Theranostic Nanoparticles for the Treatment of Cancer

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THERANOSTIC NANOPARTICLES FOR THE TREATMENT OF CANCER

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Bioengineering

by
Thomas Lee Moore
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Accepted by:
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ABSTRACT

The main focus of this research was to evaluate the ability of a novel multifunctional nanoparticle to mediate drug delivery and enable a non-invasive approach to measure drug release kinetics in situ for the treatment of cancer. These goals were approached by developing a nanoparticle consisting of an inorganic core (i.e. gadolinium sulfoxide doped with europium ions or carbon nanotubes). This was coated with an external amphiphilic polymer shell comprised of a biodegradable polyester (i.e. poly(lactide) or poly(glycolide)), and poly(ethylene glycol) block copolymer. In this system, the inorganic core mediates the imaging aspect, the relatively hydrophobic polyester encapsulates hydrophobic anti-cancer drugs, and poly(ethylene glycol) stabilizes the nanoparticle in an aqueous environment.

The synthesis of this nanoparticle drug delivery system utilized a simple one-pot room temperature ring-opening polymerization that neglected the use of potentially toxic catalysts and reduced the number of washing steps. This functionalization approach could be applied across a number of inorganic nanoparticle platforms. Coating inorganic nanoparticles with biodegradable polymer was shown to decrease in vitro and in vivo toxicity. Nanoparticles could be further coated with multiple polymer layers to better control drug release characteristics. Finally, loading polymer coated radioluminescent nanoparticles with photoactive drugs enabled a mechanism for measuring drug concentration in situ. The work presented here represents a step forward to developing theranostic nanoparticles that can improve the treatment of cancer.
DEDICATION

This work is dedicated to my family: Mom, Dad, Matthew, and David. Thank you for all the years of support and love.
ACKNOWLEDGMENTS

I would like to acknowledge my advisor Dr. Frank Alexis for his guidance and patience through this journey. Thank you for always pushing me to achieve beyond the required, and to learn as much as possible. Thank you to my committee members for your input at each critical stage of this process. Thank you to the faculty and staff in the Department of Bioengineering at Clemson University, which has served as my home for the past eight years. Much thanks to my many collaborators: Dr. Rao and Dr. Podila (Department of Physics at Clemson University), Dr. Whitehead and Dr. Anker (Department of Chemistry at Clemson University), Dr. Brown (Department of Pharmacology and Toxicology at East Carolina University), Dr. Van Horn (Department of Chemistry and Biochemistry at the College of Charleston), and Dr. Globus (Bone and Signaling Laboratory at the NASA Ames Research Center). All of your guidance, experience, and wisdom has proven invaluable. A special thanks to Dr. Guzeliya Korneva, Dr. Terri Bruce, Linda Jenkins, and Kim Ivey for all forms of technical guidance. Thank you to Maria Torres for always tempering my anxiety.

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CHAPTER ONE

INTRODUCTION

Although nanoparticle controlled drug delivery systems have significantly increased the clinical efficacy of chemotherapeutic drugs [1], cancer remains the second leading cause of mortality in the United States [2, 3]. Chemotherapy fails in part due to: (1) diagnosis of disease at a late, aggressive, and metastatic stage (Figure 1.1), (2) physiological barriers to drug delivery such as poor drug pharmacokinetics, poor tumor penetration, development of multidrug resistance, and genetically different cancer cell populations in tumors [4-7], and (3) effective methods for non-invasively determining drug dose at the tumor site [8]. Although the fundamental understanding of cancer biology is growing, diagnostic tools are becoming more advanced, and novel therapies are becoming more effective; the World Health Organization projects that global cancer diagnoses will increase by 45% from 2007 to 2030.

Figure 1.1 Prognosis for the most deadly cancers remains poor, especially when diagnosed at later stages. Therefore development of more effective diagnostic and therapeutic is expected to improve patient outcome.
Nanoparticles are one platform with potential to improve both cancer diagnostics and therapy. The National Nanotechnology Initiative (NNI) defines nanotechnology as the research and utilization of materials on the size of 1-100 nm, however a broader definition applies this label to technologies on the 1-1000 nm scale [9, 10]. Nanotechnology has been shown clinically to improve the delivery of drugs to solid tumors [1, 11]. Furthermore, nanoparticles have been shown both in the lab and clinically to have applications for cancer imaging [12]. In order to completely dissect challenges in modern oncology, especially in the context of nanoparticle-mediated drug delivery and imaging, a robust understanding of tumor physiology and specific challenges in drug delivery is required.

1.1. Tumor Physiology and Challenges in Modern Chemotherapy

Understanding the anatomy and physiology of solid tumors is pertinent for framing nanoparticle design parameters to optimize therapeutic efficiency. Nanoparticles, due to their unique physical and chemical properties, are able to mediate both drug delivery and imaging [1, 8, 9, 11]. Moreover, nanoparticles are able to overcome physiological, cellular, and intracellular barriers to drug delivery. The structural anomalies of solid tumors and aberrations in cellular markers allow clinicians and researchers to actively or passively target cancer cells. Active targeting employs tumor-specific ligand binding to localize nanoparticle at the tumor site, whereas passive targeting takes advantage of natural anomalies in tumors to increase localization at the tumor site. During tumor growth, blood vessel development is irregular and the vascular
network within tumors is heterogeneous [13-15]. Tumor vasculature is irregularly spaced, exhibits ill-defined branching hierarchy [16], and is fairly tortuous [10]. Vessel dilation and tortuosity causes a decrease in blood flow rate within the tumor. An increase in tumor vasculature permeability is caused by widened intercellular defects and the lack of a regular basement membrane [16]. Tumor vasculature is highly fenestrated with pores between 400 and 600 nm [17, 18], and these fenestrated vessels allow small molecules and nanoparticles to cross the endothelial layer. Maeda first described extravasation and retention of small molecules and nanoparticles in tumors in 1986 as the enhanced permeability and retention (EPR) effect [19, 20]. This phenomenon is generally restricted to molecules with diameter <400-600 nm. The EPR effect has been employed as a mechanism to passively target nanoparticles to tumors due to the extravasation and retention within the tumor. The EPR effect is further enhanced by the lack of lymphatic vessel development near tumors. However, reduced lymphatic drainage coupled with fluid extravasation leads to high interstitial fluid pressure (IFP) within tumors. Tissues with normal vascular architecture and healthy lymphatic drainage generally have an interstitial fluid pressure of approximately 0 mm Hg, whereas tumor tissue will have an IFP ranging from 7 to 60 mm Hg [16, 21]. The high IFP within tumors creates a hydrostatic barrier to the delivery of drugs or nanoparticles into the tumor center. Nanoparticles may overcome this hydrostatic barrier either through active targeting [22] or via modification of nanoparticle physical properties (i.e. size and shape) [23-26].

Given that diffusion across the endothelial layer occurs, drug molecules or nanoparticles must then navigate the dense tumor extracellular matrix (ECM). Tumor
microenvironment, specifically the ECM, serves as a physical barrier to the diffusion and convection of nanoparticles or drugs to the tumor center. Studies have shown that collagen acts as a barrier to transport into tissue \([26, 27]\), and Gullino, et al. \([28]\) showed that collagen content in eleven hepatomas in rats was between 1.2 and 6.4 times higher than in healthy liver tissue. Nanoparticles must penetrate the dense ECM of solid tumors in order to deliver their therapeutic payload to cancer cells. Nanoparticle size and surface properties will influence the ability to navigate through the ECM. Krol et al. \([29]\) investigated the available volume fraction \((K_{AV})\), the interstitial space that can physically contain a therapeutic agent, by measuring the diffusion of fluorescently labeled inulin, bovine serum albumin (BSA), and dextran in ex vivo fibrosarcoma ECM and model ECM gels. The molecular weight cut-off (MWCO) was found to be roughly \(>40,000 \text{ Da}\). More recently, Perrault et al. \([30]\) showed in vivo that the diffusion of gold nanoparticles was directly related to degree of PEGylation and inversely related to nanoparticle size.

Lieleg et al. \([31]\) assert that nanoparticle size did not control the diffusion through the ECM, rather electrostatic interactions between ECM components and the nanoparticle surface charge determine diffusivity. Studies in ECM hydrogels show that \(~1000 \text{ nm}\) polystyrene particles with cationic amine, neutral PEG, and anionic carboxyl coatings exhibited similar behavior to \(~170 \text{ nm}\) liposomal formulations with corresponding surface charges. Negatively charged carboxyl and positively charged amine liposomes and polystyrene particles were relatively immobile in ECM gels, whereas the neutral particles diffused freely. Thus it appears that nanoparticle diffusion through solid tumors may be mediated by size, shape, and surface properties.
Decreased intratumoral pH can act to reduce drug efficacy and cellular uptake [32, 33]. As stated previously, tumor vasculature development is heterogeneous and irregular. Thus, cells often grow at distances far enough from capillaries such that oxygen and nutrient diffusion is insufficient [34, 35]. The diffusion limit of oxygen in a model tissue cylinder is between 100-200 µm [36], and tumor cells growing beyond 180 µm from blood vessels were observed to undergo necrosis due to insufficient nourishment [16, 35]. Oxygen-deprived cancer cells may become chronically hypoxic and undergo phenotypic mutations, inducing aggressively growing cancer cells. Chronic hypoxia also results in changes to cellular metabolism, resulting in an acidic microenvironment. Thus, nanoparticles must shield therapeutic agents from acidic environment and improve delivery into the cell. If the primary cancer is not completely eliminated, cancer cells may develop resistance to therapeutic agents. Thus, the most effective theranostic agents will be able to navigate all of the physical and chemical barriers associated with solid tumors, and completely eradicate all cancerous cells.

1.2. Nanomedicine

Nanoparticles may be broadly classified as inorganic or organic in nature, and are able to take advantage of unique, size-dependent physico-chemical properties. Inorganic nanoparticles include gold nanoparticles (AuNP), iron oxide nanoparticles (IONP), carbon nanotubes (CNTs), quantum dots (QDs), or mesoporous silica. This class of nanomaterial has found widespread biomedical application as local regulators of hyperthermia, MRI contrast, or as fluorescent markers for cell labeling or imaging.
Furthermore, inorganic nanoparticles have found application as drug delivery vehicles. Drug molecules are covalently bound or physically adsorbed to the nanoparticle surface. However, inorganic nanoparticles generally are limited as drug delivery vehicles due to short drug release time (< 6hr) [37-39]. Moreover, covalent attachment or surface adsorption of drug molecules to a nanoparticle is expected to alter in vivo pharmacodynamics and biodistribution. Liu et al. [39] showed that CNTs functionalized with poly(ethylene glycol) (PEG) had a circulation half-life of 3.3 hr. However functionalization of PEG chain ends with paclitaxel (PTX) effectively mitigated the PEG surface properties, and circulation time decreased to 1.1 hr. Another approach to loading drugs into inorganic nanoparticles is to encapsulate drugs within a hollow core, or a porous shell. Chen et al. [40] loaded doxorubicin (DOX) into hollow spindle-shaped nanoparticles made of gadolinium sulfoxide and doped with terbium (Gd₂O₂S:Tb). These particles were in turn coated with a thin layer of SiO and then coated layer-by-layer with poly(styrenesulfonate sodium) (PSS), and poly(allylamine hydrochloride) (PAH) to impart pH-dependent release of drug. While the polymer coating was able to effectively low the release at pH 7.4, release at a lower pH 5 occurred within 48 hours. Similarly, Kang et al. [38] synthesized sodium yttrium fluoride nanoparticles doped with terbium and erbium (NaYF₄:Tb,Eu) and coated with varying thickness layers of mesoporous silica. Thus, while inorganic NP have found use for diverse imaging applications, organic NP offer advantages for drug delivery through more efficient drug encapsulation, controlling drug release, and biodegradability.
Organic nanoparticles include polymers (polymeric micelles, solid polymeric nanoparticles, polymer-drug conjugates, dendrimers, filomicelles) [22, 25, 41-43], lipids (liposomes) [44], solid-lipid polymeric nanoparticles [45], or self-assembled peptide nanoparticles [46]. Polymeric nanoparticles show much promise as drug delivery vehicles. Genexol-PM (Samyang), a PLA-PEG polymeric micelle loaded with paclitaxel, has entered phase IV clinical trials in the U.S. BIND Therapeutics, a U.S. company spun out of Dr. Robert Langer’s lab at MIT, is currently developing their prostate cancer targeted, docetaxel-loaded PLGA-PEG nanoparticle platform (Accurins™). Polymeric PLGA-PEG nanoparticles have been shown in vivo to increase blood circulation time, improve tumor reduction efficacy, and act as targeted therapies [22, 47-49]. With advanced polymers, nanoparticles may utilize pH- or temperature-sensitive release [50, 51]. For imaging, polymeric nanoparticles generally require loading with a contrast agent such as a fluorescent dye, iron oxide nanoparticles, or quantum dots [52]. However, polymer dots have been described which have inherent fluorescent capabilities [53-55].

Robert Langer reported biodegradable, polymeric nanoparticles intended for drug delivery as early as 1994 [48]. PCL-PEG and PLGA-PEG particles 90-150 nm in diameter and capable of loading up to 45 w/w% of lidocaine, a model drug, were synthesized. These particles were able to release lidocaine over 14 hours. PEGylation increased circulation time, and 66% of uncoated PLGA particles were cleared via the liver within 5 minutes while only 30% of PLGA-PEG particles were cleared after 2 hours. More recently, docetaxel (DTX)-loaded PLGA-PEG particles functionalized with PSMA-targeted aptamer were shown to improve tumor targeting and therapeutic efficacy.
in vivo [22, 47]. After intratumoral injection in subcutaneous xenografts of LNCaP in nude Balb/c mice, PLGA-PEG(DTX)-Apt were significantly more effective than PLGA-PEG(DTX) and free DTX at reducing tumor volume. Histological tumor sections at 109 day endpoint showed that PLGA-PEG(DTX)-Apt completely reduced the tumor and tumor was replaced by fibrotic tissue. In vivo studies by Kim et al. [56] discovered that loading paclitaxel (PTX) in PLA-PEG particles increased the drug maximum tolerated dose (MTD) from 20 mg/kg for free PTX to 60 mg/kg for PLA-PEG(PTX) in female SPF C3H/HeNerj mice. In tumor reduction models, PLA-PEG(PTX) administered at the MTD of 60 mg/kg decreased the volume of SKOV3 human ovarian cancer xenografts in athymic nude mice to 20% of initial tumor volume at ~25 days. Free PTX initially limited tumor growth, but did not shrink tumor volume. Human MX-1 breast cancer xenografts in Tac:Cr:(NCr)-nu athymic mice were completely reduced by treatment with 60 mg/kg of PLA-PEG(PTX). Phase I clinical trials of PLA-PEG(PTX) in patients with metastatic breast cancer determined the MTD to be 390 mg/m² for 3 hours every 3 weeks with no adverse effects [57]. Thus, nanoparticles are suitable as imaging agents and drug delivery vehicles. While inorganic nanoparticles may be better suited for imaging applications and organic nanoparticles better suited for controlled release drug delivery applications, some nanoparticle platforms aim to merge these two applications into a single package. Theranostics is a new field that aims to marry these two functionalities: therapy and diagnostics.
1.3. Theranostic Nanomedicine: Imaging Drug Delivery

Theranostic nanomedicines, systems that combine therapeutic and diagnostic modalities into a single package, are expected to provide real-time information of a drug delivery system’s biodistribution, drug concentration, or drug release kinetics. The National Institute of Health (NIH) Challenge Grants in Health and Science Research, part of the American Recovery and Reinvestment Act of 2009 outlined the urgent goal of finding effective systemic treatments that can validate the localization of drug at the tumor site in real time, i.e. smart, theranostic biomaterials.

First generation theranostic nanomedicines have focused on combining both therapeutic and imaging modalities to image the localization of the drug delivery vehicle at the tumor. These systems generally rely on nanoparticles with discrete imaging and drug delivery components. In this approach, the imaging modality is independent of drug delivery and may comprise a number of different nanoparticles (NPs). Magnetic NPs are of considerable interest for theranostic applications due to their capacity for drug delivery, controlled localization in vivo [58], and MRI contrast [59, 60]. Moreover, magnetic NPs may be combined with other nanocarriers to impart optimal drug delivery properties [61, 62]. Other systems capable of combined imaging and release include gold nanoparticles (AuNP) [63], carbon nanotubes (CNT) [39, 64-66], quantum dots (QD) [37, 67], polymeric nanoparticles [52, 53, 68-71], nanobubbles [72], and liposomes [73]. While these exciting technologies offer approaches to combine imaging and drug delivery, many lack the capacity to quantitatively measure drug release kinetics in real time.
Figure 1.2 Theranostic nanomedicine with real-time monitoring of drug biodistribution, in situ concentration, and therapeutic efficacy would provide tools to clinicians to monitor therapeutic efficacy and adjust treatment regimens accordingly.

Traditional pharmacokinetics approaches such as quantification of radiolabelled drugs in tissue using positron emission tomography (PET), single photon emission tomography (SPECT), or radiology of excised tissues are valuable for measuring total drug accumulation rates in tissue. However, they cannot distinguish free drug that has been released from a NP from encapsulated drug because they produce the same signal. Consequently, a frontier of theranostics is to measure in situ release rates. Below, we describe methods to detect drug release through tissue using MRI and optical techniques. Table 1.1 shows a summary of the techniques advantages and limitations.
Table 1.1 Summary of methods for measuring the release of drug from particles.

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<td>Reflection: Porous silicon photonic crystals change color upon drug release.</td>
<td>Biodegradable, the pore size and surface chemistry can be tuned to change release rate, multiple materials may be encapsulated e.g. magnetic materials for targeting.</td>
<td>Reflection spectrum not observable through thick tissue, requires microstructures for photonic crystal effect, size is a limitation for some applications.</td>
<td>90</td>
</tr>
<tr>
<td>Fluorescent drug accumulation /singlet oxygen luminescence</td>
<td>Instrument is inexpensive and could be made portable.</td>
<td>Accumulation in tumor is nonspecific, monitoring through deep tissue is limited by autofluorescence background and light attenuation in tissue, spatial resolution is limited by optical scattering.</td>
<td>87</td>
</tr>
<tr>
<td>Fluorescent nanoparticles</td>
<td>The instrument is inexpensive and could be made portable. Allows targeting drug delivery and release monitoring.</td>
<td>In vivo monitoring is limited by the autofluorescence background and light attenuation in tissue. Spatial resolution is limited by optical scattering.</td>
<td>37</td>
</tr>
<tr>
<td>Upconverting nanoparticles</td>
<td>Upconverting nanoparticles have high photostability and low toxicity, NIR excitation allows good tissue penetration depth and produces no autofluorescence background.</td>
<td>Penetration depth is limited by the excitation and emission wavelength, the spatial resolution of image is limited by optical scattering.</td>
<td>38, 88</td>
</tr>
<tr>
<td>Radioluminescent nanoparticles</td>
<td>Excitation source (X-ray) generates no autofluorescence background, provides high spatial resolution images and allows deep soft tissue penetration depth in vivo.</td>
<td>X-ray dose limits detection depth and image resolution.</td>
<td>43, 114-115</td>
</tr>
</tbody>
</table>
1.3.1. Magnetic Resonance Imaging of Drug Release

Theranostic nanomedicines can employ drugs that are complexed with an imaging agent. Thus, when the drug releases from the NP, the imaging agent is also released and quantification of the imaging agent reports the release of drug. Viglianti and Dewhirst [74-76] reported liposomes that encapsulated doxorubicin (DOX), an anthracyclines anticancer drug, and manganese sulfate, an MRI contrast agent. Temperature sensitive liposomes less than 200 nm in diameter were prepared to destabilize and release drug at hyperthermic temperatures (39 °C to 40 °C) [77]. In this study, DOX molecules were complexed with manganese ions (Mn$^{2+}$), and these ions are able to act as $T_1$-shortening MRI contrast agents. Within the liposome, MRI contrast is diminished because $T_1$-shortening is dependent upon interaction of water with the Mn$^{2+}$, and sequestration of DOX-Mn$^{2+}$ within the liposomes reduces the interaction of Mn$^{2+}$ with water (Figure 1.3).

