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I, Christopher Huth, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Materials Science.

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Development of Multifunctional Nanoparticles for Cancer Therapy Applications

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Development of Multifunctional Nanoparticles for Cancer Therapy Applications

by

Christopher Huth

Abstract

The focus of this thesis is the functionalization and tailoring of nanoparticle surfaces to perform specific objectives in a biological environment. The nanoparticles examined include carbon nanotubes (CNTs), superparamagnetic iron oxide nanoparticles and superparamagnetic iron oxide nanocomposites. The unique nanomaterials have been developed to address continued issues in cancer therapy, including cancer diagnosis and efficient drug delivery.

CNT surfaces were modified by plasma polymerization, providing functional groups for conjugation. Luminescent amine labeled quantum dots were fixed to the surface of the CNTs to aid in cancer diagnosis by in vivo imaging. Mice, injected with the quantum dot functionalized carbon nanotubes, were imaged displaying the in vivo imaging capability. In addition, the drug loading and drug release capabilities were examined by incorporating the drug paclitaxel into PLGA-coated CNTs, which showed much higher cytotoxicity to PC-3MM2 human prostate carcinoma cells compared to CNTs without paclitaxel. Paclitaxel was loaded at 112.5 µg/mg of PLGA-coated CNTs.

Iron oxide nanocomposites were functionalized with quantum dots for diagnosis applications. Because the nanocomposites contain iron oxide, the nanoparticle provides the opportunity for magnetic hyperthermia, creating a unique material for diagnosis and therapy. Mice, injected with the quantum dot functionalized iron oxide nanocomposites, were imaged displaying the in vivo imaging capability. The magnetic hyperthermic property of the quantum dot functionalized nanocomposites was observed with the attainment of temperatures above 50°C during exposure to an alternating magnetic field.

Thermoresponsive nanoparticles were prepared by immobilizing a 2 – 3 nm thick phospholipid layer on the surface of superparamagnetic Fe₃O₄ nanoparticles via high affinity avidin/biotin interactions. Morphological and physicochemical surface properties were assessed using TEM, confocal laser scanning microscopy, differential scanning calorimetry, and ATR-FTIR. The zeta potential of Fe₃O₄ colloids in phosphate buffered saline (PBS) decreased from -23.6 to -5.0 mV as a consequence of phospholipid immobilization. Hyperthermia-relevant temperatures greater than 40°C were achieved within 10 – 15 min using a 7-mT magnetic field alternating at a frequency of 1MHz. Loading of the surface-associated phospholipid layer with the hydrophobic dye dansylcadaverine was accomplished at an efficiency of 479 ng/mg Fe₃O₄. Release of this drug surrogate was temperature-dependent, resulting in a 2.5-fold greater release rate when nanoparticles were exposed to temperatures above the experimentally determined melting temperature of 39.7°C.
**In vitro** cytotoxicity studies by release of the cytotoxic drug, doxorubicin, from the thermoresponsive nanoparticles was lastly intended. However, colloidal stability became an issue, prompting a thorough review of nanoparticle stabilization. Factors affecting stabilization, including dispersant, the nanoparticle, and the thermoresponsive coating, were characterized by dynamic light scattering and zeta potential. PBS was compared to two dispersants containing lower ionic concentrations, HBSS and HEPES, using the original iron oxide nanoparticles compared to an iron oxide nanocomposite. The nanocomposite in the HEPES buffer displayed the greatest stability with a zeta potential of -30.47 mV and particle size of 155.4 nm. Stabilization of the immobilized phospholipid bilayer was examined with and without incorporation of the cationic lipid stearylamine. Zeta potential (33.6 mV) and size (315 nm) data indicate that stearylamine incorporated DPPC coated nanoparticles provide better stability.
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Chapter 1 - Introduction

1.1 Motivation and Approach

According to the American Cancer Society, in 2008 it was estimated that nearly 12 million Americans have or had cancer in their lifetime, while in 2012 it is anticipated that 1.6 million individuals from the United States will be diagnosed with cancer. Of the 1.6 million, over 550,000 people or about one third, are expected to die. The rampant problems with cancer are the issues of diagnosis and treatment. Unfortunately, there is no single method or test that can accurately diagnose cancer, not only because of the wide varieties of cancers, but due to human factors such as the environment or family history. Any accurate cancer diagnosis requires extensive history and a multitude of physical examinations. After a cancer diagnosis has been made, it is often unknown whether treatment will be successful or not, depending on the severity of the cancer. In many cancer cases, such as pancreatic cancer where early diagnosis is difficult, there is little therapy that can be done to terminate metastasis of the cancer. In cases where cancers can be treated by methods including chemotherapy, radiation therapy and surgery, there are side effects that drastically reduce the quality of life for the individuals. Some of these side effects include anemia, decreased appetite, diarrhea, fatigue, hair loss, nausea and vomiting. Many of these side effects are a result of passive and random distribution of the therapeutic agent. Drugs or cytotoxic agents are administered into the blood circulation with the aim at destroying the tumor cells. However, the nonselected and uncontrolled biodistribution of these therapeutic moieties result in the undesired killing of healthy cells.
The possible solution to the issues of diagnosis and passive drug distribution for cancer may be in the development of nanosystems that provide a variety of functionalities. The multifunctional nanosystems include nanoparticles conjugated with biological moieties and cytotoxic agents for proper cancer therapies, and luminescent molecules to aid in diagnoses. For example, the development of a multifunctional drug delivery system that relies on the controlled release of a cytotoxic agent will help to reduce the negative effects associated with cancer drugs. Release of the drug, controlled or initiated by an external stimulus, will result in high drug concentrations only at the tumor site. This will result in patients requiring reduced concentrations of the therapeutic agents, because the drug release is limited to the affected region. In other words, less drug is needed due to the increased drug concentration at the tumor site.

In this paper, the development of multifunctional nanoparticles to aid and improve cancer therapies will be examined. A few nanoparticles will be examined for improvement in diagnosis and cancer therapies. The construction of the systems will be systematically described and characterization data will be presented to support the creation of the novel materials.

1.2 Nanoparticles and Nanotechnology

Nanotechnology is a multidisciplinary science that can be described as the engineering of workable materials at the molecular level, with at least one dimension in the nanometer range (1nm = 10^{-9}m) where properties of materials with dimensions at the nano level are significantly different from those of atoms as well as those of bulk materials. Nanotechnology has been studied across many science fields including
physics, biology, chemistry, engineering, and materials science with the objective of controlling the arrangement of atoms and molecules to perform a specific function.\textsuperscript{2} The nano-engineering of materials can potentially provide a wide range of products and applications ranging from medicine, biomaterials, electronics, transportation and agriculture. For example, membranes designed with nanosized pores can allow for ultrafiltration of many fluids including blood for hemodialysis.\textsuperscript{3} The life and durability of many of the products we used can be increased using nanotechnology as seen by the addition of carbonitride nanoparticles into steel, which has been shown to prolong the life of steel by making it more creep-resistant extending the time-to-rupture.\textsuperscript{4} The aerospace industry looks to take advantage of nanotechnology to develop and design spacecraft with lighter and smaller materials, reducing the amount of fuel, and subsequently, the cost required in launching a vehicle off the ground.\textsuperscript{5}

1.2.1 Nanoparticles

Nanoparticles are typically defined as a particle having dimensions of 100 nanometers or less, a size range in which the properties of the materials differ from the bulk. However, it is known that there is no concrete dividing line between nanoparticles and “non” nanoparticles. Properties of nanoparticles differing from the bulk can certainly be above 100 nanometers. In addition, a clear definition of nanoparticles become even more difficult when observing particles that are not spherical, such as rods as in the case of carbon nanotubes. The lengths of carbon nanotubes are often longer than 100 nanometers, sometimes microns in length and have even been observed in the centimeter range.\textsuperscript{6} This has lead to the proposal that nanoparticles be defined as materials that have
at least one dimension in the 100 nanometer range that cause their properties to differ from the bulk. There is an extensive variety of nanoparticles that exist for a myriad of applications. Some of the nanoparticles that will be examined include magnetic nanoparticles, carbon nanotubes, liposomes and quantum dots.

1.2.1.1 Magnetic Nanoparticles

Magnetic nanoparticles are materials that can be manipulated by a magnetic field, and are usually comprised of the magnetic element iron, but can also contain cobalt and nickel. To date, iron oxide nanoparticles have been the most studied magnetic nanoparticle and exists in two main forms, magnetite ($\text{Fe}_3\text{O}_4$) or maghemite ($\gamma\text{-Fe}_2\text{O}_3$) with magnetite being the more researched of the two. Magnetic nanoparticles are envisioned to improve applications in wide range of disciplines including magnetic fields, catalysis, biomedicine, magnetic resonance imaging, and data storage.$^{7,8,9,10,11}$ For the majority of applications that magnetic nanoparticles are envisioned, the nanoparticle size will be required to be below a critical value, which is dependent on each material. It is at this critical size range that each nanoparticle becomes a single magnetic domain and shows superparamagnetic behavior when the temperature is above the so-called blocking temperature. Each superparamagnetic nanoparticle displays a large constant magnetic moment and behaves like a giant paramagnetic atom that has a rapid response to applied magnetic fields with negligible residual magnetism and coercivity. These two properties make superparamagnetic nanoparticles very attractive for biomedical applications because the risk of forming agglomerates is negligible at room temperature.
Iron oxide (Fe$_3$O$_4$), typically a ferromagnetic material, can undergo a change to the superparamagnetic state when the iron oxide becomes small enough to consist of a single magnetic domain, around 128nm.$^{12}$ At this nano-level, iron oxide does not retain any residual magnetization in the absence of a magnetic field, yet the nanoparticles are still rendered magnetic in the presence of the field, similar to paramagnetism. However, the magnetic susceptibility of the superparamagnet is much larger than that of a paramagnet, defined by the equation, $M = \chi H$, where $M$ is magnetization, $H$ is the magnetic field strength and $\chi$ is the magnetic susceptibility. In other words, the degree of magnetization, or net magnetic moment, is much higher in a superparamagnet than that of a paramagnet due to a larger magnetic susceptibility.$^{13}$

Superparamagnetism can be more understood by considering the behavior of an isolated single-domain particle. The magnetic anisotropy energy per particle, which is responsible for holding the magnetic moments along a certain direction, can be expressed as follows: $E(\theta) = KV\sin^2\theta$, where $V$ is the particle volume, $K$ is the anisotropy constant, and $\theta$ is the angle between the magnetization and the easy axis, or the energetically favorable direction of magnetization. In the equation, the energy barrier, $KV$, separates the two energetically easy directions of magnetization. With a decrease in particle size, the thermal energy, expressed as $kT$, will exceed the energy barrier $KV$ and the magnetization can be easily flipped, where $k$ is the Boltzmann constant and $T$ is temperature. When $kT > KV$, the material will behave similarly as a paramagnet, but instead of the atomic magnetic moments seen in paramagnets, a giant moment will exist in each particle and will display no magnetic hysteresis, hence no net magnetization in
the absence of a field. The relaxation time, or the time it takes for two flips of a magnetic moment, can be expressed by the Néel relaxation equation,

\[ t_N = t_0 e^{KV/kT} \]

where \( t_N \) is the Néel relaxation time, \( t_0 \approx 10^{-9} \text{ s} \), \( k \) is the Boltzmann constant and \( T \) is the temperature.

In an alternating magnetic field (AMF), the magnetic moment will experience multiple and rapid relaxations. These rapid Néel relaxations at a high frequency, where the magnetic moment will quickly oscillate through the energy barrier, will generate heat. The heat response caused by the rapid flipping of the magnetic dipole is referred to as magnetic hyperthermia. Magnetic hyperthermia can also be attributed to Brownian motion. The particle’s magnetic moment not only oscillates, but the nanoparticle experiences whole body rotation due to the magnetic oscillations. The particle rotation within the surrounding medium produces frictional energy released as heat. The energy barrier for reorientation of the particle in this scenario is determined by the rotational friction with the surrounding solution and can be characterized by the Brownian relaxation time, \( t_B \)

\[ t_B = 3\eta V_B / kT \]

where \( \eta \) is the viscosity of the surrounding solution and \( V_B \) is the hydrodynamic volume of the nanoparticle. With an increase in viscosity, one would expect more rotation
friction resulting in more dissipated heat.

The unique properties of superparamagnetic nanoparticles, namely high magnetization, no magnetic hysteresis, and magnetic hyperthermia, make the nanoparticles great candidates for drug delivery and cancer therapy applications. The heat generated by superparamagnetic nanoparticles has many uses including ablation of tumor tissue due to magnetic hyperthermia and as a trigger for release of therapeutic biomolecules. Coating the nanoparticles with a thermoresponsive layer that solubilizes a cytotoxic agent, it should be possible to develop a nanocarrier that can release the drug in a controlled manner through magnetic hyperthermia.

1.2.1.2 Carbon Nanotubes

Since the discovery of carbon nanotubes (CNTs), a wide range of applications including structural, electromagnetic, electroacoustic, chemical, mechanical and medicinal has been researched. CNTs can be imagined as a single graphene sheet rolled cylindrically forming a single walled CNT or rolled up many layers to form concentric cylinders forming multi-walled CNTs. The graphene lattice structures can be rolled up in different angled orientations, or chiralities, resulting in the CNTs having metallic or semi-conducting properties depending on the rolled angle. In Figure 1-1 below, an illustration is shown for conformations of CNTs rolled at various angles. CNTs that are considered semi-conduction or, “metallic,” provide very good electrical properties. For example, metallic CNTs are thought to carry an electric current density 1000 times more than copper. In addition to their electrical properties, CNTs are good thermal conductors and are very strong in terms of tensile strength and elastic modulus. In medicine, CNTs
are viewed as a possible vector for cancer diagnosis and therapy and have been demonstrated *in vitro* to effectively shuttle a variety of biomolecules into cells including drugs, peptides, proteins, plasmid DNA, and small interfering RNA via endocytosis.\textsuperscript{15}

Controlling the surface properties of CNTs, such as coatings and modifications for functionalization, is key for all CNT applications.\textsuperscript{15,16}

*Figure 1-1:* Illustration showing the CNT chiralities based on rolling angles.

