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Multifunctional Nanoparticles for Theranostic Applications

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

MULTIFUNCTIONAL NANOPARTICLES FOR THERANOSTIC APPLICATIONS

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOMEDICAL ENGINEERING

by

Supriya Srinivasan

2015

To: Interim Dean Ranu Jung
College of Engineering and Computing

This dissertation, written by Supriya Srinivasan, and entitled Multifunctional Nanoparticles for Theranostic Applications, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Florida International University, 2015

DEDICATION

I want to dedicate this dissertation to my husband Karthikeyan who lent never ending love and support throughout my journey in graduate school. He had immense belief in me that I would be able to make my way through graduate school. There was not a single day in four years of our married life that he complained about my absence. He always had more confidence in me than I did in myself. I regretted those times when I could not attend his call or wasn't able to hear to his problems during his difficult times. However, he was patient and was there for me, no matter what the circumstances were. He was always there when I was close to giving up, trying to make me understand the importance of what I was doing. I also want to dedicate my dissertation to my mother, father, mother-in-law and father-in-law. I would like to thank them for all the support they extended during my journey.

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ABSTRACT OF THE DISSERTATION
MULTIFUNCTIONAL NANOPARTICLES FOR THERANOSTIC APPLICATIONS

by

Supriya Srinivasan

Florida International University, 2015

Miami, Florida

Professor Anthony J. McGoron, Major Professor

Multifunctional agents for the management of highly heterogeneous diseases, like cancer, are gaining increased interest with the intent of improving the diagnostics and therapy of cancer patients. These agents are also important because more than one treatment modality is typically used for cancer therapy in the clinic. Further, nanotechnology offers a platform where more than one agent can be combined to help provide improved cancer diagnosis and therapy. Near-infrared light-activatable phototherapeutic agents have great potential *in vivo*. Body tissues have minimum absorption in the near- infrared range. They also have been shown to enhance the cytotoxic effect of chemotherapeutic drugs when used in combination with them. We have, hence, investigated the potential of two multifunctional targeted nanoparticles for combined chemo-phototherapy (employing near- infrared light activable agent) and for understanding their underlying cellular responses. The first is employing polymeric Poly-lactic acid-co-glycolic acid (PLGA) nanoparticles with simultaneous incorporation of Indocyanine Green (ICG) (a near-infrared light-activatable photothermal agent) and Doxorubicin (DOX) and surface conjugated with anti-Human Epithelial Receptor-2 (HER-2). The PLGA nanoparticles were subjected to two modes of hyperthermia, incubator and laser hyperthermia, to

mimic whole-body and localized hyperthermia used clinically. These nanoparticles upon laser exposure showed a rapid heat shock protein 70 (HSP70) response in comparison to the cellular HSP70 response upon incubator hyperthermia exposure. However, 12h post-treatment, downregulation of HSP70, was observed, thus, causing cellular apoptosis or necrosis based on the degree of thermal insult. These targeted nanoparticles, simultaneously incorporating agents, suffer from the limitation of release of both the agents from the nanoparticles and the need to control their release for bringing in effective therapy. Therefore, the second multifunctional nanoparticle employing silver nanoparticles (AgNPs) conjugated with Doxorubicin was formulated. AgNP serve as a near-infrared activatable agent itself, other than serving as a drug delivery vehicle. Thus, these nanoparticles only require the need to control the release of DOX alone. We further studied their mechanism of action, which included enhanced reactive oxygen species (ROS) production and reduction of intracellular thiol levels.

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LIST OF ABBREVIATIONS

PLGA	Poly-lactic acid co-glycolic acid
ICG	Indocyanine Green
DOX	Doxorubicin
NMR	Nuclear Magnetic resonance
IDNP	ICG and DOX loaded PLGA nanoparticles
AIDNP	antibody conjugated ICG and DOX loaded PLGA nanoparticles
PDT	Photodynamic therapy
PTT	Photothermal therapy
AgNPs	Silver nanoparticles
SERS	Surface enhanced Raman Spectroscopy
FP	Folate-PEG
DC	DOX-cbm-PEG
DH	DOX-hyd-PEG
FHAgNPs	Folate-PEG and DOX-hyd-PEG self-assembled AgNPs
FCAgNPs	Folate-PEG and DOX-cbm-PEG self-assembled AgNPs

1. INTRODUCTION AND REVIEW OF LITERATURE:

1.1 Background and Relevance:

Cancer is the second leading cause of death in USA next to cardiovascular disease. With increasing annual cancer incidence and related mortality, it is evolving as a major public health problem across the globe. The 5-year relative survival rate for all cancers diagnosed has increased from 49% between 1975-1977, to 68% in 2014. In children, a higher improvement in survival rates was found due to an increase in current treatment methodologies (ACS 2014). Thus, this increasing survival rate among adults and children reiterates the need for the development of strategies with higher therapeutic effects at the tumor site and lesser negative side effects to normal cells.

The current treatment modalities for cancer include chemotherapy, radiotherapy and surgery. These, however, lack specificity towards cancer. Several investigators have found that a combination of two or three treatment modalities often have enhanced therapeutic outcome compared to the application of a single modality alone (Bozic 2013). Delivery of these adjuvant therapies in conjunction with traditional therapies employing advanced technologies, can help in an accurate and precise diagnosis through imaging and early cancer detection. Novel technologies developed with intent to reduce the side effects of primary therapies (such as chemotherapy) and enhance their therapeutic effects at the cancer site are thus required. Research efforts geared towards the development of multifunctional platforms aim at combining several therapies while providing control

over drug release profiles. For instance, a chemotherapeutic agent and a phototherapeutic agent could be simultaneously delivered to a target site and activated for a combined effect. This approach can help customize treatment schedules, enhance treatment planning, and monitor responses to therapy for improved therapeutic outcomes.

Anthracycline drugs are one among the most commonly used chemotherapeutic agents in the clinic. These drugs mediate their toxic effect by binding to DNA and impairing its replication, and subsequently affecting cell growth. These drugs, however, lack specificity, and therefore, exhibit high systemic toxicity (Ferrans 1978). Recent studies have shown the application of these drugs in conjunction with several adjuvant treatment therapies such as radiotherapy and hyperthermia (Ragaz 1997; Gori 2005). Hyperthermia is one among the emerging neo-adjuvant therapies that when used in conjunction with chemotherapy is found to have synergistic anticancer effect.

Phototherapy is a therapeutic modality involving light, preferably near-infrared (NIR) light, with enhanced tissue penetration and less scattering. These therapeutic agents exhibit toxicity only when irradiated. Selectivity of these agents can be gained through two methods:

- (1) Engineered phototherapeutic agents (nanoscale agents or photosensitizers) can selectively get into tumor cells through passive or active means.
- (2) Illumination can be limited only to the diseased site, leaving normal cells unaffected.

Thus, the selectivity of these agents reduces the systemic toxicity of traditional treatment modalities such as chemotherapy. Rapid advancement in the field of nanotechnology has raised interest in nanomaterial-based phototherapies (Cheng 2014).

Two modes of phototherapy are currently used for cancer treatment: photothermal therapy (PTT) and photodynamic therapy (PDT):

PTT employs agents that absorb light and convert it into heat, leading to cell death. Ideal PTT agents absorb light in the NIR regime, to which biological tissues are mostly transparent. PTT is a form of hyperthermia, which is thermal dose-dependent. The preferred temperature for cell killing is 41-43 °C as these temperatures have shown selective toxicity towards tumor cells in comparison to normal cells (Cavaliere 1967). Clinical hyperthermia often involves a wide range of temperatures from 39-60 °C, with high temperatures being applied in ablation procedures. Lower temperatures (41-43 °C) often are used as an adjuvant therapy to radiation or chemotherapy

Treatment outcome based on hyperthermia is also highly dependent on cancer cell type, rate of temperature change, duration of exposure, and mode of heat delivery (Urano 1999). Hyperthermia is found to disrupt proteins and the structure of cancer cells (Hildebrandt 2002), and tumor cells are found to be more susceptible to hyperthermia-based cell killing (van der Zee 2002). Hyperthermia agents for cancer therapy can be activated through several ways, such as application of microwave heat, radiofrequency waves, and laser (Colombo 1996; Curley 2008; Tang 2013). Among several hyperthermia agents under study, organic dyes are gaining wide popularity due to their combined hyperthermia and NIR-imaging capabilities. When these organic dyes interact with NIR

laser light, they generate heat (a phenomenon which is dye concentration-dependent) and can reach temperatures that are cytotoxic to tumor cells (43 °C). These organic dyes can be used as both imaging and photothermal agents, and hence are good candidates for theranostic applications. Inorganic nanomaterials such as gold, carbon nanotubes, palladium sheets, and copper sulfide nanoparticles have also been reported for their photothermal capabilities (Zhou 2010; Gou 2011; Huang 2011; Yang 2013). PTT agents can be used in conjunction with other traditional therapies to bring about synergistic cancer therapy (Tang 2010), and nanoparticles further decorated with antibodies can help improve therapeutic drug indices by enhancing delivery to target sites (Lei 2011; Srinivasan 2014).

Photodynamic therapy (PDT) utilizes singlet oxygen or reactive oxygen species (ROS) to bring about its cytotoxic effect. The ROS is generated when a photosensitizer interacts with light of suitable wavelength, and aids in cell death and tissue destruction (Dolmans 2003). A wide range of photosensitizers have been applied to PDT. However, extensive research has proved that nanoparticle based PDT agents are better alternatives over traditional PDT agents (Bechet 2008). Various nanomaterials such as polymeric nanoparticles (Son 2011), magnetic nanoparticles (Yu 2012), and mesoporous silica nanoparticles (Tu 2009) have been used for the delivery of photosensitizing agents. Recent advances in nanotechnology have included the development of metal plasmonic nanoparticles as PDT agents. These nanoparticles exhibit several positive attributes over traditional organic dye-based PDT agents, such as higher molar extinction (Brulot 2012) and lack of photobleaching (Bonnett 2001). Silver and gold nanoparticles are plasmonic

nanoparticles that can induce a photodynamic effect when interacting with a laser of appropriate wavelength and fluence rate. The phototoxicity of a silver nanoparticle (AgNPs) is through its oxidation to ions. The ions generated produce reactive oxygen species, which in turn cause further degradation of cellular DNA and cytotoxicity (Mfouo-Tynga 2014).