Figure 1.3 Thermal sensitive liposomes are destabilized when temperature is increased. This results in the release of manganese-doxorubicin complexes that are measured via magnetic resonance imaging.

In this study, rats were implanted subcutaneously with rat fibrosarcoma tumors, and liposomal treatments were administered intravenously [76]. Treatment groups
included hyperthermia (HT) + temperature-sensitive liposomes (TSL), no hyperthermia (nHT) + TSL, HT + non-temperature-sensitive liposome (nTSL), and nHT + nTSL. $T_1$-weighted MRI images were compared to HPLC analysis and fluorescent histological analysis of explanted tumor tissues after treatment with liposomes. HPLC data showed a strong correlation in drug concentration with image analysis of MRI contrast agents in tissue, indicating that MRI was a sensitive, non-invasive modality to measure the hyperthermia-mediated release of DOX in vivo. This approach was able to measure DOX release in vivo at $\mu$g/ml concentrations. This approach has the benefit of being able to quantify drug release in deep tissue.

Because some research has raised questions about the cytotoxicity of manganese ions, work has pushed for a gadolinium-based theranostic system [78, 79]. Gadolinium is in clinically approved MRI contrast agents at concentrations as low as 0.1 mmol per kg body mass [80].

**Figure 1.4** Temperature sensitive liposomes also encapsulated the imaging agent Gd-DTPA and anticancer drug doxorubicin. Upon application of heat, liposome integrity is ruptured which releases both drug and imaging agent.
Tagami et al. [81] reported temperature sensitive liposomal formulations that co-encapsulated DOX and a gadolinium-diethylene triamine pentaacetic acid complex (Gd-DTPA). In this study DOX is not complexed with the gadolinium ions, and DOX release is instead modeled by the release of Gd-DTPA. Gd-DTPA showed similar release kinetics to DOX in vitro. At 30 °C and 37 °C there was no detectable release of Gd-DTPA over 30 minutes, while at 40 °C and 42 °C there was total Gd-DTPA release within 3 minutes. DOX release showed similar results where 100% DOX was released within 3 minutes at 40 °C and 42 °C. Fluorescent readings of explanted tumor tissue showed a strong correlation between DOX in tissue and Gd-DTPA content measured via MRI. Furthermore, liposomal formulations combined with hyperthermia-mediated DOX release were able show a dose-dependent inhibition of tumor growth in vivo. Thus, these two studies illustrate a potential mechanism whereby MRI could measure drug release non-invasively.

1.3.2. Optical Approaches to Measuring Drug Release

Theranostic nanotechnologies with the ability to quantitatively measure the release of drug would provide critical data regarding delivered drug dose in real time. Non-invasive optical methods are often used to monitor concentration of fluorescent drug in tumors [82-86]. In order to measure drug release rate independent of NP accumulation, the free drug must have a different spectral signature when released versus when encapsulated. The most common approach relies on fluorescence quenching of the drug when encapsulated in the NP due to self-quenching at high concentration or energy
transfer to a quencher such as another dye or a gold NP. Release of the drug from the NP results in increased fluorescence intensity. Other methods rely upon distance dependent inner filter absorption effects, changes in the local refractive index in photonic crystal structures, and methods to magnetically modulate the dye only when it is attached to the particle [37, 40, 76, 87, 88].

1.3.2.a. Porous silicon photonic crystals

Silicon is a material that can be patterned with electrochemical and lithographic technique to generate very versatile drug carriers. For example, silicon can be electrochemically etched to generate nanopores that can be loaded with drugs. The width of the pores can be controlled during electrochemical etching to produce rugate structures with sharp spectral reflection features, similar to the photonic patterns in beetles and butterflies [89]. Porous silicon (PSi) microparticles have been shown to quantitatively measure the release of the anticancer drug daunorubicin (DAU) [90]. Plate-shaped microparticles approximately 12 µm by 35 µm were loaded with daunorubicin by covalently linking the drug to the microparticle surface via 1-undecylenic acid. Dissolution of the PSi microparticle resulted in zero-order release kinetics of DAU and orthosilic acid. Moreover, PSi dissolution resulted in a shift in the $\lambda_{\text{max}}$ reflectivity spectra of the particles. Thus, the colorimetric shift in $\lambda_{\text{max}}$ could be directly correlated with the release of daunorubicin. To further verify that daunorubicin release was mediated by the dissolution of PSi microparticles, DAU-conjugated microparticles were incubated in pH 5, pH 7, pH 9. More alkaline pH catalyzed the dissolution of PSi, and DAU release
studies showed that increasing pH lead to more rapid release of orthosilicate and daunorubicin. Increasing pH also lead to more rapid spectral peak shift. Thus, porous PSi microparticles enable the quantitative measure of drug release.

**Figure 1.5** Porous silicon (PSi) particles can be loaded with daunorubicin via covalent conjugation. PSi particles exhibit narrow reflectance spectra, and when loaded with drug reflect visible light at distinct wavelengths. PSi dissolution causes a blue shift in the spectrum.

PSi has also demonstrated inherent photoluminescent properties which may be amenable for in vivo imaging applications [91-95]. Park et al. [92] described luminescent porous silicon NPs approximately 126 nm in diameter and coated with dextran. PSi NPs were able to load 4.4 wt% DOX via the electrostatic interaction between drug and the porous silicon nanoparticles. NPs mediated the release of doxorubicin over a 10-hour period. Furthermore, PSi NPs were photoluminescent with an excitation wavelength in the ultraviolet region (370 nm), and emission maximum around 800 nm. PSi NPs were also biodegradable and dissolved within 8 hours in phosphate buffered saline. As PSi NP degraded their photoluminescent intensity not only decreased, but also exhibited a
blueshift of the luminescence spectrum. However, PSi NPs were not shown to enable quantitative imaging of doxorubicin release.

Thus, PSi particles are another platform for imaging drug release. While use of photoluminescence to image was not shown to quantitatively measure drug release from PSi NPs, [92] spectral reflectance was shown to directly measure the release of DAU from PSi microparticles [90]. However, imaging reflectance in vivo to measure drug release would be limited.

1.3.2.b. Fluorescent drug accumulation and singlet oxygen luminescence

In some instances, the drug itself serves as the imaging and therapeutic agent. This can be attributed to inherent imaging properties of the drug. For instance, pH-sensitive NPs comprised of poly(ethylene glycol)-co-poly(β-amino ester) (PEG-PBA) were shown to deliver the photosensitizer protoporphyrin IX (PpIX) for combined photodynamic therapy and imaging [87]. In this system, PEG acts as a NP-stabilizing corona and PBA loads the hydrophobic drug. PBA is protonated at its tertiary amine at lower pH, causing the polymeric micelle to destabilize and release its PpIX payload. Aside from being a photoactive drug, PpIX is also a fluorescent molecule and can therefore be used for imaging. The PpIX fluorescence is quenched by molecular oxygen, thereby generating singlet oxygen, which believed to be the primary effector responsible for killing cells. This system confers three types of information: the PpIX can be localized based upon fluorescence microscopy and optical tomography, the local dissolved oxygen concentration can be measured based upon the fluorescence lifetime
[96], and the amount of singlet oxygen generated during photodynamic therapy can be measured by detecting the singlet oxygen luminescence at 1270 nm [97, 98]. In vitro fluorescence measurements showed a pH-dependent release of PpIX over a 72-hour period with up to 60% of drug released within the first 24 hours at pH 6.4. At physiological pH of 7.4 only 20% of drug was released after 72 hours. Fluorescent imaging of SCC7 squamous cell carcinoma in vitro showed more uptake of PpIX at lower extracellular pH. This is ostensibly due to the destabilization of PBA-PEG micelles and release of the PpIX payload when internalized via lysosomes. Subcutaneous SCC7 tumor xenografts showed accumulation of PpIX-loaded PBA-PEG NPs in tumors. 5 mg PpIX per kilogram of mouse mass was injected via the tail vein as either free PpIX or PpIX encapsulated in PBA-PEG NPs. PpIX was imaged fluorescently using a 670 nm pulsed laser diode excitation source. Kinetic fluorescent biodistribution studies showed an increased accumulation of PpIX in tumor tissue over a 48-hour period when delivered via the PBA-PEG nanoparticles, as opposed to free PpIX. The relative fluorescent intensity of PpIX was calculated as 10 fold higher in tumors when administered via NP compared to free drug. Explanted tissues showed an appreciable accumulation of PpIX in the liver tissue for the free PpIX while PpIX delivered via PBA-PEG showed strong fluorescent signal in the explanted tumor. This platform shows potential for the quantitative monitoring of drug delivery in vivo based on the fluorescent properties of the drug molecule. It was shown to be effective in SCC7 squamous cell carcinoma to treat superficial, subcutaneous tumor grafts. However, feasibility of this therapy in a deeper tissue was not explored and is likely to be limited because the 670 nm excitation
wavelength is attenuated by a factor of between 10 and 1000 per cm of tissue depth, depending on the type of tissue [99]. Treatment of tumor 1 cm and deeper would therefore require either an endoscopic light source or a method to apply light over a period of days.

Figure 1.6 pH-sensitive PBA-PEG nanoparticles are loaded with the photoactive drug protoporphyrin IX (PpIX). This drug also has a fluorescent spectrum and measuring fluorescent signal in tissue can quantitate the relative amount of drug. Decrease in intratumoral pH causes protonation of PBA, which results in destabilization of the nanoparticle and release of PpIX.

1.3.2.c. Fluorescent Nanoparticles

A different approach to employing fluorescence as a theranostic modality is to utilize fluorescent NPs. Bagalkot et al. [37] reported a QD drug delivery system in which fluorescence resonance energy transfer (FRET) allows for fluorescent imaging of both nanoparticles and delivery of drug. DOX was employed as the drug model due to its inherent fluorescence. Here, the QD are functionalized with a double-stranded A10 RNA aptamer that intercalates DOX and specifically binds prostate specific membrane antigen (PSMA). The QD-aptamer-Dox construct (QD-Apt(DOX)) is “turned off” because DOX quenches the fluorescent emission of the QD, and the aptamer quenches the fluorescent
emission of DOX. Upon the release of drug both entities become fluorescent, allowing for the visualization of both nanoparticle and drug location. PSMA is overexpressed in LNCaP human prostate cancer cells but not PC3 human prostate cancer cells; therefore, LNCaP cells were qualitatively shown to uptake more QD-aptamer (QD-Apt) conjugates than PC3 cells in vitro. After incubating LNCaP cells for 30 minutes with QD-Apt(DOX), cells were washed and imaged with confocal microscopy. At this time, cells showed no fluorescence, indicating a lack of drug release. However, after incubating for 1.5 hours more fluorescent signal could be seen in the cell from both Dox and QD. Thus, the QD-Apt(Dox) conjugate could be used to image the release of drug in vitro. Moreover, QD-Apt were sensitive to the quantity of Dox loaded. QD-Apt held at a fixed concentration (1 µM) were incubated with increasing amounts of Dox, and maximum FRET quenching was observed at a QD-Apt:Dox ratio of 1:7. As a chemotherapeutic agent, QD-Apt(Dox) conjugates at 1.6 µM were approximately as effective as free Dox at 5 µM. Savla et al. [100] employed a similar system to improve the delivery of free Dox to A2780/AD multidrug resistant human ovarian cancer in vitro and in female athymic nu/nu mice. QD were conjugated to an aptamer (MUC1) which targets mutated Mucin 1, a cell surface-associated mucin overexpressed in late stage epithelial ovarian cancer. Dox was conjugated via an acid-degradable hydrazone bond. Similar to the Bagalkot study, QD-MUC1(Dox) conjugates displayed FRET extinction of QD fluorescence. Free Dox, QD-Dox, and QD-MUC1(Dox) showed similar treatment efficacy in vitro. In vivo, targeted QD-MUC1 showed higher uptake compared to untargeted QD. No tumor volume reduction data was reported for in vivo efficacy. The primary utility of these two
systems from a theranostic drug delivery system standpoint is as an “on/off” switch indicating drug loaded or released. Compared to polymeric systems in which drug is encapsulated, these systems load much lower amounts of drug and have relatively quick release. One limitation related to in vivo imaging is associated with poor penetration of excitation wavelengths through tissue. Although this system is unable to measure the release kinetics based on FRET extinction, other systems capable of dynamically measuring release rates have been developed.

Rare earth-doped nanophosphors are a class of inorganic materials that are able to convert various forms of electromagnetic radiation into visible light. Different classes of nanophosphors can convert ultraviolet light (fluorescent phosphors), infrared light (upconversion phosphors), or x-ray (radioluminescent phosphors) into visible light.

**Figure 1.7** Bi-fluorescence resonance energy transfer enables the measure of drug loading onto quantum dot-aptamer conjugates. Doxorubicin, a fluorescent anticancer drug, binds the prostate cancer targeting aptamer. Doxorubicin quenches the fluorescent signal from the quantum dot, and the targeting aptamer quenches fluorescence from doxorubicin. Upon release, both entities regain their fluorescence.

Nanophosphors are advantageous compared with organic dyes because these rare earth-doped inorganic phosphors have a high chemical stability and do not photobleach or fade
Nanophosphors also have narrow and distinct spectra, similar to quantum dots [103], however their low toxicity draws attention to their biological applications [103-105]. Xu et al. [106] developed a system that employs NPs with short ultraviolet (256 nm) excitation wavelength and emission of visible light (613 nm) to measure drug loading and release. NPs were composed of a spherical europium-doped gadolinium oxide core (Gd$_2$O$_3$:Eu$^{3+}$) approximately 270 nm within a mesoporous silica (@nSiO$_2$@mSiO$_2$) shell approximately 50 nm thick. DOX is able to load at 8.56 wt% within the pores of the mesoporous silica and quench the luminescent emission signal of the Gd$_2$O$_3$:Eu$^{3+}$. Release studies revealed that Gd$_2$O$_3$:Eu$^{3+}$@nSiO$_2$@mSiO$_2$ NPs released 80% of loaded DOX over 12 hours. The photoluminescence of the Gd$_2$O$_3$:Eu$^{3+}$ was measured as DOX was released. These simultaneous studies of NP luminescence and DOX release showed a direct relationship between the amount of Dox released and increasing NP photoluminescent intensity. These results indicate that the Gd$_2$O$_3$:Eu$^{3+}$ quenching effect of DOX is mitigated as it is released from the mesoporous silica shell, and photoluminescence returns. In vitro studies of Gd$_2$O$_3$:Eu$^{3+}$@nSiO$_2$@mSiO$_2$ NPs in HeLa cervical cancer cells showed no concentration dependent toxicity up to 2.5 µg/ml. Free Dox and DOX-loaded NPs showed equivalent efficacy in vitro against HeLa cells. This system is limited for translation to in vivo diagnostics because the UV excitation wavelengths are strongly absorbed by melanin in skin [107-109]. Furthermore, high energy UV radiation has deleterious effects on DNA and may lead to protein-DNA crosslinking, oxidative damage, and gene mutations [110].
1.3.2.d. Upconversion Nanoparticles

Visible and UV light phosphors are expected to have limited application for deep tissue imaging due to light scattering and the poor penetration depth of shorter excitation wavelengths [111]. Recent research is focused on upconversion NPs, particles that can convert longer near-infrared (NIR) wavelength light to visible light. Upconversion probes capable of excitation at longer wavelengths and emission within the optical transmission window (650-950 nm) are promising due to low tissue auto-fluorescence, deeper tissue penetration of excitation wavelengths, and lower light scattering [112]. Chen et al. [112] showed that upconversion particles could image as deep as 3.2 cm through pork tissue with excitation at 980 nm and emission at 800 nm. Upconversion NPs have been investigated as a means to track drug release by measuring luminescent intensity [38, 113].

Figure 1.8 Nanoparticle visible light luminescence, which may be excited by NIR light, is attenuated by loaded drug. As drug is released, nanoparticle luminescence returns. These systems enable quantitative measure of drug release.

Kang et al. [38] described an upconversion NP composed of sodium yttrium fluoride (NaYF₄) doped with ytterbium and erbium (Yb³⁺, Er³⁺). Similar to the previously
mentioned Gd$_2$O$_2$:Eu$^{3+}$ NP, the NaYF$_4$:Yb$^{3+}$/Er$^{3+}$ were coated with mesoporous silica (@nSiO$_2$@mSiO$_2$). In this system Ibuprofen (IBU) was chosen as a model drug and loaded into mesoporous silica. The NaYF$_4$:Yb$^{3+}$/Er$^{3+}$ core was 120 nm in diameter and coated with a tunable level of mesoporous silica (10, 20, or 45 nm). These NPs were capable of loading 11, 21, or 34 wt % IBU in the 10, 20, or 45 nm mesoporous silica coatings, respectively. All three formulations demonstrated controlled release over a 10-hour period, with changes in release kinetics altered by coating thickness. Thicker coatings were hypothesized to slow release time due to the IBU having to travel farther through the mesoporous silica. IBU also effectively quenched the photoluminescence from the upconversion NaYF$_4$:Yb$^{3+}$/Er$^{3+}$@nSiO$_2$@mSiO$_2$ nanoparticles. Nanoparticles were excited by 980 nm wavelength light and had three emission peaks at 520, 550, and 650 nm. Release kinetics were directly correlated to the return of photoluminescence, indicating that as IBU was released, the quenching effect of IBU diminished. This system demonstrates a mechanism by which the release of drug can be dynamically measured due to the quenching of NP luminescence by the drug. This system successfully shows a robust method for quantitatively measuring drug release based on the upconversion luminescence of NPs. However, as with most inorganic delivery systems, the release kinetics are not optimized for controlled release. With the thickest mesoporous silica coating, 75% of IBU was released within 12 hours. Upconversion NPs are expected to overcome some of the challenges for in vivo imaging associated with fluorescent molecules or NPs, but will still be limited in the penetration depth of the excitation and
emission wavelength. Methods to improve drug loading and release kinetics have been investigated using organic coatings on inorganic NPs.

Upconversion NPs have also been coated with organic layers to improve lipophilic drug loading and aqueous dispersion [88]. In this study, cubic 20-30 nm NaYF₄:Yb/Er NPs were capped with oleic acid and further functionalized with α-cyclodextrin (αCD). Oleic acid was able to load lipophilic photosensitizers such as Chlorin e6 (Ce6), Zinc phthalocyanine (ZnPc) and Methylene blue (MB). α-Cyclodextrin improves the aqueous solubility of this system, and cyclodextrin has been used in clinical trials as part of a nanoparticle drug delivery system [69, 70]. In this system, the upconversion particles were excited by a 980 nm laser, then they emitted strongly in the 650-670 nm range. Ce6, ZnPc, and MB had strong absorptions in this region. Loading capacity for all three photosensitizers was dependent on the loading photosensitizer concentration, and total loading between drugs was variable. This is possibly due to differences in drug chemical structure. Ce6, ZnP, and MB were able to load at 0.158, 0.165, and 0.129 mmol/g, respectively. Luminescent intensity of the NPs was quenched by loading the photosensitive drug, and the release of photosensitizer allowed for a dynamic method to measure drug release. Release studies showed that approximately 10% of drug was released in 48 hours.

