-tubes from the bromomalonate and NaH to form 24. The diethyl esters were then hydrolyzed to produce carboxylic acid functionalized US-tubes, designated as US-tube(COOH) which were converted to the acid chloride 25 using oxalyl chloride. Finally, PEG was attached to the US-tube through a nucleophilic substitution of the acid chloride to yield 26.

The degree of functionalization and exfoliation of the US-tube derivatives were determined using XPS, TGA and AFM. XPS was used to confirm that functionalization occurred. The atomic percent nitrogen in unfunctionalized US-tubes is ≤0.5%, but after the Bingel reaction with protected malonodiserinolamide,39 the atomic percent of nitrogen increased to ~6.0%. This can be attributed to the amide functionalities from the nitrogen on malonodiserinolamide. Assuming that the average US-tube contains 120 carbons/nm, approximately 5% of the US-tube was functionalized. TGA was also performed on the US-tube(Ser) sample and found that the mass gradually decreased from 350-500 °C (Appendix A-43). The free serinol malonate showed a sharp decrease in mass at 250 °C, confirming covalent bond attachment of the malonodiserinolamide adduct. For comparison, a TGA of US-tube(PEG) was obtained (Appendix A-43), showing a gradual mass loss of approximately 55%, which agrees with the amount of functionalization observed by the US-tube(Ser). This also implies that the US-tube(COOH) derivatized to a similar extent. This degree of functionalization compares
favorably with previous work that determined SWNT-PEG graft polymers functionalized 1% of carbons and SWNT-PABS 4% of carbons.\textsuperscript{126}

Tapping-mode AFM was used to show that exfoliation of US-tube(Ser) and US-tube(PEG) occurred. AFM images (\textbf{Figure 19}) and z-scan analyses (\textbf{Figure 20}) illustrated that indeed the US-tube(Ser) and US-tube(PEG) materials had been individualized. The z-height analyses of the two US-tube samples displayed ranges from 0.97-1.79 nm for US-tube(Ser) and 1.00-1.89 for US-tube(PEG), which coincide with diameters of individual HiPco US-tubes (0.5-2.0 nm)\textsuperscript{118} tubes with an expected slight increase in height as a result of the functionalization. In addition, it can be seen that over 90% of the functionalized US-tubes have heights that correspond to individualized tubes, with the remaining fraction corresponding to small bundles.

\textbf{Figure 19}. AFM images of (a) US-tube(Ser) and (b) US-tube(PEG)

\textbf{Figure 20}. Z-scan resolution height analysis of (a) US-tube(Ser) and (b) US-tube(PEG)
The water solubility and partition coefficients (K_{ow}) of functionalized US-tubes were determined using UV-vis-NIR spectroscopy at a physiological pH of 7.4 (Table 7). Samples were dissolved in water at several concentrations up to 2.0 mg/mL. The absorbance of each sample was then determined as the spectra were recorded sequentially. The solubility was taken as the point at which the absorbance ceased to increase in intensity linearly with concentration. This method produced solubilities of 1.00 mg/mL for the US-tube(PEG), 0.25 mg/mL for US-tube(Ser) and 0.05 mg/mL for US-tube(COOH). Each of the 2.0 mg/mL samples were centrifuged at 3200 rpm for 30 min, whereby both the US-tube(Ser) and US-tube(COOH) samples spun down. In contrast, the US-tube(PEG) sample remained in solution at an impressive 0.50 mg/mL (as measured by UV-vis). In a separate experiment, free PEG was added to pristine, individualized US-tubes in water and the mixture was sonicated for one hr. After sonication, this PEG/US-tube mixture showed no solubility (colorless solution) and all the US-tube material spun down when centrifuged. This result established that the PEG groups in the US-tube(PEG) sample are indeed covalently attached to (and not just physically wrapped around) the US-tube.