PTT and PDT mediate their toxicity through different mechanisms. PTT involves heat which interacts with cells through disruption of proteins, whereas PDT involves generation of reactive oxygen species that elicits cytotoxicity through cellular DNA damage and lipid peroxidation (Rim 2013). Hence, the mechanisms behind both therapies provide novel and complementary strategies to cancer therapy and diagnosis. Both PTT and PDT involve the disruption of the balance in cancer cells in order to bring about their cytotoxic effects. PTT involves disruption of proteins, so the levels of pro-apoptotic proteins and anti-apoptotic proteins can help us determine the efficacy of the treatment outcome. PDT involves the production of ROS, and hence the balance between cytotoxic oxygen species and the anti-oxidant potential of cancer cells needs to be disrupted in order to bring about effective treatment outcome.

Heat shock protein 70 (HSP70) and caspase-3 (C3) are important classes of proteins which are most widely involved in cell apoptosis. When cells are exposed to external environmental stressors, such as chemotherapeutic or hyperthermia agents, protein denaturation occurs causing them to be misfolded resulting in an abnormal state. This provides stimulus to the cells to express heat shock proteins. These HSP70 help correct

misfolded proteins, reverting them to their original state, which further promotes cell survival (Ciocca 2005). Caspase-3 (C3), on the other hand, is an important pro-apoptotic protein. It is secreted in inactive form in the cells and can be easily converted to active apoptotic proteins by death signals provided by death receptor ligands attached to death receptors such as Fas, TNF α R, etc., onto tumor cell membranes (Lavrik 2005). A positive correlation between cell death and expression of C3 has been found (Porter 1999). Thus, the expression of HSP70 and C3 affects the therapeutic efficacy of PTT in combination with chemotherapy.

1.2. REVIEW OF LITERATURE:

1.2.1. Mode of action of chemotherapeutic agent (effects vs. side-effects):

Anthracycline drugs such as Doxorubicin (DOX), Daunorubicin etc., form a class of chemotherapeutic agents widely used for the treatment of a broad spectrum of cancers such as breast, ovarian and colorectal (Buzdar 1985; Singal 1998). These anthracycline drugs mediate their cytotoxicity by intercalating with the DNA in the nucleus of cancer cells. These drugs inhibit the activity of DNA topoisomerases which plays a major role in DNA replication (Tewey 1984). Despite their potent antitumor activity, their clinical application is limited by their dose-dependent toxicity to normal cells (Jeyaseelan 1997). One of the main mechanisms of toxicity of DOX in normal cells is through the generation of reactive oxygen species, which in turn damages mitochondrial DNA (Menna 2007). Among all the healthy cells, cardiac cells are most affected by DOX dosing due to their high mitochondrial content (Chen 2007).

Increasing dose administration of these drugs has often been correlated with increasing occurrence of drug resistance among tumors. Several non- transport and transport based mechanisms are implicated for this drug resistance, of which the role of drug efflux proteins expressed on tumor cell surfaces is the most studied model for such phenomena (Feng 1992). Anthracycline drugs such as Doxorubicin are important substrates to these efflux protein pumps which thereby reduce their anti-tumor efficacy. Thus development of approaches in order to reduce the non-specific toxicity and revert the drug resistance of these agents is essential for enhanced chemotherapeutics- based treatments.

1.2.2. Approaches to reduce side-effects and improve treatment efficacy:

1.2.2.1. Drug- antibody conjugates:

Cytotoxic chemotherapeutic agents have clinically improved the survival of patients with several classes of cancer such as breast, ovarian, uterine and lung (Loehberg 2005; Pisano 2005). Mixtures of several chemotherapeutic drugs are used to treat several aggressive and malignant tumors (Kalwinsky 1991). However, these drugs exhibit low therapeutic index due to their non-specificity to tumor cells. These agents also accumulate in normal cells and thus their availability at the tumor site is reduced. A narrow margin between the efficacious and toxic doses of these drugs is created which prevents the administration of a higher dose of these drugs required to eradicate the tumor.

This problem can, however, be circumvented by a strategy proposed in 1906 by Ehrlich using the so-called “magic bullet” (Ehrlich 1906). According to Ehrlich, a magic bullet would comprise of a therapeutic agent conjugated to an organ specific carrier. The organ specific carrier can be an antibody, aptamer or other small molecule that specifically recognizes a tumor specific antigen, often overexpressed on the surface of tumor cells. This strategy would help in the accumulation of drug at the tumor site and minimize exposure to normal cells (Ehrlich 1906). Some examples of tumor associated antigens (TAAs) are Human Epithelial Receptor-2 (Molinaa 1997), Folate receptor (Sudimack 2000; Reddy 2005), transferrin (Högemann-Savellano 2003), CD22 (Matsushita 2008), and Prostate Specific Membrane Associated antigen (PSMA) (Gao 2004). A few therapeutic agents targeted to tumor associated antigens which have been approved by the FDA are Mylotarg for acute myelogenous leukemia (Sievers 1999), Ontak for cutaneous T-cell lymphoma (Kreitman 2003) and Bexxar for non-Hodgkin's lymphoma (Zelenetz 2003).

However, conjugation of a chemotherapeutic agent directly onto antibodies or small ligands (like folic acid) allows for attachment of very few drug molecules. Thus, this method requires a higher amount of drug immunoconjugate (or ligand- based conjugates) to be administered for the desired therapeutic effect at the target tumor site (Jaracz 2005). The potency of these conjugates depends on the specificity of the antibody or small molecule ligand to the TAA, the potency of drug molecule and the linker between the antibody/small molecule ligand and drug molecule (Carter 2001). High molecular weight drug immunoconjugates (or ligand- based conjugates) can be targeted to tumors through

the Enhanced Permeation Retention Effect (EPR) effect. However, they are often unable to penetrate solid avascular tumors. Diffusion is the major mode of transport in solid avascular tumors. Flux (diffusion) inside solid tumors largely depends on the diffusion coefficient of the molecule itself. According to the Stokes- Einstein equation, a direct relationship exists between diffusive flux and the diffusion coefficient of molecules (Miller 1924). High molecular weight molecules have smaller diffusion coefficients and thus, have a lower penetration in solid avascular tumors (Pluen 2001). Low molecular weight drug-immunoconjugates (or ligand-based conjugates) created by employing truncated antibodies (those formed by truncating the Fc portion of the antibody to have Fab conjugated to drug molecules), on the other hand, can more easily penetrate solid tumors. However, they are cleared faster by the kidneys (Hansson 1988).

1.2.2.2 Prodrug- based therapies:

A prodrug is a drug which is non-toxic and inert, and becomes pharmacologically active when metabolized inside the body. Prodrugs are used when the active form of drug is insoluble in plasma or unstable and easily metabolized before it reaches the target. However, this concept is different from drug loaded carrier or drug vehicle. Some of the advantages of prodrug-based cancer therapies are increased drug stability, overcoming drug resistance, improved pharmacokinetics, and selective activation in target cells (Connors 1986).

Prodrugs in cancer therapy help in improving selectivity of the drug to tumor cells by being selectively activated in tumors. Prodrug-based strategies for selectively delivering

chemotherapeutics are based on the unique aspects of tumor physiology, such as selective enzyme expression (Xu 2001), hypoxia (Denny 2000), and low intracellular pH (Fan 2013). Prodrugs are based on externally-supplemented compounds which can selectively be activated in tumors. Exogenously supplemented prodrug-based strategies include those directed through monoclonal antibodies (Antibody directed enzyme prodrug therapy, ADEPT) or DNA constructs (Gene directed enzyme prodrug therapy, GDEPT) (Xu 2001).

Several prodrug based strategies for selective delivery of DOX into tumors have been investigated. For instance, Albright and coworkers investigated tumor-selective delivery of DOX through a peptide-based DOX prodrug. The prodrug was activated by the combined proteolytic action of extracellular and intracellular enzymes MMP-2, MMP-9 and MMP-14. Briefly, the prodrug was comprised of an Ac–Glu–Pro–Cit–Gly–homo-Phe–Tyr–Leu–DOX sequence, which under the combined action of the above mentioned enzymes could selectively cleave the prodrug to release DOX in tumor cells. The prodrug demonstrated selective delivery of DOX in *in vitro* and *in vivo* in xenograft models. It also exhibited a 10-fold tumor/heart distribution, thus reducing the inherent cardiotoxicity of DOX (Albright 2005). Another example of a prodrug-based strategy for DOX delivery was reported by Biela and coworkers. Their group utilized an antibody-directed enzyme prodrug based strategy, based on the application of Tumor Necrosis Therapy (TNT) monoclonal antibodies (MAbs) for selective delivery of DOX to necrotic tumor regions. Briefly, fusion proteins of single chain Fv (scFv), Fab or F(ab')₂ fragments of chimeric TNT-3 and the human beta-glucuronidase (betaG) enzyme were constructed. These

constructs were used to make a glucuronide prodrug conjugate of DOX. When the efficacy of the prodrug was tested *in vivo*, the results showed that the BetaG enzyme in the construct could specifically localize in tumors and was active for up to 9 days. Fusion protein dose administration after prodrug administration demonstrated active tumor cytotoxicity without systemic toxicity (Biela 2003).

Thambi and coworkers reported a tumor hypoxia-based prodrug for selective delivery of DOX. Hypoxia-responsive nanoparticles were created by conjugating hydrophobically modified 2-nitroimidazole derivative to the backbone of carboxymethyl dextran (CM-Dex). DOX was encapsulated into these nanoparticles. These nanoparticles exhibited sustained release under normoxic conditions, and significantly increased drug release under hypoxic conditions. These drug encapsulated nanoparticles exhibited high antitumor efficacy *in vivo* (Thambi 2014).