In vitro toxicity of photosensitizer-loaded upconversion NPs was shown in A-549 adenocarcinomic human alveolar basal epithelial cells. Photosensitizer-loaded NPs without laser irradiation showed no significant toxicity in cells. However, when irradiated with the 980 nm laser (1 W/cm²) for 3 minutes, upconversion luminescence activates the
photosensitive drug, creating reactive oxygen species and the irradiated NPs showed
dose-dependent toxicity in vitro. Toxicity was also dependent on the duration of laser
irradiation. This photodynamic therapy could be combined with traditional chemotherapy
by loading Dox in the nanoparticle. The combination of Dox and photodynamic therapy
significantly improved therapeutic efficacy of the NP. For example, NPs loaded with
0.077 mmol/g of Ce6 and 0.082 mmol/g Dox irradiated with 980 nm laser showed
approximately 15% cell viability with A-549 cells in vitro. This was significantly better
than NPs loaded with Ce6 and Dox without irradiation (~40% cell viability) or Ce6 NPs
with irradiation (~40% cell viability). While these treatments are effective in vitro, near-
infrared and infrared wavelengths are still limited in translation to deep tissue imaging
because the penetration depth is on the scale of millimeters [111]. Technologies with
greater potential to penetrate deep into tissue are expected to improve theranostic
modalities.

1.3.2.e. Radioluminescent Nanoparticles

Radioluminescence is a phenomenon whereby radiation energy is converted into
visible light. The benefit of using this type of system for measuring drug release is that x-
ray has excellent soft tissue penetration. Thus, the excitation wavelength is not limited in
penetration depth. Radioluminescent nanophosphors have been investigated for in vivo
imaging and as quantitative sensors for drug delivery [40, 114, 115]. Chen et al. [40]
described radioluminescent NPs coated with poly(styrenesulfonate sodium) (PSS) and
poly(allyl-amine HCl) (PAH) via a layer-by-layer assembly, which imparts a pH-
sensitive drug release. The hollow nanoparticle was comprised of gadolinium oxysulfide (Gd$_2$O$_2$S) doped with terbium (Tb) and europium (Eu). NPs were ellipsoidal in shape with an average length of 420 nm and a width of 150 nm. Higher aspect ratio NPs have been shown previously to extend plasma circulation time in vivo [25, 116, 117]. The Gd$_2$O$_2$S:Tb-PSS/PAH NPs were able to load ~5 wt% DOX, and release measured by high performance liquid chromatography directly correlated with peak quenching of the NP radioluminescence. Moreover, DOX release was shown to be pH dependent with rapid release at pH 5.0 and slow release at pH 7.4. The goal of pH dependent release is to exploit intracellular release of drug in endosomes or lysosomes after endocytosis. NP uptake was shown in MCF-7 breast cancer cells, and in vivo biodistribution showed the ability to dynamically and non-invasively monitor radioluminescent nanoparticle accumulation in the liver of Balb/c mice. Previous studies have shown the ability of radioluminescent NPs for deep tissue imaging in chicken breast after injection deeper than 1 cm [114]. This system offers a promising platform for theranostic nanomedicine due to excellent penetration depth of x-rays through soft tissue.

**Figure 1.9** Radioluminescent Gd$_2$O$_2$S nanoparticles doped with terbium and europium (Gd$_2$O$_2$S:Tb/Eu) were coated layer by layer with poly(styrenesulfonate sodium) (PSS) and poly(allyl-amine HCl) (PAH). These hollow nanoparticles were able to encapsulate doxorubicin and exhibit pH dependent response. Doxorubicin attenuates radioluminescence. Luminescence returns in intensity proportional to doxorubicin release.
A key advantage of this approach is that there is almost no autofluorescence background and the radioluminescence is only generated where the x-ray beam irradiates the tissue. By irradiating the tissue with a collimated or focused x-ray beam, it is possible to map the spectrum point-by-point in order to generate high resolution images, limited in resolution by the x-ray beam width. This is in contrast to fluorescence excitation, wherein the excitation beam is scattered with a typical mean free path of ~100 µm, and the almost all the light passing through more than 1 mm of tissue propagates diffusively providing a point spread function with a width similar to the tissue depth [118, 119].

The main limitation of the x-ray excited optical luminescence imaging is that radioluminescent systems may be limited by the penetration depth of visible light emissions, even though x-rays have excellent soft tissue penetration. In general, there is a tradeoff between the required x-ray dose and the scintillator concentration, the depth of the scintillators in the tissue, and the spatial resolution of the image. For example, in a numerical simulation, Carpenter and co-workers calculate picomolar (ng/ml) concentrations of 10 nm X-ray phosphor are detectable for a mammographic-like dose with a signal/noise level of 10 [120]. For applications such as tumor-resection where the X-ray excitation angle is limited by geometry, and where increased rapid acquisition is critical, a limited-angle X-ray luminescence tomography (XLCT) can be applied based on a hybrid X-ray/optical reconstruction. In XLCT X-rays pencil-like X-rays are used to excite scintillators in a narrow region defined by the beam diameter, and diffuse optical spatial discrimination for the axial dimension along the beam. According to their model, µg/mL particle concentrations may be observed through 5 cm of tissue with ~10 mGy
doses [121]. Even more rapid images can be acquired using a cone-angle geometry with multiple angle views, but the resolution will be limited by the optical scattering in the tissue [122].

Nanophosphors (luminescent nanoparticles) exist as a potential platform for a theranostic system able to quantitatively measuring drug delivery has been shown at the bench. These NPs can be tailored to have excitation wavelengths over a broad range, from x-ray to near infrared light. Employing NPs with luminescence that overlaps with the optical properties of drugs has been shown to quantitatively measure the release of drug. The use of these NPs in a clinical sense is still limited by specific challenges, namely nanoparticle-associated toxicity and physical limitations. Currently optical imaging in tissue is limited to the millimeter or centimeter depth. MRI imaging techniques rely on indirect approaches which model drug delivery based on the co-delivery of MRI contrast agents, or rely on the conjugation of imaging agent to drugs which could alter release kinetics or efficacy. Thus, identifying and addressing current imaging challenges will guide decisions for future research.

1.4. Challenges in Theranostic Nanomedicine

Real challenges exist with the development of theranostic nanomedicines to enable non-invasive measurement of drug delivery. Nanomedicines must demonstrate reasonable safety. Many particles used for imaging have shown success in vitro for cellular labeling but are limited in translation to more complex living systems due to toxicity.
1.4.1. Factors Affecting Nanoparticle Toxicity

A primary challenge surrounding the use of nanoparticles for biomedical applications is the selection of material that optimizes the NP’s intended function, but mitigates nanomaterial-related toxicity. For example, quantum dots (QD) have garnered much excitement for use as in vitro fluorescent tags, however concerns about their toxicity arise because QDs are generally made out of heavy metal elements such as cadmium selenium (CdSe), cadmium tellurium (CdTe), and zinc selenium (ZnSe) [123]. While QD generally have a core-shell structure to reduce cytotoxicity, investigations into the leaching of heavy metals from QD via oxidation showed toxicity in vitro [124]. CdSe QD oxidized in air or with UV light showed a red shift in fluorescence, indicating a change in size because of the leaching of surface atoms due to oxidation. QD kept in an inert environment, i.e. no oxidation, showed no toxicity in vitro against primary rat hepatocytes. However, when oxidized in air for 30 minutes cell viability decreased to 21% at a QD concentration of 62.5 µg/ml. Free Cd\(^{2+}\) concentrations were determined from oxidized QD samples via inductively coupled plasma optical emission spectroscopy. QD oxidation in air resulted in a 21-fold increase in Cd\(^{2+}\) concentration, from 6 ppm to 126 ppm. Therefore it can be surmised that toxicity is in part related to nanomaterial composition and stability.

Some nanomaterials have a historic track record for biocompatibility and have entered clinical trials. Gold, for example, has long been used as a biomaterial because it is relatively bioinert and stable [125], and gold nanoparticles (AuNP) have been employed as a therapeutic since the 1930s as treatment for rheumatoid arthritis [126, 127]. Gold
nanoshells meant to mediate hyperthermia have seen success in vivo, and a company developed on this technology platform (Nanospectra Biosciences, Inc.) has begun clinical trials of AuNP for thermal ablation of solid tumors [128-130]. In fact, tissue necrosis factor α (TNFα)-bound AuNP functionalized with PEG showed no adverse events in human clinical trials with concentrations from 50 µg/m² to 600 µg/m² [131]. Magnetic iron oxide nanoparticles (IONP) have also shown promise as non-toxic nanomaterials. In vitro studies with RAW 264.7 rat macrophages indicated that 9 nm IONP induced oxidative stress, however cell viability and growth was not hindered [132]. Clinical trials for superparamagnetic and ultra-small IONP exist, and the Food and Drug Administration (FDA) has approved Ferumoxtran-10 (AMAG Pharmaceuticals), a formulation of mono-dispersed 5 nm IONP with a ~15 nm coating of dextran, for MRI contrast. Phase II clinical studies of intravenously administered Ferumoxtran-10 showed no significant events with doses up to 1.7 mg Fe/kg [133, 134]. Thus, nanomaterial stability in vivo seems to be important in determining NP toxicity. AuNP are considered bioinert and relatively non-toxic. QDs, however, have been shown to leach toxic metal ions leading to cytotoxicity. Thus, determining the stability and degradation of a nanomaterials will be key to preventing unintended toxicity.

NP size and shape is also relevant in determining toxic response. Altering NP size and shape has been shown to greatly influence circulation time, biodistribution, toxicity, and cellular uptake [25, 30, 41, 116]. Carbon nanotubes (CNT) are a prime example of nanomaterials in which toxicity is closely related to size and morphology. Several studies indicate that CNTs are safe when injected intravenously, and it appears that toxicity,
fibrosis, and chronic inflammatory response are mediated by the size and morphology, route of administration, dose, and surface functionalization [135-141]. Tang et al. [142] showed that carbon-based nanomaterials such as carbon nanocapsules (CNCs), C\textsubscript{60} fullerene (C\textsubscript{60}), multi-walled carbon nanotubes (MWCNT), and single-walled carbon nanotubes (SWCNT) had different toxicity profiles due to differences in size and shape. Carbon nanomaterials were dispersed in 1 wt% polyvinyl alcohol (PVA) and administered via tail vein injection. In both MWCNT and SWCNT no mice survived after receiving 50 µg/g, however 50% of mice receiving C\textsubscript{60} and 91.7% of mice receiving CNCs survived at this dose.

Gold nanoparticles, on the other hand, show little toxicity associated with size and shape. In vitro studies with 4, 12, and 18 nm diameter nanoparticles tested against K562 leukemia cells showed no toxicity up to 250 µM concentration [143]. Moreover shape does not seem to play as large a role in AuNP-mediated toxicity. PEGylated gold nanorods approximately 65 nm long and 11 nm in diameter were injected at a concentration of 0.9 mM Au atom into male ddY mice, and at various time points, mice were euthanized and organs were collected [144]. Gold content was quantified via ICP mass spectrometry, and gold nanorods had a circulation half-life of approximately 1 hour, and were found up to 24 hours in the blood. At 72 hours, nanorods were absent from blood and 35% of initial injected dose had accumulated in the liver. In summary, nanoparticle-mediated toxicity appears to vary significantly between nanomaterial, size, shape, aggregation, and degradability.
1.4.2. Optical Challenges in Theranostic Nanomedicine

Furthermore, theranostic particles must overcome physical limitations with respect to penetration depth of light due to scattering, and developing sensitive methods to image drug or nanoparticle. The previously described theranostic approaches are limited by drug modification, interaction of NPs and drugs, and optical imaging capabilities in deep tissue. With optical techniques in tissue imaging, viable wavelengths are generally restricted to the “biological window,” a range of wavelengths from 650-1400 nm; we will refer to the spectral region from 650-1000 nm as the first optical window, and the region from 1000-1400 nm as the second optical window [66, 111, 145-148]. Wavelengths before 650 nm are strongly absorbed by hemoglobin, and those above 950 nm light begin to be absorbed by water [145, 149]. Imaging in the first optical window can be hindered by autofluorescence and tissue scattering [111]. However, imaging in the second optical window has shown promise with low incidence of autofluorescence and tissue scattering. Hongjie Dai’s group has developed single-walled carbon nanotubes capable of sensitive in vivo imaging in the second optical window, [65, 66] and Won et al. [148] showed significant improvement with imaging quantum dots in the second optical window compared to the first optical window. Moreover, upconversion NPs for deep tissue imaging have been developed which are capable of imaging as deep as 3.2 cm through tissue [112]. However, approaches to image drug release in the biological window must rely on drugs and NPs that are either photoactive or absorb within this range of wavelengths. In the previously stated examples of NP systems that measure drug release based upon FRET, the NP must be excited by a light
which can penetrate deep into tissue. The drug must also be such that its absorption profile overlaps with the nanoparticle’s emission wavelength within this window. Moreover, to get meaningful data from a complex and dynamic in vivo system, the emitted light must overcome tissue scattering and be detected with enough sensitivity to measure drug release. The power of the excitation wavelength source and its distance to the NPs, the spatial distribution of the NP drug delivery system within the patient, and the sensitivity of the luminescent detection will complicate detection sensitivity in a deep tissue living system.

A relevant goal for drug delivery aims to non-invasively determine drug concentration or release in tissue. This is possible with sensitive molecular imaging techniques. Preclinical studies with the cyclodextrin polymer based nanoparticle (CRLX101) with polymer-conjugated camptothecin showed that biodistribution could be monitored using $^{64}$Cu-labeled polymer [70]. While CRLX101 has advanced to early clinical trials, no efforts to pursue the positron emission tomography (PET) imaging aspect for a theranostic drug delivery approach have been reported. Use of PET is also limited due to cost and the short half-lives of radioisotopes: from 2 min with $^{15}$O to 109.8 min with $^{18}$F [150, 151]. Positron emitters with longer half-lives (e.g. 8.3 h for $^{52}$Fe and 4.2 days for $^{124}$I) are less commonly used because these elements are not found in many drugs and require a higher energy synchrotron to produce. Ideally, a theranostic nanomedicine to measure drug release will avoid modification of the drug, as this may affect release kinetics, biodistribution, and activity. Measuring the release of drug using MRI required complexing DOX with manganese ions [76], and the study employing Gd-
DTPA only modeled the release of DOX but did not measure its release from liposomes directly [81]. Thus, finding a way to measure drug release without drug modification remains a challenge in theranostic nanomedicine.

1.5. Conclusions

Theranostic nanoparticles for non-invasive measurement of in situ drug release are being realized in laboratory studies. Current approaches are able to utilize drugs complexed with MRI contrast agents or take advantage of optical properties of drugs to measure drug release non-invasively. Nanotechnologies offer a platform to realize non-invasive measurement of drug concentrations, improve drug delivery and imaging of solid tumors, and improve drug biodistribution and efficacy. However, significant challenges in theranostic nanomedicine must be overcome, namely the physical limitations of optical methods, complexities in imaging sensitivity and resolution in deep tissue, and toxicity of nanomaterials.
1.6. References


CHAPTER TWO
MULTIFUNCTIONAL NANOPARTICLES TO IMPROVE THE SAFETY OF DRUG DELIVERY

Current challenges in chemotherapy include the non-ideal biodistribution of drugs, limited dose due to systemic toxicity, and excessive dosing regimen required because of rapid drug clearance. These challenges can be overcome with nano-sized drug delivery vehicles capable of prolonging drug release, increasing circulation time, and localizing delivery at the site of interest. However, hesitance to employ nanomaterials for medical applications often stems from concerns regarding nanomaterial toxicity. Though some nanoparticle (NP) formulations have successfully reached clinical use, significant NP toxicity persists. This may generally be attributed to choice of nanomaterial, degradation, size, shape, and in vivo stability. Using carbon nanotubes as a proof-of-concept, we show that coating inorganic nanoparticles with a multifunctional, amphiphilic polymer coating comprised of poly(lactide)-poly(ethylene glycol) (PLA-PEG) decreases NP toxicity in vitro and in vivo. Moreover, such a coating enables the controlled delivery of the anticancer drug paclitaxel (PTX).

2.1. Methods to Coat Inorganic Nanoparticles

Inorganic NP are often unsuitable for drug delivery applications due to their propensity for aggregation and difficulty in sustaining drug release. However, coating inorganic NP with polymer may improve their suitability for drug delivery applications. Carbon nanotubes (CNTs), nano-hydroxyapatite (nHA), nano-graphene sheets (nGr), and
europium-doped gadolinium sulfoxide (Gd$_2$O$_2$S:Eu) radioluminescent nanoparticles were coated with amphiphilic, biodegradable polymers. Here we will focus on CNTs as a proof-of-concept to show that inorganic nanoparticles may be coated with polymer to improve drug delivery and decrease nanotoxicity.

Recent research indicates the utility of carbon nanotubes as drug delivery vehicles [1-7]. In cancer nanomedicine, carbon nanotubes are currently investigated for their ability to act as drug delivery vehicles, mediators for hyperthermia, or act as imaging agents because they can be easily internalized into cells [2, 3, 5, 8-10]. Though the large specific surface area of CNTs provides the potential for delivering high drug doses, pristine CNTs are limited in their applications as drug delivery vehicles because their hydrophobicity causes aggregation and toxicity [11-16].

Most approaches for CNT drug loading utilize adsorption or covalent conjugation of drugs on the surface of CNTs [2, 17]. Hongjie Dai’s group has developed a drug delivery system whereby a phospholipid-PEG molecule is adsorbed onto the CNT surface [2, 3, 18, 19]. Here, tumor targeting moieties or modified drugs may be either chemically conjugated to the PEG chain ends, or drugs may be adsorbed (doxorubicin) onto the nanotube surface due to pi-stacking of benzene rings. Previously, Pastorin et al. [20] directly functionalized the sidewall of CNTs with methotrexate, an anti-cancer drug, via 1,3-dipolar cycloaddition. Surface adsorption or conjugation is controlled by the available CNT surface functional groups and reactivity, however this reduces the availability of functional groups for further attachment of PEG or targeting ligands.
Table 2.1 Experimental plans to test effect of polymer coating on toxicity and drug delivery

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Objective</th>
<th>Methods</th>
</tr>
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<tbody>
<tr>
<td>(A) PLA-PEG coating on inorganic nanoparticle</td>
<td>Determine the parameters to successfully functionalize and characterize polymer-coated inorganic nanoparticles.</td>
<td>TGA, NMR, FT-IR, TEM</td>
</tr>
<tr>
<td>(B) NP uptake in vitro</td>
<td>Determine if fluorescently tagged CNT-PLA-PEG is uptaken into cells.</td>
<td>confocal microscopy</td>
</tr>
<tr>
<td>(C) NP toxicity and inflammatory response in vitro</td>
<td>Investigate dose-dependent toxicity of coated and non-coated NP.</td>
<td>PrestoBlue cell viability assay, RT-PCR</td>
</tr>
<tr>
<td>(D) Drug loading and release. In vitro drug delivery efficacy</td>
<td>Investigate viability of coated NP as a drug delivery vehicle.</td>
<td>HPLC, PrestoBlue cell viability assay</td>
</tr>
<tr>
<td>(E) In vivo biodistribution</td>
<td>Determine changes in biodistribution due to polymer coating.</td>
<td>IVIS live animal imaging, histology</td>
</tr>
<tr>
<td>(F) In vivo maximum tolerated dose and inflammatory response</td>
<td>Determine changes in maximum tolerated dose and inflammatory response.</td>
<td>Bronchoalveolar lavage, histology</td>
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Moreover, the surface properties and pharmacokinetics of the CNT is expected to change with surface adsorbed drugs, drugs conjugated to the surface, or drugs conjugated to PEG chain ends [3, 21]. In a study of CNTs functionalized with PEG-PTX, the conjugation of PTX to the PEG molecule decreased the circulation half-life of CNT-PEG from 3.3 hr to 1.1 hr for CNT-PEG-PTX. Here we describe an amphiphilic copolymer, PLA-PEG, which functionalizes the CNT surface. In this system, hydrophobic PLA acts as a reservoir to sequester hydrophobic drugs and mediate their release, while hydrophilic PEG stabilizes CNTs to reduce aggregation in an aqueous solution. While current CNT-based therapies for drug delivery and imaging have shown encouraging results, the translational success of this system is expected to be facilitated by preventing aggregation due to surface properties, and improving drug loading to increase therapeutic efficacy.