**1.2.1.3 Liposomes**

Liposomes are tiny, spherical shaped vesicles made up of amphiphilic lipids, enclosing an aqueous core. The amphiphilic lipids are predominantly phospholipids, analogous to the materials that form bilayers found in biomembranes. The term amphiphilic refers to the polar, hydrophilic and non-polar, hydrophobic regions of the lipid molecules. In the presence of water, the hydrophobic regions orient themselves toward the interior away from the aqueous phase, with the polar, hydrophilic regions
oriented toward the exterior and in contact with the water. The varieties of phospholipids that constitute liposomes affect its properties and characteristics. There are diverse arrays of natural and synthetic phospholipids and analogs available that exhibit different physicochemical properties.\textsuperscript{17} The bilayer surface chemistry determines charges the liposomes carry while the acyl chain chemistry determines the bilayer phase behavior. Polar phospholipid head groups determine if a liposome carries a positive, negative or neutral charge, and there are a variety of head groups available to tailor to the desired charge. For example, a liposome comprised solely of the phospholipid dioleoylphosphatidylcholine (DOPC) will yield a negative charge while a liposome comprised of ethyl phosphatidylcholine will yield a positive charge. Additional molecules can be added to liposomes to induce surface charge as well as stabilize the liposomes. These include stearylamine, dicetylphosphate, solulan C-24 and diacylglycerol.\textsuperscript{18} The internal lipid bilayer chemistry, specifically acyl chain length and saturation, will determine the phase behavior of the liposomes, including melting temperature ($T_m$).\textsuperscript{19} At different temperatures, the phospholipid bilayer membranes can exist in different phases and states of fluidity. The $T_m$ is a reflection of the passage of the bilayer from an ordered gel-like state to a fluid phase. The bilayer fluid phase does retain some order and therefore is termed the liquid crystalline phase. However, in the liquid crystalline phase, there is more freedom of movement and conformational changes of the individual acyl chains than in the gel-like state. With an increase in temperature, the fatty acid chains in the gel-like state begin to adopt conformations other than the all-\textit{trans} straight chain arrangement. This results in the expansion of the area occupied by the acyl chains, reducing the length of the hydrocarbons, and decreasing the bilayer thickness.
upon transition from the gel-like state to the liquid crystalline state. Liposomes made of a single component phospholipid, especially saturated hydrocarbon chains of phospholipids with a chain length of C12-C20, will demonstrate a melting temperature transition over a narrow temperature range above room temperature, with the $T_m$ increasing with as the chain length increases.\textsuperscript{20} For unsaturated phospholipid acyl chains, the $T_m$ is usually below room temperature with a broad transition.\textsuperscript{20} In multicomponent liposomal systems, the $T_m$ is more broadened due to the variety of hydrocarbon chains. Additives, such as cholesterol, will change the melting temperature as well, increasing the $T_m$ by making the membrane more ordered.\textsuperscript{18}

\textbf{1.2.1.4 Quantum Dots}

Quantum dots are tiny nanoparticles in the range of 2-10 nanometers in diameter that consist of chalcogenides of metals, usually zinc or cadmium. Quantum dots have very unique optical and electrical properties due to their small size. The most apparent is the emission of photons under excitation, which the wavelength of light that is emitted is dependent on the dot diameter. Smaller quantum dots emit visible light in the blue end of the spectrum while larger dots emit light in the red end of the spectrum. Figure 1-2 below shows an illustration of the quantum dot size and emission color. Quantum dots are currently being researched for a range of applications including medical imaging, solid-state quantum computation, photovoltaic devices and light emitting devices.
1.3 Synthesis Methods of Nanoparticles

1.3.1 Magnetic Nanoparticles

Synthesis of magnetic nanoparticles and magnetic fluids involves the formation and stabilization of the nanoparticle. Stabilization involves synthesis of the nanoparticle conducted in the presence of a surfactant or polymer, and is very important as it prevents agglomeration during synthesis. Magnetic fluid synthesis is usually performed in the eventual dispersion medium, however sometimes the nanoparticles are synthesized in one solvent and then transferred to another. Four common synthesis procedures for magnetic nanoparticles will be reviewed in this section.

1.3.1.2 Co-precipitation
An easy and convenient method for the production of ferrite nanoparticles, specifically magnetite ($\text{Fe}_3\text{O}_4$) and maghemite ($\gamma$-$\text{Fe}_2\text{O}_3$), is the co-precipitation of $\text{Fe}^{2+}$/Fe$^{3+}$ salt solutions through the addition of a base that induces nucleation. Size, shape and composition of the nanoparticles is dependent upon the type of salts used (chlorides, sulfates or nitrates), the $\text{Fe}^{2+}$/Fe$^{3+}$ ratio, the reaction temperature, the pH value and ionic strength of the media.$^{21}$ A typical stoichiometric reaction is shown below for the use of ammonium hydroxide as the precipitating base.

$$2 \text{FeCl}_3 + \text{FeCl}_2 + 8 \text{NH}_4\text{OH} \rightarrow \text{Fe}_3\text{O}_4 + 8 \text{NH}_4\text{Cl} + 4 \text{H}_2\text{O}$$

The base is typically added in excess so the pH of the reaction medium is highly basic, usually between 8 and 14. Reaction temperature is generally carried out at 80°C in a non-oxidizing environment to prevent the transition of $\text{Fe}_3\text{O}_4$ into $\gamma$-$\text{Fe}_2\text{O}_3$. The synthesis of nanoparticle quality is highly reproducible once the parameters are fixed. Magnetite nanoparticles with diameters ~ 10 nanometers were synthesized by Gokon et al. using co-precipitation. Briefly, an aqueous solution of iron (III) chloride and iron (II) chloride in a 2:1 molar ratio was used with nucleation induced by the addition of NaOH, forming a spinel structure that resulted in a net magnetic dipole made up of Fe$^{3+}$ and Fe$^{2+}$ ions. Nanoparticles synthesized by co-precipitation are usually performed in the presence of a dissolved stabilizing surfactant or polymer that binds to the particles just after nucleation, limiting the growth of the particles as well as preventing aggregation. It has been shown that the surfactant, oleic acid, is one of the best candidates for stabilization of $\text{Fe}_3\text{O}_4$.\textsuperscript{22,23}
The main advantage of the co-precipitation process is that a large amount of nanoparticles can be synthesized, however the control of particle size distribution is difficult because only kinetic factors control the growth of the nanoparticles. Separating the two stages of precipitation, nucleation and growth, will lead to production of monodisperse nanoparticles. When nanoparticle growth is occurring, nucleation should be avoided and while nucleation is occurring, nanoparticle growth should be prevented.

1.3.1.2 Thermal Decomposition

Monodisperse magnetic nanoparticles can be prepared through the thermal decomposition of organometallic compounds in high-boiling organic solvents. In this synthesis method, an organometallic compound combined with a stabilizing surfactant are dissolved in a solvent and heated to high temperatures. Temperatures can be as high as 200-300 °C depending on the compound. Upon reaching the high reaction temperature, the organometallic species decomposes and the insoluble metal precipitates. Just after nucleation, the surfactant will bind to the particles, limiting the growth and stabilizing the formed nanoparticles. The organometallic compounds include metal acetylacetonates, which include iron, manganese, cobalt and nickel, metal cupferronates, or carbonyls. Typical surfactants used are fatty acids, oleic acid, and hexadecylamine. A negative aspect to thermal decomposition synthesis is the long reaction times that take place compared to co-precipitation which can take hours or days compared to minutes for co-precipitation.

1.3.1.3 Microemulsion
Magnetic nanoparticles can be synthesized through the creation of a microemulsion, which is a thermodynamically stable, isotropic dispersion of two immiscible liquids, where an interfacial film of surfactant molecules stabilizes the liquids. Mixing two identical water-in-oil microemulsions containing the desired reactants and surfactants, induce the formation of micelles. During mixing, the micelles will continuously collide, coalesce and break apart, eventually forming a metal precipitate within the micelle. Filtration or centrifugation of the mixture allows for extraction of the precipitates or nanoparticles. Nanoparticles that have been synthesized by the microemulsion process include metallic cobalt, cobalt/platinum alloys, and gold-coated cobalt/platinum nanoparticles. This synthesis technique allows for very good shape control of spherical and rod/tube nanoparticles but results a low yield and requires large amounts of solvents, which drive up the cost for scaled-up production.

1.3.1.4 Size Reduction

The most basic and oldest nanoparticle preparation is the size reduction method. This technique relies on a ball mill to grind bulk materials mixed with a dispersion medium and surfactant for stabilization into nanoparticles. V. M. Chakka et al. produced magnetic nanoparticles in a ball mill by grinding powders that ranged from 10 – 45 µm mixed with oleic acid and oleyl amine as surfactants. Ball milling for 10-25 hours produced nanoparticles with diameters under 10 nm. Production of nanoparticles with the size reduction method is simple and flexible, in that any type of particle can be produced if a bulk powder is available. However, it is a time consuming and energy
intensive process, requiring many hours of grinding to produce the particles with the appropriate dimensions.

1.3.2 Carbon Nanotube Synthesis

A variety of methods for the synthesis of carbon nanotubes have been developed since their first observation in 1991. These methods include arc discharge, laser ablation and chemical vapor deposition (CVD). Each of these methods will be briefly reviewed.

1.3.2.1 Arc Discharge

The most widely used process of producing CNTs is the arc discharge method with CNTs being first discovered in the carbon soot of graphite electrodes during an arc discharge. During this process, carbon in the negative electrode sublimates due to the high-discharge temperatures. To produce CNTs, two carbon rods are placed end to end, separated by 1 mm, in an enclosed area that is often filled with inert gas at low pressure. A direct current of 50 to 100 A, driven by a potential difference of 20 V, creates a high temperature discharge between the two electrodes. The surface of one of the carbon electrodes is vaporized by the discharge, forming small rod-shaped deposits on the other electrode. A drawback to this method is that purification of CNTs from the carbon soot is required, however CNTs can be produced in a high yield in a short period of time. Both single walled and multi-walled CNTs can be synthesized using the arc discharge method. However, it has been found that formation of single walled CNTs require the use of a metal catalyst, such as iron or cobalt.
1.3.2.2 Laser Ablation

The laser ablation process uses a pulsed laser to vaporize a graphite target in a high-temperature reactor, approximately 1200 °C, in the presence of an inert gas such as argon, that yields about 70% pure CNTs. The vaporization of the graphite target is done in the presence of a catalyst that is a mixture of cobalt and nickel. During the ablation process there are two laser pulses, with the second allowing for uniform vaporization of the graphite target, in addition to breaking up larger particles ablated by the first pulse. The CNTs produced by laser ablation have 10-20 nm diameters and can be up to 100 µm in length. This procedure is a great method for producing high quality CNTs, however is difficult to scale up to the industrial level due to the small carbon source. In addition, it is difficult to purify the CNTs from the carbon soot that is produced during the ablation process.

1.3.2.3 Chemical Vapor Deposition

CVD is a chemical process in which high-purity materials are produced or deposited onto a substrate from gaseous precursors. During the CVD process, a substrate, usually porous, is prepared with a nickel, cobalt or iron catalyst. The diameters of the produced CNTs are related to the size of the catalysts etched to the substrate. At temperatures ranging between 500 and 1200 °C, gases are introduced to the CVD reactor. The gases introduced are a non-carbon containing gas such as ammonia, nitrogen or hydrogen and a carbon containing gas such as acetylene, ethylene or methane. The gas containing carbon is decomposed at the catalyst with the carbon diffusing to the edges of the catalyst, initiating the growth of the nanotubes. Impurities in the form of amorphous
carbon, fullerenes, and the catalyst material are often formed during the CVD process, which requires purification.

1.3.3 Liposomal Synthesis

Phospholipids, a major component of cellular membranes, are amphiphilic molecules containing two hydrophobic acyl tails attached to a polar, hydrophilic head group. In an aqueous environment, phospholipids will self-assemble into unilamellar or multilamellar vesicles called liposomes, containing an aqueous core and a lipophilic bilayer region. The driving force behind liposomal formation is the interaction between water and phospholipids. The hydrophilic head groups of phospholipids prefer the aqueous phase, while the hydrophobic acyl tails of the lipid prefer each other’s company to that of water. There are a large variety of methods for generating liposomes and several of these procedures will be briefly discussed.

1.3.3.1 Lipid Hydration

The most widely used method for preparation of liposomes is lipid hydration. This method involves drying a mixture of lipids, often in a rotary evaporator, to form a thin film at the bottom of a round bottom flask. The thin film is hydrated by adding an aqueous liquid to the flask, followed by sonication of the dispersion. Heat is applied during the hydration step at a temperature above the $T_m$ during sonication to promote a better dispersion. During hydration and sonication, the phospholipids spontaneously form liposomes. Compounds such as drugs can be incorporated into the liposomes by
adding to the mixture of lipids during the drying stage or to the aqueous solution during hydration, depending on the solubility of the compounds.

1.3.3.2 Solvent Injection

Solvent injection is another method for the preparation of liposomes. There are two approaches for the creation of liposomes by solvent injection, one using diethyl ether and the other using ethanol. In the diethyl ether method, lipids dissolved in diethyl ether are slowly injected into warm water (50-60 °C), typically with the aid of a syringe infusion pump. After injection, the diethyl ether is removed from the preparation under a high vacuum environment. The removal of the solvent leads to the production of liposomes. In the ethanol injection method, lipids dissolved in ethanol are rapidly injected into a large amount of aqueous solution, resulting in liposomal formation immediately. However, removal of ethanol from the solution by vacuum or distillation can prove to be difficult since the alcohol forms an azeotrope with water.

1.3.3.3 Detergent Removal

In the detergent removal method, phospholipids and a detergent are mixed together to form micellar solutions. The detergent is removed from the preparation by column chromatography or adsorption onto biobeads, leading to the micelles containing higher phospholipid content. The micelles will then coalesce and form liposomes. Commonly used detergents are those that have a high critical micelle concentration (10-20mM), such as octylglycoside, sodium cholate and triton X-100.
1.4 Stabilization of Nanoparticles

Stabilization of nanoparticles, especially in solution, is very important due to flocculation that occurs because of the van der Waals attractive force that exists between particles. For iron oxide nanoparticles, the van der Waals force is even more important than particle-to-particle magnetic attraction at short-range distances. Stabilization layers added during nanoparticle synthesis is aimed to prevent flocculation by exerting a repulsive force between particles in a short range. For stabilization, steric, electrostatic or a combination of the two can be used to prevent the nanoparticles agglomerating. Figure 1.3 below shows an illustration of electrostatic and steric stabilization. Steric stabilization involves covering the nanoparticles with a highly branched polymer that uses the molecular chemistry to prevent the particles from attracting to each other. The stabilization agents are required to be well solvated by the dispersion medium to induce repulsive interactions when stabilizing layers of two particles overlap. Electrostatic stabilization relies on the mutual repulsion of like charges by coating the nanoparticles with charged molecules. The stabilizing agents require a means of attachment to the nanoparticles, which can be physical attachment to the nanoparticle surface or through the incorporation of a functional group.
Figure 1.3: Illustrations representing stabilization of nanoparticles. Panel A shows electrostatic repulsion through surface associated cationic charges while panel B shows steric repulsion of branched molecules attached to the nanoparticle surface.