The n-octanol/water partition coefficient (K_{ow}), which is useful in the determining biological structure-activity relationships, was obtained from K_{ow} = c_{o}c_{w}^{-1}, where c_{o} and c_{w} are the equilibrium concentrations of the analyte in n-octanol and water, respectively, at 25 °C. A 0.25 mg/mL solution of each of the three US-tube samples was shaken with an equal volume of n-octanol and water. The UV-vis absorbance of the aqueous layer and organic layer were then measured independently for each sample. In the case of US-tube(PEG), K_{ow} = 1.21, for US-tube(COOH), K_{ow} = 0.83 and for the US-tube(Ser),
$K_{ow} = 0.26$ at pH = 7.4. In comparison, $K_{ow} = 0$ for a malonodiserinolamide derivative of C$_{60}$. A $K_{ow}$ value of 0, which indicates negligible lipophilicity, is typical of drugs which are restricted to extracellular space and rapidly clear from the body. This data suggests that the most lipophilic US-tube derivative, US-tube(PEG), would likely internalize into cells. Even the US-tube(Ser) agent, with the lowest $K_{ow}$ value (0.26) in Table 7, would also likely internalize, since a similar $K_{ow}$ value for a polyarginine-containing Gd(DOTA) MRI CA resulted in internalization.$^{131}$

**Table 7.** Water solubility and n-octanol/water partition coefficient ($K_{ow}$) for three derivatized US-tubes species at pH = 7.4

<table>
<thead>
<tr>
<th></th>
<th>Solubility (mg/mL)</th>
<th>$K_{ow}$</th>
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<tbody>
<tr>
<td>US-tube(PEG)</td>
<td>1.00</td>
<td>1.21</td>
</tr>
<tr>
<td>US-tube(Ser)</td>
<td>0.25</td>
<td>0.26</td>
</tr>
<tr>
<td>US-tube(COOH)</td>
<td>0.05</td>
<td>0.83</td>
</tr>
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</table>

The focus of this research has been initial steps toward nano-based radioimmunotherapy (RIT) treatments for cancer.\textsuperscript{132,133} The interest in RIT stems from recent discoveries of Bexxar\textsuperscript{TM}, a $^{131}$I-labeled B1 mAb and RituxiMAb\textsuperscript{TM}, an Y-90 labeled mAb, which have shown high efficacies and have been approved as therapeutic agents against cancer. Currently, delivery methods of RIT agents are incapable of delivering to cancer cells, without causing excessive radiation damage to healthy cells. This radiation damage is instigated by \textit{in vivo} metabolic attack of the antibody radionuclide linkage, causing radiation damage to occur in the bone marrow, liver, kidney and intestinal tract, thus limiting the amount of radiation dose administered to patients. Therefore the further development of US-tube based radiopharmaceuticals could eventually supersede the metal chelates presently employed to deliver radiotherapeutic agents for RIT. These US-tube agents would be advantageous due to the tube cage chelated around the radionuclide.

The work detailed herein demonstrated that nanomaterials, specifically C\textsubscript{60}, when derivatized properly, could form interactions with antibodies for use in cell specific drug delivery. The next step is to develop US-tube based immunoconjugates in conjunction with biocompatible iodine encapsulated US-tubes in order to demonstrate iodine delivery to specific cells. This type of delivery would serve as a proof of principle for delivery of US-tube-based astitine (as $^{31}$At-Cl) $\alpha$-emitters (\textbf{Scheme 15}), since astitine, iodine and the other halogens exhibit similar properties. Development of $\alpha$-RIT is an important endeavor for the treatment of single cells, small cell clusters, micrometastases, leukemias and lymphomas, which are not ideally treated with $\beta$-emitters that focus on solid tumors.
Current efforts at creating α-RIT have been problematic due to major release of the radionuclide before the radiolabeled-antibody conjugate reaches its target. This occurs because the α-emitters possess a large metal-ion size, resulting in smaller stability constants with existing chelating ligands and a weak carbon-halogen bond (e.g., the carbon-At bond is weaker than the carbon-I bond). Therefore, implementing US-tube materials as chelating agents could eliminate the "leakage" problems, possibly improving the chances for α-RIT to become a clinical reality.