2.2.1. Synthesis of Coated Nanoparticles

Nanomaterials were covalently coated with PLA using a room temperature, ring-opening polymerization. In a typical reaction, 40 mg of MWCNT-OH (Sun Innovations, Fremont, CA, USA) and varying amounts of d,l lactide (Purac Biomaterials, Lincolnshire, IL, USA) were dissolved in 4 ml of acetonitrile. Phosphazene base P$_2$-t-Bu at 2M in tetrahydrofuran (Sigma-Aldrich) was added as a catalyst. This reaction resulted in CNT coated with PLA (CL). Next, methoxy-PEG-isocyanate (Nanocs Inc, New York, NY, USA) was dissolved in 2 ml of solvent and added directly to the reaction mixture. The reaction then proceeded under nitrogen and was washed via centrifugation. The product, CNT coated with PLA-PEG (CLP), was lyophilized and stored at -20 °C under...
nitrogen. This reaction scheme could be followed using a number of inorganic nanoparticle templates. To characterize nanomaterials, NMR was performed with a Bruker Avance 300, and ATR FT-IR was performed with a Thermo-Nicolet Magna 550 equipped with a Thermo-SpectraTech Foundation series Endurance Diamond ATR. \(^1\)H-NMR (300 MHz, CDCl\(_3\), \(\delta\)): 7.26 (s, CDCl\(_3\)), 5.17 (q, -C(=O)-CH(CH\(_3\))-), 3.65 (s, -CH\(_2\)CH\(_2\)-O-), 1.59 (d, -CH(CH\(_3\))-); IR 2881 cm\(^{-1}\) (-CH\(_2\)CH\(_2\)-O-), 1745 cm\(^{-1}\) (C=O).

Thermogravimetric analysis was performed on a TA Instruments Hi-Res TGA 2950 thermogravimetric analyzer under nitrogen from 25 °C to 600 °C at 20 °C/min. Transmission electron microscope images were taken on a Hitachi HD2000 at 200 kV.

2.2.2. Cell Culture

Human umbilical vein endothelial cells (Lonza, Walkersville, MD, USA) were grown on collagen-1 coated flasks (Thermo Scientific, Rockford, IL, USA) in endothelial growth media (Lonza, Walkersville, MD, USA). U-87 glioblastoma cells (American Type Culture Collection, Manassas, VA, USA) were grown on CellBind-treated flasks (Corning, Tewksbury, MA, USA) in EMEM (American Type Culture Collection) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA). Cells were subcultured at 37°C and 5% CO\(_2\). Rat lung epithelial cells RLE-6TN (American Type Culture Collection) were grown in Ham’s F12 medium with 2 mM L-glutamine supplemented with 0.01 mg/ml bovine pituitary extract, 0.005 mg/ml insulin, 2.5 ng/ml insulin-like growth factor, 0.00125 mg/ml transferrin, 2.5 ng/ml epidermal
growth factor, 1000 U/ml penicillin-G, 100 µg/ml streptomycin, and 10% fetal bovine serum.

2.2.3. CNT-PLA-PEG Uptake In Vitro

CLP uptake in HUVEC and U-87 cells was determined via confocal microscopy. Cells were seeded at 12,00 cells per chamber in a Lab-Tek 8-chambered slide (Thermo Scientific). Nanoparticles were loaded with Alexa Fluor 647 cadaverine (Life Technologies, Grand Island, NY, USA), a fluorescent dye, by dissolving Alexa Fluor 647 (AF647) in a 50/50 dimethylformamide/acetonitrile (DMF/ACN) solution. CLP was dissolved in the AF647 solution on a rotisserie. Next, fluorescent dye-loaded CLP solution was prepared by solvent evaporation in HyPure water (Thermo Scientific). Samples were washed via centrifugation twice in HyPure water and once in sterile phosphate buffered saline (Sigma-Aldrich). Samples were re-suspended at 0.25 mg/ml in cell culture media and added to cells. After 24 hours, media was removed and cells were washed twice with sterile phosphate buffered saline (PBS). Cells were fixed with a 4% formaldehyde solution in PBS for 30 minutes, and then washed with PBS. Cells were incubated with a 0.1% Triton-X solution for 2 minutes, washed with PBS, and stained with Alexa Fluor 488 phalloidin. Finally, cells were mounted with VectaShield fluorescent mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). Slide chambers were removed and the slide was cover slipped. Confocal microscope images were taken with a Nikon Eclipse Ti confocal microscope.
2.2.4. Paclitaxel Release from CNT-PLA-PEG

Release of PTX from CLP was measured using high performance liquid chromatography (HPLC). Paclitaxel (LC Laboratories, Woburn, MA, USA) was encapsulated by dissolving CLP and PTX in ACN and rotating on a rotisserie protected from light. The CLP/PTX solution was then dropped in HyPure deionized water and PTX was encapsulated via solvent evaporation. Samples were washed three times in HyPure water via centrifugation. CLP/PTX was re-dispersed in HyPure water and added to the top of a 3.5 kD Slide-a-Lyzer MINI dialysis unit (Thermo Scientific). Each dialysis unit was placed in a vial with HyPure deionized water. Five repeats were used in this release study and samples were placed in an incubator at 37°C and 5% CO₂ for the duration of the study. At each time point dialysate was removed and frozen, and fresh HyPure water was replaced. Lyophilized aliquots were analyzed via HPLC on a Waters 1525 Binary HPLC pump with a 2998 photodiode array detector. An Alltima C18 column (Grace, Deerfield, IL, USA) was used with dimensions 4.6 x 25 mm with 5 µm pores. Samples and standards were dissolved in acetonitrile. The mobile phase used was 60% acetonitrile and 40% water. The mobile phase flow rate was 1 ml/min and PTX was detected at a wavelength of 227 nm with an average elution time of 11.5 minutes.

2.2.5. In Vitro Inflammation and Toxicity Studies

RLE-6TN cells were plated at 100,000 cells per well in a 12-well plate (Corning) and incubated at 37 °C and 5% CO₂ for 24 hours. Media was exchanged and returned to temperature before being dosed with NP at 1 or 10 µg/cm² for 6 hr. Media was then
removed and cells were lysed by Trizol (Life Technologies) and stored at -80°C. RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA, USA) and quantitated on a Nanodrop (Thermo Scientific). Reverse transcription of 1 µg of RNA was performed using a QuantiTect reverse transcription kit (Qiagen). QuantiTect primer assays (Qiagen) and SYBR green master mix (Qiagen) were utilized for Quantitative real-time PCR to examine mRNA expression levels of IL1r1l, IL-6, ICAM1 in RLE cells. Cycle threshold (Ct) values and internal reference cDNA levels for the target genes were determined by an Applied Biosystems StepOnePlus Real-Time PCR System (ABI). The cDNA levels of each treatment were then normalized to GAPDH, used as an internal reference, by the equation $C_{t_{\text{target}}} - C_{t_{\text{gapdh}}}$. The relative expression of each target was then calculated by $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ is defined as $\Delta Ct_{\text{treatment}} - \Delta Ct_{\text{control}}$.

HUVEC and U-87 cells were seeded at 10,000 cells per well in a 96-well plate (Corning) with five repeats per test concentration. CNT and CLP were prepared by solvent evaporation in HyPure water. Solutions were stirred for 2 hours and washed by centrifugation. NP were then re-dispersed in media and added to cells at different concentrations. Cells were incubated with the NP for 24 hours at 37°C and 5% CO2. Next, NP media was removed and cells were gently washed twice with PBS. A Presto Blue cell viability assay (Life Technologies) was performed by mixing Presto Blue reagent at a 1:9 ratio with cell culture media. 100 µl of working solution was added to the cells and incubated protected from light for 45 minutes at 37°C and 5% CO2. Fluorescent intensity was measured with a BioTek Synergy 4 plate reader at an excitation wavelength of 560 nm and emission wavelength of 590 nm. Cell viability was determined by
normalizing fluorescent intensity to the average intensity of five wells of cells only treated with media.

For Live/Dead staining, U-138 human glioblastoma cells were seeded at 10,000 cells/chamber in an 8-chambered slide and allowed to attach overnight. The next day nGr and PLA-PEG coated nGr (nGrLP) were dispersed in cell culture media and added to cells at varying concentrations. Cells were incubated with nanomaterials for 24 hours and then washed gently with sterile phosphate buffered saline. Live/Dead working solution was made by diluting ethidium homodimer-1 (EthD-1) and calcein AM to final concentrations of 2 nM and 1 nM, respectively, in Dulbecco’s PBS. One control well was killed by treating with 70% ethanol for 1 minute. Working solution was added to cells and incubated for 30 minutes. Cells were washed with PBS, fixed with 4% formaldehyde for 30 minutes, and coverslipped. Widefield fluorescent images were taken on a Nikon Eclipse Ti.

Efficacy studies of PTX-loaded CLP were performed using U-87 cells, in which cells were seeded at 10,000 cells per well in a 96-well plate. CLP was loaded with PTX as previously described and CLP/PTX was added to cells at 2.5, 5, and 10 µg/cm². Estimated PTX concentrations, based upon the release data were 70, 140, and 280 nM, respectively. Cells were incubated for 6 hours or 24 hours, and washed twice with sterile PBS. Fresh media was added to cells and they were grown for a total of 72 hours after initial exposure to NP. Cell viability was measured with a Presto Blue assay.
2.2.6. In Vivo Biodistribution Studies

Animals were housed at Clemson University’s Godley-Snell Research Center or within the Department of Comparative Medicine at East Carolina University. All studies were done in accordance with Clemson and East Carolina University Institutional Animal Care and Use Committee (IACUC) approved protocols. For the CNT biodistribution studies, Balb/c mice were used and no tumor xenografts were implanted. CL or CLP was loaded with AF 647 as previously described. The final wash in the centrifugal filter unit was done in sterile PBS. Samples were re-dispersed at 1 mg/ml in sterile PBS, and 200 µl of NP was administered via a tail vein injection. Live animal imaging was done with an IVIS Lumina XR small animal imaging system. At the final time point of 24 hours, animals were euthanized and kidney, liver, spleen, muscle, fat, heart, lung, and brain tissue was retrieved. Tissues were fixed in 4% formaldehyde and stored protected from light at 4 °C. For tissue staining, organs were incubated overnight in a 30% sucrose in PBS solution. Next, samples were embedded in optimal cutting temperature (O.C.T.) compound (Sakura, Torrance, CA, USA) and sectioned at 10 µm on a Microm HM 505N cryostat microtome. Sections were then stained with haematoxylin and eosin. Histology images were taken on a Nikon Labopho-2 microscope with a Sony HDR-HC9 camera.

Tumor xenograft biodistribution studies were performed to observe differences between CLP and nGrLP. U-138 cells were washed with sterile PBS. Cells were collected and concentrated in serum-free media at 40,000,000 cells/ml. 100 µl of cell suspension was added to 100 µl of matrigel. Athymic nude Balb/c mice were anesthetized with ketamine-xylazine. Skin was disinfected with chlorohexidine and eyes were
lubricated with Puralube ophthalmic ointment. A 1 cm sagittal incision was made across the top of the skull. The skull was sterilized with hydrogen peroxide. A hole was made 2 mm anterior and 1 mm lateral of the bregma using a 25-gauge needle. 3 µl of cell/matrigel solution was injected ~3 mm deep into the brain over 1 min. The skull was closed using dental cement and the incision site was stapled shut. Tumors were allowed to grow for 10 days. After 10 days, biodistribution studies were performed. nGrLP or CLP was prepared via previously described fluorescent loading method. Samples were washed in sterile HyPure H₂O and re-dispersed in sterile PBS at 1 mg/ml. 200 µl of NP was administered via a tail vein injection. Animals were euthanized at 24 hours and fluorescent images were taken with an IVIS Lumina XR small animal imaging system. Saline was injected as a control.

2.2.7. In Vivo Inflammatory Response and Maximum Tolerated Dose

C57BL/6 mice were obtained from Jackson Labs at 4 weeks of age. Mice were instilled with 4 mg/kg of CNT or CLP by oropharyngeal aspiration, and euthanized 30 days following aspiration of CNT or CLP. Mouse lungs underwent in situ bronchoalveolar lavage (BAL). The right lung was lavaged 4 times (26.25 ml/kg body weight) with ice-cold Hank’s balanced salt solution (HBSS). Bronchoalveolar lavage fluid (BALF) was collected and centrifuged at 1000 g for 10 min at 4 °C. Cell pellets were re-suspended in 1 ml HBSS and counted. In order to obtain differential cell counts, 20,000 cells from each BALF sample were centrifuged using a Cytospin IV (Shandon Scientific Ltd, Cheshire, UK) and stained with a three-step hematology stain (Richard
Allan Scientific, Kalamazoo, MI). Cell differential counts were determined and imaged for presence of CNTs or CLPs. Left lungs from CNT or CLP treated mice were perfused with 10% neutral buffered formalin fixative and stored for 24 h, then processed and embedded in paraffin. Samples were then sectioned at a thickness of 5 µM and were mounted on slides for staining with Masson’s trichrome to detect morphological changes and collagen deposition.

For the maximum tolerated dose studies, NPs were prepared as previously described. After washing, NPs were re-dispersed in sterile PBS at concentrations determined by mouse body mass. NP solutions were sonicated just prior to injection due to rapid aggregation of pristine CNTs. Following injection mice were monitored for 6 hours for signs of distress or deteriorating conditions. Mouse body mass and food consumption was monitored every day for one week following injection. Subjects whose body mass went below 20% of initial body mass or who were non-responsive were euthanized immediately. All subjects were euthanized at the final time point per IACUC protocol requirements.

2.2. Results and Discussion

2.2.1. Preparation and Characterization of Coated NP

The CLP drug delivery system was synthesized by using non-toxic organocatalyst phosphazene base P2-t-Bu. We eliminated the need for high temperature polymerization methods and potential toxicity from tin-based catalysts [22-24]. PLA was grafted on the surface of hydroxyl-functionalized MWCNT with an inner diameter of 5-15 nm, outer
diameter of 30-50 nm, and lengths from 10-20 µm. CNTs were used as provided and were dissolved in the presence of d,l lactide in anhydrous acetonitrile. CL particles were PEGylated by adding methoxy-PEG5k-isocyanate directly into the reaction. Our rationale for this approach was to utilize the high reactivity of isocyanate groups to chemically cap PLA with the PEG molecule on the surface of CNTs without using a purification step [25-27]. The CLP particles were washed through centrifugation to remove residual monomer, PEG, and catalyst, and the amount of polymeric coating was quantified by thermogravimetric analysis under N₂ flow (Figure 2.1A).

Pristine CNT showed no decomposition over the entire temperature range, from 30 °C to 600 °C, whereas CL exhibited a single stage of decomposition around 280 °C, revealing that approximately 60 wt.% of CL was the polymer PLA. CLP samples showed a two-stage decomposition, with PLA decomposing at approximately 280 °C and PEG decomposing at approximately 430 °C. This two-stage decomposition indicates the presence of two polymers: approximately 60 wt.% PLA and 20 wt.% PEG. The chemical bonds in the CNT composites were characterized via Fourier transform infrared spectroscopy (Figure 2.1B). The carbonyl stretch peak at 1745 cm⁻¹, present in both the CL and CLP samples, corresponds to the ester bond present in PLA. In the CLP sample there is a peak at 2881 cm⁻¹, which corresponds to the ether bond in PEG (Figure 2.1B, inset). ¹H NMR was taken on a Bruker Avance 300 NMR (300 MHz, CDCl₃) for CL (Figure 2.1C) and CLP (Figure 2.1D). Peaks for PLA were observed at 1.59 and 5.17 ppm. PEG had a single peak at 3.65 ppm. This data indicates the successful coating of CNT with an inner layer of PLA and an outer layer of PEG. Transmission electron
microscope images of CNT (Figure 2.1E) and CLP (Figure 2.1F) show that the polymer coating was approximately 10-15 nm.

Successful synthesis of nGrLP using the one-pot room temperature approach was also successful. PLA was grafted on the surface of hydroxyl-functionalized nGr. PLA coated nGr (nGrL) was PEGylated by introducing mPEG-isc directly into the reaction mixture. Polymer coating on nGr was confirmed via TGA, TEM, and NMR (Figure 2.2). Uncoated nGr showed little decomposition (~5 wt%) across the temperature range of 20 °C to 600 °C, while nGrL showed decomposition of approximately 20 wt% at 280 °C. PEG was shown to decompose around 430 °C. Thus, nGrLP was coated with approximately 20 wt% PLA and 70 wt% mPEG. TEM showed qualitative surface differences between nGr and nGrLP (Figure 2.2B), and nGr was plate shaped with dimensions of 5 µm by 5 µm. NMR confirmed the presence of PLA (Figure 2.2C) and PLA-PEG (Figure 2.2D).

Thus, this approach provides a means for covalently functionalizing inorganic NP with a multifunctional, polymeric coating. Our rationale was to encapsulate hydrophobic drug within the hydrophobic PLA matrix, which would then mediate the drug’s controlled release. The PEG layer is intended improve the aqueous dispersion of the nanomaterials, reduce NP clearance, and mediate the interaction of the NP with the body. Since PLA and PEG are FDA-approved polymers, we expected the polymers to decrease toxicity associated with the inorganic NP.
Figure 2.1 Chemical characterization of coated CNTs. (A) Thermogravimetric analysis (TGA) of CNT, CNT-PLA (CL), and CNT-PLA-PEG (CLP) showing the percent of polymer coating. (B) FTIR spectra of coated particles with expanded view to show the corresponding peaks for PEG at 2881 cm\(^{-1}\) (inset). (C,D) \(^1\)H NMR spectra for CL and CLP, with an inset of the polymer structure for PLA-PEG. (E,F) TEM images of CNT and CLP. CLP shows a 10-15 nm thick polymer coating. Scale bars represent 150 nm.
2.2.2. In Vitro Uptake, Toxicity, Inflammatory Response, and Controlled Release

CNTs were coated with PLA and PEG, two FDA-approved polymers, to decrease toxicity. The rationale is based on controlling the surface properties of CNTs, allowing them to disperse in an aqueous solution and allowing the polymer to mediate the interactions of carbon nanomaterials with cells. By improving aqueous dispersion, the interaction of CNT with cells is reduced and aggregation of the nanoparticles in blood is
subsequently reduced. Nanoparticles loaded with a hydrophobic fluorescent dye (Alexa Fluor 647) were used to study the uptake of CLP in two different cell lines: U-87 human glioblastoma cells and human umbilical vein endothelial cells (HUVEC) as a control. Cells were incubated with dye-loaded CLP for 24 hr in a chambered glass slide. Cells were washed, fixed, and stained for actin filaments and nuclei. Figure 2.3A and Figure 2.3B show the confocal images of the uptake of CLP in HUVEC and U-87 cells, respectively.