Willis et al. demonstrate that nanoparticles that have oleic acid adsorbed onto the surface of the nanoparticle during synthesis of $\gamma$-Fe$_2$O$_3$ by thermal decomposition leads to spherical and monodisperse nanoparticles of 11 nm in diameter.$^{22}$ The capping with oleic acid prevents nanoparticle flocculation by providing steric repulsion. Willis et al. also demonstrated that the addition of oleic acid gives rise to nanoparticles with higher monodispersity and uniformity compared to other ligands. F. Cousin et al. synthesized $\gamma$-Fe$_2$O$_3$ nanoparticles using the co-precipitation method. The particles, dispersed in an acidic aqueous medium (pH 2) were electrostatically stabilized with trisodium citrate molecules adsorbed to the surface that resulted the dispersion medium having a pH of 7.$^{30}$
1.5 Nanoparticle Applications

There are many potential opportunities for the use of nanoparticles in a variety of applications. Some of the areas currently being researched include medicine, biomaterials, electronics, transportation and agriculture. In this section, applications for magnetic nanoparticles, carbon nanotubes and liposomes will be briefly reviewed.

1.5.1 Magnetic Nanoparticle Applications

Magnetic nanoparticles are one of the more intriguing materials because of their unique properties and are potentially very useful for biomedical applications. They have controllable size ranges that have dimensions comparable to biological cells (10-100 µm), viruses (20-450 nm), proteins (5-50 nm), or genes (2 nm wide and 10-100 nm long), which indicates that they can get near to a biological moiety of interest. Because magnetic nanoparticles obey Coulomb’s law, they can be directed to different parts of the body through the application of an external magnet. Finally, magnetic nanoparticles can be tailored to generate heat in the presence of an alternating magnetic field, also known as magnetic hyperthermia. These unique magnetic nanoparticle properties have lead to research for applications in magnetic separation, drug delivery, hyperthermia, and contrast agents for imaging.

Magnetic separation is a process in which a magnetically susceptible material is extracted from a mixture using a magnetic force. Magnetic separation is used in many industries including food, recycling and biomedicine. In the food industry, foods and
drinkable liquids can be cleaned by the removal any contaminating magnetic materials through the use of magnetic separation. In biomedicine, magnetic separation can be used to separate out specific biological entities from their native environment in order to make concentrated samples. This involves tagging a desired biological molecule with a magnetic nanoparticle, separating the nanoparticle – biological molecule complex from its environment and finally de-tagging the magnetic nanoparticle from the biomolecule. This process has been successfully applied to the selection of rare tumor cells in blood, the enhanced detection of malarial parasites in blood samples, and a pre-processing technique for amplification of DNA by polymerase chain reactions.32,33,34

Magnetic nanoparticles have been studied vigorously as drug delivery vehicles, mostly for cancer therapies. The objective behind using nanoparticles is to reduce the amount of systemic distribution of the drug, reducing malicious side effects, and reducing the dosage by more efficient, localized targeting of the drug. Magnetic nanoparticles are used because it is possible to target by the use of an external, high gradient magnetic field to concentrate a drug – nanoparticle complex at a specific site within the body. Once at the site of importance, the drug would be released by a change in physiological conditions such as pH or temperature.

An alternative treatment to cancer, as opposed to chemotherapy or radiation therapy, is by the artificial induction of hyperthermia. Cancer cells have been shown to be more susceptible to a lower temperature compared to healthy tissues. Magnetic nanoparticles, within a certain size range, can generate heat in the presence of an alternating magnetic field. Injecting a tumor site with magnetic nanoparticle along with the application of the alternating magnetic field, can lead to the ablation of cancer tissue.
while the surrounding tissue remains unharmed. Magnetic hyperthermia is also believed to aid in chemotherapy and radiation therapy, by damaging the cells with elevated temperatures first followed by the selected therapy.\textsuperscript{35,36}

Magnetic resonance imaging (MRI) is an imaging tool that allows for the visualization of internal structures of the body, specifically the contrast between soft tissues. MRI works by measuring changes in the magnetization of hydrogen protons in water molecules sitting in a magnetic field after a pulse of radio frequencies has hit them. The protons from tissues react differently, providing a picture of bodily structures. The images from an MRI can be enhanced through the addition of contrast agents during the scanning procedure. The idea of contrast agents is that they increase the contrast in the tissues and bloodstream, providing more detailed images leading to more accurate diagnoses. Magnetic nanoparticles, such as iron oxide, have been used to overcome many of the issues that occur with the use of other contrast agents and can provide a very large signal from a MRI scanner.

\textbf{1.5.2 Carbon Nanotube Applications}

Carbon nanotubes offer a wealth of opportunity for applicable use across a wide range of fields. Researchers have been examining ways to take advantage of their unique properties that include good electrical and thermal conductivity, high tensile strength and relative inertness. Some CNT applications that are currently used as well as being developed are CNT composites, electrochemical devices, field emission devices, nanometer-sized electronic devices, and chemical sensors and probes.\textsuperscript{37}
CNT composites are currently commercially used as electrically conducting components, which depending on the polymer matrix, conductivities of 0.01 to 0.23 S/cm can be obtained.\textsuperscript{38} For example, in commercial automotive gas lines and filters, the nanotube filler dissipates charge buildup that can lead to explosions and better maintains barrier properties against fuel diffusion than do plastics filled with carbon black. Also, plastic semiconductor chip carriers and reading heads made from nanotube composites avoid contamination associated with carbon black sloughing. Incorporation of CNTs into plastics can also potentially produce materials that structurally have increased modulus and strength. The main issues with these types of materials however, are uniformly dispersing the CNTs, the interfacial interaction between CNTs and the matrix to provide effective stress transfer, and the sliding that occurs between concentric tubes within multi-walled CNTs.

The high electrochemically accessible surface of porous nanotube arrays, along with their high electronic conductivity, make CNTs attractive materials as electrodes for devices that use electrochemical double-layer charge injection. This includes giant capacitors that have larger capacitances in comparison to electrochemical actuators and dielectric-based capacitors. Supercapacitors that contain carbon nanotube electrodes can be used in applications, such as hybrid electric vehicles, that require much higher power capabilities than batteries and much higher storage capacities than ordinary capacitors.\textsuperscript{39}

Both industry and academic institutions have been researching single-walled CNTs and multi-walled CNTs as field emission electron sources.\textsuperscript{40,41} These field emission devices would be incorporated into flat panel displays, lamps, gas discharge tubes providing surge protection, and x-ray and microwave generators.\textsuperscript{42,43,44,45}
potential is applied between a CNT coated surface and an anode, a high local field is produced, causing electrons to tunnel from the nanotubes. For flat panel displays, electric fields will direct the field-emitted electrons towards the anode, where a phosphor produces light.

It is believed that CNTs can potentially be used to downsize circuit dimensions at the nanometer level. As conventional metal wire diameters decrease in size, current-induced electromigration causes interconnects to fail. Using CNTs would possibly prevent this breakdown from occurring. Non-metallic CNTs can possibly be used as chemical sensors, due to their sensitivity to changes in voltages. These sensors, because of their small size, would require a very minute amount of material for a response. However, these devices need to be able to differentiate between absorbed molecules in complex mixtures.

1.5.3 Liposomal Applications

Due to their unique structure, liposomes have the capability of storing a drug with varying polarities. Hydrophilic drugs can be encapsulated in the aqueous core or a hydrophobic therapeutic agent can be incorporated in the bilayer region of a liposome. Incorporation of hydrophobic drugs is important in many therapeutic delivery vector applications due to the aqueous environment of biological systems. Liposomes constituted of phospholipids provide an opportunity for these drugs to be sufficiently administered in the body and more importantly, reduce the toxicity of the drug without decreasing the effectiveness of the drug.
An attractive characteristic for the use of liposomes as drug delivery systems is the opportunity for the functionalization of molecules to the polar head groups.\textsuperscript{48} PEGylated phospholipids, or phospholipids that have a polyethylene glycol (PEG) molecule functionalized to the head group, are one of the more important and popular phospholipids available. Incorporation of a PEGylated phospholipid into a liposome will provide the liposome with “stealth” characteristics, reducing the uptake of liposomes by the immune system.\textsuperscript{47,48} In addition, the PEG group on phospholipids can be modified and further functionalized with molecules such as biotin, allowing for interaction with the protein avidin. Phospholipid head groups can additionally be functionalized with fluorescent markers, MRI agents, peptides and antibodies providing opportunity for a variety of applications.\textsuperscript{49}

Stimulus-induced release of a therapeutic agent from colloidal phospholipid based systems can be accomplished by a variety of methods including changes in pH, ultrasound, hyperthermia, and optical.\textsuperscript{46,47,50-57} pH sensitive liposomes developed from the observation that certain enveloped viruses infect cells following acidification of the endosomal lumen to infect cells, and from the fact that some tissues have a more acidic environment compared to normal tissues.\textsuperscript{50} The liposomes, stable at physiological pH, will destabilize in acidic conditions, inducing the release of contents stored in the aqueous core. Liu et al. have shown that liposomes made of phosphatidylcholine, cholesterol, and PEGylated 1,2-distearoyl-\textit{sn}-glycero-3-phosphoethanolamine (DSPE) loaded with the cytotoxic agent, doxorubicin, will release the drug upon a 7.4 to 5 pH change.\textsuperscript{51} When the liposomes loaded with doxorubicin enter a cell endocytotically, lysosomes with a pH of 5 will encapsulate the drug carrier. The production of CO\textsubscript{2} inside
the acidic environment of the lysosome increases the permeability of the liposomes, releasing the drug. Liu et al. demonstrated that the release rate of the doxorubicin increased significantly with the pH change.

Ultrasound-induced drug delivery of phospholipid colloidal systems release drug through cavitation, or rupturing of the liposomal structure. Ultrasound causes cavitation, which is a process of nucleation, rapid growth, and collapse of bubbles or sustained oscillatory motion of bubbles. This leads to the destruction of the liposomal bilayer, releasing contents stored within the bilayer or aqueous core. In addition, when cavitation occurs, shock waves are emitted that have the capability to disrupt cellular membranes and tissues, enhancing transport of a therapeutic agent. The use of high intensity ultrasound however, can disrupt healthy tissue as well, despite the scarcity of endogenous gases in blood and tissue. Liposomes that are designed to contain gas bubbles, made during liposomal formation, can be used for lower power ultrasound, reducing the damage risk. “Bubble liposomes,” developed by Suzuki et al., are modified PEGylated liposomes containing perfluoropropane gas. Placing PEGylated liposomes in vials super-charged with perfluoropropane gas, and sonicating them in a bath sonicator induced gas incorporation. Suzuki et al. demonstrated that the bubble liposomes, containing plasmid DNA could almost instantaneously transfect extracellular plasmid DNA into COS-7 cells, kidney cells from the African green monkey, using an ultrasound frequency of 2 MHz. Kheirolomoom et al. developed another liposomal system capable of ultrasonic induced release. The group assembled a hybrid particle of microbubbles linked to liposomes through an avidin-biotin interaction. More than 1000 liposomes containing either a hydrophobic or hydrophilic drug could be linked to a single micron
sized microbubble. Drug release would be induced by the cavitation of the microbubble in response to ultrasound, producing a localized high concentration of drug.

Release of drug from liposomes can occur through an increase of temperature, or hyperthermia. Heating the phospholipid bilayer to its melting temperature will induce release of a contained therapeutic moiety. Upon attainment of the colloidal $T_m$, the liposomal bilayer will transition from the gel-like state into a liquid crystalline state, increasing the permeability of the bilayer. Hydrophobic drugs incorporated into the bilayer and hydrophilic drugs stored in the aqueous core will more readily be released. Many phospholipid liposomal drug systems that rely on hyperthermic release have dipalmitoylphosphatidylcholine (DPPC) as its main constituent due to its 41°C $T_m$. It must be noted that temperatures above 45°C will lead to permanent thermal damage of healthy tissues. Therefore, it is important to design temperature responsive systems with a melting temperature under 45°C. In addition to drug being released by increase in temperature, it has been demonstrated that hyperthermia can also increase the efficiency of a drug, thereby potentially reducing the need for higher concentrations of drug.\textsuperscript{47} Temperature sensitive liposomes developed by Lindner et al. show induced release of drug surrogate carboxyfluorescein (CF) upon mild hyperthermia (41-42°C). Liposomes comprised of DPPC, DPPG, and a new lipid, 1.2-dipalmitoyl-sn-glycero-3-phosphoglyceroglycerol (DPPGOG), containing CF were incubated in fetal bovine serum for 5 minutes, 60 minutes, and 14 hours at temperatures between 37 and 45°C. The release of CF was monitored during these time periods. It was demonstrated that the longer the liposomes were exposed to the higher hyperthermic temperatures above the $T_m$ (41°C), the greater the release of CF. Another temperature sensitive liposome, developed
by Chen et al. examines the rapid release of doxorubicin from lysolecithin-containing liposomes.\textsuperscript{55} Chen et al. reports that the liposomes can completely release the cytotoxic agent within 20 seconds upon heating to 42°C, allowing for the potential of vastly higher concentrations of the drug at a tumor site.

Drug release from phospholipid colloidal systems can be induced optically by light. Release of therapeutic moieties from liposomes can occur by multiple methods optically. Near-infrared irradiation of hollow gold nanoparticles, liposomes containing light-sensitive polymeric materials or the irradiation of liposomes with visible light in the presence of photosensitizers are examples of optically induced drug release. Pashkovskaya et al. studied the release of carboxyfluorescein (CF), sulforhodamine B and calcein from liposomes comprised of saturated phospholipids in the presence of the photosensitizer trisulfonated aluminum phthalocyanine (AlPcS\textsubscript{3}) after irradiation with red light.\textsuperscript{56} They showed pronounced release of CF upon irradiation, however release of sulforhodamine B and calcein was weak to negligible which could be due to the size and shape of each fluorescent molecule. Pashkovskaya et al. believe that photosensitized liposome permeabilization was a result of the oxidation of lipid double bonds, which is the main route of photodynamic lipid peroxidation. However, they are unsure how the oxidation leads to formation of pores within the liposome. An et al. have recently examined drug release from thermosensitive liposomes by using hyperthermia induced by the near-infrared irradiation of gold nanoparticles incorporated into the liposomal bilayer.\textsuperscript{57} Upon irradiation of the gold nanoparticles, the optical energy is converted into heat and causes the liposome to go through a phase transition, releasing a drug stored within its aqueous core. An et al. were able to demonstrate the release of the drug
berbine from liposomes containing gold nanoparticles in the phosphatidylcholine and cholesterol bilayer upon exposure to UV light.
Chapter 2 – Development of Multifunctional CNTs for Cancer Diagnosis and Drug Therapy

2.1 Background

A key challenge of nanotechnology for cancer diagnosis and therapy has been the design and development of a multifunctional nanostructure. Because biological systems are so complex, these nanostructured materials will require the ability to control the surface properties. Functional groups must be present on the nanosurface for the attachment of specific biomolecules and luminescent particles for \textit{in vivo} imaging. The nanoparticle geometry is also important for the storage and release of an anticancer drug. In addition, the nanoparticle must be biocompatible in order to avoid uptake by the immune system as well as avoiding any toxic response of the body. CNTs are a good nanoparticle candidate for the development of a multifunctional system because of the ability to control surface properties and the nanoparticle geometry that supports drug storage.