Scheme 15. The cell-specific astitine encapsulated US-tube immunoconjugate
11. Conclusions

The first carbon nanostructures-based immunoconjugates have been prepared and characterized with fullerene (C₆₀ and Gd@C₆₀) and the ZME-018 mAb for use in fullerene immunotherapy (FIT).

The C₆₀-immunoconjugates can be prepared without covalent bond formation, which should allow for the facile development of FIT.

The targeting efficacy and cell internalization behavior of the C₆₀-immunoconjugate are only minimally affected (relative to the free ZME-018 mAb), even though the immunoconjugate is ~20% fullerene by weight.

Ultra-short carbon nanotubes (US-tubes ~20 nm long) have been individualized, functionalized and water-solubilized as a universal platform for the containment and delivery of medically useful materials for diagnostic and therapeutic medicine. Eventually, this platform might be developed into US-tube immunoconjugates in a parallel fashion to the C₆₀-immunoconjugates of this thesis.
### Appendix I (Abbreviations and Symbols)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ABTS</td>
<td>2,2’-Azino-bis(3-ethylbenzthiazoline-6-Sulfonic Acid</td>
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<tr>
<td>Ac₂O</td>
<td>acetic anhydride</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>CA</td>
<td>contrast agent</td>
</tr>
<tr>
<td>conc</td>
<td>concentrated</td>
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<tr>
<td>CP-MAS</td>
<td>cross polarization-magic angle spinning</td>
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<td>CT</td>
<td>computed tomography</td>
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<tr>
<td>DBU</td>
<td>1,8-Diazabicyclo[5.4.0]endec-7-ene</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's phosphate-buffered saline</td>
</tr>
<tr>
<td>DI</td>
<td>deionize</td>
</tr>
<tr>
<td>EI-MS</td>
<td>electron ionization mass spectrometry</td>
</tr>
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<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<td>ethyl acetate</td>
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<tr>
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<td>Ethanol</td>
</tr>
<tr>
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<td>fast atom bombardment (MS technique)</td>
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<td>free induction decay</td>
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<td>fullerene immunotherapy</td>
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<td>fourier transform-infrared spectroscopy</td>
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<tr>
<td>H₄EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>HiPco</td>
<td>high pressure carbon monoxide</td>
</tr>
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IC_{50}    50% inhibitory concentration
ICP-AE    inductively coupled plasma-atomic emission spectrometer
ICP-MS    inductively coupled plasma-mass spectrometer
IgG       immunoglobulin G
kDa       kilodalton
kHz       kilohertz
K_{ow}    n-octanol/water partition coefficient
L         liter
M         molar
mAb       monoclonal antibody(s)
MeOH      methanol
mg        milligram(s)
MHz       megahertz
min       minute(s)
/mL       milliliter(s)
/mM       millimolar
/mmol     millimole(s)
/mp       melting point
MRI       magnetic resonance imaging
/ms       millisecond(s)
MW        molecular weight
/μg       microgram(s)
<table>
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<tr>
<th>Symbol</th>
<th>Definition</th>
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</thead>
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<tr>
<td>μL</td>
<td>microliter(s)</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
</tr>
<tr>
<td>μs</td>
<td>microsecond(s)</td>
</tr>
<tr>
<td>NIT</td>
<td>nanotube immunotherapy</td>
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<tr>
<td>nm</td>
<td>nanometer(s)</td>
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<tr>
<td>nM</td>
<td>nanomolar</td>
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<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<td>acetate</td>
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<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<tr>
<td>ppb</td>
<td>parts per billion</td>
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<td>rpm</td>
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<tr>
<td>T₁p</td>
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<tr>
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<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
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<td>US-tubes</td>
<td>ultra-short carbon nanotube(s)</td>
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<tr>
<td>SPDP</td>
<td>N-succinimidyl 3-(2-pyridyldithio) propionate</td>
</tr>
<tr>
<td>SWNT</td>
<td>single-walled carbon nanotube(s)</td>
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<tr>
<td>XPS</td>
<td>x-ray photoelectron spectroscopy</td>
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</table>
Appendix II (Spectra)