Toxicity in both HUVEC and U-87 cell lines was measured with a Presto Blue cell viability assay in which metabolically active cells reduced the non-fluorescent resazurin in the Presto Blue working solution into a red-fluorescent resorufin [28]. Cells were treated with varying concentrations of pristine CNT or CLP, and percent cell viability was determined by normalizing the fluorescent intensity of each test group with the average intensity of cells receiving only media. CNT showed a dose dependent toxicity in both HUVEC (Figure 2.3C) and U-87 (Figure 2.3D) cell lines. A Student’s t-test (p-value < 0.05) was performed to compare toxicity between CNT and CLP at each concentration. CNTs were significantly more toxic than CLP in HUVEC cells at all concentrations, up to 700 µg/cm². U-87 cells exhibited similar results where CNTs were significantly more toxic than CLP, from 140 µg/cm² to 700 µg/cm². Moreover, Tukey’s Honestly Significant Difference (HSD) test determined a demonstrable significance between each concentration of CNT and the 0 nM concentration (cell control). Though pristine CNT was significantly toxic in both HUVEC and U-87 cell lines in concentrations of approximately 70 µg/cm², no significant toxicity was evident in CLP.
using concentrations 10 times higher than pristine. Thus, coating our samples with the biocompatible PLA-PEG polymer reduces the toxicity associated with CNTs in vitro, which is critical for medical applications.

Inflammatory responses to CNT and CLP were tested in rat lung epithelial cells in vitro. Expression of interleukin-6 (IL-6), a cytokine involved in the inflammatory response, and intercellular adhesion molecule-1 (ICAM-1), a cell surface molecule involved in leukocyte extravasation, was measured following exposure to CNT and CLP at concentrations of 1 µg/cm² and 10 µg/cm² [29-31]. The fold difference in expression of IL-6 between CNT and CLP when normalized to the 1 µg/cm² CNT group is illustrated in Figure 2.3E. At 10 µg/cm² the expression of IL-6 for CNT was nearly three fold higher than for CLP. Moreover, ICAM-1 expression following CNT exposure at 10 µg/cm² was approximately 50% higher than the CLP (Figure 2.3F), indicating that coating CNT with PLA-PEG significantly decreased both toxicity and inflammatory response compared to non-coated CNTs.

nGr was also coated with PLA and PEG. Dose-dependent toxicity studies of nGr and nGrLP with U-138 glioblastoma showed that PLA-PEG coating decreased the toxicity associated with nGr. Figure 2.4 shows Live/Dead staining of U-138 cells after 24 hr exposure to uncoated nGr, and nGrLP. Cells treated with nGr showed dose dependent toxicity compared to the live cell control. With increasing concentrations of nGr there appeared to be not only more dead cells (red), but also a decreased cell population. Cells treated with nGrLP did not appear to have significantly more dead cells compared to the live cell control, and also did not display a decrease in cell population.
Figure 2.3 In vitro studies illustrate the uptake, toxicity, and inflammatory response of CNT-PLA-PEG (CLP). (A,B) Confocal microscope images show the uptake of fluorescently loaded CLP particles (red) in HUVEC and U-87 cells stained for actin filaments (green) and cell nuclei (blue). Scale bars represent 50 µm. (C,D) Toxicity studies of CNT and CLP in HUVEC and U-87 cells showed that polymer coating decreased NP toxicity. *indicates significant difference between each sample and the 0 µg/ml concentration, determined by Tukey’s HSD test. (E,F) Polymer coating on CNTs also decreased expression of inflammatory cytokines IL-6 and ICAM-1.
Figure 1.4 Live/Dead assay showed live U-138 cells (green), and dead cells (red). nGr showed dose-dependent toxicity both by increased presence of dead cells, and lower cell population density. nGrLP seemed to be relatively unaffected even at doses up to 25 µg/ml. Scale bars represent 500 µm.

In a controlled release study performed to quantify the amount of PTX released from CLP (Figure 2.5A), we determined that CNT-PLA-PEG could load 1.65 wt.% PTX and release the drug in a controlled manner for at least one week. To carry out in vitro efficacy experiments, U-87 cells were incubated for 6 hours and at 24 hours with drug loaded CLP to allow for nanotube uptake and intracellular release. Three different amounts of NP were tested containing the equivalent concentrations of 70, 140, and 280 nM of PTX (Figure 2.5B) compared to free drug. At the end of the time point, cells were washed with sterile PBS and allowed to grow for a total of 72 hours following initial exposure to the nanoparticles. This data emphasizes the importance of rapid nanoparticle uptake and controlled release due to short circulation time of free drugs.
Figure 2.5 (A) Release of paclitaxel (PTX) from CNT-PLA-PEG (CLP) was monitored by high performance liquid chromatography with the release sustained over a 1-week period. (B) PTX toxicity with free drug compared to drug loaded CLP shows that NP are more effective with shorter incubation time, apparently due to NP uptake and controlled intracellular release. *indicates significant difference between each sample and the 0 nM concentration, determined by Tukey’s HSD test.

Thus, this figure illustrates that the nanotubes are uptaken into the cells in as little as 6 hours and can mediate drug release over an extended period of time. Free paclitaxel efficacy is significantly reduced when the cells are exposed to drug for a short period of time (i.e. 6 hours). Free PTX at 140 nM incubated for 6 hours was only able to kill 12% of cells, compared to the 50% viability after 24 hours of exposure to free drug. However with the 6 hour incubation, CLP at a concentration equivalent to 70 nM of PTX was as effective as free PTX at twice the concentration (140 nM). This demonstrates that, with a shorter incubation time, CLP loaded with PTX was twice as effective as free PTX. A Tukey’s HSD test showed that at 6 hr incubation with free drug or drug-loaded CLP, only CLP loaded with the equivalent of 140 nM and 280 nM were significantly different than
the 0 nM control (cell blank). CLP loaded with an equivalent of 280 nM, however was as efficient after 6 hours of incubation as it was at 24 hours, perhaps due to the rapid uptake of the drug delivery vehicle saturation and the intracellular controlled release of PTX. To achieve a PTX concentration of 280 nM, a concentration of only 10 µg/cm² CLP was required. Therefore, only 3 µg of CLP loaded with PTX was able to eliminate 50% of U-87 cells in a 96-well plate. These results illustrate the potential of CLP as a safe, potent mediator of drug delivery and cancer therapy using low doses of CNTs.

2.2.3. In Vivo Biodistribution in Mice

Biodistribution studies in Balb/c mice showed clearance of fluorescently loaded CLP through the kidneys and bladder, as well as through the reticuloendothelial system, i.e. the liver and spleen (Figure 2.6). NPs loaded with a fluorescent dye Alexa Fluor 647 were administered via tail vein injection at a dose of 10 mg/kg to enable live animal imaging with an IVIS Lumina XR small animal imaging system. Animals were imaged at 5 minutes, 30 minutes, and 24 hours post injection (Figure 2.6A), and fluorescent images show fluorescent signal from CLP in the bladder. Kostarelos et al. [11, 32-35] determined that dispersed single-walled CNTs are cleared through the kidneys due to the alignment of thin diameter tubes with the pores of the glomerulus, approximately 5-20 nm in diameter. Our results suggest that some of the CLP nanotubes may be cleared via this mechanism while the majority of tubes are cleared via the liver and spleen similar to other nanomaterial drug delivery systems. Mice were euthanized and organs were explanted for fluorescent imaging after 24 hours. Figure 2.6B shows representative
images of the fluorescent signal in the spleen (iii) and liver (iv) from three repetitions. In addition to fluorescent biodistribution studies, mice were injected with saline, pristine CNT, CL, and CLP to analyze tissues using histology after 24 hours. Figure 2.7 shows the representative haematoxylin and eosin (H&E) staining of the liver, spleen, lung, and brain. Bright field microscope images show the accumulation of CNT aggregates in the lungs, liver, and spleen, with CL aggregates also in the liver, lung, and brain. In contrast, though CLP particles were found in the lungs and brain, the images suggest no CLP aggregation.

**Figure 2.6** (A) Representative images of live Balb/c mice injected intravenously with saline, fluorescently loaded CNT-PLA (CL), or fluorescently loaded CNT-PLA-PEG (CLP) showed clearance through the bladder. (B) Fluorescent imaging of explanted organs after 24 hr shows accumulation of fluorescent signal in the spleen, liver, and brain. Organs shown are (i) muscle, (ii) fat, (iii) spleen, (iv) liver, (v) kidneys, (vi) heart, (vii) lung, and (viii) brain.
Figure 2.7 Hematoxylin and eosin (H&E) staining of mouse organs following intravenous administration of saline, CNT, CNT-PLA, and CNT-PLA-PEG showed some deposits of NP in tissue. NPs are indicated with black arrows and white scale bars indicate 50 µm.
Biodistribution studies of CLP and nGrLP mice showed the passive accumulation of NP within intracranial U-138 tumor xenografts in nude Balb/c mice. Tumors were implanted within mice brains, and fluorescently tagged CLP or nGrLP were administered intravenously. Time-course studies with fluorescent imaging showed that both CLP and nGrLP accumulated within brains within 1 hr after injection (Figure 2.8A). There also appears to be strong fluorescent signal for nGrLP localized in the mouse spleen. Fluorescent imaging of explanted organs showed most nanoparticle accumulation in the liver, and spleen (Figure 2.8B). There was strong signal from the brain for both CLP and nGrLP, indicating passive accumulation of NP within tumors. There was also some accumulation of nGrLP in lungs. Thus, it appears that difference between fibrillar NP (CLP) and plate-shaped NP (nGrLP) leads to differences in NP biodistribution and accumulation in tissues.

2.2.4. In Vivo Inflammatory Response and Toxicity

Inflammatory response in C57BL/6 mice was monitored following the pulmonary instillation of both CNT and CLP. Lung tissue was collected 30 days after pulmonary instillation and stained with Masson’s trichrome (Figure 2.9A-C). Figure 2.9B shows that mice instilled with CNT clearly had aggregates throughout the lung tissue, with some inflammation, slight granuloma formation around the CNT aggregates, and a small amount of collagen deposition. No CNTs were visible with the CLP group when the entire lung was analyzed with histology (Figure 2.9C). Thus, the CLP particles effectively reduced the inflammation following instillation in the lungs.
Figure 2.8 In vivo biodistribution of fluorescently tagged CLP and nGrLP showed accumulation in intracranial tumors. (A) Time-course study of intravenously administered CLP and nGrLP showed accumulation in tumors and in organs such as the spleen, kidney, and liver. (B) Explanted tissues showed accumulation in the liver, kidneys, some lung, and brain (tumor). (i) fat, (ii) spleen, (iii) liver, (iv) kidneys, (v) heart, (vi) lungs, (vii) brain.
The inflammatory response was further investigated following pulmonary instillation in mice via bronchoalveolar lavage (BAL). Though macrophages collected from BAL were shown to uptake pristine CNT (Figure 2.9E), none containing CLP were found following pulmonary instillation. Figure 2.9G shows the percent of alveolar macrophages containing CNTs, comparing pristine CNT to PLA-PEG coated CNT. The results demonstrate that coating CNT in PLA-PEG can mitigate the inflammatory response associated with CNTs, possibly due to decreased NP aggregation and by imparting a hydrophilic superficial coating.

The polymer coating also increased the CNTs maximum tolerated dose. In a dose escalation study, Balb/c mice were injected intravenously via the tail vein with increasing amounts of either pristine CNT or CLP. Five mice were injected at each concentration. At 25 mg/kg; 85% of mice injected with pristine CNT expired in less than two minutes. However, all CLP mice injected at 25 mg/kg (not shown) and 40 mg/kg survived. Importantly, 60% of mice injected with CLP at 50 mg/kg survived. Figure 6h shows a Kaplan-Meier survival curve of mice injected with pristine CNT or CLP. Coating CNT with PLA-PEG reduces acute toxicity associated with intravenous administration of CNTs, possibly due to decreasing particle aggregation and thus decreasing capillary blockage and NP accumulation in the lungs as shown with histology analysis [36-38]. Moreover the maximum tolerated dose of coated CNT was twice greater compared to the pristine CNT.
Figure 2.9 C57BL/6 mice underwent pulmonary aspiration of either saline or 2 mg/kg of CNT or CNT-PLA-PEG (A-G). Masson’s trichrome staining of lung tissue of (A) saline, (B) CNT, and (C) CLP showed that CNT caused mild inflammation. Alveolar macrophages were collected via bronchoalveolar lavage following pulmonary instillation of NP (D-G). Arrows in (E) indicate macrophages containing CNT. No CLP particles were detectable in macrophages (F, G). Kaplan-Meier survival curves for Balb/c mice receiving intravenous tail vein injections of CNT or CLP (H) show that the polymer coating increases the maximum tolerated dose of CNT.
2.3. Conclusions

In this work we showed that an amphiphilic polymer coating on the surface of CNTs could reduce toxicity and increase therapeutic efficacy via the controlled delivery of the anticancer drug PTX. Here, the PLA layer serves to encapsulate and mediate the controlled release of hydrophobic drugs, while the PEG layer serves to improve aqueous solubility and improve pharmacokinetic properties. This CLP drug delivery system was significantly less toxic in both in vitro and in vivo when compared to pristine CNTs with a maximum tolerated dose of approximately 50 mg/kg and 25 mg/kg, respectively. Moreover, PTX-loaded CLP at equivalent concentrations of 70 nM Ptx was as effective as free PTX incubated for 6 hr at 140 nM. This approach for functionalizing inorganic nanoparticles (e.g. CNTs) illustrates how multifunctional polymer coatings can enable a previously unsuitable nanomaterial for biomedical applications.
2.4. References


CHAPTER THREE

MULTILAYERED POLYMER COATINGS TO CONTROL DRUG DELIVERY

Nanoparticles (NP) have been the subject of significant research as a methodology for controlled drug delivery. However, monolayered nanoparticles generally exhibit an initial non-ideal “burst” release, which limits the delivery of drug for prolonged periods [1, 2]. Utilizing multilayered particles may improve drug delivery capabilities. Here we report a one-pot room temperature ring-opening polymerization approach to functionalize carbon nanotubes (CNT) with multiple polymer layers composed of poly(glycolide) (PGA), poly(lactide) (PLA), poly(lactide-co-glycolide) (PLGA) and poly(ethylene glycol) (PEG) to tailor drug delivery and improve therapeutic efficacy compared to single layer coated CNTs.

3.1. Principles of Controlled Drug Release

Multilayered particles offer a solution to impart better control over drug release characteristics when compared to single layer particles. Loo, Lee, and Widjaja [3-7] reported multilayered microparticles composed of PGA, PLA, PLGA, poly(caprolactone) (PCL), poly(styrene) (PS), and poly(ethylene-co-vinyl acetate) (EVA) that controlled the release of the model drugs ibuprofen, metoclopramide HCl, and lidocaine [3]. Multilayered particles were fabricated via an emulsion solvent evaporation method resulting in an EVA core, with PS, PLA, and PLGA shells. These particles not only
altered the release kinetics, but the polymers exhibited spatial separation of drug loading within distinct layers [3, 4]. While single layered microparticles generally exhibited an initial burst release, closer to zero-order release kinetics were achieved with the use of multilayered particles. Multilayered microparticles have been previously fabricated using one-step solvent evaporation [3], biomimetic solution polymerization [8], spray draying [9], layer-by-layer assembly [10], and liquid jets with acoustic disruption [11, 12]. With CNTs as a nanoparticle template, we created a controlled drug delivery system for the anticancer drug dasatinib (DAS) by polymerizing multiple polymer layers directly onto CNTs.

3.2. Methods for Multilayered Coatings to Control Drug Release

A variety of methods have been developed to functionalize CNTs as mechanisms for delivering drugs [13-19]. Liu et al. [16, 17, 20, 21] functionalized CNT surfaces using a phospholipid-PEG molecule, whereby the phospholipid strongly adsorbs to the CNT surface and the PEG improves aqueous dispersion. Drug molecules could be further adsorbed to the CNT surface [17] or PEG could be modified to attach drugs [16]. Another approach involves the direct conjugation of drug to the CNT surface. Dhar et al. [22] conjugated a prodrug that is reduced intracellularly to release cisplatin. Previously, we have demonstrated that monolayer polymer coated CNTs can release paclitaxel for one week, improve therapeutic efficacy against U-87 glioblastoma compared to free drug, and reduced CNT-associated toxicity in vitro and in vivo. Here, we report a one-pot “grafting from” approach to sequentially add either block copolymers or random copolymers, i.e.
PGA-PLA or PLGA, where the polymer coating thickness can also be controlled. Capping the ring opening polymerization with PEG improved the aqueous stability of these coated CNTs. The significance of this work is that (1) synthesis occurs via a one-pot reaction at room temperature using organocatalysts; (2) CNTs are coated with multiple layers to control drug release kinetics. **Figure 3.1** illustrates the approach for polymerizing multiple polymer layers onto carbon nanotubes at room temperatures in a one-pot reaction.

*Figure 3.1* Hydroxyl-functionalized carbon nanotubes (CNTs) were coated with multiple layers of poly(glycolide) (green) or poly(lactide) (blue) via a one-pot synthesis approach. Monomers were added sequentially into the reaction to yield distinct layers of polymer coating on CNTs. Methoxy-poly(ethylene glycol)-isocyanate (red) was added to cap the reaction and impart an amphiphilic coating able to sequester hydrophobic drugs and disperse in aqueous solution.
Table 3.1 Experimental plans for multilayered nanoparticles.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Objective</th>
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<tbody>
<tr>
<td>(A) Synthesize multilayered polymer coated NP</td>
<td>Determine factors influencing the multilayered coating of NP.</td>
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<tr>
<td><img src="image1" alt="Diagram" /></td>
<td>Methods: TGA, TEM</td>
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<tr>
<td>(B) Determine drug release kinetics from multilayered NP</td>
<td>Investigate the effect of different polymer coatings on dasatinib release.</td>
</tr>
<tr>
<td><img src="image2" alt="Diagram" /></td>
<td>Methods: HPLC</td>
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<tr>
<td>(C) In vitro drug delivery efficacy</td>
<td>Determine the efficacy of different coatings on in vitro efficacy for delivery of dasatinib.</td>
</tr>
<tr>
<td><img src="image3" alt="Diagram" /></td>
<td>Methods: PrestoBlue cell viability assay</td>
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3.2.1. **One-pot Room Temperature Ring-opening Polymerization**

Hydroxyl-functionalized multi-walled carbon nanotubes (MWCNT-OH) were purchased from Sun Innovations, Inc. (Fremont, CA). D,l lactide was purchased from Purac Biomaterials (Lincolnshire, IL). Methoxy-poly(ethylene glycol)₅k-isocyanate (mPEG-isc) was purchased from Nanocs, Inc (New York, NY). Glycolide, ε-caprolactone, phosphazene base 1-tert-butyl-2,2,4,4,4-pentakis(dimethylamino)-2Λ⁵4Λ⁵- catenadi(phosphazene) (P₂-t-Bu), and all solvents were purchased from Sigma-Aldrich. All reagents were dried overnight under 28 in Hg. Next, both CNT and monomer were dissolved in 4 mL of acetonitrile (ACN) by stirring for 30 min. Phosphazene base P₂-t-Bu
was added as a catalyst and the reaction was continued overnight. The next day an aliquot of sample was removed, washed, and lyophilized for analysis. The second monomer was then dissolved in 2 ml of ACN and added directly into the reaction vessel. More P$_2$-t-Bu was added, the reaction vessel, and the reaction was continued overnight. Next, mPEG-isc was dissolved in 2 ml of ACN and added directly into the reaction vessel. The product was lyophilized and stored at -20 °C under N$_2$. TGA was performed on a TA Instruments Hi-Res TGA 2950 thermogravimetric analyzer under nitrogen from 25 °C to 600 °C at 20 °C/min. Transmission electron microscopy was performed on a Hitachi H7600T at 115kV.