Surface functionalization of CNTs has been examined previously by altering the physical and chemical properties through chemical surface modification, doping, and coating.\textsuperscript{58} These methods have lead to the creation of many unique and novel nanomaterials. For biomedical applications, functionalization of biomolecules or other nanospecies is required. Through a unique plasma polymerization process developed in Dr. Shi’s lab, the surface of carbon nanotubes can be coated with an ultrathin polymer film that provides functional groups on the surface.\textsuperscript{59,60,61,62} The functional groups available via the polymer film allows for the covalent attachment of biomolecules and
luminescent particles to the CNT surface. For example, quantum dots (QDs) provide excellent optical properties that do not photobleach. The QDs can be conjugated to the surface of the CNTs through available functional groups, allowing the luminescent QDs to be imaged in deep tissues when excited in the near infrared range (>700nm).

The anti-cancer agent, paclitaxel, has been demonstrated to show great resistance to the growth of tumor cells in clinical studies.\textsuperscript{63,64} However, the low-solubility of paclitaxel restricts the therapeutic applications of the drug. Solubilizing paclitaxel within a nanosystem and corresponding release at the tumor site can allow for proper clinical use of anticancer agent. Some paclitaxel-carrier nanosystems that have been researched include liposomes, nanoparticles, and soluble polymers.\textsuperscript{65,66,67,68} Due to their stability and availability of functional groups, polymers have gained interest as a drug carrier. For example, the polymer poly(lactic-co-glycolic acid) (PLGA), biodegradable and FDA approved, is amongst some of the polymers that can solubilize paclitaxel.

For this study, a unique nanoassembly was developed. CNTs were functionalized with monomers to provide carboxyl groups, specifically lactic acid (LA) and glycolic acid (GA) to make PLGA and acrylic acid (AA) through plasma polymerization, which allowed for the conjugation of amine labeled QDs. In addition, CNTs plasma coated with PLGA were loaded with paclitaxel and loading efficiency was determined by high performance liquid chromatography (HPLC). Cytotoxicity studies were also completed by testing the therapeutic effect of the paclitaxel loaded CNTs in human PC-3MM2 prostate carcinoma cells.

\textbf{2.2 Results and Discussion}
2.2.1 Plasma Polymerization and Functionalization

Plasma polymerization is an effective process for providing surface functional groups on nanoparticles and CNTs. The key fundamental of plasma polymerization is that ionized and excited monomer molecules, created by an electrical field, bombard and react with the surface of the substrate. The monomers AA or LA and GA, are introduced from a gas inlet, enter a gas vacuum chamber and are ionized by a radio frequency source. The monomers are then etched, sputtered, or deposited onto the surface of CNTs. Below in Figure 2.1, two high-resolution transmission electron microscopy (HRTEM) images are shown of CNT surfaces functionalized by plasma polymerization. In the first image, a thin AA film of ~3nm is seen on the surface of the CNT. The contrast between the amorphous AA film and the lattice spacing for carbon is evident. The second image shows a CNT coated with PLGA by plasma polymerization of the monomers LA and GA. The layer thickness of the polymer in this image is 6-7 nm. The polymer coatings have been previously confirmed by time of flight secondary ion mass spectroscopy and these results can be found in the references.\textsuperscript{59,60,61,62}
Figure 2.1. a) HRTEM image showing plasma deposited AA polymer film (~3nm) near the open end of the CNT, b) HRTEM image of the coating layer of PLGA on CNT by plasma polymerization.\textsuperscript{69}

Covalent conjugation of QDs to CNTs plasma coated with AA was next achieved. Amine functionalized QDs were bonded to the carboxyl groups on the AA-functionalized CNTs when in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and \textit{N}-hydroxysuccinimide (NHS). The covalent conjugation of QDs to CNTs was confirmed by HRTEM and energy dispersive spectroscopy (EDS). In the HRTEM image below (Figure 2.2), it can be seen that the QD shows a dark contrast to the CNT, with an average particle size of 5-10nm randomly distributed on the CNT surface. A Z-contrast TEM image shows the QDs as bright aggregated clusters. The EDS measurement of the CNT-QD construct is shown below in Figure 2.3. Large peaks of Cd, Se, Te, Zn, and S can be seen within the spectrum and are the elemental make up of QDs. The Cu and C peaks in the spectrum represent the TEM grids and CNT substrate. The EDS spectrum confirms the presence of CdSe/ZnS QDs conjugated to the CNT surface.
Figure 2.2 a) TEM image of CNT with surface coupled QD, b) and Z-contrast TEM image showing the light gray QD on the CNT’s surface.\textsuperscript{69}

Figure 2.3 EDS acquired from QD attached on CNT’s surface. It shows elemental signals from the CdSeTe/ZnS QD and CNTs.\textsuperscript{69}
2.2.2 Paclitaxel Loading

Next, paclitaxel-loaded CNTs were prepared by mixing 20-35 mg of PLGA-coated CNTs with the drug dissolved in methanol at a concentration of 0.7 mg/mL at a final CNT/drug ratio of 2.5w/w. The suspension was sonicated with an energy output of 5 W in a pulse mode for 5 minutes under cooling and then vacuum dried. The organic solvent was completely removed after 2 hours followed by three washes in distilled water. After the last wash, the paclitaxel-loaded CNTs suspended in water were frozen and lyophilized for use. The drug loading efficiency, measured by HPLC, was determined to be 112.5±5.8 μg/mg CNT. Previous research of paclitaxel encapsulation into polymeric microparticles reported loading of 14 and 24 μg/mg carrier material.66,67 This means nearly a five-fold increase of drug loading when the polymer PLGA is thinly coated onto CNTs by plasma polymerization. It should be noted that PLGA is highly soluble in an aqueous environment and one would expect the coating to be dissolved during the drug loading process. In this case, the PLGA coating remains intact. This observation can be explained by the cross-linkage of the polymer chains during the plasma polymerization process, reducing the water solubility of the coating.

2.2.3 Efficacy of Paclitaxel Loaded CNTs

The therapeutic efficacy of the paclitaxel loaded CNTs was determined in vitro by quantifying drug induced viability changes of human PC-3MM2 prostate cancer cells using the MTT assay. Briefly, 1000 cells in 100 μL cell media were seeded in a 96 well plate and exposed to either varying concentrations of paclitaxel or different amounts of
paclitaxel loaded CNTs for four days. Figure 2.4 below shows that exposure of the prostate cancer cells to paclitaxel for 96 hours produced a dose-dependent inhibition of cell viability essential mitochondrial dehydrogenase enzymes. The IC$_{50}$, or drug concentration leading to 50% cell viability is ~5 ng/mL. Exposure of paclitaxel loaded onto PLGA-coated CNTs to the cells shows an IC$_{50}$ dosage of 100 ng/mL while exposure of the PLGA-coated CNTs without paclitaxel only showed significant cell death at doses greater than 1000 ng/mL. By comparing the IC$_{50}$ of the drug in the absence of CNTs (5ng/mL) with the volume of media added per well (100 µL), the amount of paclitaxel released from the PLGA-coated CNTs within 96 hours can be estimated to be 0.5 ng. Because the average drug load is 112.5 µg per mg of CNT, the estimated 0.5 ng paclitaxel corresponds to nearly 50% of the total amount of the drug incubated with the cells at the CNT dilution of 100 ng/mL.

Figure 2.4 Dose-dependent effects of paclitaxel and various CNTs on viability of human PC-3MM2 prostate cancer cells. Tumor cells were treated for 4 days with various
concentrations of paclitaxel (Ø), paclitaxel-loaded, PLGA-coated CNTs (■), or PLGA-coated CNTs without drug (□) either dissolved or dispersed in culture media supplemented with 3% FBS for 96 hours. Each data point represents the mean of six replicate experiments.69

2.2.4 In Vivo Imaging of CNTs

To determine the utility of in vivo imaging, the CNT-QDs were i.v. injected via the tail vein into mice, and the emission at 800 nm was monitored in live animals directly after injection, and 1, 2, 4, and 6 days after injection. Using the Kodak 4000MM whole mouse imaging system, in vivo images from the front, side and back of the mouse were acquired. Some select images are shown below in Figure 2.5 with the red spots representing the location of the CNT-QDs. QDs with longer wavelengths in the 800nm range compared to QDs in the 600nm range show a much brighter image due to the mouse auto-fluorescence, which occurs near the 600nm range. Directly after injection of the CNT-QDs, it is seen that the CNT-QDs are located only in the tail vein. After 2-6 days of blood circulation, the images express strong signals of the CNT-QDs in the organs of the mice, including the liver, kidney, stomach and intestines. The contrast in the images indicates that the CNT-QDs can be seen against an essentially back background with little to no interference of auto-fluorescence of the mouse show that the QDs with emission at 800nm are good candidates for fluorescence imaging of an animal. Sacrificing the mice and harvesting the liver, kidneys, stomach and intestines for ex vivo imaging confirmed uptake of the CNT-QDs. The strong luminescent emissions within the organs support the conclusion that the CNT-QDs can be used for deep tissue imaging.
Inductively coupled plasma mass spectrometry (ICP-MS) was conducted on the organ samples to determine where the CNT-QD mostly accumulated. Results of the ICP-MS are reported in Figure 2.6. It was determined that the Cd amounts (from the QDs) were distributed mostly within the liver with lesser amounts in the kidney, stomach and intestines.

**Figure 2.5** *In vivo* fluorescence images of CNT-QD IV injected into nude mice and imaged after circulation at various time intervals; (a=0 to a=4) the images taken from the side of mice, and d), e), f), g) organ images taken after having sacrificed the mice on the 6\(^{th}\) day of post-injection. These pictures show prominent uptake in the liver, kidney, stomach, and intestine.\(^{69}\)
Figure 2.6  *In vivo* distribution of CNT coupled with CdSeTe/ZnS (QD) after IV injected in the nude mice for 1, 3, and 6 days. The liver, kidney, stomach, and intestine of the mice were collected, respectively. The digested organ samples were analyzed for Cd mass by ICP-MS. 

2.3 Conclusion

After examination of the data, it can be concluded that QDs were successfully conjugated to the surfaces of AA coated CNTs for *in vivo* imaging. It was determined that plasma polymerization of CNTs with acidic polymers facilitated the coupling of amine functionalized QDs to the carboxyl coated AA CNTs. The CNT-QD exhibited emission in the near infrared range of 800 nm, making the CNT-QD construct a suitable candidate for non-invasive optical *in vivo* imaging. ICP-MS studies showed predominant CNT-QD uptake in the liver, kidneys, stomach and intestines following i.v. administration in mice.
It is also concluded that the anti-cancer drug paclitaxel was efficiently loaded in PLGA-coated CNTs. The polymer PLGA was chosen due because it is an FDA-approved biodegradable polymer that is widely studied in drug delivery. The PLGA-coated CNT demonstrated high paclitaxel loading efficiency, which exhibited \textit{in vitro} anti-tumor efficacy against human PC-3MM2 prostate cancer cells.
Chapter 3 – Development of Fluorescent Magnetic Nanoparticles for In Vivo Imaging

3.1 Background

A primary objective for many research programs has been the development of a material that can improve the clinical diagnosis of cancers, allowing for better and faster treatment.\textsuperscript{71-82} The surface functionalization of quantum dots (QDs) is widely acknowledged as a tool to improve diagnoses. However, QD conjugations have been limited only to perform the function of imaging. Functionalization of QDs to materials such as iron oxide, provide additional opportunities including therapeutic treatment using magnetic hyperthermia. This work presents a novel material that simultaneously combines the efforts of in vivo imaging with local therapy by magnetic hyperthermia.

The new dual functionality material concept is based on a unique nanostructure of 100 nm diameter polystyrene nanospheres that are surface modified by polyethylene oxide, along with 5-10nm diameter Fe\textsubscript{3}O\textsubscript{4} nanoparticles embedded in their matrices. The polyethylene-oxide-modified polystyrene-Fe\textsubscript{3}O\textsubscript{4} nanospheres were synthesized by miniemulsion/emulsion polymerization.\textsuperscript{83} The iron oxide nanoparticles embedded in the nanospheres will collectively generate heat, raising the external temperature of the surround environment, in response to an external alternating magnetic field. QDs are immobilized on the surfaces of the nanosphere composites, providing fluorescent imaging capabilities. A schematic representation of the design is provided in Figure 3.1 below.
Figure 3.1: Schematic illustrating the surface-immobilized QDs on magnetic nanosphere composite. EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl; NHS: N-hydroxysuccinimide; PEG: polyethylene glycol

The synthesis of the nanosphere composite containing a high fraction of iron oxide within its matrix can effectively maintain large magnetic moments in an externally applied field for both hyperthermia and magnetic guiding. The functionalization of QDs on the surfaces of the magnetic nanospheres provides additional properties that are desirable for localized cancer diagnosis. First, near infrared QDs conjugated to the nanosphere surfaces will display bright emissions in the near infrared range of the electromagnetic spectrum, ideal for deep-tissue imaging. Second, the addition of multivalent, surface-functionalized QDs provides coupling opportunities of unique cancer targeting ligands, such as peptides, making the nanosphere composite more selective of its destination. Finally, the high fraction of iron oxide embedded in the polystyrene matrix displays a larger and stronger magnetic moment in comparison to individual Fe$_3$O$_4$ nanoparticles, improving magnetic manipulation and magnetic hyperthermia.
In this study near-infrared QDs were functionalized to the surfaces of the magnetic nanocomposite. Optical microscopy and TEM images were taken of the QD-magnetic nanosphere (QD-MNS) constructs. To explore the potential biological applications of the novel QD-MNS concept, fluorescence imaging was performed in live mice. After imaging, the mice were sacrificed and organs harvested, sliced and imaged to confirm the bio-distribution of the MNS-QDs.

3.2 Experimental

Qdot 800 ITK amino (PEG) QDs with an emission wavelength of 800 nm were supplied by Invitrogen Corporation (Carlsbad, CA). These QDs have a CdSeTe core and a ZnS shell with a covalently attached, amine functionalized layer of PEG. The CdSeTe/ZnS QDs were dispersed in borate buffer (8 nmol/mL). In order to assure effective surface immobilization of the QDs on the MNSs, a combined strategy of covalent coupling and electrostatic adsorption was pursued. Under physiological conditions, the positively charged amine group of the Qdot 800 ITK amino (PEG) is predicted to form electrostatically stabilized association with the electric double layer surrounding the polymer core of the MNS. Short-range, electron donor/acceptor interactions between the two surfaces are hypothesized to stabilize this association complex, which is consistent with extended DLVO (Derjaguin, Landau, Verwey, Overbeek) theory. In addition, carboxyl groups resulting from auto-oxidation and partial cleavage of ethylene-oxide subunits of MNS-incorporated polysorbate allow covalent coupling of the amine-functionalized QDs using conventional carbodiimide chemistry.
Briefly, 200 mL of a freshly prepared solution of 30mg NHS and 10mg EDC in 1mL of phosphate buffered saline (PBS) (pH 7.4) was mixed with 100 mL of a suspension containing 2mg of MNSs and 160 pmol of QDs. The reaction mixture was incubated for 12 h at 50 °C, cooled to room temperature, and centrifuged at 12 000 rpm for 5 min. The QD–MNSs were washed three times with PBS and stored in 200 mL PBS until used.