Tumor pH has also been investigated as a target strategy to selectively deliver chemotherapeutic drugs, especially DOX. One of the first attempts to employ pH-sensitive linkages for tumor selective delivery of DOX was made by Zhou and coworkers. They developed poly(ethylene oxide)-*graft*-doxorubicin (PEO-*g*-DOX) prodrugs, with DOX covalently conjugated to PEO via a pH-sensitive hydrazone bond. When compared with free DOX *in vivo*, these prodrugs had significantly prolonged circulation time and enhanced drug accumulation in the tumor (Zhou 2011). Several other investigators have developed pH-targeted prodrug systems employing different pH-

responsive polymers and pH-sensitive linkages in particular for tumor delivery of DOX (Du 2013; Zhang 2014).

HPMA-doxorubicin-galactosamine (known as PK2) is a targeted prodrug for DOX that has entered clinical trials. This prodrug is targeted towards asialoglycoprotein receptor (ASGPR), which is selectively expressed in hepatocytes and hepatoma cell lines. Preclinical trials employing rodent models have shown selective release of DOX in tumors, with a fivefold reduction in cardiotoxicity in comparison to free DOX after intravenous or intraperitoneal administration at several doses. The prodrug also showed several positive attributes over its corresponding drug DOX in Phase I/II trials, with 12-to-50 fold increased tumor concentration compared to free DOX. Thus, this prodrug holds huge promise for advancing to the next stages of clinical trials (Seymour 2002).

Based on current research, a major advantage of prodrug based strategies is their target selectivity and reduced systemic toxicity, which makes them ideal for clinical applications

1.2.2.3. Adjuvant therapies:

Adjuvant therapies are referred to as those therapeutic modalities which are often co-administered along with a primary mode of treatment. These therapies, when administered along with the primary treatment method, can help enhance the therapeutic effect of the primary therapy (i.e., synergism). Several adjuvant therapies are used in

conjunction with chemotherapy, such as radiotherapy (Ragaz 1997), hormonal therapy (Solomayer 2003) and surgery (Felip 2010).

Hyperthermia is a neo-adjuvant therapy, and when applied along with chemotherapy and other traditional therapies bring about a synergistic therapeutic effect (Issels 2001). Hyperthermia (41-43 °C) when used as an adjuvant therapy selectively kills tumor cells. Healthy cells are less susceptible to heat than tumor cells (van der Zee 2002). Thus, lower hyperthermia temperatures can also help in tumor cell killing, when used in conjunction with other treatment modalities. Hyperthermia often denatures proteins and disrupts structures inside the cell (He 2004). Hyperthermia can either be administered to the whole body or localized (by administering photothermal agents directed to local areas) (Xi 2001; Latorre 2009). Several inorganic nanoparticles, such as gold or magnetic nanoparticles, have been investigated for the application of heat (Latorre 2009). However, these nanoparticles often suffer from the limitation of poor biodegradability. Near infrared (NIR) dyes are widely investigated due to their good biocompatibility, biodegradability and ability to produce NIR fluorescence light and heat upon excitation, using a laser of appropriate wavelength. Indocyanine Green (ICG) is one such dye (Dzurinko 2004; Sakka 2007). The only challenge in using ICG clinically is its poor aqueous stability and short plasma residence time. Research has focused towards entrapping this drug into nanoparticles (Saxena 2004; Manchanda 2010). In our lab, we developed multifunctional nanoparticles entrapping ICG and DOX simultaneously into PLGA nanoparticles, which were successfully able to revert P-gP resistance mechanisms (Tang 2010).

Photodynamic therapy (PDT) has also been used by some investigators as an adjuvant therapy, including PDT and chemotherapy (Rigual 2013), PDT and surgery (Baas 1997) and PDT and immune therapy (Castano 2006). PDT has been used in combination with several classes of chemotherapeutic drugs, such as alkylating agents (He 2009), drugs targeting topoisomerases (Peng 2009), drugs targeting the cytoskeleton (Dima 1990), antimetabolites (Hatse 1999), and antitumor antibiotics (Ma 1992). Among several antitumor antibiotics, DOX is the most widely used chemotherapeutic agent for the treatment of a broad spectrum of tumors, and has been utilized in conjunction with PDT. For example, Canti and coworkers investigated the effects of combinatorial therapy consisting of disulfonated aluminium phthalocyanine (a known PDT agent) along with DOX on leukemia-bearing mice, and found a significant additive antitumor effect for the combination treatment (Canti 1998).

Photodynamic therapy employs photosensitizers which mediate their toxicity upon laser exposure. Photosensitizers in PDT help transform light energy to a type II chemical reaction. The reactive end products in the reaction pathway result in rapid cytotoxicity. Recently, several researchers have helped in creating imaging and laser technologies to measure effective dose of photosensitizers with high ROS turnover *in vivo*. There are several photosensitizers that are clinically available, for example, Photofrin (Axcan Pharma inc, Birmingham, IL), Metvix (Galderma, Ft. Worth, TX) Photochlor (Medkoo Biosciences, Chapel Hill, NC), etc. The different photosensitizer families that have been widely investigated for PDT include the porphyrin and chlorophyll platforms, and dyes such as phthalocyanine and naphthalocyanine (Allison 2004). These photosensitizers are referred to as first-generation photosensitizers, and suffer from several limitations such as

poor light absorption, cutaneous photosensitivity, and possible photobleaching effects. Thus, there is a need for the development of novel photosensitizers to address these problems and improve the efficacy of PDT. This could be achieved by structural modification of existing photosensitizers, or by developing new photosensitizers with different cytotoxicity mechanisms. Other researchers are exploring the development of new non-porphyrin, non-traditional photosensitizer agents (O'Connor 2009). For example, plasmonic nanoparticles can be used as photosensitizers upon appropriate laser irradiation and fluence rate (Vankayala 2013).

1.2.2.4. Image- guided therapy (IGT):

Surgery is the first line of treatment for most solid tumors. During resection, some tumors do not require a clear demarcation between tumor and normal tissues because a portion of healthy tissue can be resected without complications, or because the boundary of the tumor is distinct. For example, this is the case in early skin cancer. Certain surgeries, however, require much more precise differentiation between tumor and normal cells. This is true in tumors where cancer cells invade nearby tissues, such as breast cancer; or in cases where critical structures may be located near tumor cells so that a meticulous choice of resection boundaries is crucial, for instance in brain tumors. Probes that can provide visual differences between tumor and normal tissue would help in such surgeries. Image-guided therapy, or IGT, exploits imaging modalities to help localize and target therapeutic agents in the diseased tissue and monitor and control treatment. Targeted labeled probes (mostly guided through different imaging modalities) also help reduce the chances of leaving residual tumorous tissue behind after surgery, and facilitate the

assessment of tumor prognosis. Over the past decade, image-guided surgeries have evolved from traditional invasive approaches into applying minimally invasive imaging modalities. Advanced imaging modalities such as Magnetic Resonance Imaging (MRI), Computer Tomography (CT) and Positron Emission Tomography have replaced the routine X-ray (Jolesz 2014). Different imaging modalities that can be used in image-guided therapy or surgery are described in the next sections.

1.2.2.4.1. Image-guided Radiation Therapy (IGRT):

The specific containment of radiation doses in a clinical target has been a longstanding challenge in the practice of radiation oncology. Recent technological advances have helped in estimating dose distribution in tumors with great accuracy and precision. The appropriate choice of imaging modality is of utmost importance, since resolution and contrast of images can greatly alter the assessment of treatment outcomes and the ability to accurately monitor the progress of radiotherapy. There are two imaging modalities which are currently available for use in IGRT: Ultrasound (US) and Computerized Tomography (CT). Ultrasound is a technology which provides soft-tissue contrast at low cost with no known side effects, in comparison to more expensive modalities such as Computerized Tomography. However, it suffers from the limitation of required acoustic coupling, which necessitates physical contact between the probe and the patient's external contour (Djemil 2007). CT-based scanners help in imaging and helical delivery of Intensity Modulated Radiation Therapy (IMRT). Image-guidance in this technique is achieved by employing low energy x-ray and a CT-detector. The imaging capacity of this

technology is highly integrated with automatic alignment, and has the capacity to correct for rotations about the superior-inferior axis of the patient (Mackie 1999; Welsh 2002).

A major drawback of IGRT is the use of ionizing radiation and potential high cost of some of the technologies. This has caused researchers to investigate other forms of image-guided therapy which do not have these shortcomings.

1.2.2.4.2 Fluorescence- guided therapy:

Optical imaging is a technology which uses light produced by biological or chemical moieties, and is gaining utmost importance in biomedical applications because it employs non-invasive and non-ionizing radiation (Balas 2009). Fluorescence imaging is the most widely used form of optical imaging, which is described in the subsequent section with respect to its application in guiding surgeries.

The phenomenon of fluorescence was first described by British scientist Sir George G. Stokes, who coined the term after observing fluorescence from the mineral “fluorspar”. Fluorescence is characterized by the emission wavelength being longer than the excitation (absorbance) wavelength, also known as Stokes shift. It could be argued that fluorescence imaging has now emerged to be the most important phenomenon in modern biology and medicine. Specific fluorescence staining boosted its application in early last century, and fluorescence microscopy raised its prominence further with the invention of immunofluorescent staining and functional imaging. The possibility to create living

material with targeted expressible fluorescent proteins revolutionized fluorescence microscopy to the next level.