(1) Tetrahydro-1,3-thiazine-2-thione

Figure A.1. 400 MHz $^1$H NMR spectrum of 1 in CDCl$_3$

Figure A.2. 400 MHz $^{13}$C NMR spectrum of 1 in CDCl$_3$
Figure A.3. FT-IR spectrum of 1

Figure A.4. FAB mass spectrum of 1 showing the [M+H]^+ peak at 134.0 m/e
(2) 3-Amino-propane-1-thiol

Figure A.5. 400 MHz $^1$H NMR spectrum of 2 in D$_2$O

Figure A.6. 400 MHz $^{13}$C NMR spectrum of 2 in D$_2$O
Figure A.7. FAB mass spectrum of 2 showing the [M+H]^+ peak at 91.0 m/e
(3) 3-tert-Butylsulfanylpropylamine

Figure A.8. 400 MHz $^1$H NMR spectrum of 3 in D$_2$O

Figure A.9. 400 MHz $^{13}$C NMR spectrum of 3 in D$_2$O
Figure A.10. FAB mass spectrum of 3 showing the [M+H]^+ peak at 148.0 m/e
(4) \(N\)-(3-tert-butylsulfanyl-propyl)-malonamic acid ethyl ester

Figure A.11. 400 MHz \(^1\)H NMR spectrum of 4 in CDCl\(_3\)

Figure A.12. 400 MHz \(^{13}\)C NMR spectrum of 4 in CDCl\(_3\)
Figure A.13. FAB mass spectrum of 4 showing the $[\text{M+H}]^+$ peak at 261.1 m/e
(5) Asymmetric protected thiol fullerene derivative

Figure A.14. MALDI TOF mass spectrum of 5a and 5b
(6) Malonic acid 3-tert-butoxycarbonylamino-propyl ester ethyl ester

Figure A.15. 400 MHz $^1$H NMR spectrum of 6 in CDCl$_3$

Figure A.16. FAB mass spectrum of 6 showing the [M+H]$^+$ peak at 290.4 m/e
(7) Asymmetric protected amine fullerene derivative

Figure A.17. 400 MHz $^1$H NMR spectrum of 7 in CDCl$_3$

Figure A.18. MALDI TOF mass spectrum of 7 [$M^+$] = 1007
(8) Asymmetric amine fullerene derivative

Figure A.19. 400 MHz $^1$H NMR spectrum of 8 in DMSO-d6

Figure A.20. MALDI TOF mass spectrum of 8 [M$^+$] = 908
(9) Asymmetric SPDP fullerene derivative

Figure A.21. 400 MHz $^1$H NMR spectrum of 9 in CDCl$_3$

Figure A.22. 400 MHz $^{13}$C NMR spectrum of 9 in CDCl$_3$
Figure A.23. MALDI TOF mass spectrum of $9 \ [M^+] = 1106$
(10) \(N,N'-\text{bis}[2-(\text{acetyloxy})-1-[(\text{acetyloxy})\text{methyl}]\text{ethyl}]-\text{malonamide}

Figure A.24. 400 MHz \(^{13}\text{C}\) NMR spectrum of 10 in CDCl\(_3\)

Figure A.25. FAB mass spectrum of 10 showing the \([\text{M}+\text{H}]^+\) peak at 418.1 m/e
(11) Asymmetric protected amine + protected serinol fullerene derivative

Figure A.26. MALDI TOF mass spectrum of 11 \([M^+] = 1423\) for bisadduct, 1840 for trisadduct, 2257 for tetraadduct