3.3 Results & Discussion

3.3.1 Bright-field Characterization

Following conjugation of the QD-MNS construct, a drop of the suspension was applied to a glass slide for optical evaluation using an Olympus BX51 equipped with fluorescence illumination capability. Figure 3.2 (a) below shows a bright-image of the QD-MNS particles sans fluorescence. The dark nanoparticles are visibly noticeable in contrast to the white background. In Figure 3.2 (b), the fluorescence emission is displayed of the same area shown in the bright field image, after excitation with light of wavelength, $\lambda = 540-580$ nm and emission at $\lambda = 600-660$ nm. The particles exhibit a bright red emission in contrast to the dark background, indicating the successful functionalization of QDs to the surface of the magnetic nanosphere composites.
**Figure 3.2:** a) Bright-field optical image of dispersed MNSs with surface conjugated Qdot 800 ITK amino (PEG) QDs. b) Fluorescence image of QD-MNSs obtained under excitation at 560 nm using a Texas Red emission filter set.

### 3.3.2 TEM and EDS Characterization

Transmission electron microscopy (TEM) was used to characterize the surface structures of the QD-MNSs with a JEOL 2010F microscope. The samples were prepared by dispersing the QD-MNS particles directly on porous-carbon films supported on Cu grids. The Figure 3.3 (a) below confirms the spherical shape of the magnetic nanosphere composite in the bright-field. The darker spots are the iron oxide nanoparticles while the lighter grey areas are the polystyrene matrix. High-resolution TEM of the QD-MNS construct in Figure 3.3 (b) further supports the random distribution of the surface immobilized QDs on the nanocomposite surface. In Figure 3.3 (c), a Fourier transformed imaged of a randomly selected nano-construct, indexed as ZnS. Finally, in Figure 3.3 (d), an EDS spectrum is shown. The spectrum is consistent with the elemental signals
from CdSe/ZnS QDs. As a whole, this data provides further experimental evidence that conjugation of amine-functionalized QDs onto the surfaces of the magnetic nanosphere composite was successful.

**Figure 3.3:** a) Bright-field TEM image clearly illustrating surface structure of the QD-MNSs. b) High-resolution TEM image depicting surface properties of fabricated QD-MNSs. c) Fourier-transformed image of a randomly selected nanoparticle, which can be indexed either by hexagonal CdS or quartzite, ZnS. d) Energy dispersive X-ray (EDS) spectrum showing chemical signals of surface-associated CdSe/ZnS QDs.\(^\text{84}\)
3.3.3 In Vivo Imaging

The biological applications of the nanocomposite, specifically fluorescence imaging, was performed in live mice. In vivo fluorescence was monitored using the Kodak Imaging station (Carestream Health, Inc., Rochester, NY; excitation: 725 nm, emission 790 nm) before and after intravenous injection of the QD-MNSs (10 mg/mL in PBS, 100 µL per animal) in nude mice through the tail vein. Figure 3.4 below shows the gray-scale fluorescence images from both the control and QD-MNS injected mice. The image acquisition time was 1 minute. In Figure 3.4 (a), auto-fluorescence in clearly visible in the control mouse in certain ventral regions prior to the QD-MNS injection. One day following injection, fluorescence attributed to the QD-MNSs was localized in the region of the spleen (Figure 3.4 b). The spleens were harvest from the animals and ex vivo fluorescence imaging was conducted as seen in the insets of Figure 3.4 a and b. The control spleen shows no fluorescence as expected. The spleen of the mouse injected with the MNS-QDs shows the fluorescence. To further support the in vivo and ex vivo images, cryosections of the spleens were prepared and imaged. Briefly, frozen tissue was embedded in an optimal cutting temperature compound (EMS, Hatfield, PA), and 10 µm cryosections were prepared at -20 °C using an UltroPro 5000 cryostat (Vibratome Comp., St. Louis, MO). Figure 3.4 c and d show the microscopic bright-field and fluorescence images of the accumulation of the QD-MNSs. This data shows evidence that in vivo administration of the QD functionalized nanocomposites results in detectable fluorescence signals in a live animal.
Figure 3.4: *In vivo* and *ex vivo* images after intravenous injection of QD-MNSs in nude mice. Red fluorescence signals indicate QD-MNS accumulation in spleen; a) *in vivo* fluorescence before injection; b) *in vivo* fluorescence one day after injection; c) bright-field microscope image of a histological spleen section, and d) corresponding fluorescence image of the same section of tissue shown in (c).  

### 3.3.4 SQUID and Magnetic Hyperthermia Characterization

Magnetic hysteresis and magnetic hyperthermia experiments were next conducted on the QD-MNS. Hysteresis curves were generated using the Quantum Design MPM-5 superconducting quantum interference device (SQUID) magnetometer. The field dependent magnetization was measured at 300 K. Figure 3.5 below shows the sample hysteresis, typical of a superparamagnetic material. The magnetic saturation can be seen at about 1000 Oe and displays reversible hysteresis. The hyperthermic rate of the QD-
MNSs was measured using the Hilger’s technique. The magnetic field frequency and amplitude used was 63 kHz and 7 kA/m respectively. 100 µL of a 500 mg/mL magnetic fluid sample was placed in the sample container. The temperature was monitored with an optical fiber thermometer probe (FTI-10; FISO Co., Ltd., Canada). Upon application of the alternating magnetic field, the temperature of the magnetic fluid rose to 52 °C after 30 minutes. The heating curve can be seen in the Figure 3.6 below.

![Figure 3.5: Magnetization vs. applied field for the QD-MNS taken at 300 K.](image)

Figure 3.5: Magnetization vs. applied field for the QD-MNS taken at 300 K.
3.4 Conclusion

It can be concluded from the data that the novel design of a nanostructure consisting of QDs conjugated to magnetic nanosphere composites for concurrent cancer diagnosis and treatment was successfully achieved. The QD-MNS constructs displayed strong and bright fluorescence emission in the near-infrared range of the electromagnetic spectrum. In vivo administration of the QD-MNSs in live mice and subsequent fluorescence imaging provided images that support further evaluation of the novel composite. The ability to generate heat through magnetic hyperthermia displays the multifunctional capability of the novel design. Functionalization of targeting ligands to the nanostructure would further increase the utility of the design by increasing diagnosis accuracy and providing a greater concentration of nanoparticles for magnetic hyperthermia therapy.
Chapter 4 – Thermoresponsive Coatings on Magnetic Nanoparticles

4.1 Background

Controlled release drug delivery systems have evolved from conventional formulations designed to maintain predefined pharmacokinetic drug profiles over prolonged periods of time to more responsive drug delivery systems engineered with stimulus-induced release mechanisms. Among those “smart” drug delivery technologies, open-loop systems constitute formulations whereby dissociation of a therapeutic moiety from its carrier is regulated externally, most frequently by physical stimuli such as light, ultrasound, or magnetic fields.\textsuperscript{54,63,65,90,91}

Stimulus-controlled drug delivery systems are predicted to particularly benefit patients diagnosed with diseases including bronchial asthma, arthritis, peptic ulcer, and diabetes.\textsuperscript{92,93} In addition, these drug systems are expected to improve the care of disease states dominated by distinct, local occurrences such as inflammation and cancer. Conventional drug delivery strategies usually rely on passive drug distribution that achieves therapeutically effective concentrations at the desired site of action. Using cancer chemotherapy as an example, highly potent cytotoxic agents are distributed throughout the body through the blood circulation aimed at destroying tumor cells. The nonselective and uncontrolled biodistribution of these agents, however, lead to undesired killing of normal cells with high proliferation rates. These “off-target” effects are responsible for drug-induced nausea, vomiting, diarrhea, hair loss, and bone marrow suppression.\textsuperscript{94} Controlled, stimulus-triggered release of an anticancer drug at the tumor site is believed to limit undesired side effects and effectively improve the quality of life for cancer patients.
The design of bioresponsive matrices that facilitate stimulus-activated drug delivery critically depends on a capable sensing functionality within the system. As a consequence, drug release can be effectively controlled via a wireless, externally applied “on-off” switch to meet the spatial and/or temporal requirements of therapeutic treatment. For many years, materials that change properties in response to temperature were explored for fabrication of stimulus-induced drug delivery systems. Biocompatible analogs of poly(N-substituted acrylamide), poly(ethylene glycol), and phospholipids have emerged as leading polymers since they undergo reversible volume or sol-gel phase transition within 36-45°C, which corresponds to a temperature range spanning physiological environment to mild hyperthermia.\textsuperscript{95,96,97,98} Clinical hyperthermia, in particular, has gained attraction for drug delivery applications in cancer patients after several clinical studies demonstrated that heating of tumor tissue up to 45°C significantly enhances therapeutic efficacy of chemotherapy and radiation.\textsuperscript{98}

Magnetic nanoparticles, including superparamagnetic materials such as Fe\textsubscript{3}O\textsubscript{4} or liposomes/polymeric micelles containing encapsulated magnetic nanoparticles, have been evaluated for a diverse array of drug delivery, biomedical diagnostic, and analytical applications.\textsuperscript{97,98,99} Appropriately tailored magnetic properties facilitate accumulation in a target tissue guided by a strong, external magnetic field (i.e., magnetic drug targeting), while exposure of magnetic nanocarriers to an AMF induces local heating that effectively translates into therapeutic hyperthermia.\textsuperscript{98,100,101,102,103} Combination of these magnetic nanocarriers with thermosensitive polymers have resulted in bioresponsive nanocomposites suitable for stimulus-induced drug delivery applications. To date, coprecipitation of superparamagnetic Fe\textsubscript{3}O\textsubscript{4} in the presence of thermosensitive polymers
is an accepted standard for fabrication of bioresponsive nanocomposites.\textsuperscript{83,98} Using this methodology, processing variables such as Fe\textsubscript{3}O\textsubscript{4}/polymer ratio, solvent system, and stirring rate critically affect Fe\textsubscript{3}O\textsubscript{4} encapsulation efficiency and, consequently, the overall magnetic moment of the nanocomposite, particle size distribution, and thermoresponsive properties. Considering the added requirement for incorporation of a therapeutic payload into these bioresponsive systems, it becomes evident that alternative fabrication methods need to be explored to reproducibly prepare thermoresponsive magnetic nanocarriers suitable for drug delivery applications.

In this study, a unique phospholipid assembly immobilized as bilayer on superparamagnetic Fe\textsubscript{3}O\textsubscript{4} nanoparticles via high-affinity avidin-biotin binding interactions was explored (Figure 4.1). To investigate loading efficiency and temperature-dependent release of a carrier-associated payload, the lipophilic dye dansylcadaverine was incorporated into the phospholipid bilayer as a drug surrogate. Material properties of the thermoresponsive nanoparticles were assessed by differential scanning calorimetry (DSC) and attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR). Confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM) were used to visualize these novel nanocomposites. Zeta potential was determined by dynamic laser light scattering (DLS). Magnetically induced hyperthermia was monitored using a field strength of 7 mT at a frequency of 1 MHz. Payload incorporation efficiency and temperature-dependent release was quantified by fluorescence spectroscopy.
Figure 4.1. Schematic overview of layered phospholipid assembly on superparamagnetic nanoparticles. Phospholipid bilayer is immobilized on the surface of avidin-coated Fe₃O₄ nanoparticles using high-affinity binding interactions with DSPE-PEG2000-biotin anchors embedded at 5% (w/w) within the DPPC layer.¹⁰⁴

4.2 Results and Discussion

4.2.1 TEM and EDS Characterization

Phospholipid bilayers were immobilized on the surface of superparamagnetic colloids via high-affinity avidin-biotin binding interactions using a protocol adapted from the literature.¹⁰⁵ Deposition of thermoresponsive phospholipids on the surface of Fe₃O₄ colloids was attempted by anchoring DPPC bilayers via a biotin bridge to avidin-coated superparamagnetic nanoparticles. This methodology was shown previously to allow immobilization of phospholipid bilayers on spherical silica substrates using neutral, negatively, or positively charged lipid compositions.¹⁰⁵ Critical to this process is avidin adsorption onto the polar Fe₃O₄ surface, which is most likely facilitated by strong
hydrogen bonds.\textsuperscript{106,107} During sonication of the lipid film in the presence of the aqueous suspension of avidin-coated iron oxide, it is hypothesized that DPPC bilayer vesicles with exterior facing DSPE-PEG2000-biotin anchors are formed and rearrange into spherically supported bilayers on Fe\textsubscript{3}O\textsubscript{4} colloids stabilized by strong avidin-biotin interactions. TEM combined with EDS was used to investigate morphology and composition of control and phospholipid-modified nanoparticles. Figure 4.2A shows the spherical magnetite structure with a diameter of about 100 nm. The surface of this colloid seems smooth without visible associations. The dominating Fe peak in the corresponding EDS spectrum (Figure 4.2B) confirms composition. In contrast, TEM of colloids isolated after immobilization of phospholipids clearly demonstrates the presence of an amorphous layer associated with the Fe\textsubscript{3}O\textsubscript{4} surface (Figure 4.2C). The EDS spectrum of these modified colloids includes a carbon peak around 0.2 keV and a phosphorus peak around 2 keV, which is indicative of the presence of phospholipids (Figure 4.2D). As compared to the Fe and Cu peaks associated with the nanoparticle and TEM grid, respectively, phospholipid signals exhibit lower strength suggesting deposition of a thin lipid layer. Experimental predictions and computational simulations estimate the thickness of a fully hydrated DPPC bilayer between 4-6 nm.\textsuperscript{108,109} From TEM images obtained with phospholipid-modified Fe\textsubscript{3}O\textsubscript{4} nanoparticles (Figure 4.2C), it appears that the surface-immobilized DPPC/DSPE-PEG2000-biotin layer is only around 2-3 nm in diameter. Most likely, limitations associated with TEM technology prevented satisfactory visualization of lipid-associated water layers.
Figure 4.2. TEM images and EDS spectra of control and phospholipid-modified Fe$_3$O$_4$ nanoparticles. Panels A & B show morphology and materials composition of purified Fe$_3$O$_4$ nanoparticles. Panels C & D show structure and materials properties of phospholipid-modified colloids prepared by sonication of an aqueous, avidin-coated Fe$_3$O$_4$ nanoparticle suspension with a dried DPPC/DSPE-PEG2000-biotin lipid film.$^{104}$