In vivo and *in vitro* fluorescence imaging is similar in several aspects, such as the use of a low-light camera and appropriate filters to collect fluorescence from samples. However, *in vivo* fluorescence imaging is more complex than *in vitro* fluorescence imaging. Practical concerns during *in vivo* imaging involve strong absorption and scattering effects in animal tissues which obscure signal collection and quantification. The biologically complex *in vivo* environment presents additional challenges in terms of stability of fluorescent probes and their selective accumulation in target tissues (Rao 2007). Fluorescence-guided surgery is currently under investigation in clinical trials. It has been proved that 5-aminolevulinic acid (5-ALA) induces localization of fluorescent porphyrins in glioblastoma, which can be exploited for potential tumor resection (Stummer 2000; Stummer 2006). Clinical trials have been conducted to analyze the effects of fluorescence-guided resection on postoperative magnetic resonance (MR) imaging and survival in patients undergoing surgery (Stummer 2000; Stummer 2006). Additional examples include the application of fluorescently labelled protease-sensitive probes to improve tumor detection in animal models (Sheth 2009), and the use of activatable cell-penetrating peptides responsive to tumor-associated matrix metalloproteinases (MMP) which have been shown to improve tumor resection in breast tumor and melanoma-based animal models (Jiang 2004).

Fluorescence can be measured if fluorophores are expressed by specific modified genes or tagged onto specific molecules and/or biomolecules. This technique, however, has certain limitations such as photobleaching in long-term studies, as well as the potential for disadvantageous modifications in the properties of the tagged molecule of interest.

1.2.2.4.3 Raman Spectroscopy based image – guided therapy:

Raman spectroscopy is a non-invasive, label-free technique which is a feature of vibrations of molecular bonds. Monochromatic light interaction with materials results in two types of scattering: elastic and inelastic. When the energy of the scattered photons is different from that of the incident photon, this is known as inelastic or Raman scattering, and Raman spectroscopy is the method of measuring and analyzing these scattered photons. Fourier Transform Infrared Spectroscopy employs a similar technique for molecular characterization. However, it is dependent on low power sources and noisy detectors. Infrared spectroscopy suffers from low penetration in aqueous solution, making it unsuitable for biological specimens. Raman spectroscopy can employ lasers with wavelength in the near-infrared region, which facilitates *in vivo* imaging. The sub-cellular resolution achieved through this technique is comparable to fluorescence imaging and far superior to several medical diagnostic techniques, such as ultrasound, magnetic resonance imaging, Positron Emission Tomography, or X-ray imaging. The light emitted is red-shifted to lower wavelengths and can be recorded by a charge-coupled device (Downes 2010).

Near-infrared Raman Spectroscopy is a powerful analytical tool used in detecting important differences between biological samples without interference from autofluorescence. It has been suggested as a tool for cancer screening (Mahadevan-Jansen 1998; Huang 2003). Raman spectroscopy in the near-infrared range is also a very useful technique in providing quantitative diagnostic information about the tissue under study. Progression of a disease is accompanied by chemical changes in the tissue associated with it, making Raman spectroscopy a useful tool to diagnose disease progression. Raman spectroscopy can be performed *in vivo* using optical fibers incorporated in catheters, endoscopes and cannulas (Hanlon 2000). Raman signals generated from samples are often very weak due to strong background emission due to fluorescence, which would make them completely undetectable (Kim 2010). However, statistical algorithms can be employed to distinguish the spectra of different samples.

Raman spectroscopy has been used as an *in vivo* image-guiding tool to assess spectral features of nasopharyngeal and laryngeal tissue during clinical endoscopic examination. A 785-nm Raman endoscopy system along with a miniaturized Raman probe was used for real time Raman measurements of different anatomical regions of the head and neck. Raman spectral differences between different components of the nasopharynx and larynx were used as important parameters for interpretation, and utilized to analyze decision algorithms for *in vivo* tissue diagnosis and characterization (Bergholt 2012). Raman spectroscopy with a diagnostic detection algorithm has also been used for nasopharyngeal carcinoma diagnosis and detection *in vivo* (Vankayala 2013).

Surface-Enhanced Raman Scattering (SERS) is a method that can be used to increase Raman signals without employing complex algorithms. This technique uses metal nanoparticles to enhance the Raman signal. A massive enhancement of the electromagnetic field around the nanoparticle surface occurs based on size and geometry, causing an enhancement up to the order of 10^{14} recorded till date (Huh 2009).

SERS is a newly emerging field for biomolecule detection (Li 2004; Han 2009). It is based on the principle of interaction of electromagnetic waves with a metal surface. When metal surfaces are rough or occur at junctures, the wave may excite the surface plasmons on the surface causing amplification of electromagnetic fields near the metal surface. This causes a large enhancement of the Raman scattering. Chemi-adsorbed molecules exhibit chemical SERS enhancement in their Raman signal (Moskovits 1985).

SERS technology exhibits several positive attributes, such as high sensitivity and multiplexing capabilities that help in *in vitro* cancer detection. The most common approach involves immunoassays for recognition of cancer cell-surface biomarkers with their complimentary antibody-conjugated SERS substrates. This technique overcomes the limitations of currently used immunoassays, such as extended incubation time and the need for repeated washings (Chon 2009). SERS was initially investigated as a non-invasive modality for *in vivo* cancer imaging using colloidal gold nanoparticles with organic dye based Raman reporters in mice (Qian 2008). Single-walled carbon nanotubes selectively targeting integrin-positive U87MG tumors can be imaged *in vivo* in mice using SERS (Keren 2008). Gold nanoparticles with selective NIR SERS reporters can

selectively image HER-2 positive tumors *in vivo* (Maiti 2012). Current research approaches are intended to exploit the multiplexing capabilities of SERS nanotags to track tumors *in vivo* (Zavaleta 2009).

1.2.2.5. Nanotechnology based methods:

Drug delivery research focuses on the development of targeted delivery of chemotherapeutics and other cancer therapeutic drugs. An ideal drug delivery vehicle is the one which entraps high drug payload and is able to deliver it specifically at the tumor site with minimal accumulation in normal cells. Nanotechnology is a fast-developing field with the potential to converge detection, analysis and treatment of cancer in a simultaneous manner (Srinivas 2002; Ferrari 2005). Theranostics is a term that refers to the combination of diagnostics and therapeutics on a single platform (Fernandez-Fernandez 2011; McGoron 2014). Drug entrapped delivery vehicles have been found to overcome multi-drug resistance in several cancers (Soma 2000). The drugs which are substrates of drug efflux proteins enter the cells entrapped, hence evade exposure to these proteins (Szakács 2006). Several nanoparticles entrapping chemotherapeutic agents have been found to possess the potential to avoid resistance in drug resistant cells, thus increasing intracellular accumulation of the drug and hence inducing a higher toxic effect (Bennis 1994). Nanoparticles with size 100-200 nm possess the ability for controlled drug release, longer plasma residence, and exit leaky tumor vasculature to be taken up by tumor cells (Yuan 1995). The gaps in endothelial lining in tumor capillaries range from 100-600 nm. Thus nanoparticles in this range are suitable to penetrate through tumor capillary linings and avoid uptake by macrophages of the reticulo-endothelial system

(RES) (Cho 2008). Targeted nanoparticles have also been prepared by modifying the surface of the particles with antibodies so that they can selectively target tumor cells over expressing the corresponding receptor (Lei 2011). However, these nanoparticles have much less diffusion into solid tumors with high packing density due to their low diffusivity (Kostarelos 2004).

The drug delivery vehicles used so far for cancer applications are often composed of natural and synthetic polymers in the form of nanoparticles, metal and semiconductor nanoparticles (Huff 2007; Sarah 2010), micelles (Iyer 2007; Gou 2011), dendrimers (Majoros 2005; Peer 2007; Yellepeddi 2009) and liposomes (Takeuchi 2003; McGoron 2014). Polymeric nanoparticles are widely used due to their several favorable characteristics such as biocompatibility, biodegradability, reduced toxicity and availability of functional groups for further surface modification (Pridgen 2007; Bhattarai 2010; Rejinold 2011). Polymeric nanoparticles are prepared through different methods which can be broadly classified into two classes; first, by polymerizing monomers and further emulsifying the polymers at an interface between aqueous/ organic phase; second, based on emulsifying pre- formed polymers (Ganapathy 2009). Nanoparticles that are formed by pre-formed polymers can be prepared using different techniques such as salting- out, double emulsion preparation, emulsification diffusion and emulsification - solvent evaporation methods (Allémann 1992; Desgouilles 2003; Cohen-Sela 2009). The ideal method of preparation of polymeric nanoparticles depends on the hydrophobicity/hydrophilicity of the polymer and drugs.

Polyesters are widely used synthetic polymers for delivery of cancer therapeutics; because ester bonds are highly acid-labile. This property of these polymers aids in their higher degradation in acidic vacuoles and hence higher drug release inside the cells after their uptake (Anderson 1997). PLGA, which is an FDA approved polymer for various biomedical applications, is one such polyester polymer (Lü 2009). This polymer has been found to be favorable to carry drugs due to its high biodegradability and biocompatibility. The lactic acid and glycolic acid (the monomers of the polymer, PLGA) ratio can be easily engineered. The biodegradation products of the polymer; lactic acid and glycolic acid; are important components of cell metabolism and hence, nontoxic. PLGA has been used for delivery of drugs/genes and proteins (Ruhe 2003; Bivas-Benita 2004; Klose 2008). This polymer can entrap both hydrophilic and hydrophobic drugs. Hydrophobic drugs such as doxorubicin, paclitaxel etc., and hydrophilic molecules such as genes and proteins have been extensively studied by researchers (Ruhe 2003; Bivas-Benita 2004; Klose 2008; Xu 2008; Lei 2011).

Noble metal nanoparticles, especially gold (Au) and silver (Ag) formulations, exhibit unique and tunable optical properties on account of their surface plasmon resonance (SPR). The strongly enhanced SPR scattering from Au and Ag nanoparticles makes them useful as bright optical tags for molecular-specific biological imaging and detection using simple dark-field optical microscopy. The SPR absorption of the nanoparticles has allowed their use in selective laser phototherapy of cancer. SPR frequencies are sensitive to the dielectric constants of local medium and can be used for sensing of chemical and biological analytes (Jain 2007).