3.2.2. Dasatinib loading and release.

Dasatinib (DAS) was purchased from LC Laboratories (Woburn, MA). High performance liquid chromatography (HPLC) was used to determine the release kinetics of DAS from coated CNTs. DAS was encapsulated by dissolving coated CNTs at 5 mg/ml in a solution of DAS. This CNT/DAS solution was then dropped at a 1:2 ratio in HyPure water and stirred for 2 hours, and DAS was encapsulated via solvent evaporation. Samples were washed three times in HyPure water via centrifugation. DAS-loaded CNTs were re-dispersed in HyPure water and added to the top of a 3.5 kD Slide-a-Lyzer MINI dialysis unit. Each dialysis unit was placed in a 1.5 ml microcentrifuge tube containing HyPure water. Five repeats per formulation were used and samples were stored at 37 °C. At each time point dialysate was removed and frozen, and fresh HyPure water was replaced. Lyophilized aliquots were analyzed via HPLC on a Waters 1525 Binary HPLC
pump with a 2998 photodiode array detector. An Alltima C18 column (4.6 x 25 mm, 5 µm) was used with mobile phase of 50/50 solution of ACN and 0.4 v/v% triethylamine (TEA) in water. The mobile phase flow rate was 0.8 ml/min and DAS was detected at a wavelength of 280 nm with an average elution time of 7.5 minutes.

3.2.3. Cell Culture.

U-87 glioblastoma and Eagle’s minimum essential medium (EMEM) were purchased from American Type Culture Collection (Manassas, VA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). U-87 glioblastoma cells were grown in EMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were subcultured at 35 °C and 5% CO₂. To test DAS efficacy in vitro, U-87 cells were seeded at 10,000 cells/well in a 96-well plate. DAS was loaded as previously described and drug loaded CNTs were incubated with cells at 10 µg/ml. Free DAS was prepared by first dissolving at 0.1 mg/ml in DMSO and diluting 1:1000 in EMEM. Fresh media, free DAS, or DAS-loaded CNTs were added to cells and incubated for 6hr or 24 hr. There were five repeats per formulation. After the initial time point, media was removed and cells were washed gently with EMEM. Cells were incubated in in fresh EMEM and incubated for a total of 72 hr after initial exposure to free drug or CNTs. Cell viability was measured with a Presto Blue assay by comparing fluorescent intensity of each formulation to the average fluorescent intensity of cells grown in only media.
3.3. Results and Discussion

3.3.1. Preparation and Characterization of Multilayered Carbon Nanotubes

Commercially available CNTs functionalized with surface hydroxyl groups were coated by initiating the ring-opening polymerization of glycolide directly on the CNT surface using phosphazene base P$_2$-t-Bu in anhydrous acetonitrile [23-25]. A CNT coated with a layer of PGA (CNT-PGA-OH) was the result. As PGA has a terminal hydroxyl group, PLA was directly polymerized from CNT-PGA-OH as a second layer by introducing d,l lactide into the reaction vessel. A multilayered coating of PGA-PLA on the CNT surface (CNT-PGA-PLA-OH) was the result. Finally, the polymer chain ends were capped by introducing methoxy-poly(ethylene glycol)-isocyanate (mPEG-isc) into the reaction vessel. Here the reactive isocyanate groups form a urethane linkage with terminal hydroxyl groups on the polymer chain ends, thereby coating CNTs with an external PEG layer (CNT-PGA-PLA-PEG) [26]. This capping step effectively terminated the polymerization. Multilayer polymer coating was confirmed via thermogravimetric analysis (TGA). Single layer CNTs was synthesized by simply introducing mPEG-isc directly into the reaction after the first polymerization step (Figure 3.2). Single layer CNTs comprised of PLA-PEG, PGA-PEG, copolymer PLGA-PEG, and poly(caprolactone)-poly(ethylene glycol) (PCL-PEG) were synthesized in this manner. However, the synthesis of PCL-PEG encountered issues with repeatability, possibly due to the liquid nature of the ε-caprolactone monomer. Therefore this formulation was not pursued further.
Figure 3.2 Thermogravimetric analysis of (A) CNT-PLA-PEG, (B) CNT-PGA-PEG, (C) CNT-PLGA-PEG, and (D) CNT-PCL-PEG shows the successful synthesis of single layered CNTs.

Polymer coating thickness can be controlled by varying the ratio of monomer (i.e. glycolide or d,l lactide) to initiator (i.e. hydroxyl-functionalized CNT), and tunable levels of PLA and PGA were polymerized onto CNTs. Moreover, the relative amounts of polymer in the multilayered CNT were controlled in the same manner. Figure 3.3A shows multilayered CNTs coated with a thin base layer of PGA (12 wt%) and a thick outer layer of PLA (42 wt%). By changing the reaction conditions, however, a thick base layer of PGA (48 wt%) and a thin outer layer of PLA (12 wt%) can be polymerized on CNT surfaces (Figure 3.3B). The isocyanate capping was also validated via TGA; Figure 3.3C shows TGA data for a multilayered CNT composed of all three polymer
layers: 25 wt% PGA, 25 wt% PLA, and 6 wt% PEG. The order of the monomer polymerization can also be changed. **Figure 3.3D** shows a multilayered CNT with 58 wt% PLA, 20 wt% PGA, and 10 wt% PEG. Single layer and multilayer coated CNTs were further characterized via transmission electron microscopy (**Figure 3.4**). While the thin single layer coating is difficult to discern, the multilayer coating clearly shows aggregated CNT with polymer coating. This data further confirms the presence of a polymeric coating on the carbon nanotubes.

**Figure 3.3** Thermogravimetric analysis shows successful control of multilayered coatings on CNTs. (A) By decreasing the initial monomer to initiator ratio it is possible to coat a thin layer of PGA on CNTs, followed by a thick layer of PLA. (B) Conversely it is possible to coat a thick layer of PGA and then have a relatively thin layer of PLA. (C) PEG capping imparts a fully functional multilayered CNT with a PGA, PLA, and PEG coating. (D) By altering the order of monomers added into the reaction it is possible to get a CNT coated with PLA, then PGA, and finally PEG.
By holding the mass of CNT constant and varying molar ratio of glycolide or d,l lactide, the weight percent of polymer coating could be controlled (Figure 3.5). Thus, this synthesis approach enables control over not only the thickness of the multiple layers, but also the spatial organization of these layers.

**Figure 3.4** Transmission electron microscope images at 100k magnification of CNT, CNT-PGA, and CNT-PGA-PLA coated carbon nanotubes. The polymer coating can clearly be seen with the multilayered coating. Scale bars represent 100 nm

### 3.3.2 Multilayered Nanoparticle Release Kinetics

Drug release kinetics of dasatinib (DAS), an Src/Abl kinase inhibitor [27, 28], were measured with high performance liquid chromatography (HPLC), and single layered CNTs were compared to multilayered formulations. Drug loading efficiency for CNT-PLA, CNT-PGA, CNT-PLGA, and CNT-PGA-PLA was 0.4, 4.4, 1.7, and 3.5 wt%, respectively. Thus, drug-loading efficiency of DAS is influenced by polymer composition of the coating. Each formulation also showed different release kinetics (Figure 3.6). Though DAS released immediately from PGA (instant), there was a lag time (delayed) in the release of DAS from PLA coatings. This variation in DAS release kinetics is possible
attributable to interactions between either the drug and the polymer matrix, or the polymer degradation kinetics [29-33].

Figure 3.5 The thickness of polymer coated onto CNT surface can be controlled by varying the ratio of monomer (i.e. glycolide or lactide) to CNT. (A) Poly(glycolide) (PGA) coating thickness on CNT was shown to increase by increasing the molar amount of glycolide in the reaction. (B) Poly(lactide) (PLA) exhibited a similar control over the PLA thickness by varying the molar amount of d,l lactide in the reaction.

Random copolymer PLGA showed a steady release profile similar to PGA (instant). The multilayered CNT-PGA-PLA released drugs in a near linear manner, however, suggesting that multilayered coatings offer a means of controlling both the loading and release profile.

3.3.3. In Vitro Efficacy of Drug-loaded Multilayered Particles

Controlling the release kinetics of DAS is expected to alter the therapeutic efficacy of these CNT drug delivery vehicles. Cell viability data using U-87 glioblastoma suggests that controlling release kinetics is critical for improving therapeutic efficacy of DAS, which is being investigated as a treatment against recurrent glioblastoma [27, 34].
Figure 3.6 Dasatinib release from polymer coated CNTs shows that a difference in the polymer coating exhibits control over the release kinetics. PGA coated CNTs show the quickest release. CNT-PLA had an initial lag period before release, and CNT-PLGA showed release similar to that of CNT-PGA. Multilayered CNT-PGA-PLA showed mostly linear release.

In Figure 3.7A, we show the efficacy of different DAS-loaded polymer coated CNTs compared to free DAS. Cells were exposed to different formulations at 10 µg/ml for either 6hr or 24hr. The different incubation times ensures CNT uptake into cells. Cells were washed after each time point and cell viability was determined at 72hr. The therapeutic efficacy was measured by normalizing all formulations to cells treated with cell culture media only. Multilayered CNTs showed significant improvement (40% viability) over DAS loaded CNT-PLA-PEG (55% viability), and free DAS (75% viability). Results showed that at both time points the PGA, PLGA, and multilayered formulations had significantly higher cytotoxicity than the free DAS and PLA coated CNTs. This is ostensibly due to the lag in release of DAS from PLA, and the low therapeutic loading in the PLA-coated CNTs. Thus it is apparent that altering the release kinetics by controlling the multilayered coatings on CNTs is critical to improving therapeutic efficacy.
Figure 3.7 Cell viability assays using U-87 glioblastoma indicated that polymer coating and dasatinib release kinetics result in different therapeutic efficacy. Cells were incubated with different formulations at 10 µg/ml for 6hr or 24hr and viability was tested at 72hr after initial exposure to drug loaded CNT formulations. (A) Multilayered CNT-PGA-PLA-PEG drug loaded formulations had significantly higher cytotoxicity to U-87 compared to monolayer formulations at 24hr exposure. (B) Multilayered CNT formulations also improved efficacy compared to spherical PLA-PEG nanoparticles.

After 24hr incubation, the DAS-loaded multilayered CNT-PGA-PLA-PEG formulation had lower cell viability viability than the monolayer formulations. The in vitro efficacy of polymer coated CNTs was compared to spherical polymeric PLA-PEG nanoparticles (Figure 3.7B). This data suggests that multilayered CNTs are also more effective than polymeric PLA-PEG nanoparticles to kill U-87 cells with DAS.

3.4. Conclusions

In conclusion we have reported a one-pot, room temperature synthesis to coat multiple polymer layers on CNTs for drug delivery applications. These multilayered coatings on the surface of CNTs are able to sequester hydrophobic drug, tailor drug release kinetics of DAS, and improve therapeutic efficacy against U-87 glioblastoma in
vitro. The synthesis occurs through a surface initiated ring opening polymerization using phosphazene base catalysts. Step-wise addition of different monomers allows for multiple polymer layers on the CNT surface. Multilayered coatings adopted release characteristics of both homopolymers PGA and PLA. Moreover, multilayered coating of PLA-PGA-PEG significantly improved therapeutic efficacy in vitro against U-87 compared to monolayered coatings. Coating nanomaterials with multiple layers of polymers has broad impact for drug delivery applications.
3.5. References


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CHAPTER FOUR
RADIOLUMINESCENT NANOPARTICLES FOR IMAGING DRUG DELIVERY

Theranostic nanomedicines are expected to improve cancer therapy by monitoring drug delivery, drug release, and drug efficacy. Continuous monitoring of drug biodistribution and intratumoral concentration would provide critical real-time feedback, and allow clinician’s to adjust treatment regimens in a personalized manner [1, 2]. Moreover, nanoparticle drug delivery systems (NPDDS) have been shown to increase localization of drug to tumor sites passively through the enhanced permeability and retention effect [3, 4], or through active targeting [5-7]. NPDDS are subsequently able to subvert some of the more limiting side effects of chemotherapy by decreasing systemic toxicity, increasing intratumoral concentration of drug, and improving therapeutic efficacy due to nanoparticle (NP) uptake into cells. Early theranostic nanoparticle systems were able to combine drug delivery with nanoparticle imaging [8-10]. However current technologies are aiming to develop theranostic nanomedicines able to quantitatively report in situ drug doses [5, 11-14].

4.1. Quantitative Approaches to Measure Drug Release

Recently, luminescent nanoparticles have been investigated as a means for measuring drug release kinetics in situ. Radioluminescence is a phenomenon whereby radiation energy (e.g. X-ray) is converted into visible light. As X-rays possess excellent soft tissue penetration, radioluminescence offers an appealing approach for measuring drug release. Radioluminescent nanoparticles have been previously investigated for drug
delivery and biomedical imaging applications [14-17]. This approach provides deep tissue penetration, and avoids complications from tissue autofluorescence. Previously, porous nanocapsules of gadolinium sulfoxide (Gd₂O₂S) doped with terbium (Gd₂O₂S:Tb) or europium (Gd₂O₂S:Eu) were shown to exhibit radioluminescence at excitation wavelengths of 544 nm or 621 nm, respectively [14]. Moreover by choosing optically absorbent drugs (e.g. doxorubicin), it was possible to measure the release of drug from radioluminescent nanocapsules due to the return of luminescent intensity as the drug is released. Radioluminescence of drug-loaded nanocapsules is quenched because there is overlap in the x-ray luminescent spectrum of the nanocapsules and the drug absorption spectrum. Thus, as drug is released, radioluminescent intensity of the particle returns. With optically absorbent drugs it is therefore possible to develop a theranostic nanoparticle system capable of delivering drug and quantitatively measuring drug loading.

Protoporphyrin IX (PpIX) is a photosensitizer (PS) used clinically in photodynamic therapy (PDT) [18, 19]. Clinically, PpIX is traditionally administered as its prodrug 5-aminolevulinic acid, which is biosynthetically converted into PpIX in vivo. NPDDS have been used to both deliver photosensitive drugs, and act as the stimulus for drug activation [13, 20-23]. Cui et al.[22] reported upconversion nanoparticles (UCNP), NP able to convert near infrared (NIR) light into visible light, to deliver zinc(II) phthalocyanine (ZnPc). Deep-tissue activation of ZnPc, a photosensitive drug, was possible because of the improved penetration of NIR light through tissue to activate the UCNP, which in turn activated ZnPc.
To develop theranostic nanoparticles, spherical Gd$_2$O$_3$:Eu nanoparticles were coated with poly(glycolide)-poly(ethylene glycol) (PGA-PEG) for the controlled delivery of PpIX. Quenching of Gd$_2$O$_3$:Eu-PGA-PEG (XGP) radioluminescence by PpIX was dose dependent, and NP could be used to measure drug loading concentration. XGP maintained radioluminescence after polymer coating and could be used for imaging in tissue ex vivo. Intracranial U-138 glioblastoma xenografts in nude mice showed that systemically administered NP accumulated in brain tumors and could be imaged non-invasively using radioluminescence. Finally, coated and uncoated NP were shown to be relatively non-toxic at a dose up to 25 µg/ml in U-138 glioblastoma in vitro, and in Balb/c mice after injections up to 100 mg/kg.

4.2. Methods for Theranostic Radioluminescent Nanoparticles

4.2.1. Gd$_2$O$_3$:Eu Nanoparticle Synthesis

Gadolinium(III) nitrate, europium(III) nitrate, urea, glycerol, sodium fluoride, sulfur powder, ammonium hydroxyide, glycolide, tetraethyl orthosilicate, and all solvents were purchased from Sigma-Aldrich. Gadolinium(III) nitrate (Gd(NO$_3$)$_3$) was dissolved in 2L distilled water (diH$_2$O). Europium(III) nitrate (Eu(NO$_3$)$_3$) was added at a concentration of 200 mM. Nanoparticles were collected via centrifugation and washed with diH$_2$O. Nanoparticles were re-dispersed in 20 ml diH$_2$O. Sodium fluoride (NaF) at was dissolved in diH$_2$O and 4 ml of NaF solution were added to the nanoparticle solution. After stirring for 1hr, solution was cooled to room temperature and washed with diH$_2$O.
**Table 4.1** Experimental plans for radioluminescent theranostic nanoparticles

(A) **Mechanism for Measuring Drug Release**

![Diagram showing the mechanism for measuring drug release](image)

- Red: Protoporphyrin IX
- Blue: Poly(ethylene glycol)
- Green: Poly(glycolide)
- Yellow: Gd$_2$O$_3$:Eu nanoparticle

(B) **Spectrometer Measurements**

- X-ray source
- Sample
- Luminescent detector
- In chambered slide
- Through pork tissue

(C) **Ex vivo imaging in tissue**

- X-ray source
- NP injection site
- IVIS Luminescent Camera
- Ex vivo liver tissue

(D) **In Vivo Studies**

- Biodistribution
- Maximum Tolerated Dose

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Nanoparticles were re-dispersed in 200 ml diH$_2$O and sonicated. Nanoparticles were heated to 80 °C to dry. Nanoparticles were heated at 600 °C for 2 hours to remove impurities. Nanoparticles were collected and cooled to room temperature.

4.2.2. Amine-functionalization of Gd$_2$O$_2$S:Eu Nanoparticle Functionalization

20 ul TEOS was added to the solution after the solution was stirred for 10 min. The particles were stirred and aged at room temperature for two hours. After that, 20 ul (3-aminopropyl) triethoxysilane (APTES) was added to the suspension and the reaction continued stirring for one more hour. The resulting particles were separated by centrifugation and washed three times with ethanol.

4.2.3. Poly(glycolide)-poly(ethylene glycol) coating of Gd$_2$O$_2$S:Eu(NH$_2$)

Nanoparticles were covalently coated with poly(glycolide) (PGA) using a room temperature, ring-opening polymerization. In a typical reaction, 40 mg of Gd$_2$O$_2$S:Eu(NH$_2$) and varying amounts of glycolide were dissolved in 4 ml of acetonitrile (ACN). 2M phosphazene base P$_2$-t-Bu in tetrahydrofuran was added as a catalyst. Methoxy-PEG-isocyanate (mPEG-isc), MW=5,000 Da, was purchased from Nanocs, Inc. (New York, NY). Next, mPEG-isc was dissolved in 2 ml of ACN and added directly to the reaction mixture. The product was lyophilized and stored at -20 °C under nitrogen.
4.2.4. Nanoparticle characterization

Chemical composition was analyzed FT-IR was performed with a Thermo-Nicolet Magna 550 equipped with a Thermo-SpectraTech Foundation series Endurance Diamond ATR. Thermogravimetric analysis was performed on a Hi-Res TGA 2950 thermogravimetric analyzer (TA Instruments, New Castle, DE) under nitrogen from 25 °C to 600 °C at 20 °C/min. Transmission electron microscopy was performed on a Hitachi H7600T transmission electron microscope at 115 kV. For radioluminescence experiments, X-ray was generated by a mini X-ray tube (Amptek Inc, Bedford, MA, USA) operated at a tube voltage of 40 kV and a tube current of 99 µA. Radioluminescence measurements were taken by a DMI 5000M microscope (Leica, Wetzlar, Germany) equipped with a DNS 300 Spectrometer (Intevac-DeltaNu, Laramie, WY, USA) with a 150 lines/mm grating blazed at 500 nm and with a cooled iDUS-420BV CCD camera (Andor, South Windsor, CT, USA). Ex vivo and in vivo radioluminescence images were captured with an IVIS Lumina-XR imaging system (Caliper Life Sciences, Hopkinton, MA, USA).