4.2.2 Confocal Laser Scanning Microscopy Characterization
To assess uniformity of surface-supported phospholipid assembly the fluorescent lipid analog NBD-DSPE was incorporated into the lipid film at 2.5% (w/w) for CLSM experiments. Instead of Fe$_3$O$_4$ nanoparticles, which were too small to visualize surface properties using this technology, magnetic microbeads (~ 5 µm diameter) were subjected to the same coating protocol as described above. Figure 4.3A shows a representative micrograph of a fluorescence and brightfield overlay for bare control colloids affirming absence of significant autofluorescence. Similar images obtained for phospholipid-modified microbeads visually reveal almost complete coverage of the colloid surface with the NBD-DSPE-labeled lipid phase represented by the pseudogreen color (Figure 4.3B & C). Previously, Gopalakrishnan and colleagues used similar CLSM technology to demonstrate that incubation of polar silica particles with a fluorescent avidin analog achieves uniform surface coating. Combined with our data obtained with a fluorescent lipid analog, these results underline successful immobilization of phospholipid bilayers on polar surfaces stabilized by high-affinity binding interactions between colloid-adsorbed avidin and lipid-associated biotin anchors. In this study, phospholipid layers were assembled on polar, avidin-coated colloid surfaces using ultrasonication dispersion of a dried lipid film in an aqueous environment. Thermodynamically, immobilization of phospholipid bilayers on colloid surface is predicted to occur in parallel with undesired formation of liposome-like phospholipid vesicles. The intrinsic magnetic properties of Fe$_3$O$_4$ nanoparticles and microbeads used in this study successfully facilitated separation of phospholipid-modified colloids from liposome-like phospholipid vesicles.
Figure 4.3. Confocal images of phospholipid-modified microbeads. (A) representative overlay of brightfield and fluorescence images of ~5 µm control microbeads. (B) representative overlay of brightfield and fluorescence image of microbeads following immobilization of DPPC/DSPE-PEG2000-biotin/NBC-DSPE phospholipid layer. NBD-associated fluorescence emission is displayed in the pseudocolor green. (C) Increased magnification of representative phospholipid-modified microbead.104

4.3.3 Differential Scanning Calorimetry Characterization

Thermal properties of fabricated nanocomposites that control temperature-dependent release of carrier-associated payload were determined by DSC (Figure 4.4). The thermogram of pure DPPC shows a broad endothermic behavior with an estimated melting temperature ($T_m$) of 42.0°C, which is consistent with reported literature values for the main ($P_{β′}/L_α$) phase transition.110,111 In contrast, the gel/liquid-crystalline phase transition of the surface-immobilized phospholipid layer occurred at a peak $T_m$ of 39.7°C. The reduced enthalpy measured for the colloid-associated lipid layer can be explained by the significant contribution of the Fe$_3$O$_4$ particle core to the sample weight. Reduction in
temperature may be explained by the 5% (w/w) addition of DSPE-PEG2000-biotin to the DPPC lipid phase. Interestingly, Kastantin and co-worker were unable to detect a distinct transition temperature of DSPE-PEG2000 between 0-90°C. Consequently, the hypothesis is that the small shift in $T_m$ results from increased lateral tension of surface-immobilized phospholipids that energetically favors lower melting temperatures. Furthermore, greater radius of curvature due to the small size of phospholipid-supporting nanoparticles is also expected to lower $T_m$.

![DSC thermograms illustrating gel/liquid-crystalline phase transition of pure DPPC (“DPPC”) and DPPC/DSPE-PEG2000-biotin (95:5) phospholipid layer immobilized on avidin-coated Fe$_3$O$_4$ nanoparticles (“Fe$_3$O$_4$ lipid”).](image)

**Figure 4.4.** DSC thermograms illustrating gel/liquid-crystalline phase transition of pure DPPC (“DPPC”) and DPPC/DSPE-PEG2000-biotin (95:5) phospholipid layer immobilized on avidin-coated Fe$_3$O$_4$ nanoparticles (“Fe$_3$O$_4$ lipid”).

### 4.3.4 ATR-FTIR Characterization
ATR-FTIR was used to compare chemical features of surface-immobilized phospholipid layers to pure DPPC (Figure 4.5). The most prominent features were divided into four spectral regions. Panel A shows intense IR bands around 2850 and 2915 cm\(^{-1}\) that correspond to CH\(_2\) asymmetric and symmetric stretching modes of the acyl chains in DPPC. It is notable that the intensity of these bands is significantly reduced in colloid-associated phospholipids. As the amount of lipids deposited on Fe\(_3\)O\(_4\) nanoparticles is presumably small, it is conceivable that FTIR was not sensitive enough to detect these stretching modes. Similarly, the ester C=O stretching band near 1735 cm\(^{-1}\) is distinct for pure DPPC but broadened from about 1730 to 1830 cm\(^{-1}\) in the colloid-associated lipid layer, with an apparent peak at 1790 cm\(^{-1}\) (Figure 4.5, Panel B). In general, it is accepted that incorporation of water into DPPC layers results in broader IR bands due to formation of hydrogen bonds.\(^{115}\) In the phospholipid head group region, the characteristic asymmetric stretching band of the PO\(_2^-\) around 1240 cm\(^{-1}\) is clearly visible in both preparations (Figure 4.5, Panel C). The most prominent difference in IR spectra, however, is the dramatic increase in relative intensity of the CH\(_2\) scissoring bands at 1466 and 1357 cm\(^{-1}\), respectively. This behavior is consistent with changes in direction of the transient dipole vector of an adsorbed lipid molecule through electrostatic interactions.\(^{116}\)
Figure 4.5. ATR-FTIR absorbance spectra of pure DPPC (“DPPC”) and DPPC/DSPE-PEG2000-biotin (95:5) immobilized on the surface of avidin-coated Fe₃O₄ nanoparticles (“Fe₃O₄ lipid”). Expanded regions are shown for CH₂ asymmetric and symmetric stretching of acyl chains (Panel A), C=O stretching (Panel B), and CH₂ scissoring and asymmetric PO₃⁻ stretching (Panel C).¹⁰⁴

4.2.5 Surface Charge Characterization

The surface charge of colloidal systems measured as zeta potential directly correlates with chemical properties of surface-associated coatings and, in addition, defines dispersion stability in aqueous or nonaqueous media. Purified control Fe₃O₄ nanoparticles exhibited a zeta potential of -23.6 ± 1.3 mV in PBS, which is consistent with previous reports for this material.¹¹⁷ The dominant negative charge associated with these colloids facilitates effective repulsion of individual nanoparticles, which translates into measurable suspension stability in PBS for up to two hours. In contrast, the zeta potential measured for phospholipid-modified Fe₃O₄ nanoparticles was only -5.0 ± 3.0 mV, which is comparable to values reported for DPPC dispersion in aqueous solutions.¹¹⁸ This significant change in surface charge supports successful assembly of a phospholipid layer on these superparamagnetic nanoparticles. However, it also implies substantially decreased dispersion stability as compared to control nanoparticles, which was experimentally confirmed. Phospholipid-coated Fe₃O₄ nanoparticles begin to sediment within 5-10 min after dispersion. Nevertheless, colloids with slightly positive or negative zeta potentials demonstrate reduced adsorption capacities for serum components such as
proteins and lipoproteins.\textsuperscript{117} This may result in more consistent biodistribution pattern as compared to colloids with dramatically stronger surface charges.

### 4.2.6 Magnetic Hyperthermia Characterization

Thermoresponsive release of a payload incorporated into phospholipid-modified nanoparticles depends on heating properties of these superparamagnetic colloids within an alternating magnetic field. Figure 4.6 compares the temperature time course of 1 mL of control and phospholipid-coated Fe$_3$O$_4$ nanoparticles suspensions prepared in PBS (0.24 mg/mL) following exposure to 7 mT magnetic field pulses alternating at a frequency of 1 MHz. After a 3 min lag time, both preparations show fairly linear increases in temperature within the first 15 min. The slope calculated for this steady state hyperthermia was 1.50 ± 0.04°C/min and 1.38 ± 0.05°C/min for bare and phospholipid-modified nanoparticles, respectively. As mentioned previously, heating effects of magnetic nanoparticles within an alternating magnetic field are theoretically consequences of the loss processes Néel and Brownian relaxation.\textsuperscript{89,119,120} As the magnitude of magnetically induced heat changes strongly depends on particle size, comparable heating rates of the two colloid populations suggest only minimum changes in particle size due to surface immobilization of a phospholipid layer. This conclusion is consistent with TEM data shown in Figure 4.2C.
Figure 4.6. Heating properties of superparamagnetic Fe$_3$O$_4$ suspensions within an alternating magnetic field. Bare control (□) and phospholipid-modified (●) Fe$_3$O$_4$ nanoparticles were suspended at 0.24 mg/mL in PBS. Temperature of suspension was monitored for 30 min while repeatedly exposed for 90 s to a 7 mT magnetic field alternating at a frequency of 1 MHz. Data are shown as mean ± SD (n = 3).$^{104}$

Therapeutically desired hyperthermia conditions (>40°C) were achieved within 10-15 min using both control and phospholipid-modified Fe$_3$O$_4$ nanoparticles. DSC analysis identified a $T_m$ of 39.7°C for the lipid layer immobilized on Fe$_3$O$_4$ nanoparticles (Figure 4.4). It is, therefore, anticipated that hyperthermia-induced release of colloid-associated payload from these phospholipid-modified colloids will require exposure to an AMF of similar strength as used in this simple in vitro experiment for at least 10 min. If more rapid release profiles are desired, smaller Fe$_3$O$_4$ nanoparticles could be used due to the exponential relationship between Néel relaxation time and particle volume.$^{121}$
Phospholipid bilayers immobilized on the surface of superparamagnetic nanoparticles are predicted to serve as carriers for predominantly lipophilic guest molecules that are stabilized within the hydrophobic fatty acid tails of the phospholipids. In contrast to liposomes, which contain a hydrophilic core suitable for encapsulation of hydrophilic guest molecules,\textsuperscript{122,123} the geometry of surface-immobilized phospholipid bilayers does not allow sequestering of a significant water volume between colloid surface and phospholipid head groups.\textsuperscript{124} To determine the loading capacity of phospholipid-modified Fe\textsubscript{3}O\textsubscript{4} nanoparticles, the lipophilic dye dansylcadaverine was used as drug surrogate. The amount of colloid-associated dye was quantified by fluorescence spectroscopy following dissolution of lipid layer in methanol. On average, 479 ± 124 ng of dansylcadaverine were recovered per mg of Fe\textsubscript{3}O\textsubscript{4} nanoparticles from different fabrication batches (n = 10). Future experiments using therapeutically effective drugs will reveal whether the loading capacity of phospholipid-modified Fe\textsubscript{3}O\textsubscript{4} nanoparticles will be sufficient to prepare clinically required doses. Currently, FDA-approved formulations of superparamagnetic Fe\textsubscript{3}O\textsubscript{4} nanoparticles used as MRI contrast agents, including Endorem\textsuperscript{®} and Resovist\textsuperscript{®}, are administered in doses between 50-100 mg Fe\textsubscript{3}O\textsubscript{4}.

4.2.7 Drug Surrogate Incorporation

To assess thermoresponsive release properties of phospholipid-modified Fe\textsubscript{3}O\textsubscript{4} nanoparticles \textit{in vitro}, dye-loaded iron oxide suspensions prepared in PBS were exposed for 60 min to 25°C and 70°C using a temperature-controlled water bath. Dansylcadaverine concentrations released from the carrier were quantified after magnetic separation by fluorescence spectroscopy. At 25°C, which is below the experimentally
determined $T_m$ of the colloid-immobilized lipid layer, $8.4 \pm 1.7\%$ ($n = 4$) of the loaded dye was recovered in the dissolution media after 1 hour. In contrast, release rate at $70^\circ C$ was significantly greater reaching on average $23.4 \pm 6.8\%$ ($n = 4$). This corresponds to approximately 106 ng of dansylcadaverine released per mg of Fe$_3$O$_4$ nanoparticles. Similar to conventional thermoresponsive drug delivery systems such as liposomes or polymeric nanoparticles, release of the encapsulated payload occurs even at temperature below $T_m$. Considering the hydrophobic properties of dansylcadaverine, we speculated that bilayer imperfections and/or dye associated with the phospholipid surface contribute to these results. Most importantly, however, the data underline that increased mobility of phospholipids at a temperature $>T_m$ translates into accelerated payload release. Time-dependent release profiles in the presence of magnetically induced hyperthermia will facilitate delineating kinetic aspects of this thermoresponsive payload release from phospholipid-modified Fe$_3$O$_4$ nanoparticles in greater detail.

4.3 Conclusion

The results from this study demonstrate for the first time successful immobilization of a phospholipid assembly on the surface of superparamagnetic nanoparticles via high-affinity avidin/biotin interactions. Physicochemical and morphological evaluations revealed deposition of a 2-3 nm layer of DPPC/DSPE-PEG2000-biotin (95:5) on avidin-coated Fe$_3$O$_4$ nanoparticles with a gel/liquid-crystalline phase transition temperature of 39.7$^\circ$C. The zeta potential of Fe$_3$O$_4$ colloids in PBS decreased from -23.6 to -5.0 mV as a consequence of phospholipid immobilization. Nevertheless, heating properties of these superparamagnetic nanoparticles exposed to an
alternating magnetic field were not significantly affected. Hyperthermia-relevant temperatures >40°C were achieved within 10-15 min using a 7 mT magnetic field alternating at a frequency of 1 MHz. Loading of the surface-associated phospholipid layer with the hydrophobic dye dansylcadaverine was accomplished at an efficiency of 479 ng/mg Fe$_3$O$_4$. Release of this drug surrogate was significantly dependent on temperature. On average, release rate of this dye was 2.5-fold greater when nanoparticles were exposed to a temperature above the T$_m$ of the colloid-associated lipid layer.

These data underline the feasibility of preparing novel, stimulus-induced drug delivery systems where payload release from a colloid-immobilized phospholipid assembly is triggered by hyperthermia in response to an AMF. In contrast to previously described thermoresponsive polymers, including analogs of poly(N-substituted acrylamide) and poly(ethylene glycol), deposition of a phospholipid lipid layer on the surface of superparamagnetic nanoparticles offers the opportunity to incorporate a multitude of lipid-based targeting ligands and hydrophilic shielding groups using conventional technology that has been established for liposomal drug carriers. This may significantly accelerate clinical translation of phospholipid-modified superparamagnetic nanoparticles as alternative drug delivery systems to improve inadequate response to conventional therapeutic regimens.
Chapter 5 – *In Vitro* Studies of Magnetic Hyperthermia Induced Drug Release & Design Changes

5.1 Experimental Design and Goals

The natural progression for further development of the stimulus-induced drug delivery system would be observing the effect that magnetic hyperthermia and subsequent drug release has on tumor cells *in vitro*. Selection of the cytotoxic drug and cell line is important to the experimental design. Drug factors that need to be addressed are the ease of drug monitoring, the cytotoxicity of the drug to cells, and the hydrophobicity of the drug, which affects the drug loading, and release kinetics. Factors for cell selection include the cellular response to changes in temperature and the design setup for magnetic hyperthermia exposure.