Nanoparticles can help deliver CT, MRI, nuclear, and optical imaging agents (Lee 2008; Kang 2010). Multifunctional nanoparticles further allow for simultaneous *in vivo* delivery of diagnostic (or imaging) and therapeutic agents (Kim 2010). The intrinsic properties of nanoparticles can be exploited in some cases for imaging and/or hyperthermia or photodynamic therapy (Fernandez-Fernandez 2014; Lei 2014; Mfouo-Tynga 2014; Lei 2014). This helps in generating integrated theranostics systems which allow for image-guided therapy. For example, Au nanoparticles have been simultaneously used for dark field microscopy, photoacoustic imaging, and hyperthermia applications (Huang 2006; Eghtedari 2007).

1.2.2.6 Multifunctional nanoparticles based approaches:

Multifunctional nanoparticles serve as a unique platform for simultaneous entrapment of an imaging molecule and therapeutic molecule, thus creating a new phase of developing theranostic agents (McGoron 2014). Thus, these multifunctional nanoparticles can allow for improved diagnostics and therapeutics. Further, surface engineering of these nanoparticles with antibodies can also achieve active targeting. Traditional *in vivo* imaging probes include Positron Emission Tomography (PET) (Phelps 2000), Single Photon Emission Computed Tomography (SPECT) (Chatziioannou 2005) and Magnetic Resonance Imaging (MRI) (Abraham 1996). Recently developed nano-sized imaging probes such as quantum dots and near infra- red dyes are gaining popularity as there is no exposure of ionizing radiation (Joshi 2010). Optical imaging systems based on light have higher temporal resolution and sensitivity and are less expensive in comparison to

traditional techniques (Fujimoto 1999). However, visible light is absorbed and scattered by tissue to a much greater extent than is ionizing radiation (Simpson 1998). Imaging probes based on quantum dots are often found to be toxic *in vitro* and *in vivo*, depending on their characteristics such as size, charge etc., (Hardman 2006). Therefore, near infra-red (NIR) light is preferred as they evade absorption by biological chromophores and hence have higher tissue penetration and many are non-toxic to biological tissues (Lammertyn 2000). Thus, nanoparticles entrapping NIR dyes can be easily used for *in vivo* imaging, tailored for surface modification and easily extravasate into tumors through their leaky vasculature (Yuan 1995). Some of the dyes belonging to the class of NIR dyes can also be used for phototherapy due to their unique heat generation properties (Genina 2003). Tricarbocyanine dyes such as pthalocyanine, is one such class of dyes which possess both imaging and heat generation capabilities (Nelson 1988). Indocyanine Green (ICG), which is FDA approved for several clinical applications, is another NIR dye belonging to the same class (Dzurinko 2004; Sakka 2007). This dye has photodynamic, photothermal and imaging capabilities (Chen 1995; Bäumlér 1999; Kitai 2005). Cronaine dyes are another class of NIR dyes which have photothermal capabilities and can be utilized for *in vivo* imaging. (Spence 2013,). Therefore, nanoparticles entrapping these dyes which have simultaneous imaging and therapeutic properties serve as ideal agents for cancer theranostics. Nanoparticles can also serve as a common platform wherein such theranostic NIR dyes can be simultaneously entrapped with other therapeutic agents such as chemotherapeutics to synergistically control tumor cell growth (Tang 2010; Srinivasan 2014; Manchanda 2010).

Targeted photodynamic therapy based on multifunctional nanoparticle platforms is gathering increased interest for *in vitro* and *in vivo* applications. These nano-platforms exhibit high surface to volume ratios which help encapsulation and/or coupling of several photoactivatable units for PDT, and also allow for tumor sequestration via passive and active targeting. Further, contrast agents can also be incorporated into these structures (Roy 2003).

1.2.2.6.1. PLGA nanoparticles based multifunctional approaches:

PLGA (Poly-L-lactide-co-glycolide) is an FDA approved copolymer, widely used for drug and gene delivery (Bala 2004). Several multifunctional approaches to converge different treatment therapies have been investigated by researchers. PLGA nanoparticles can be functionalized with Polyethylene glycol (PEG), which provides more stealth, wherein the circulation time of nanoparticles can be enhanced. These stealth nanoparticles can also be entrapped or conjugated with anticancer agents and further surface functionalized with ligands corresponding to the tumor associated antigens for active tumor targeting (Song 2005). Multifunctional PLGA nanoparticles incorporating anti-cancer agents along with other treatment agents aid in synergistic anticancer therapy. For example, Wang and coworkers simultaneously entrapped Doxorubicin and Paclitaxel into PLGA nanoparticles for enhanced anti-tumor efficacy (Wang 2011). PLGA nanoparticles have also been used for the co-delivery of drugs such as Vincristine and Quercetin, Indocyanine Green and Doxorubicin etc., in order to achieve synergistic cancer therapy (Song 2008; Tang 2010; Srinivasan 2014; Manchanda 2010). Several antibodies, such as

Human Epithelial Receptor-2, transferrin etc., have been conjugated to this polymer in order to achieve active targeting to tumor. Antibody-polymer conjugates are highly immunogenic based on the molecular weight of the antibody and are prone to reticulo-endothelial system capture (Duncan 2003; Srinivasan 2014).

1.2.2.6.2 Plasmonic- based multifunctional nanoparticles:

Nanoparticles with more than one functionality often prove to be advantageous for several bioapplications. Multifunctional plasmonic nanostructures frequently combine desirable plasmonic properties along with other functionalities, such as aqueous dispersibility and high optical stability, and can be detected through more than one sensing modality. The performance of such nanoparticles can be enhanced by the combination of therapy and diagnostic agents, making them good candidates for *in vivo* theranostic applications and non- or minimally-invasive cancer therapies (Kreibig 1995).

1.2.2.6.2.1. Silver nanoparticles for cancer therapy:

In the past few decades, there has been increasing interest in the use of Silver nanoparticles (AgNPs) in the field of biomedicine and healthcare. These nanoparticles have found their applications in rapid wound healing and as potent antimicrobial agents (Ip 2006; Tian 2007). The potential of these nanoparticles as drug delivery vehicles is also promising, as their large surface area can help deliver large drug doses (Yih 2006). Both AgNPs and AuNPs can be used for cell imaging due to their unique property of SPR

(Paul 2011), and these imaging capabilities can be used to support early diagnosis. Early detection of tumors helps manage and prevent invasion of surrounding tissues and improve subsequent prognosis. AgNP with folate conjugation and p-aminothiophenol reporter could selectively produce SERS signals in folate receptor overexpressing ovarian cancer cells (Boca-Farcau 2014). AgNPs have been used as contrast agents for photoacoustic imaging and image-guided therapy, and can be detected *ex vivo* in pancreatic tissue using combined photoacoustic and ultrasound imaging when exposed to an 808 nm laser (Homan 2010). These nanoparticles hold promise to selectively hone in on tumor cells through the enhanced permeation-retention effect, and may further be selectively targeted to tumor cells through the conjugation of tumor-selective ligands onto the nanoparticle surface (Liu 2007).

Mahmood and coworkers showed that AgNPs, when used in conjunction with chemotherapeutic agents such as etoposide, enhance tumor toxicity in comparison to AgNP or the drug alone. However, the drugs applied along with AgNP could not confer tumor selectivity (Mahmood 2010). Therefore, specific conjugation of tumor selective ligands to such treatment can help bring about selective response in tumor cells.

Angiogenesis is the process of development of new blood capillaries to supply oxygen and nutrients to growing tissue, and it is critical for cancer progression (Carmeliet 2005). Hence, considerable research has been focused towards the development of angiogenesis inhibitors that could reduce the supply of oxygen and nutrients to tumors and further aid in tumor cell reduction. Vascular endothelial growth factor (VEGF) and fibroblast growth

factor -2 are crucial factors in the process of angiogenesis. Thus, targeting therapies to inhibit angiogenesis can aid in preventing cancer metastases (Cross 2001). Gurunathan and coworkers investigated the potential of AgNPs (diameter: 50nm) to inhibit cell proliferation, migration and capillary-like tube formation induced by VEGF in bovine retinal endothelial cells (BRECs) (Gurunathan 2009; Kalishwaralal 2009). The authors further proved that heparin-derivatized polysaccharide-reduced AuNPs and AgNPs exhibited similar FGF-2 induced anti-angiogenesis. The anti-angiogenic property of AgNPs is attributed to inhibition of P13K/Akt signaling (Mukherjee 2005).

Nallathamby and coworkers observed growth inhibition of mouse fibrosarcoma cells by AgNPs in a time and dose-dependent manner. They also observed that these nanoparticles caused segregation of chromosomes and thus assist in inhibiting tumor cell growth (Nallathamby 2010).

AgNPs have also shown the ability to be used in conjunction with several therapies to bring about additive toxic effects. AgNPs, when used along with DOX, have shown deleterious effects on cellular DNA by changing DNA conformation and hence affecting viability in tumor cells (Hekmat 2012). These nanoparticles have also shown radiation sensitizing effects in glioma cells (Xu 2009). AgNPs have also shown extensive *in vivo* applications in tumor models (Sukirthaa 2012; Lara-González 2013; Park 2013), and recent investigations have suggested that AgNPs have the potential for use as a photodynamic agent (Mfouo-Tynga 2014). Thus, AgNps hold great promise in being used as multifunctional drug delivery vehicles.

Many anticancer drugs (in free form or particles form) often target apoptosis (Fulda 2006). Therefore, understanding their mechanisms in this process would help in understanding the response of therapies (alone or in combination) and also help in developing new strategies of therapies.

1.2.3. Role of apoptosis proteins in evaluating treatment efficacy of PTT based treatments:

Apoptosis is a natural cellular process to check uncontrolled cell proliferation. This term was first introduced by Kerr, Wyllie, and Currie in 1972; however the components of the process were discovered long before the coining of this term (Kerr 1972). This process has been widely accepted as a form of programmed cell death wherein, it is genetically determined to eliminate old and worn- out cells. It is a part of the cell homeostatic mechanism for maintaining cell populations in tissues. This is also an important defense mechanism, wherein, cells damaged by any disease or harmful external agents are eliminated (Norbury 2001).