4.2.5. Radioluminescent Drug Quenching and Release Measurements

Radioluminescence measurements were taken to compare empty NP and PpIX-loaded NP. Briefly, NPs were loaded with PpIX via solvent evaporation. PpIX was dissolved at varying concentrations in an 80/20 solution of acetonitrile/dimethyl sulfoxide. NP were then dissolved in this PpIX solution at 5 mg/ml. NP were dropped at 1:2 ratio of PpIX/NP solution in HyPure H2O. Mixture was stirred for 2 hours to
evaporate solvent. NP were washed 5x in HyPure H\textsubscript{2}O to remove excess drug. Drug loaded NP were re-dispersed at 25 mg/ml in HyPure water and added to a chambered slide. Radioluminescent signal was measured with the spectrometer. Signal was analyzed by comparing the average intensity across the 630 nm peak to the average intensity across the 700 nm peak. PpIX is attenuates luminescence at 630, however has little absorbance at 700 nm. Therefore, comparing the 630 nm intensity to the 700 nm intensity provides a normalize measurement of signal attenuation.

Radioluminescence measurements were achieved similarly in pork tissue. Pork tissue was diced into cubes and placed into an 8-chambered glass slide. PpIX was loaded as previously described and NP were injected at 20 mg/ml into pork tissue. Radioluminescent intensity was measured with the spectrometer and signal ratios were calculated as previously described.

To measure drug release kinetics, coated NP were loaded with PpIX as previously described at a PpIX concentration of 1 mg/ml. After washing via centrifugation, NP were re-suspended at 10 mg/ml in HyPure water and placed into the top of 3.5kD Slide-A-Lyzer MINI dialysis unit. These were placed in 2 ml microcentrifuge tubes with HyPure water and stored at 37 °C protected from light. At each time point, microcentrifuge tubes were exchanged with fresh water and dialysate was lyophilized for future analysis. 50 µl of the top NP solution was removed and placed in a chambered slide for X-ray spectrometer measurements. X-ray spectrometer measurements were taken with 15 second exposure, and radioluminescent intensity for PpIX-loaded NP was compared to a blank control (unloaded NP).
4.2.6. In Vitro Cellular Uptake in U-138 Glioblastoma

Uptake of coated NP in U-138 glioblastoma was measured via radioluminescent intensity. Cells were seeded at 1,000,000 cells per T-25 flask. After allowing cells to attach overnight, coated NP were added to cells at 25 µg/ml and incubated for varying time points. At each time point cells were washed 2x with sterile PBS. Cells were then treated with 1 ml of trypsin and then centrifuged at 1,000 rpm for 5 minute. Trypsin supernatant was removed, being careful not to disturb the cell pellet. Samples were stored at 4 °C protected from light until all time points were completed. NP uptake was measured by taking radioluminescent images in the IVIS Lumina XR with the mini X-ray tube operated at a tube voltage of 40 kV and a tube current of 99 µA as the excitation wavelength and a DsRed emission filter. Uptake was quantified by taking region of interest measurements compared to a cell blank (i.e. no NP).

4.2.7. Ex Vivo and In Vivo Radioluminescence Imaging

Radioluminescent imaging in deep tissue was accomplished using the IVIS Lumina XR live animal imaging system. Porcine liver was a gift from Snow Creek Meat Processing (Seneca, SC). Nanoparticles were injected 1 cm deep into liver tissue and imaged with the IVIS small animal imaging system. Fluorescent Trilite Red quantum dots (Crystalplex, Fremont, CA) with an emission wavelength of 630 nm were injected as a control. Fluorescence images were taken using the IVIS Lumina XR with excitation wavelength of 430 nm and DS red filter. Radioluminescent images were taken using the bioluminescence setting of the IVIS system with the filter set at DSRed. X-ray was
generated using a mini X-ray tube operated at a tube voltage of 40 kV and a tube current of 99 µA.

For in vivo biodistribution studies, nude Balb/c mice were implanted with intracranial tumors of U-138 glioblastoma following the protocol of Ozawa et al [24]. Briefly, U-138 cells were washed with sterile phosphate buffered saline (PBS). Cells were collected and concentrated in serum-free media at 40,000,000 cells/ml. 100 µl of cell suspension was added to 100 µl of matrigel. Athymic nude Balb/c mice were anesthetized with ketamine-xylazine. Skin was disinfected with chlorohexidine and eyes were lubricated with eye ointment. A 1 cm sagittal incision was made across the top of the skull. The skull was sterilized with hydrogen peroxide. A hole was made 2 mm anterior and 1 mm lateral of the bregma using a 25-gauge needle. 3 µl of cell/matrigel solution was injected ~3 mm deep into the brain over 1 min. The skull was closed using dental cement and the incision site was stapled shut. Tumors were allowed to grow for 10 days. After 10 days, biodistribution studies were performed. Nanoparticles were prepared via the solvent evaporation approach without drug as described previously. Samples were washed in sterile HyPure H₂O and re-dispersed in sterile PBS at 1 mg/ml. 200 µl of NP was administered via a tail vein injection. Animals were euthanized at 24 hours and radioluminescent images were taken with the IVIS.

4.2.8. In Vitro Toxicity

U-138 cells were seeded at 10,000 cells per well in a 96-well plate (Corning) with five repeats per test concentration. Gd₂O₂S:Eu and Gd₂O₂S:Eu-PGA-PEG were prepared
by solvent evaporation in HyPure water. Solutions were stirred for 2 hours, washed by centrifugation. NP were then re-dispersed in media and added to cells at different concentrations. Cells were incubated with the NP for 24 hours at 37°C and 5% CO2. Next, NP media was removed and cells were gently washed twice with PBS. A Presto Blue cell viability assay (Life Technologies) was performed by mixing Presto Blue reagent at a 1:9 ratio with cell culture media. 100 µl of working solution was added to the cells and incubated protected from light for 45 minutes at 37°C and 5% CO2. Fluorescent intensity was measured with a BioTek Synergy 4 plate reader at an excitation wavelength of 560 nm and emission wavelength of 590 nm. Cell viability was determined by normalizing fluorescent intensity to the average intensity of five wells of cells only treated with media.

4.2.9. In Vivo Maximum Tolerated Dose

Animals were housed at Clemson University’s Godley-Snell Research Center. All studies were done in accordance with Clemson Institutional Animal Care and Use Committee (IACUC) approved protocols. For the maximum tolerated dose studies, Balb/c mice were injected with NP at varying concentrations relative to body mass. NPs were prepared as previously described via solvent evaporation. After washing, NPs were re-dispersed in sterile PBS at concentrations determined by mouse body mass. NP solutions were sonicated just prior to injection. Following injection mice were monitored for 6 hours for signs of distress or deteriorating conditions. Mouse body mass and food consumption was monitored every day for one week following injection. Subjects whose
body mass went below 20% of initial body mass or who were non-responsive were euthanized immediately via CO$_2$ asphyxiation, followed by cervical dislocation as a secondary means for euthanasia. All subjects were euthanized at the final time point per IACUC protocol requirements.

4.3. Results and Discussion

4.3.1. Preparation and Characterization of Polymer Coated Radioluminescent Particles

Radioluminescent nanoparticles were fabricated by doping the precursor Gd$_2$O(CO$_3$)$_2$·H$_2$O with the rare earth ions via urea precipitation in aqueous solution. Particle surfaces were then functionalized with amines via the adsorption of APTES. Next, surface amine groups were used to initiate the ring opening polymerization of glycolide, as previously described in Chapters 2 and 3. PEG capped the reaction resulting in Gd$_5$O$_2$S:Eu particles coated with PGA-PEG. Figure 4.1 shows the characterization of coated and uncoated particles. Thermogravimetric analysis (TGA) reveals a polymer coating with approximately 20 wt% PGA and 10 wt% PEG. Fourier transform infrared spectroscopy (FTIR) shows the presence of a polymer coating, with a carbonyl stretch peak from PGA at 288 cm$^{-1}$. Moreover, transmission electron microscopy images show aggregates of uncoated nanoparticles, and PGA-PEG coated nanoparticles. Photographs under white light, and under X-ray radiation showed that radioluminescence was preserved even with polymer coating. Uncoated and coated samples were photographed under visible light, and under X-ray excitation in the dark.
Figure 4.1 Characterization of radioluminescent nanoparticles. (A) Thermogravimetric analysis indicates multiple polymer coatings of ~20 wt% poly(glycolide) (PGA), and ~10 wt% poly(ethylene glycol) (PEG). (B) Fourier transform infrared spectroscopy (FTIR) of uncoated and PGA-PEG coated particles confirms the presence of a polymeric coating. (C) Transmission electron microscope images show aggregation of multiple uncoated nanoparticles (left), and two aggregated nanoparticles coated with PGA-PEG (right). Black scale bars represent 500 nm. (D) White light photograph (left) and X-ray photoluminescent photograph (right) of coated and uncoated nanoparticles shows that radioluminescence is maintained even after polymer coating.
Thus, the synthesis of NP and subsequent coating via surface-initiated ring-opening polymerization resulted in a system capable of imaging due to the inorganic Gd$_2$O$_2$S:Eu core and drug delivery due to the PGA-PEG coating.

4.3.2. Quantifying Drug Loading in Coated Nanoparticles

Nanoparticles with spectral characteristics that overlap with those of drugs have been previously used to measure drug release kinetics. Since drugs “quench” the luminescent signal from the nanoparticles, drug release is directly related to the return, or increase, in NP luminescence [13, 14]. Figure 4.2 illustrates the ability of the photoactive drug protoporphyrin IX (PpIX) to quench Gd$_2$O$_2$S:Eu-PGA-PEG (XGP) radioluminescence. UV/Vis measurements of PpIX in dimethylsulfoxide (DMSO) showed an overlap between PpIX absorbance at 630 nm and XGP radioluminescence at 627 nm. By increasing the concentration of PpIX loaded into the XGP particles, radioluminescent signal at 627 nm could be quenched. Radioluminescence was quantified by averaging the luminescent intensity across the full-width half-maximum (FWHM) of the 627 nm and 700 nm peaks. The ratio of these two values for each formulation (loaded with varying concentrations of drug) was normalized by the XGP with no PpIX loaded. This value gave a “percentage of signal” quenched, and Figure 4.2C shows dose dependent quenching of XGP with increasing PpIX loading. This data indicates that the XGP can be utilized as a mechanism to measure the amount of drug loaded in XGP based on the quenching of luminescence. This study was repeatable in pork tissue as shown in Figure 4.2D.
Figure 4.2 (A) UV/Vis spectrum of protoporphyrin IX (PpIX) in DMSO overlaid with the radioluminescent spectrum of XGP. PpIX absorbance at 630 nm overlaps with the luminescent peak of XGP at 627 nm. (B) XGP was loaded with PpIX using solvent evaporation and varying the loading concentration of PpIX. Increased loading concentration resulted in attenuation of the XGP radioluminescence due to PpIX quenching of the 627 nm peak. XGP luminescent signal at 700 nm was not affected by PpIX absorbance. (C) 627 nm peak signal was compared to the signal intensity of the 700 nm signal. The ratio of these signals was normalized by the radioluminescent signal of XGP with no PpIX. Normalizing gives a percentage of XGP signal quenched, and loading with a concentration of 5 mg/ml showed attenuation of approximately 50% signal. (D) Radioluminescent quenching could be similarly measured in pork tissue.
Pork tissue was diced and placed in an 8-chambered slide on the X-ray spectrometer. Unloaded XGP, and XGP loaded at 2.5 and 5.0 mg/ml PpIX were injected approximately 1 cm deep into pork tissue. Radioluminescent measurements were made and radioluminescent quenching in water and pork was compared as previously described. These data suggest that the radioluminescent, polymer-coated NP maybe be used to measure drug loading concentrations in XGP in deep tissue.

![Graph showing release kinetics and PpIX content](image)

**Figure 4.3** Release kinetics were measured via return of radioluminescent signal intensity (red) or via PpIX content in release study dialysate (blue). Data shows that increase in radioluminescent intensity can be correlated to the release of PpIX from XGP particles.

### 4.3.3. Kinetic PpIX Release Measurements

In a controlled release study performed to quantify the release of PpIX from XGP (Figure 4.3), it was shown that PpIX release could be measured by the radioluminescent signal of XGP. As PpIX was released from XGP over a 48 hr period, the luminescent
intensity of XGP increased. This indicated that the radioluminescent peak ratio of XGP was sensitive to the release of XGP. Figure 4.3A shows the radioluminescent intensity of drug-loaded XGP over time compared to a blank, non-loaded sample. At 48 hr 100% of blank luminescent intensity has returned. Measurement of PpIX content in dialysate from the release study further confirmed the release of PpIX (Figure 4.3B). Thus, XGP may have use as a sensor for not only measuring drug loading concentration, but also measuring drug release kinetics due to radioluminescent properties.

![Figure 4.3A](image)

**Figure 4.4** Radioluminescent IVIS imaging of XGP uptake into U-138 cells. (A) Luminescent images show increased uptake of XGP into cells over time. (B) Quantification of luminescent intensity normalized by a cell blank show the fold increase in luminescent intensity over time.
4.3.4. Cellular Uptake of Coated NP

The kinetic uptake of XGP in U-138 glioblastoma was determined by measuring radioluminescent intensity. T-25 cell culture flasks containing 1,000,000 cells were incubated with XGP at 25 µg/ml for varying time points. At each time point, cells were washed with PBS and collected by trypsinizing and centrifugation. Radioluminescent images were taken on an IVIS Lumina XR live animal imaging system with X-ray radiation used as the excitation wavelength and 1 second luminescent exposure through a DsRed emission filter. Luminescent intensity was measured with the Lumina XR software using the region-of-interest (ROI) tool. Luminescent intensity of cells incubated with XGP was compared to a cell blank. Figure 4.4 shows the kinetic uptake of XGP at 3, 6, and 24 hr. Radioluminescent images show increasing signal strength of XGP in U-138 (Figure 4.4A), and Figure 4.4B shows the fold increase in intensity over the cell blank as measured by ROI intensity.

4.3.5. Ex Vivo Imaging in Porcine Liver and Biodistribution in a Xenograft Model

Radioluminescent imaging is advantageous for imaging due to no autofluorescence, and deep penetration of X-ray due to minimal tissue scattering. It was possible to image radioluminescent NP injected as 1 cm deep in tissue. Quantum dots, fluorescent nanoparticles with narrow emission ranges that are excited by shorter wavelength ultraviolet or visible light, are limited for in vivo imaging applications due to tissue autofluorescence, and poor penetration of excitation wavelengths. Thus, by testing the luminescent intensity of quantum dots (control) and radioluminescent nanoparticles
injected deep into porcine liver tissue, it was possible to illustrate the advantages radioluminescent NP. Fluorescent quantum dots ($\lambda_{\text{ex}}$ 430 nm, $\lambda_{\text{em}}$ 630 nm) or radioluminescent NP were injected into porcine liver tissue at 1 cm deep. As seen in Figure 4.5, radioluminescent nanoparticles were clearly visible under X-ray excitation through porcine tissue. However quantum dots, while vividly fluorescent under 430 nm excitation (inset microcentrifuge photograph), were not visible through tissue. This is possibly due to the attenuation of the 430 nm excitation wavelength in tissue.

**Figure 4.5** IVIS luminescent images of saline, QD, and radioluminescent NP injections 1 cm deep in porcine liver tissue. Inset images of QD tubes show QD fluorescence under 430 nm excitation, but no luminescence after X-ray excitation. Radioluminescent inset images show luminescence after X-ray excitation, and little fluorescent activity after excitation at 430 nm. Luminescent images show that, while imaging QD in deep tissue is not feasible, radioluminescent NP are visible.
Figure 4.6 Biodistribution studies of athymic nude Balb/c mice with intracranial U-138 xenografts following intravenous tail vein injection of Gd$_2$O$_3$:Eu-PGA-PEG NP showed accumulation of nanoparticles in brain tissue. Dorsal and ventral images were taken with the X-ray source beam focused superior and inferior to the transverse plain. Strong luminescent signal was evident due to accumulation of NP in tumor xenografts by the enhanced permeability and retention effect. X-ray source is evident on the right of each photograph, and red circles emphasize the region excited by the X-ray beam.

Aside from imaging ex vivo, it was also possible to image accumulation of NP in tumors in vivo (Figure 4.6). Athymic nude Balb/c mice were implanted with intracranial U-138 glioblastoma xenografts, and mice were administered NP via tail vein injection. Mice were euthanized 24 hr post-injection. Radioluminescent images were taken superior and inferior of the transverse plain on the dorsal and ventral sides. Strong accumulation of NP is shown in the U-138 glioblastoma xenografts. This is most likely due to the accumulation of NP via the enhanced permeability and retention effect. Moreover, it was possible to detect nanoparticles accumulating in the spleen and liver. Thus, nonspecific mechanisms for tumor targeting (i.e. passive accumulation) are capable of accumulating
NP in tumors, and a non-invasive radioluminescent method for imaging NP biodistribution is possible.

4.3.6. In Vitro and In Vivo Toxicity

Toxicity in U-138 glioblastoma was determined in vitro. Cells were seeded in a 96-well plate and incubated with varying doses of Gd$_2$O$_2$S:Eu or polymer coated Gd$_2$O$_2$S:Eu-PGA-PEG for 24 hours. Viability was determined using a Presto Blue cell viability assay. Percent viability was measured by normalizing the fluorescent intensity of cells treated with NP to the average fluorescent intensity of cells treated with only media. Five repeats were used for each treatment group. As seen in Figure 4.7, both coated and uncoated NP were relatively nontoxic at 25 µg/ml. At higher concentrations (100 to 250 µg/ml) both treatment groups were significantly more toxic than the media only control (p-value < 0.05), and at 50 µg/ml the polymer coated NP was significantly more toxic than the control. However, at all concentrations there were no significant differences between the two formulations as determined by a Student’s t-test.

Maximum tolerated dose studies of Gd$_2$O$_2$S:Eu-PGA-PEG in Balb/c mice showed no systemic toxicity in doses up to 75 mg/kg following intravenous tail vein injection. A higher dose at 100 mg/kg lead to adverse side effects and the subject was euthanized at 72 hr (data not shown). NP toxicity is mediated by material, size, shape, surface characteristics, and porosity. Previous maximum tolerated dose studies of inorganic nanoparticles have shown dose-dependent toxicity ranging from 30-1000 mg/kg for silica
NP [25, 26], or 25-50 mg/kg for carbon nanomaterials [27]. Thus, the toxicity of the polymer coated Gd$_2$O$_2$S:Eu NP falls within ranges similar to other inorganic NP.

![Graph A](image1.png)  ![Graph B](image2.png)

**Figure 4.7** (A) In vitro toxicity of coated and uncoated NP in U-138 glioblastoma showed no significant difference between the two formulations. (B) In vivo maximum tolerated dose studies of Gd$_2$O$_2$S:Eu-PGA-PEG in Balb/c mice showed no systemic toxic effects after intravenous administration after 1 week up to 75 mg/kg. Systemic toxicity was determined by monitoring changes in percent body mass following injection. A drop in <20% body mass was designated as toxic.