Doxorubicin (dox), an anti-cancer drug, was selected as the cytotoxic agent for drug loading and drug release experiments. Nicknamed “red death” or “red devil,” the highly cytotoxic drug displays significant red fluorescence, allowing for easy of monitoring by fluorescence analysis. However, dox is weakly hydrophobic which may cause drug loading issues. Jurkat cells, a free-floating blood cancer used for leukemia research, have been selected for *in vitro* studies. The design of the magnetic hyperthermia machine, which requires free floating cells, lead to the selection of Jurkat cells. In addition to their floating nature, Jurkat cells are also known to be very susceptible to dox.\(^{125}\)

The goal for this work was to confirm the cytotoxicity of the nanosystem by stimulus induced drug release due to magnetic hyperthermia. To accomplish this goal, specific experiments were designed. These experiments are aimed to determine the IC\(_{50}\)
dosage of dox, or the concentration of dox that is cytotoxic to 50% of Jurkat cells, the encapsulation and loading efficiency of dox within the nanosystem, the release profile of dox in response to hyperthermia, the hyperthermic effects on Jurkat cells, and the combination of dox and magnetic hyperthermia on Jurkat cells. Some of these goals were accomplished while others were not due to problematic issues that will be discussed. Solutions to these issues will be examined as well as suggestions for future work.

5.2 Results and Discussion

5.2.1 Doxorubicin Loading and Release

Encapsulation of doxorubicin was accomplished in the same manner as dansylcadaverine encapsulation. Briefly, 10 μL of a 740 μM solution of dox was added to the DPPC and DSPE-PEG2000-biotin mixture. After the mixture was dried, a lipid film containing the drug was combined with avidin coated iron oxide, sonicated for 15 minutes and incubated for 30 minutes. Following incubation, the samples were washed three times with PBS to remove excess dox, resulting in dox incorporated nanoparticles. Dox loading of the nanoparticles was determined as follows. After dispersion in PBS, the drug-loaded samples were magnetically separated and re-dispersed in methanol, sonicated for 15 minutes and placed in an orbital shaker overnight. After the overnight period, the samples were magnetically separated and the methanol, now containing the dox, was plated in a 96-well plate. The drug loading was determined by fluorescence analysis using the POLARstar Optima (BMG Labtech, Ortenberg, Germany). On average, 760 ± 366 ng of dox was recovered per mg of iron oxide (n=10).
The amount of dox released from drug incorporated nanoparticles due to hyperthermia was next determined. Dox incorporated samples in PBS were subjected to four different temperatures (35 °C, 40 °C, 42 °C and 45 °C) in a water bath. At specified time points (0, 10, 20, 30, 40, 50, 60, 75 and 90 minutes), the samples were magnetically separated and 200 µL of PBS containing released dox was extracted and placed in a 96 well plate. Before being placed back into the water bath, 200 µL of PBS warmed to the temperature of the water bath, was added to the sample replacing the amount removed. The cumulative release at each temperature is shown in Table 1 below. The data shows that dox release is correlated to the increase in temperature. As the temperature increases, dox release increases, implying that at and above the melting temperature of the DPPC phospholipid bilayer coating (39.7 °C), the better the release rate of dox. However, the amount released may or may not be clinically relevant and needs to be evaluated.

Table 5.1: Hyperthermia induced doxorubicin release.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Dox Release (ng / mg Fe3O4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>22 ± 12.91</td>
</tr>
<tr>
<td>40</td>
<td>96.1 ± 15.96</td>
</tr>
<tr>
<td>42</td>
<td>98.35 ± 34.96</td>
</tr>
<tr>
<td>45</td>
<td>141.34 ± 21.08</td>
</tr>
</tbody>
</table>

5.2.2 Doxorubicin Cytotoxicity to Jurkat Cells

To determine if the amount of drug cumulatively released from the nanosystem in
response to hyperthermia is clinically applicable, Jurkat cells were subjected to varying concentrations of doxorubicin and the cell viability was ascertained. Clinically speaking, the aim is to find the drug concentration at which 50% of the cells are viable, also known as the IC$_{50}$. The IC$_{50}$ will be compared to the amount of dox released from the nanosystem, with the goal of having similar volume drug concentrations released from the system. Jurkat cell viabilities following drug exposure were determined fluorescently using the Live/Dead assay (Invitrogen, Carlsbad, CA), which uses two fluorescent reagents, calcein AM and ethidium homodimer-1, that yield two colors to allow for discrimination of live cells from dead. In live cells, calcein AM fluoresces green to indicate intracellular esterase activity, while in dead cells, ethidium homodimer-1 fluoresces red to indicate a loss of membrane integrity. These two colors are easily distinguishable using the POLARstar Optima plate reader, allowing for cell viabilities to be calculated.

Jurkat cells were counted to $2 \times 10^5$ cells per mL in RPMI (Roswell Park Memorial Institute) media supplemented with FBS (fetal bovine serum). 500 µL of the cell mixture was added into 11 microcentrifuge tubes and each tube was combined with 500 µL of varying dox concentration in supplemented RPMI media. The dox concentrations used were 0 nM (control), 10 nM, 50 nM, 100 nM, 250 nM, 500 nM, 750 nM, 1 µM, 2.5 µM, 5 µM and 10 µM. The combination of the cells and the dox concentrations gave a final volume of 1 mL with $10^5$ cells per mL. The Jurkat cells and dox incubated for 24 hours in a 5% CO$_2$ environment at 37 °C. After 24 hours, the microcentrifuge tubes were centrifuged at 400 rcf for 5 minutes and the supernatant was removed. The cells were washed with 1 mL of HBSS (Hank’s balanced salt solution) to remove excess dox and
centrifuged again at 400 rcf for 5 minutes. The supernatant was removed and re-suspended in 1 mL supplemented RPMI media. The cells were then incubated for 48 hours. Following incubation, the Jurkat cells were centrifuged at 400 rcf for 5 minutes and the media was removed and replaced with 1 mL of HBSS. Live and dead Jurkat cells were prepared at this time as reference points for cell viability calculations. For preparation of the Live/Dead assay, the 100% live cells and 100% dead cells are plated in a 96 well plate. Calcein AM is added to a well of live cells to give a maximum fluorescence intensity reading of live cells and calcein AM is added to a well of dead cells to give a minimum intensity of live cells. Ethidium homodimer-1 is added to a well of dead cells to give a maximum fluorescence intensity for dead cells and a well of live cells to give a minimum intensity reading. The cells from the microcentrifuge tubes are next added to the 96 well plate and both calcein AM and ethidium homodimer-1 is added to the wells. After 40 minutes of incubation, the plate containing the cells and the fluorescent reagents are analyzed by the POLARstar Optima, giving fluorescent intensities for calcein AM and ethidium homodimer-1. These fluorescent intensities are inserted into the following equations to give the percent of viable cells or the percent of dead cells:

\[
\% \text{ Live Cells or Dead Cells} = \frac{F(\text{sample}) - F(\text{min})}{F(\text{max}) - F(\text{min})} \times 100
\]

Where \(F(\text{sample})\) is the fluorescence intensity of the sample, \(F(\text{min})\) is the minimum fluorescence intensity (calcein AM in 100% dead cells for calculation of % live cells or ethidium homodimer-1 in 100% live cells for calculation of % dead cells) and \(F(\text{max})\) is
the maximum fluorescence intensity (calcein AM in the 100% live cells for calculation of % live cells or ethidium homodimer-1 in 100% dead cells for calculation of % dead cells).

Figure 5.1 below shows the cell viabilities against the log of the dox concentrations. From the figure, the IC$_{50}$ was determined to be 1.1 µM. This is the concentration of doxorubicin that is cytotoxic to 50% of Jurkat cells. Comparing this to the amount of dox released in a water bath at 42 °C, nearly 27 L of dox incorporated iron oxide nanoparticles at 0.24 mg per mL would be required to deliver a dox concentration of 1.1 µM in a 1 mL sample of $10^5$ cells. Of course, a more concentrated nanosystem could prepared, but this involves injecting a patient with nearly 6.5 grams of iron oxide per $10^5$ cells, assuming all the nanoparticles arrive at its destination. This absurdly high dosage requires a re-examination of the experimental design.

![Graph of cell viability vs. doxorubicin concentration](image_url)

**Figure 5.1**: IC$_{50}$ curve showing doxorubicin cytotoxicity to Jurkat cells. The IC$_{50}$ or concentration that is cytotoxic to 50% of the Jurkat cells is approximately at 1.1 µM.
5.3 Design Changes: Nanoparticle, Dispersant and Phospholipid Bilayer Coating

A quick visual observation of the lipid bilayer immobilized nanoparticles in solution leads to a conclusion that there is significant nanoparticle aggregation. This is confirmed by particle sizing by dynamic light scattering (DLS), which shows the average hydrodynamic diameter of the DPPC coated nanoparticles to be 4580.33 ± 1337.98 nm (~4.6 µm) in PBS with a polydispersity index of 0.742. First, this particle size is too large for in vivo experiments, which require particle sizes of 200-300 nm on the large end. Second, because the formation of aggregates of varying sizes and polydispersities, any drug loading and subsequent drug release can not be measured accurately. Leading to the possibility that significantly more drug has been incorporated into the DPPC coatings than the 760 ± 366 ng of dox per mg of iron oxide that was measured. The loading problem directly affects release measurements, with only the drug incorporated on the outside of the aggregates being released in response to the change in temperature. The issue of particle size requires the coating parameters of the project to be reexamined.

There are a few material factors that possibly attribute to the flocculation of the DPPC immobilized nanoparticles. These include the ionic concentration of the solution in which the particles are dispersed, the surface properties of the particles, and the steric and electrostatic charge of the phospholipids used for coating. The issue first inspected was the dispersant used for all experiments, PBS. The zeta potential of the iron oxide nanoparticles in PBS without DPPC coating is -23.6 ± 1.3 mV, indicating that there should be some stability for a short period of time in solution. However, the absence of
stability can be seen by examining the particle size of the uncoated iron oxide nanoparticles in PBS by DLS, giving $3786.33 \pm 668.95$ nm. Comparing this number to sizes observed by TEM imaging (largest particle size of ~ 40 nm in diameter) as shown in Figure 5.2 below, it can be concluded that aggregation in PBS occurs. The conceivable reason for aggregation is the high chloride ion content found in PBS. The chloride ions in the dispersant may be forming a “bridge” between particles, leading to the flocculation. If a liquid that has little to no ions in concentration is used as a dispersant, such as DI water, a change in particle size and zeta potential should be observed. This does occur as DLS of iron oxide nanoparticles in water show a much lower particle size of $327.52 \pm 51.43$ nm. A change is also seen in the zeta potential, changing from ~ -24 mV to 34.3 $\pm$ 5.38 mV. The change in particle size and zeta potential measurements, indicate that the highly ionic concentration of PBS leads to aggregation and suggests a change in the dispersion medium is needed.
The second potential material factor leading to aggregation of the nanoparticles is the nanoparticle surface properties. The nanoparticles selected for these experiments are naked, without any stabilizer associated with the surface. Because of the lack of stabilizers, the nanoparticles experience weak electrostatic and no steric repulsion between the particles, leading to nanoparticle flocculation. This is evident by examining the particle size of the iron oxide nanoparticles in water. The size, $327.52 \pm 51.43$ nm is more than eight times larger than the 40 nm observed by TEM. A nanoparticle with surface stabilizers may provide better dispersion in solution, preventing flocculation. Therefore, switching to a nanoparticle with surface stabilizers is a possible route to reducing aggregation.

A final material issue is the chemistry of the phospholipids used for the immobilized bilayers. DPPC, the main constituent in the bilayer coating, is a zwitterionic phospholipid, a material that carries both positive and negative charges resulting in an overall neutral charge. The neutral charge of the DPPC is evident by examining the zeta potential before and after coating. As mentioned previously, the zeta potential of the iron oxide nanoparticles in PBS are $-23.6 \pm 1.3$ mV before coating. After coating, the zeta potential is nearly neutral at $-5.0 \pm 3.0$ mV. The neutrality of the DPPC, like the naked nanoparticles, does not promote any electrostatic repulsive forces. There are two approaches to fixing the stability of the phospholipid immobilized nanoparticles. The first is changing the main phospholipid used for coating to a phospholipid with a charged
head group or a phospholipid that contains functionalized molecules that promote steric repulsion. However, by changing phospholipid or adding functionalized molecules, the melting temperature of the coating changes. The second approach is introducing an additive to the DPPC coating. These additives can either be charged or provide steric repulsion. Again, introducing an additive to the nanosystem will change the melting temperature, so a balance between repulsion and melting temperature of the bilayer coating must be maintained.

5.3.1 Nanoparticle and Dispersant Changes

Two of the proposed material issues were addressed simultaneously; the change in dispersant and change in nanoparticle. Two new buffer solutions, safe for cell studies, were examined with one new nanoparticle compared to the original Fe$_3$O$_4$. The two dispersants selected were Hank’s Buffered Saline Solution (HBSS) and HEPES. Both buffers contain a lower ionic concentration than PBS, and are used regularly in cell culture and help maintain physiological pH for cellular growth. The new nanoparticle examined is a magnetic nanosphere composite coated thinly with silica (MNS-SiO$_2$). The magnetic nanosphere composite is the same material used in the experiments for functionalization of QDs, but with a thin silica coating on the outer surface.$^{84}$ Briefly, the MNS-SiO$_2$ consist of iron oxide nanoparticles, 10-20 nm in diameter, embedded into a polystyrene matrix coated with silica on the surface. Figure 5.3 below shows a TEM image of the MNS-SiO$_2$. Particle sizing and zeta potential were completed for each of the nanoparticles dispersed in DI water, HBSS and HEPES with and without the immobilized DPPC bilayer. The results are shown in Table 5.2 below.
**Figure 5.3:** TEM image of magnetic nanospheres coated with silica (A: 30 nm coating; B: 2 nm coating). The magnetic nanospheres consist of 10-20 nm diameter iron oxide nanoparticles embedded in a polystyrene matrix.\textsuperscript{83}

**Table 5.2:** \(\text{Fe}_3\text{O}_4\) and MNS-SiO\textsubscript{2} particle size and zeta potential in different solutions.