Although there are several external stimuli and conditions that can lead to apoptosis; cells need not be essentially triggered to cell death by the same stimuli. Therefore, at this juncture anti- apoptotic proteins play a major role in evading cell death. Therefore, enhanced expression of anti-apoptotic proteins and weak expression of pro-apoptotic proteins can lead to cancer cell survival (Hoepfner 2001).

The apoptosis process is carried out using three widely accepted mechanisms: extrinsic pathway, intrinsic pathway and the perforin pathway (Elmore 2007). Apoptosis mechanisms are energy- dependent and involve a cascade of molecular events. These pathways converge into cleavage of caspase-3 and further DNA fragmentation, cytoskeletal and nuclear proteins degradation, apoptotic body formation and uptake by phagocytotic cells (Martinvalet 2005).

The extrinsic pathway involves death receptors which are members of tumor necrosis factor receptor (TNF) (Walczak 2000). These receptors transmit death signals from the cell surface to intracellular signaling events. The most widely studied death receptor is TNF- related apoptosis- inducing ligand- receptor 1 and 2 (TRAIL-1 and TRAIL-2). The intrinsic pathway involves outer mitochondrial membrane permeabilization by pro-apoptotic proteins (Green 2004). Disruption of the mitochondrial membrane causes the release of pro-apoptotic proteins which trigger cell death by caspase-3 stimulation (Saelens 2004). The perforin based pathway is induced by cytotoxic T- cells, wherein caspase-10 activation finally stimulates caspase-3 expression (Martinvalet 2005).

Cancer cells often resist these death pathways by an increased expression of anti-apoptotic proteins or by a decrease or defective function in pro-apoptotic proteins, or mutations of genes regulating the mitochondrial pathway (Fulda 2006). Pro-apoptotic proteins (such as caspase-3) thus aid in cell death, and anti-apoptotic proteins (such as HSP70) aid in cell survival. The ratio of expression of both determines the fate of a cell. A defective apoptosis process involves either defective or less expressing pro-apoptotic

proteins and/or increased expression of anti-apoptotic proteins. Since, most of the cancer therapeutics target apoptosis mechanisms; such defects can affect their treatment efficacy. Thus, understanding the expression kinetics of these proteins and the development of sensors to measure their levels in real time can help in the evaluation of the efficacy of treatment modalities associated with this process. Measurement of high levels of HSP70 and low levels of caspase-3 would denote reduced efficacy and on the other hand, reduced expression of HSP70 and increased expression of caspase-3 would denote enhanced efficacy of a treatment modality.

1.2.3.1 Heat shock proteins in PTT treatment:

Heat shock protein 70 (HSP70) is one of the classes of heat shock proteins which are preferentially expressed in tumors. HSP70 levels are elevated in several tumors and their overexpression often denotes a poor prognosis in terms of response to therapy (Cornford 2000). Elevated expression of these proteins denotes spontaneous protection from malignancy and also therapy. Thus, HSP70 expression is a major obstacle in rendering effective treatment (Volloch 1999). For tumor cell survival, correct conformation and function of cellular proteins is necessary. Thus, these HSP70 proteins exert their anti-apoptotic role by inhibiting key check points of apoptotic machinery and help unfold denatured proteins inside the cell on exposure to stress, such as heat or chemotherapeutic agents (Arya 2007).

Anticancer therapy often involves denaturation of proteins. Chemotherapeutic agents have often been shown to have detrimental effects on proteins. Doxorubicin (DOX) commonly exerts its anti-tumor activity by DNA damage. Mandili et al, (2012) described a different mechanism of action of Doxorubicin. It was found to cause: i) dose-dependent over-ubiquitylation of proteins in the absence of proteasome, (ii) deplete activity of ubiquitylated enzymes and (iii) enhance binding of HSP70 with ubiquitinated proteins. Thus, silencing of these proteins can enhance the apoptotic effect of DOX (Mandili 2012). Hyperthermia-based cell killing, where temperatures are greater than 40°C causes protein denaturation (Day 2009). HSP70 is often tailored to be released only under stress and its main signal is the presence of denatured cellular proteins (Voellmy 2004). Cancer cells gain thermotolerance upon prolonged heating at a sub-lethal level. Induction of thermotolerance in pre-heat treatment also confers drug resistance to cancer cells. Thus, an elevated response of HSP70 in cancer cells is associated with resistance to both chemotherapy and hyperthermia, and hence measurements of HSP70 levels can indicate resistance to the combinatorial therapy of chemotherapy and hyperthermia. Therefore, measurement of low levels of expression of this protein implies higher chemotherapy and hyperthermia treatment efficacy.

1.2.3.2 Caspase-3 proteins in PTT treatment:

Caspase-3 (or apopain) belongs to the family of cysteine proteases, and is a major pro-apoptotic protein. Death signals generated through several stimuli converge into a common pathway involving caspase-3 as the primary signaling protein, as described in the previous section 2.3 (Martin 1995). It is produced inside the cells in an inactive form (pro-caspase). This inactive form is further stimulated to active apoptotic proteins by means of a death-inducing stimulus by cancer therapy (Hasegawa 2001). Most of the chemotherapeutic drugs exert their anti-apoptotic activity through the intrinsic pathway leading to caspase-3 expression. Thus, caspase-3 is the major pro-apoptotic signal in cancer cells undergoing chemotherapy (Fulda 2006). Chemotherapeutic agents also cause protein denaturation and DNA damage; therein caspase-3 plays a major role as a protease (Kaufmann 2000). Hyperthermia-based cell killing is also associated with expression of caspase-3 (Hermisson 2000). Thus, enhanced expression of caspase-3 has been associated with an enhanced apoptotic effect in both chemotherapy and hyperthermia. Its expression can be used to evaluate treatment efficacy of the combination of both chemotherapy and hyperthermia. Enhanced expression of caspase-3 would suggest enhanced therapeutic efficacy of chemo-hyperthermia treatment.

1.2.4. Role of intracellular redox environment in evaluating treatment efficacy:

Tumor cells possess an altered redox environment as well as an increased rate of production of reactive oxygen species (ROS) in comparison to normal cells. Redox

signaling and regulation play an important role in cancer therapeutic response, as high levels of ROS generated inside cancer cells can cause cytotoxicity. The oxidative stress in cancer cells is compensated by their increased anti-oxidant capacity. This suggests that new therapeutic strategies exploiting pro-oxidant based techniques could be the foundation for successful antitumor approaches. Thus, anticancer therapies based on increasing ROS levels and on reducing the presence of ROS scavengers may successfully eradicate tumors as described in Figure 1.

Figure 1. The “Achilles Heel” of cancer cells.

a) In both normal and tumor cells, intracellular ROS levels are maintained by creation of a balance between ROS production and elimination, establishing ROS homeostasis. This homeostasis is imperative for normal signaling within cells. In tumor cells, ROS

production and elimination rates are both much higher than those in normal cells. Thus, tumor cells are more dependent on ROS scavenging capacity. b) The dependence of cancer cells on their antioxidant potential can be exploited in anticancer therapies in order to selectively target tumor cells without affecting normal cells. c) Application of antioxidant inhibitors can impair ROS scavenging capacity in cancer cells, resulting in ROS overload which in turn causes a cytotoxic effect. d) Normal cells are also affected by a small overburden of ROS, but do not lose their cellular homeostasis. Cancer cells, on the other hand, can be easily affected even with a small overload of ROS, subsequently losing cellular homeostasis and dying from oxidative stress. (Figure adapted from (Glasauer 2014))

The following two sections will discuss the ways to modulate ROS levels to treat cancer.

1.2.4.1. Role of reactive oxygen species in PDT-based combinatorial therapy:

Chemotherapeutic drugs and ionizing radiation are known to mediate their cytotoxic effect through promotion of intracellular ROS production, thus causing irreversible oxidative damage to cells (Wondrak 2009). Drugs such as taxol and several anti-folates promote mitochondrial-based cell death by disrupting their electron transport capacity, thereby increasing intracellular ROS production (Kaufmann 2000). Other drugs such as cisplatin and DOX also mediate their anti-tumorigenic activity through elevated production of ROS (Conklin 2004). ROS-producing monoclonal antibodies such as rituximab have also been synthesized for targeted treatment of non-Hodgkin's lymphoma

(Renschler 2004). Elesclomol (STA-4783) is a ROS-producing compound in clinical trials for treatment of malignant melanoma cells. This formulation has been shown to increase tumor progression-free survival when applied as a single agent, and significantly increased antitumor effect upon administration with paclitaxel. Its mechanism of action is still under investigation, although treatment effectiveness was reduced several fold when antioxidants were administered simultaneously (Kirshner 2008). The most common problem encountered with these drugs is their selectivity to tumors. However, a more targeted ROS-producing drug can effectively induce tumor cell apoptosis. Several researchers have suggested that AgNPs and silver nanocomposites affect cellular viability through production of ROS (Arora 2008; Hsin 2008).

1.2.4.2. Role of anti-oxidants in PDT based combinatorial therapy:

A major drawback of anticancer therapies associated with elevated production of ROS is the development of resistance to elevated ROS. Tumor cells produce endogenously elevated levels of anti-oxidants that help detoxify and scavenge the ROS produced (Lenehan 1995.). Oncogene-induced tumors generate elevated levels of antioxidant need to define proteins such as NRF2 (Taguchi 2011). Glutathione (GSH) is a major antioxidant produced in elevated levels in tumor cells. GSH protects tumor cells from cell death and also prevents apoptosis in ROS-inducing therapies such as chemotherapies (Tai 2012). Thus, disabling the anti-oxidant potential of tumor cells triggers ROS-mediated cell death in numerous tumor cell types (Trachootham 2006; Glasauer 2014).