(12) Asymmetric amine + protected serinol fullerene derivative

Figure A.27. MALDI TOF mass spectrum of 12 \([M^+] = 1325\) for bisadduct, 1741 for trisadduct, 2158 for tetraadduct
(13) SPDP + protected serinol fullerene derivative

Figure A.28. MALDI TOF mass spectrum of 13 \([\text{M}^+] = 1523\) for bisadduct, 1940 for trisadduct, 2357 for tetraadduct
(14) Water-soluble SPDP fullerene derivative

Figure A.29. MALDI TOF mass spectrum of 14 $[M^+ + 1$ epoxide O] = 1620 for bisadduct

Figure A.30. 400 MHz $^{13}$C NMR spectrum of 14 in D$_2$O
(15) Protected serinol fullerene derivative

Figure A.31. MALDI TOF mass spectrum of 15 $[M^+] = 2804$ for pentadduct; lower peaks due to cleavage of -OAc

(16) Water-soluble serinol fullerene derivative

Figure A.32. MALDI TOF mass spectrum of 16 $[M^+] = 1467$ for trisadduct, 1716 for tetraadduct, 1963 for pentadduct
(17,18) C$_{60}$-SPDP and C$_{60}$-serinol ZME-018 Immunoconjugates

![Graph showing normalized induced absorbance over delay (μs)]

**Figure A-33.** Triplet state decay kinetics of C$_{60}$-SPDP and C$_{60}$-SPDP-(ZME-018), as measured at 690 nm following 532 nm excitation.

![Graph showing UV-vis spectra of C$_{60}$-derivatives]  

**Figure A-34.** UV-vis spectra of the C$_{60}$-derivatives showing negligible intensity at 595 nm (the Bio-Rad detection wavelength)
**Figure A-35.** Bio-Rad protein assay standard curve used to determine the ZME-018 concentration in the immunoconjugates.

**Figure A-36.** a) UV-vis absorption spectra of ZME-018, C$_{60}$-Ser, and their conjugate after two stages of purification and b) Calibration curve used to determine the C$_{60}$-Ser concentration in the immunoconjugate.

**Figure A-37.** UV-vis absorption spectra of a) C$_{60}$-SPDP-(ZME-018) at 6 µM and C$_{60}$-SPDP at 30 µM showing that the intensity at 440 nm is not sufficient for concentration determination in the µM range and b) C$_{60}$-SPDP absorption maximum at 282 nm at 10 µM.
(19) Malonic acid bis-(3-tert-butoxycarbonylamino-propyl) ester

Figure A.38. 400 MHz $^{13}$C NMR spectrum of 19 in CDCl$_3$
(21) Protected US-tube (Amide)

Figure A-39. a) AFM height image of US-tubes b) AFM height image of 21 from fluorination c) Z-scan resolution height analysis of US-tubes and d) Z-scan resolution height analysis of 21 from fluorination

Figure A-40. a) AFM height image of reduced US-tubes b) AFM height image of fluorinated US-tubes c) Z-scan resolution height analysis of reduced US-tubes and d) Z-scan resolution height analysis of fluorinated US-tubes
Figure A-41. FT-IR of 21

Figure A-42. TGA of US-tube(Amide) 21, 19 and Mixture
(22) Protected US-tube(Ser)

Figure A-43. TGA of US-tube(Ser), 9 and US-tube(PEG)
Bibliography

5. Licensed in the United States. Information obtained from the US Food and Drug Administration.
15. R. L. Wahl; *J. Nucl. Med.*, 2005, 46, 128S-140S.
21 G. Payne; Cancer Cell, 2003, 3, 207-212.


93. A ratio of (38:1) is reasonable even though the initial ratio of C₆₀-SPDP:antibody was only 10:1 because a large amount of antibody precipitate always occurs upon immunoconjugate formation over a 24 hour period.