<table>
<thead>
<tr>
<th></th>
<th>(\text{Fe}_3\text{O}_4) Particle Size (nm)</th>
<th>(\text{Fe}_3\text{O}_4) Zeta Potential (m\textsuperscript{V})</th>
<th>MNS-SiO\textsubscript{2} Particle Size (nm)</th>
<th>MNS-SiO\textsubscript{2} Zeta Potential (m\textsuperscript{V})</th>
</tr>
</thead>
<tbody>
<tr>
<td>DI Water</td>
<td>327.52 ± 51.42</td>
<td>34.3 ± 5.38</td>
<td>172.07 ± 3.17</td>
<td>-45.8 ± 0.65</td>
</tr>
<tr>
<td>HBSS</td>
<td>3964.83 ± 472.18</td>
<td>-8.33 ± 1.12</td>
<td>1657.33 ± 330.91</td>
<td>-18.27 ± 0.49</td>
</tr>
<tr>
<td>HEPES</td>
<td>3823.44 ± 563.16</td>
<td>-15.9 ± 0.6</td>
<td>155.4 ± 1.48</td>
<td>-30.47 ± 0.35</td>
</tr>
</tbody>
</table>

The results show that in DI water, the iron oxide particle size is lowest compared to HBSS and HEPES. In both DI water and HEPES, MNS-SiO\textsubscript{2} sizes are low and nearly the same. The small sizes seen for both particles in DI water are expected due to the absence of ions in solution that promotes aggregation. However, the iron oxide nanoparticles show an average size of 327.52 ± 51.42 nm, more than double the predicted size of 120 nm. The observed low particle size for MNS-SiO\textsubscript{2} in HEPES can be
explained by examining the zeta potential of the particle in HEPES and DI Water, -45.8 ± 0.65 and -30.47 ± 0.35 respectively. The zeta potentials show that there is sufficient electrostatic repulsion between the MNS-SiO$_2$ nanoparticles, preventing aggregation in DI water or HEPES. The particle sizes and zeta potentials for Fe$_3$O$_4$ and MNS-SiO$_2$ in HBSS indicate that aggregation is still occurring and that the bicarbonate ions in HBSS are most likely “bridging” the nanoparticles together. The HEPES buffer improves the zeta potential for Fe$_3$O$_4$ but not enough to improve overall particle stability. Because the MNS-SiO$_2$ nanoparticles show good stability in the HEPES buffer, all future experiments were conducted with these materials.

5.3.2 Phospholipid Bilayer Coating Changes

Next, MNS-SiO$_2$ nanoparticles were coated with a DPPC bilayer and dispersed in HEPES. Particle sizing and zeta potential measurements of the DPPC coated particles were completed and compared to the measurements prior to coating. Measurements are arranged for viewing in Table 3 below. As expected, there is an increase in particle size and drop in zeta potential after immobilization of the DPPC bilayer due to the charge neutrality of the phospholipid. As mentioned previously, adding a stabilizing component into the DPPC coating or replacing DPPC with a material that provides stabilization are potential modes for improving phospholipid immobilized nanoparticle stability. For these experiments, the cationic lipid stearylamine (STR) was incorporated as a stabilizing component into the coating bilayer with the aim to provide electrostatic stabilization. Briefly, a 10:1 ratio of DPPC to STR along with the biotinylated phospholipid (DSPE-PEG2000-biotin) were combined and dried to form a lipid film. The coating was then
prepared as previously described (page 64). Following coating, particle sizing and zeta potential were completed on the DPPC - STR coating. Examining the data in the Table 5.3 below, it is observed that the particle size has decreased nearly 200 nm in diameter, an indication of increased stability. However, the size is still too large for \textit{in vivo} experiments. The zeta potential also shows a rather large change from $-8.01 \pm 1.94$ to $33.6 \pm 1.77$, which is expected as STR is a cationic lipid. The data is encouraging, as a decrease in agglomeration has occurred, however, additional work needs to be accomplished to further reduce agglomeration.

\begin{table}[h]
\centering
\caption{Particle size and zeta potential of bilayer immobilized MNS-\text{SiO}_2.}
\begin{tabular}{lll}
\hline
 & Particle Size (nm) & Zeta Potential (mV) \\
\hline
Without DPPC & 155.4 $\pm$ 1.48 & -30.47 $\pm$ 0.35  \\
DPPC Coated & 533.9 $\pm$ 134.29 & -8.01 $\pm$ 1.94  \\
DPPC and STR & 314.4 $\pm$ 109.73 & 33.6 $\pm$ 1.77  \\
\hline
\end{tabular}
\end{table}

\textbf{5.4 Complimentary Work}

Due to the issues with nanoparticle stability, \textit{in vitro} testing of the nanosystem was unable to be completed. However, some additional work for the preparation of \textit{in vitro} testing was accomplished. Temperature sensitivity of the Jurkat cells for magnetic hyperthermia experiments was completed with the aim to determine the optimum temperature for magnetic hyperthermia experiments, a temperature above the $T_m$ of the DPPC coating (39.7 °C) but below the temperature at which cell death occurs via hyperthermia. Briefly, Jurkat cells, at $10^5$ cells per mL, were exposed to three different temperatures (40 °C, 42 °C and 45 °C) for five different time lengths (0, 30, 45, 60 and
90 minutes). Cells in FBS supplemented RPMI media were counted and $10^5$ cells (1 mL) were pipetted into 1.5 mL microcentrifuge tubes. The tubes were placed in a water bath at one of the three temperatures for the one of the five time lengths. After exposure, the cells were placed in an incubator at 37 °C with 5% CO$_2$ for 24 hours. Following 24 hours, the cells were centrifuged at 400 rcf for 5 minutes and the supernatant was removed and replaced with fresh supplemented RPMI media. The cells were incubated again for 72 hours and then prepared for the live dead assay. Figure 5.4 below shows the cell viability results of the live dead assay due to temperature exposure.

**Figure 5.4:** Graph showing the Jurkat cells viability to three temperatures for varying exposure times.

It can be seen that after exposure to 40 °C, there is virtually no cell death. Cell
growth actually occurs as viabilities above 100% are observed, even for the longer exposure times. Cell death begins to occur at 42 °C for the 60 minutes exposure time, with just over 60% of cells remaining viable. Viabilities decrease even further for 90 minutes. Sensitivity to temperature becomes even more apparent after Jurkat exposure to 45 °C, with no cell viabilities following any of the timed exposures. Because cell viabilities dip at 42 °C after 1 hour and are non-existent for 45 °C, there is a narrow working window using Jurkat cells for magnetic hyperthermia experiments. The goal of the magnetic hyperthermia experiment is to observe cell death due to drug release and not solely from temperature increase. Therefore, the maximum temperature and time that cells can be exposed to are 42 °C for 1 hour. An advantage of the creating cell death by temperature and dox is that cell death occurs by two different mechanisms, apoptosis and necrosis. Doxorubicin has been shown to induce necrosis on Jurkat cells while temperature induces apoptosis. There are tests that are able to determine whether cell death occurs by necrosis or apoptosis, allowing for the mechanism of cell death to be determined upon completion of magnetic hyperthermia experiments.
Chapter 6 – Conclusions and Future Work

6.1 Conclusions

Multifunctional nanosystems are nanoparticles designed and engineered to target tumors or diseased tissue, aid in *in vivo* imaging and deliver a therapeutic agent. The collaborative aim of the work presented is the design of these multifunctional nanosystems. Carbon nanotubes functionalized with quantum dots and loaded with drug were constructed for diagnostic and medicative cancer applications. Magnetic nanospheres were functionalized with quantum dots for *in vivo* imaging capabilities. The nanospheres being superparamagnetic also provide the opportunity of therapy by magnetic hyperthermia. The final project was the development of a smart, stimulus-activated nanosystem that releases drug upon the increase of temperature.

The key to the development of multifunctional carbon nanotubes is the presence of functional groups on the surface of the CNTs. Plasma polymerization, the process in which monomers are bombarded and sputtered onto the surface of a material, was the method of providing molecules for the covalent attachment of biomolecules or other nanospecies. CNTs coated with acrylic acid by plasma polymerization provided carboxyl groups for the conjugation of amine functionalized quantum dots. The CNT-QD construct was shown to provide *in vivo* imaging capability in mice after intravenous tail injection. The drug, paclitaxel, was also shown to be incorporated into a PLGA coated CNTs. The drug loaded CNTs demonstrated that they were much more cytotoxic than drug absent PLGA coated CNTs and uncoated CNTs, indicating the therapeutic efficacy of the nanosystem. As a whole, it can be concluded that CNTs have the potential of being developed into effective multifunctional nanosystems. However, due to the
cylindrical shape of CNTs, there may be issues of quicker uptake by the immune response as well as spatial issues with travel within the blood stream. These problems suggest that a more spherically shaped nanoparticle are better options for multifunctional nanosystems.

A spherical nanocomposite that consists of iron oxide nanoparticles embedded into a polystyrene matrix was functionalized with quantum dots for in vivo imaging capabilities. Amine functionalized quantum dots were immobilized on the surface of the MNS through the use of two coupling agents (EDC and NHS). The MNS-QD constructs were injected intravenously through the tail veins of mice and were monitored to determine their imaging capability. It was determined that the MNS-QDs were mostly localized in the spleen. The fluorescent imaging shows the potential capability of the MNS-QD construct for diagnostic applications. In addition to the imaging potential, the MNS-QD structure is also superparamagnetic, providing the opportunity for therapy by magnetic hyperthermia. The nanoparticles were shown to heat to temperatures above 50°C, temperatures at which thermal ablation of cells occurs. Localized heating of tumor tissue with the MNSs could aid in the reduction of cancer cells.

A major issue in cancer treatment, the effective delivery of cancer drugs, is a problem in which multifunctional nanosystems may provide a viable solution. In this work, a superparamagnetic nanoparticle, coated with a bilayer of thermoresponsive phospholipids, was developed for stimulus induced drug release applications. The phospholipid coating, anchored onto the spherical nanoparticle by an avidin-biotin interaction, can solubilize hydrophobic molecules within the lipophilic bilayer. These molecules will be released upon an increase in temperature, by magnetic hyperthermia,
upon attainment of the $T_m$ of the bilayer. The increase in temperature in conjunction with drug release provides an opportunity for dual therapies by thermally damaging cancer cells, weakening them for more efficient drug action. Immobilization of the phospholipid DPPC bilayer was confirmed various characterization methods including TEM, EDS, DSC and ATR-FTIR. The drug loading capability was demonstrated by incorporation of the lipophilic dye dansylcadaverine and later by the cytotoxic drug doxorubicin. However, due to the lack of stabilization of the nanoparticles after coating caused by the neutrality of DPPC and the high ion concentration in the buffers, proper drug loading and drug release experiments could not be completed. As a result, cytotoxicity studies were also not completed.

Because of the nanoparticle stability issues, some changes in the design were proposed and tested. Two new buffers, HBSS and HEPES, with lower ionic concentrations than the original dispersant, PBS, were tested along with DI water with the aim of reducing nanoparticle aggregation. In addition, magnetic nanospheres with a silica coating were compared to the iron oxide nanoparticle, to see if the nanoparticle substrate was an issue with flocculation. It was determined that the MNS-SiO$_2$ dispersed in HEPES buffer resulted in little to no aggregation. To further examine aggregation issues, the cationic lipid stearylamine was incorporated into the DPPC bilayer coating. The addition of STR showed significant reduction in aggregation by electrostatic stabilization, but not enough for in vivo experiments. Although aggregation was not completely prevented and particle sizing was not reduced to an optimal size, incorporation of a charged molecule shows significant potential in preventing the amount of flocculation.
6.2 Future Work

There is still much more work to be accomplished for this project. The reduction in particle size and consistent drug loading and drug release needs to be obtained before any cytotoxicity measurements are made. As mentioned previously, reduction in particle size can be accomplished by increasing the particle stability by electrostatic repulsion, steric repulsion or a combination of both. Because there are a limited number of phospholipids that have a \( T_m \) in the desired range for hyperthermia induced release (40 \(^\circ\)C - 45 \(^\circ\)C), the best route to increasing particle stability is through incorporation of stabilizers into the bilayer coating. However, changes to the coating makeup will affect the \( T_m \) of the bilayer, so the \( T_m \) must be carefully monitored. Additionally, other buffers with low ionic concentrations should be examined to keep nanoparticles from aggregating in solution.

Following good stabilization of the phospholipid immobilized nanoparticles, a reevaluation of the drug for incorporation into the bilayer should be made. The drug selected for this study, doxorubicin, is in the hydrochloric salt form. The form of this drug is very hydrophilic and does not incorporate itself very well in a hydrophobic environment, an environment like the hydrophobic region of the phospholipid bilayer. It is in this region that the majority of drug storage is desired and expected. Because the drug is highly hydrophilic, it is expected that dox incorporation will be limited by its solubility. Although aware of the solubility issue, the choice of using dox for these experiments was a monetary and availability issue. Future experiments would require a more soluble drug for incorporation. For example, paclitaxel, cisplatin or the basic form
of doxorubicin are drugs that can provide more ample incorporation and ultimately more concentrated drug release.

The design of the cytotoxicity studies has some additional design flaws outside of the drug selection. First, the IVMFG (*in vitro* magnetic field generator) requires the use of PCR tubes with a maximum volume of 250 μL. Because the use of PCR tubes are required, only free floating cells as opposed to surface adhesive cells can be used for *in vitro* experiments, limiting the types of cancer cells used for study. In addition, the IVMFG does not allow for complete control of magnetic field parameters. The frequency is unadjustable and is set to 1 MHz while the magnetic field strength range can be tuned from 0 mT to 7 mT. Currently, collaboration is underway in the development of a magnetic field generator with capabilities of *in vitro* experiments with free floating and surface adhesive cells and *in vivo* studies with mice. This new machine will offer much more flexibility in experimental design, allowing for better control of parameters and the ability to test a variety of volumes. The second design flaw for the cytotoxicity studies is the choice of Jurkat cells. The cells, as previously mentioned, are free floating cancer cells used for leukemia experiments. After exposure to small temperature changes, it was seen that Jurkat cell death was significant (a change from 40 - 42 °C). The high cell death rate within a small temperature window limits the opportunity to attribute cell death to released drug, as a lower temperature will result in lower drug concentrations released. A cell line that is less temperature sensitive could potentially provide better cytotoxicity data.

After the stability issues have been solved, the drug incorporation and release problems have been addressed, and a more appropriate cell line has been selected, the
proposed *in vitro* experiments can be started. The parameters for the new magnetic hyperthermia machine need to be determined, which include appropriate frequency and field strength. With these settings, the drug release profile from the nanosystem will need to be obtained and compared to the IC$_{50}$ for the new drug and cell line. These project objectives will allow for appropriate parameters to be set for cytotoxicity experiments of the drug incorporated nanosystem. The combination of drug and hyperthermia should elicit a synergistic cell death response, with more observable cell death from the combination as compared to drug and hyperthermia alone.
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