Phenethyl isothiocyanate (PEITC) interacts with intracellular GSH through electrophile-nucleophile interactions, depleting the GSH pool and leading to oxidative stress-mediated cell death. PEITC can cause cell death in HRAS(V12) transformed ovarian cancer cells and also prolong survival in an ovarian cancer xenograft model (Trachootham 2006). L-Bethionine-sulfoximine (BSO) depletes intracellular GSH and has also shown synergistic anticancer effects with cisplatin in preclinical studies (Bailey 1997; Engel 2006). AgNPs have been shown to cause their anti-cancer effect through large reductions in intracellular thiol concentration, especially GSH (Nguyen 2013). The plant-derived compound Piperlongumine (PL) increases intracellular ROS by modifying the expression of the antioxidant enzyme glutathione transferase, thus modifying the ROS-stress response. This compound exerts an anticancer effect in breast, bladder and lung cancer models and their respective mouse models (Raj 2011). Thioredoxin is another intracellular thiol-based antioxidant which is overexpressed in many cancers. Several inhibitors of thioredoxin have been created and investigated to show anti-tumor activity *in vivo*, such as PX-12 (Welsh 2003), Motexafin gadolinium (Magda 2006), etc. The antioxidant regulator gene NRF2 has been widely investigated as a potential target for anticancer therapy. NRF2 inhibition can reduce the antioxidant system and induce ROS-mediated cell death in tumor cells (Sato 2013).

The success of combined therapies depends on a thorough understanding of the mechanisms behind cellular responses to cytotoxic agents. The goal of my study is to generate two different multifunctional nanoparticles to explore their cellular responses and how they are mediated by agent-specific mechanisms. One type of nanoparticle

requires the simultaneous release of two drugs, and the effect of the nanoparticles on cellular systems upon exposure to two heat treatment modalities would be explained by apoptotic and anti-apoptotic protein expression kinetics. In the other type of nanoparticle, the nanoparticle itself is a drug molecule which would act via reactive oxygen species generation and intracellular thiol reduction. In the latter type of formulation, one of the therapeutic agents is actually the framework for the nanoparticle construct. Thus, once the particles reach a target site, the cytotoxic agent can be immediately activated, and the particle can release its cargo.

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CHAPTER 2: STATEMENT OF PURPOSE AND SPECIFIC AIMS

2.1. Statement of Purpose:

The overarching goal of this dissertation was to prepare multifunctional nanoparticles for combined theranostics, as well as to understand the different mechanisms and effects that derive from utilization of different multifunctional designs. We also evaluated drug delivery strategies with different co-therapy types, by designing two nanoparticle formulations that both contained DOX as a chemotherapy agent, but varied in the form of co-therapy utilized. In one formulation, we design a nanoparticle system for combined chemotherapy and photothermal therapy, whereas in another formulation we used a nanoparticle system for combined chemotherapy and photodynamic therapy.

For combined chemotherapy-photothermal therapy, we performed simultaneous incorporation of a near-infrared activatable agent (i.e. an agent that can be activated using NIR light for hyperthermia or photodynamic activity) and a chemotherapeutic agent into polymeric PLGA nanoparticles for combined chemo-laser based phototherapy. We chose ICG and DOX as the near-infrared agent and DOX as the chemotherapeutic drug. Further, we studied the cellular response to these nanoparticles upon exposure to two types of heat treatment: laser-ICG-based therapy and incubator hyperthermia. These two modes of hyperthermia emulate clinical localized and whole-body hyperthermia respectively. Photothermal therapy affects cellular proteins by disrupting their folding and/ or denaturing them. One of the major limitations of this therapy is the possible induction of thermal tolerance due to overexpression of heat shock protein 70, an anti-apoptotic protein widely implicated in cellular resistance to treatment. Based on the

severity of the thermal insult, however, cells may later exhibit downregulation of heat shock protein 70, ultimately resulting in apoptosis or necrosis, depending on the degree of cellular damage. The cellular response of these nanoparticles was hence studied using HSP70 and Caspase-3 expression kinetics because the co-therapy is based on photothermal therapy. One of the limitations of these multifunctional nanoparticles is the need to exert careful control over the release of both agents in order to obtain the desired therapeutic effect.

For combined chemotherapy-photodynamic therapy, we created multifunctional nanoparticles where the nanoparticle itself acts as a near-infrared light-activatable therapeutic agent (multifunctional AgNPs). This multifunctional nanoparticle system provides an advantageous design as the nanoparticle itself serves as a drug delivery vehicle and phototherapeutic agent. Photodynamic therapy affects the redox state of the cells. Thus, the application of photodynamic therapy or co-therapy involving photodynamic therapy will result in enhanced generation of reactive oxygen species along with reduction of the antioxidant potential of the cell. Therefore, we studied the cellular response to these multifunctional silver nanoparticles based on intracellular generation of reactive oxygen species and reduction of thiol and glutathione levels, as thiol and glutathione are part of the cell's antioxidant response system.

2.2. Specific Aims:

The specific aims corresponding to this research were:

Specific Aim#1:

- a) Prepare and characterize polymeric polyester nanoparticles (i.e., Poly-lactide-co-glycolide or PLGA) with simultaneous incorporation of indocyanine green and doxorubicin (designated as IDNP) and surface decorate with an antibody against human-epithelial receptor-2 (HER-2) (designated as AIDNP). PLGA was chosen because it is an FDA-approved copolymer widely used for drug delivery applications, whose properties can be easily engineered to simultaneously entrap both hydrophilic (ICG) and hydrophobic (DOX) agents. The surface properties of the polymer can also be easily modified to attach ligands for cell-specific receptors to achieve active targeting.
- b) Evaluate the *in vitro* cytotoxic effect, cellular uptake, and drug release kinetics of the IDNPs and AIDNPs.

Specific Aim #2:

Evaluate HSP70 and caspase-3 expression from cell lysates after exposure to incubator hyperthermia and DOX, free DOX+ICG hyperthermia, IDNP hyperthermia and AIDNP hyperthermia, using ELISA based measurements to evaluate time expression of HSP70 in cancer cells MES-SA, MES-SA/DX5 and SKOV-3.

Specific Aim# 3:

- a) Prepare and characterize AgNPs which are decorated with the folic acid (FA) targeting ligand via an amide bond, a chemotherapeutic drug doxorubicin (DOX) is conjugated to AgNPs via an acid-cleavable hydrazone bond and a polyethylene glycol (PEG) spacer.
- b) Evaluate pH-triggered release of the drug (DOX) by Surface-Enhanced Raman Spectroscopy and Fluorescence Microscopy.

- c) Evaluate the ability of AgNPs to act as a photodynamic agent upon laser irradiation *in vitro*.
- d) Evaluate the uptake and cytotoxicity of this silver nanoparticle carrier system in cancer cells (MES-SA, MES-SA/DX5 and SKOV-3) to show the combined chemotherapeutic-photodynamic effect.
- e) Evaluate cellular response to these nanoparticles by measuring intracellular generation of reactive oxygen species (ROS) and reduction of thiol and glutathione.

CHAPTER 3: COMBINED PHOTOTHERMAL THERAPY AND CHEMOTHERAPY IN CANCER USING HER-2 TARGETED PLGA NANOPARTICLES – AN *IN VITRO* STUDY

This chapter is based on my work completed and published in the article: Srinivasan, S.; Manchanda, R.; Lei, T. J.; Nagesetti, A.; Fernandez-Fernandez, A.; McGoron, A. J. Targeted nanoparticles for simultaneous delivery of chemotherapeutic and hyperthermia agents-An *in vitro* study. J. Photochem. Photobiol. B, 2014, 136, 81–90. Permission has been obtained from the journal editors to use the information and data from this article in my dissertation manuscript, and a copy of this permission has been included in the appendices.

3.1. Background

Cancer is one of the primary causes of death in the developed world, second only to cardiovascular disease. The main challenges in battling cancer include both the capability to detect it as early as possible, and the ability to treat it in a specific and effective way. As a result, the centerpiece of current cancer research involves the development of improved detection and treatment strategies that can better equip the medical team in dealing with the disease. Current cancer therapies include chemotherapy, radiotherapy, immunotherapy, and hormone therapy, among others. A common management approach that results in improved clinical outcomes is combinational therapy, i.e., the co-administration of several anticancer agents or modalities. In this case, two or more therapeutic agents or different therapies such as chemotherapy, hormone therapy,

immunotherapy, and radiotherapy, are simultaneously used in order to try to maximize the treatment effect on cancer tissue. The use of combinational therapy also helps decrease off-target and dose-dependent toxicities, as it allows a reduction in the overall drug dose given to the patient (Liotta L 2000). This is also an important consideration in achieving optimal clinical outcomes.

Doxorubicin (DOX), also known as Adriamycin, is a common anticancer drug which has been used for decades based on its effectiveness against a wide spectrum of neoplasms. DOX exerts its cytotoxic action through two mechanisms: (1) generation of free radicals and (2) DNA intercalation in the nucleus, with the latter being the most predominant mechanism of cytotoxicity in tumor cells. Despite its effectiveness in reducing the viability of cancer cells, DOX is clinically limited by its lack of tumor specificity, dose-dependent cardiotoxicity, and the development of multidrug resistance which hampers the long-term efficacy of the treatment (Sinha 1990).

A possible approach to overcoming the dose-related limitations of chemotherapy agents is to use combinational therapies such as simultaneous hyperthermia and chemotherapy in order to obtain more efficacious treatment of cancer with reduced doses of chemotherapy. Cancer cells can be selectively killed by hyperthermia in a range of temperatures that are relatively innocuous to healthy cells (41- 43 °C) (Cavaliere R 1967). Additionally, hyperthermia can result in increased membrane permeability which can in turn create increased sensitivity of cancer cells to other cytotoxic agents (Christophi C 1998). Hyperthermia mediated by near-infrared absorbing agents and triggered by laser

exposure also has the advantage of providing non-invasive treatment with greater tissue penetration of light compared to visible light (Ntziachristos V 2003). Indocyanine Green (ICG) is a near-infrared absorbing dye widely used in clinical applications such as cardiac output measurement and ophthalmic angiography (Benya R 1989). It can also be used as an agent for localized hyperthermia treatment (Chen 1996; Lucroy 2002). However, the application of ICG for in vivo hyperthermia is limited by its rapid plasma clearance and rapid degradation in an aqueous environment (Saxena 2003).