### 4.4. Conclusions

This NP system, PGA-PEG coated Gd$_2$O$_2$S:Eu, enables a 2$^{nd}$ generation theranostic approach whereby the NP is not only able to deliver drug and image NP location, but the interaction between NP and drug enables a metric for measuring the amount of drug loaded within the NP. Characterization data confirms the presence of a polymer coating comprised of 20 wt% PGA and 10 wt% PEG, and the Gd$_2$O$_2$S:Eu NP radioluminescent properties are maintained after polymer coating. Moreover, spectral overlap between the photosensitizer PpIX and the NP’s radioluminescence enables the particle to measure drug loading in a dose sensitive manner. This phenomenon was
observed both in water and through pork tissue using an X-ray excitation source and spectrometer. Moreover, release studies showed that an increase in XGP radioluminescent intensity correlated with PpIX release. Thus, these particles are an effective theranostic agent for imaging drug release. Imaging XGP following injection 1 cm deep in porcine liver tissue showed that radioluminescent imaging in deep tissue was possible, a feat not possible using fluorescent quantum dots. While X-rays have excellent soft tissue penetration that can excite the radioluminescent NP, QD are limited by the poor tissue penetration of short, UV or visible light excitation wavelengths. Moreover, non-invasive radioluminescent imaging of Gd₂O₂S:Eu-PGA-PEG NP in athymic nude Balb/c mice with U-138 glioblastoma xenografts showed strong passive accumulation of NP in intracranial tumors. Finally, coated and uncoated NP were shown to be non-toxic in vitro in U-138 glioblastoma cells at a concentration up to 25 µg/ml. There were no significant differences in toxicity between the two formulations at any concentration. XGP showed no significant systemic toxicity at concentrations up to 75 mg/kg when administered to Balb/c mice via tail vein injection. Thus this nanoparticle system is a versatile, theranostic tool for imaging drug delivery.
4.5. References


CHAPTER FIVE

CONCLUSIONS

Nanotechnology can enhance current cancer therapies by improving the delivery of drug to tumors over biological barriers, prolonging drug resistance in the body, and providing tools to measure the delivery of drugs in situ. While nanotechnologies can improve the clinical efficacy of drugs, the growing field of theranostic nanomedicine has yet to deliver a comprehensive platform capable of mediating both controlled drug delivery while also quantitatively measuring drug loading and release. Theranostic nanomedicine aims to merge both controlled drug delivery and imaging, and hybrid organic-inorganic nanoparticles offer a potentially robust platform that capitalizes on the optimal imaging properties of inorganic nanoparticles and the controlled delivery properties of polymeric materials. Moreover, polymeric coatings on inorganic nanoparticles may also improve their utility for biomedical applications by mitigating potentially toxic side effects. Challenges in development of a theranostic nanoparticle system include toxicity concerns surrounding nanomaterials and physical limitations for imaging. The presented work demonstrates an approach that may be utilized to functionalize inorganic nanoparticles that can be translated across multiple nanoparticle “templates,” and the data also points to the use of these particles to measure drug concentrations in situ. Moreover, the polymer-functionalized nanoparticles were able to mitigate nanomaterial toxicity, control delivery of anticancer drugs, and non-invasively measure the concentration of drug loaded in the nanoparticle.
5.1. Polymer Coatings Improve Nanoparticle Toxicity Profile

Nanoparticles, in particular inorganic NP, face concerns regarding toxicity mediated by NP aggregation, increased cellular oxidative stress, and toxicity due to nanoparticle interaction on a cellular and sub-cellular level. We developed a one-pot, room temperature approach to coat inorganic NP with the FDA-approved polymers poly(lactide) (PLA), poly(glycolide) (PGA), and poly(ethylene glycol) (PEG). We
hypothesized that the polymeric coating would mitigate inorganic NP toxicity, enable drug encapsulation, and prolong drug release. The rationale being that the multifunctional polymer coating would mediate the interaction between NP and cell through the less toxic polymer layer, sequester and control the release of hydrophobic drug within the polymeric core (PGA or PLA), and improve the aqueous dispersion of NP via the PEG outer layer. This functionalization approach could be applied across hydroxyl-functionalized carbon nanotubes (CNT-OH), hydroxyl-functionalized nano-graphene (Gr-OH), and amine-functionalized europium-doped gadoliniumsulfoxide radioluminescent nanoparticles (Gd$_2$O$_2$S:Eu-NH$_2$). Proof-of-concept work with PLA-PEG coated CNT (CLP), showed that the hydrophobic anticancer drug paclitaxel (PTX) could be sequestered within the hydrophobic PLA coating. PLA mediated the release of PTX over a one-week period, and CLP loaded with PTX was shown to be more effective than free PTX when treating U-87 glioblastoma in vitro.

Furthermore, the PLA-PEG coating on CNT significantly improved CNT toxicity profile. In vitro studies with U-87 glioblastoma and human umbilical vein endothelial cells (HUVEC) showed that CLP was significantly less toxic compared to pristine CNT. In vivo maximum tolerated dose studies in Balb/c mice showed that the PLA-PEG coating on CNT doubled the maximum tolerated dose from 25 mg/kg (for CNT) to 50 mg/kg. Thus, coating inorganic nanoparticles in FDA-approved polymer not only increased their functionality as a drug delivery vehicle, but also improved their safety for use in a biomedical application.
Coating inorganic NP with polymer decreases particle aggregation, and mediates the interaction of inorganic NP with cells.

**Figure 5.2** Coating inorganic NP with polymer decreases particle aggregation, and mediates the interaction of inorganic NP with cells.

### 5.2. Controlling Drug Delivery

A number of factors influence drug loading and release kinetics from nanoparticles including polymer thickness, nanoparticle size, drug chemistry, polymer degradation rates, and polymer chemistry. Multilayered particles have been shown improve control over drug release kinetics. We hypothesized that by extending the one-pot reaction approach to sequentially coat NP with multiple polymer layers (e.g. PLA-PGA-PEG, PGA-PLA-PEG, PLGA-PEG) it would be possible to control drug release kinetics.
Figure 5.31 Multilayered polymer coatings enable higher degree of control of drug release kinetics.

The rationale being that by controlling the polymerization conditions it would be possible to control polymer coating thickness, composition, enable multilayered particles, and in turn control drug release characteristics. Results showed that by controlling the synthesis conditions it was possible to tailor single polymer coating thickness, order of polymer coatings, and relative polymer coating thickness in multilayered constructs. Using dasatinib (DAS) as a model drug, we showed that multilayered polymer coatings were able to closer approximate zero-order release kinetics compared to single layer and random copolymer (PLGA) coatings. Furthermore, multilayered CNT were more efficacious when treating U-87 glioblastoma in vitro compared to free DAS, and DAS loaded into PLA-PEG NP (no CNT). The broader implications of this work are that the multilayered coating may be applied across more inorganic NP platforms, enabling a
higher degree of control over drug release characteristics. This therefore becomes a highly controllable system for creating hybrid organic-inorganic drug delivery vehicles.

5.3. Measuring In Situ Drug Concentration

Applying the one-pot room temperature approach for NP functionalization, we were able to coat radioluminescent Gd$_2$O$_2$S:Eu nanoparticles with PGA-PEG. These nanoparticles are able to convert X-ray irradiation to visible light, emitting at 630 nm. The benefit of radioluminescence over other optical approaches is the low autofluorescence in radioluminescent imaging, and excellent soft tissue penetration of X-ray radiation compared to ultraviolet (UV), visible (Vis), and near-infrared (NIR) wavelengths. Moreover, the PGA-PEG coating encapsulated the hydrophobic photosensitizing drug protoporphyrin IX (PpIX). The photoluminescent emission spectrum of Gd$_2$O$_2$S:Eu overlapped with the absorption spectrum of PpIX, and the drug could therefore be used to attenuate the luminescent intensity of the NP in a dose-dependent manner. We hypothesized that this NP could be used to measure drug concentrations in situ due to the overlap of spectral characteristics. Indeed, the attenuation of NP radioluminescence was directly correlated to loaded drug concentration. This phenomenon was observed both in water and through pork tissue, indicating that the radioluminescent NP was sensitive enough to report drug loading concentrations in deep tissue.

As an imaging agent, the PGA-PEG coated Gd$_2$O$_2$S:Eu NP (XGP) was significantly better than other imaging nanoparticles, such as quantum dots (QD). QD are
excited in the UV/Vis range of light, a range that is strongly attenuated and scattered by hemoglobin in blood and tissue. Thus, QD were used as a control and radioluminescent imaging showed that XGP was easily discernible when injected 1 cm deep in blood-rich porcine liver tissue, while QD were not visible under fluorescent imaging. This is possibly due to the attenuation of excitation wavelengths for QD, whereas X-ray can easily penetrate the porcine liver. Furthermore, the capacity for XGP to act as an imaging agent was demonstrated with intracranial U-138 glioblastoma xenograft models in athymic nude Balb/c mice. NP were administered through a tail vein injection, and noninvasive radioluminescent imaging of mice after 24 hr showed appreciable luminescent signal from the brain (i.e. the intracranial tumor).

Toxicity studies of XGP in U-138 glioblastoma in vitro, and in maximum tolerated dose studies in Balb/c mice showed that XGP was relatively non-toxic. In vitro there was no significant toxicity at 25 µg/ml, and no significant toxicity between uncoated Gd₂O₂S:Eu and XGP at any dose. In vivo, XGP showed no systemic toxicity at concentrations up to 75 mg/kg. Thus, this system offers a potential theranostic NP capable of measuring in situ drug concentrations and imaging NP biodistribution non-invasively.

5.4. Limitations

While the research presented represents steps towards the development of a quantitative theranostic nanomedicine, there are still practical challenges towards realizing a nanotechnology capable of in situ measurement of drug release kinetics.
One of the primary challenges this type of theranostic nanomedicine, one that utilizes the overlap of optical properties of nanoparticle and drug for measuring drug release kinetics, is the dependence on drugs that have distinct and strong optical properties. This research was able to show that release kinetics could be measured using protoporphyrin IX as a model drug, and the literature frequently uses doxorubicin and ibuprofen. However, such a system is not suitable for drugs that do not have strong optical absorbances in the optical transmission window of 650-950 nm. Moreover, the use of optical phenomena is suitable for imaging and quantifying drug release with superficial tumors, or tumors that are within the penetration depth of visible light. However, some tumors may have difficulty imaging through dense bone tissue, such as intracranial tumors.

Concerns also exist regarding the long-term fate of inorganic NP. Our data shows that polymer coatings can mitigate toxicity associate with inorganic NP in vitro and in
vivo. However, the long-term fate of such nanomaterials is unknown, and systematic studies of NP toxicity are relatively recent in the literature. Thus, such a theranostic nanomedicine must present robust evidence supporting its clinical use. Some nanomaterials, such as gold nanoparticles and iron oxide, are already on the market and have a history of clinical success. However new nanomaterials, such as the Gd$_2$O$_2$S:Eu NP or CNT, must be investigated for their long term biodistribution, clearance, and toxicity.

5.5. Summary

The presented results highlight the development of a theranostic NP system capable of measuring drug concentrations and noninvasively imaging in deep tissue. The results further demonstrate an approach that can improve the utility of inorganic NP for biomedical applications by via a multifunctional polymeric coating. The approach for NP functionalization can be applied across a number of NP platforms, and multifunctional coatings serve to improve the biocompatibility of nanomaterials and mediate controlled drug release. The combination of radioluminescent nanoparticle with the polymeric coating represents a small step towards more functional theranostic agents that can not only combine delivery and imaging, but also measure the concentration of drug non-invasively. Furthermore, the development of hybrid NP may lead to innovative approaches to the diagnosis and treatment of cancer.
CHAPTER SIX

RECOMMENDATIONS

Based on the current state of the relevant literature, the data presented, and the limits of these studies, the following future work is recommended:

1. **In vivo tumor reduction models**

   Previous studies have shown the improved safety of carbon nanotubes (CNT) following coating with PLA-PEG both in vitro and in vivo. Furthermore, the multifunctional polymer coating enabled controlled release of paclitaxel (PTX) and dasatinib (DAS), which improved therapeutic efficacy in vitro compared to free drug and spherical PLA-PEG NP. It would be interesting to see how CNT-PLA-PEG would improve the therapeutic efficacy in a more complex biological setting, i.e. an in vivo tumor xenograft volume reduction study. Previous data (Appendix I) has shown that CNT-PLA-PEG is able to accumulate when administered intravenously in intracranial U-138 glioblastoma xenografts in athymic nude Balb/c mice. However, observing the therapeutic efficacy of drug-loaded CNT-PLA-PEG compared to free drug or spherical PLA-PEG NP would provide further insight into the potential benefits of this drug delivery vehicle.

2. **Coat different shaped NP**

   Shape has been shown to play a critical role in the cellular uptake, biodistribution, tumor accumulation, and circulation time in nanomedicine [1-6]. It would be interesting to coat multifunctional polymers on different shaped NP and study their release kinetics, cellular
uptake, biodistribution, and efficacy in vitro and in vivo to deliver drugs. Preliminary data with plate-shaped, PLA-PEG coated nano-graphene (Appendix I) shows distinct differences between the plate shaped NP, and PLA-PEG coated CNT, which have a more fibrillar shape. It would be interesting to see how different, possibly biomimetic shaped NP may change the utility of polymer coated NP.

3. **Expand coating to other polymers**
A number of other polymers may be used for ring opening polymerization. This coating method could be attempted, optimized, and refined with different monomers such as ε-caprolactone [5] or p-dioxanone [7, 8]. For example, ε-caprolactone was shown to successfully polymerize poly(caprolactone) (PCL) on hydroxyl-functionalized CNT. However, we had limited success repeating this study. It would be interesting to see if changing reaction conditions (e.g. reaction time, monomer/catalyst ratio, monomer/initiator ratio, temperature, solvent system, etc.) could produce better yield. Incorporating new polymers into the multilayered constructs could also prove interesting towards modifying loading and release kinetics of drugs [9, 10].

4. **Expand the number of drugs used**
Currently we investigated two drugs: PTX and DAS [5, 11, 12]. However, there are many drugs in pre-clinical development, clinical trials, or in clinical use that could benefit from loading into a controlled release delivery vehicle. It would be interesting to investigate the delivery of different drugs, or drugs delivered in combination from multilayered NP.
For example, PTX is a mitotic inhibitor [13], and DAS works via the inhibition of the BCR/Abl and Src family of tyrosine kinases [14]. However the loading efficiency, release kinetics, and therapeutic efficacy of drugs that have a different mode of action or different molecular targets could improve therapeutic efficacy when delivered via the polymer coated drug delivery vehicle. Lapatinib is one such drug that inhibits the tyrosine kinases HER2/neu and epidermal growth factor receptor [15], and methotrexate (MTX) is another hydrophobic anticancer drug that works by inhibiting the enzyme dihydrofolate reductase [16]. Thus, all four of the drugs mentioned here have different modes of action, and varying drugs may lead to improved efficacy to treat different cancers. Furthermore, therapeutic efficacy of new agents could benefit from administration via a controlled release delivery vehicle [17, 18].

5. **Study polymer functionalized upconversion nanoparticles as theranostic agents.**

Upconversion NP, particles capable of converting near infrared (NIR) light into visible light, have been previously used as mediators of photodynamic therapy and as imaging agents [19-21]. While radioluminescent NP have better soft tissue penetration, upconversion NP have other advantages such as no toxicity associated with high-energy radiation. Moreover, NIR light still has decent tissue penetration (3.5 cm), low autofluorescence, and tissue can be exposed to NIR radiation at higher intensities for longer. It would be interesting to study polymer coated upconversion particles for measuring drug concentrations and release, and as mediators to activate photosensitive drugs.
6. **Radioluminescent nanoparticles for photodynamic therapy.**

Our data shows that radioluminescent NP could measure drug release kinetics. However, it would be interesting to study whether radioluminescent NP could mediate photodynamic therapy. Photosensitizers face several clinical limitations. First, systemic distribution of drug can result in accumulation and photosensitivity in non-target tissues and organs, such as the skin and eyes [22]. Secondly, activation of PS by visible light is limited by the attenuation and scattering of activation wavelengths of light. Therefore PDT is generally reserved for pathologies that are easily accessible to a light source such as melanoma or head and neck cancer. NPs offer solutions to these challenges by sequestering photosensitizers into a vector that improves their biodistribution and pharmacokinetic properties. Moreover, NPs that convert radiation wavelengths that more easily penetrate tissue (i.e. X-ray or NIR) into visible light may enable deep tissue activation of PS. Thus, utilizing the emission wavelength of radioluminescent NP to activate PS would enable treatment of tumors in deep tissue.

7. **Study the long term fate and effects of inorganic NP**

While in vitro dose escalation and in vivo maximum tolerated dose studies elucidated a safe range of use for polymer coated NP, it would be prudent to study the long term fate and effects of these NP following systemic administration in vivo. In particular, the work and data presented regarding the radioluminescent Gd$_2$O$_2$S:Eu-PGA-PEG (XGP) NP indicate safe ranges to avoid acute toxicity, however NP that remain for prolonged
periods in the body may begin to exhibit systemic toxicity due to NP degradation and leaching of constituent components (e.g. europium) [23, 24].

8. **Expansions of NP systems to broader applications**

It would be interesting to apply these inorganic-organic hybrid NP to applications beyond cancer therapy either in drug delivery, or potentially tissue engineering scaffolds. Many scaffolds in literature are comprised of a polymer matrix with some inorganic component embedded, particularly for bone regeneration [25-27]. The inorganic component may provide mechanical support, act as a sensor, or promote biological cues. For example, Mattioli-Belmonte et al. [26] showed that for PCL scaffolds loaded with CNT, varying the design of CNT the percent CNT loading significantly influenced mechanical properties, cell behavior of MG-63 human osteoblast-like cells, and cell viability. Covalently modifying the CNT with polymer, as opposed to merely encapsulating CNT within a polymer matrix, may mitigate CNT-induced toxicity or alter the interaction of cells with the scaffold. Thus, incorporation inorganic NP into polymeric scaffolds where the polymer is covalently attached to the inorganic component may show interesting applications for scaffolds or other biomedical applications.
6.2 References


9. Alexis F. Factors affecting the degradation and drug-release mechanism of poly(lactic acid) and poly[(lactic acid)-co-(glycolic acid)]. Polymer International. 2005; 54: 36-46.


CHAPTER SEVEN
LIST OF PUBLICATIONS

7.1. List of Original Contributions


7.2. List of Reviews and Book Chapters


7.3. List of Submitted Works and Works Under Preparation


7.4. List of Patents


7.5. List of Awards and Affiliations

- Page Morton Hunter Bioengineering Graduate Researcher Award for excellence in research, 2013.

- “Multifunctional polymer coated carbon nanotubes for safe drug delivery” chosen as cover art of April 2013 issue of *Particle & Particle Systems Characterization*.

- “Monitoring pH-triggered drug release from radioluminescent nanocapsules with x-ray excited optical luminescence“ selected as article of the month for the *ACS Nano* podcast.

- 1st place award for Graduate Research oral presentation at the *SFB Biomaterials Day* at Clemson University, 2012.

- Award for university with highest cumulative points for oral presentations at the *W.L. Gore Bioengineering Partnership Meeting*, 2012.

- 3rd place award for outstanding performance in scientific oral communication for Bioengineering Departmental Seminar, 2011.


- American Chemical Society, 2012 - present.

- Clemson Bioengineering Society, 2009 - present.