Some of the concerns that arise from the non-specificity of cancer therapeutics can be reduced through the use of nanoformulations. Nanomedicine is a relatively new, yet rapidly progressing area in the field of cancer therapy, which offers great potential in combining multiple therapeutic modalities and functionalities into a single platform. Combinational cancer therapy using nanoformulations of inorganic materials such as gold or iron, carbon nanotubes, and polymers is being increasingly explored (Fernandez-Fernandez 2011). In drug delivery applications, degradable polymeric biomaterials (both synthetic and natural) are preferred because their biodegradation can be easily manipulated for efficient and controlled drug release (Nair LS and Laurencin CT 2007). Poly lactide co-glycolide (PLGA) is a biocompatible polymer approved by the Food and Drug Administration for use as a drug delivery vehicle in humans. One of the advantages of PLGA in drug delivery is the fact that its degradation products, lactic acid and glycolic acid, are easily metabolized by the body. This, along with the ability to manipulate the copolymer ratio and polymer weight for controlled drug release, makes PLGA the most widely investigated polymer for drug delivery (Danhier F 2012).

Nanoparticles offer the possibility of increasing the specificity of drug delivery through both passive and active targeting. Because tumor tissues have leaky vasculature with pore sizes ranging anywhere from 100-780 nm, drug-loaded nanoparticles tend to accumulate preferentially in tumor tissues as compared to healthy tissues, although the degree of passive targeting can vary widely because of differences in tumor vasculature and the variability of its porosity depending on tumor type and staging (Hobbs 1998; Brigger 2002). Active targeting can overcome this concern by specifically directing nanoparticles for uptake into tissues which express the corresponding receptors, based on the decoration of nanoparticle surfaces with tumor-selective ligands. Many different ligands have been reported in targeted delivery applications, including monoclonal antibodies, peptides, and carbohydrates. Monoclonal antibodies in particular have excellent tumor targeting capabilities due to their high specificity to tumor markers. Several studies have used HER-2 (Human Epithelial Receptor-2) for targeting, as overexpression of HER-2 on tumor surfaces has often been correlated with poorer prognostic indicators including angiogenesis, metastasis and resistance against apoptosis-inducing therapeutic agents (Nair 2005). HER-2 is over expressed in 20-25% of ovarian cancers (Vermeij J 2008). Targeted therapy is achieved using receptors that have high copy numbers on cancer cells with respect to normal cells (10^5 - 10^6 receptors/cancer cell which is 100 fold higher than that in normal cell) (Kurebayashi J 2001).

Our group previously reported the simultaneous entrapment of ICG and DOX into PLGA nanoparticles for combined imaging and therapy (Tang 2010). This chapter

discusses further development of these agents to incorporate active targeting by surface conjugation with monoclonal antibodies against HER-2; and the potential of this targeted formulation for combined chemotherapy, hyperthermia and imaging in cancer cells with different drug resistance mechanisms.

3.2. Methods

3.2.1. Drugs and Chemicals:

Poly (DL-lactide-co-glycolide) (PLGA, L: G: 50:50; MW: 40,000–75,000; T_g: 45°C–50°C), doxorubicin hydrochloride (MW: 579.95), dimethylsulfoxide (DMSO>99.9% reagent grade), and polyvinyl alcohol (PVA, 87–89% hydrolyzed; 13KDa-23KDa) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Indocyanine Green was purchased from Acros Organics. Dichloromethane (DCM) was purchased from Burdick & Jackson (Muskegon, MI, USA).

3.2.2. Synthesis of IDNPs:

PLGA nanoparticles with dual incorporation of ICG and DOX (IDNP) were prepared using an oil-in-water emulsion solvent evaporation technique (Manchanda 2009). The protocol was previously optimized for maximum drug entrapment. Briefly, 60 mg of PLGA, 1 mg of ICG, and 1 mg of DOX were dissolved in 4 ml of methanol-dichloromethane (1:3 v/v) mixture. The organic phase was emulsified with 8 ml of PVA solution (3%, w/v) by sonication at 50 W for 1 min in an ice bath. The organic solvent was then removed under reduced pressure at 39 °C. The nanoparticle suspension was

centrifuged at 14,000 rpm (16,000 xg) for 30 min. The precipitate was further washed with equal volumes of distilled water and centrifuged again. The nanoparticle precipitate was freeze-dried and lyophilized.

3.2.3. Antibody conjugation to IDNPs to prepare AIDNPs:

The IDNPs were conjugated with anti-HER-2 using a modified protocol reported by Yang et al (Yang 2007). Briefly, the lyophilized IDNPs were resuspended in PBS (0.1 M, pH 7.4, 1 mg/mL). 1 mL of 2.0 mM NHS and 2.0 mM of EDC was added to the NPs and left to react for 20 min. Then, the activated NPs were reacted with 50 μ L (100 μ g) of IgG antibody for 1 hr. The NPs (AIDNP) were recovered by centrifugation at 12,200 rpm (13943 xg), 6°C for 30 minutes. The pellets were washed and resuspended in PBS (0.1 M, pH 7.4, equal volume of supernatant). The particles were then freeze-dried and lyophilized.

3.2.4. Characterization of NPs:

The size and size distribution of IDNPs and AIDNPs were measured by dynamic light scattering (DLS) with a Malvern Zetasizer (Malvern Instruments, Worcestershire, United Kingdom) at 25°C, using a 1:30 (v/v) dilution of the NP suspension in distilled water. The same instrument was used to measure the zeta potential of the NPs dispersed in deionized (DI) water.

3.2.5. Dye loading into NPs:

The concentrations of ICG and DOX encapsulated in the NPs (both IDNPs and AIDNPs) were determined using a Fluorolog-3 spectrofluorometer (HORIBA Jobin Yvon Inc., USA) in steady-state mode. Drug entrapment was measured after dissolving the NPs in DMSO, as previously reported by our group (Manchanda 2009). Briefly, serial dilutions of the supernatant were done in order to reach the linear range. Then, the maximum peak intensities of the supernatant solution were blank corrected and used to fit to a linear model for drug loading estimation.

3.2.6. Antibody conjugation efficiency estimation:

The amount of antibody conjugated per nanoparticle (antibody conjugation efficiency) was measured using a modified protocol reported by Mo and Lim (Mo 2005). 1 mg of lyophilized particles (AIDNP) was dissolved in 0.5 ml DMSO and 1 mL of 0.5% (wt./vol.) Sodium dodecyl sulfate (SDS)/0.05M NaOH (salinated SDS) and incubated for 4 hrs. One mL of this solution was mixed with an equal volume of Bicinchonic acid (BCA) reagent for 60 min at 60 °C. The absorbance of this mixture was estimated at 562 nm using a spectrophotometer. The instrument was calibrated with standard bovine serum albumin protein solutions (0.5-40 µg/ml) in the same proportion of DMSO and salinated SDS solution. IDNPs were used as blank. Conjugation efficiency was expressed as weight of antibody (µg) per unit weight (mg) of nanoparticles.

3.2.7. *In vitro* studies of NPs:

Human uterine sarcoma MES-SA cells, and their drug resistant counterpart (P-gp over-expressing derivative) MES-SA/Dx5 (Dx5) cells, human ovarian carcinoma SKOV-3 cancer cells, McCoy's 5A medium, and fetal bovine serum were purchased from American Type Culture Collection (Manassas, VA). Formalin, 24-well tissue culture plates and Poly-D-lysine coated coverslips were purchased from Fisher Scientific (Pittsburg, PA). Penicillin was purchased from Sigma-Aldrich. All cells were cultured in McCoy's 5A medium supplemented with 1% penicillin and 10% fetal bovine serum, and kept in a 37°C cell incubator with a humidified atmosphere of 5% CO₂ and 95% air.

3.2.8. *In vitro* drug release kinetics profile:

AIDNP (5 mg) was resuspended in 3 ml of 0.01M PBS (pH = 7.4). This sample was divided into three centrifuge tubes. The tubes were shaken at 35 rpm, 37 °C in an incubator. These tubes were removed from the incubator at regular intervals and centrifuged at 14,000 rpm (16,000 xg), 30 min. The supernatants were collected for DOX estimation and the pellet was resuspended with fresh buffer. This process was repeated for each time point up to 48 hours.

3.2.9. Subcellular localization of NPs:

MES-SA, Dx5 and SKOV-3 cells (4×10^4) cells/well were seeded onto Poly D, L-lysine coated coverslips placed into 24-well culture plates, and the cells were incubated to reach confluence. After 24 hours incubation, the medium was replaced by medium

containing IDNP or AIDNP corresponding to equivalent ICG and DOX concentrations of 6.2 μ M and 10 μ M respectively. After 24 h incubation, the cells were washed 3X with DPBS to remove free drugs (IDNP or AIDNP) and then fixed with 4% paraformaldehyde solution for 20 min. The cells were further washed 3X with DPBS. The fixed cells were viewed under fluorescence microscope (Olympus IX81, Japan) and imaged under a water-emersion 60x objective at λ_{ex} (480–490 nm), λ_{em} ($\geq$ 515 nm) for the DOX window, and λ_{ex} (775 nm), λ_{em} (845 nm) for the ICG window. The fluorescence signals were acquired using a CCD camera and merged using software to give a pseudo-color (IPLab, Qimaging, Canada). The settings of the microscope were kept constant throughout the experiment; however, different exposure times were used for the ICG and DOX windows to optimize the images. The fluorescence comparisons between IDNP and AIDNP were done within the same cell lines.

3.2.10. Cellular uptake experiments:

The cellular uptake of AIDNP and IDNP was quantified as follows. First, 2×10^4 cells were seeded in a 24 well tissue culture plate, and after the cells reached confluency, the cell medium was removed and IDNP and AIDNP resuspended in growth medium (with equivalent DOX and ICG concentrations as described in the previous section) for 24 hrs. Cells that were not subjected to any treatment were considered controls. After 24-hour incubation, the cells were washed with ice-cold DPBS (pH 7.4) and lysed using 1 ml DMSO. The cell lysis product was centrifuged at 14,000 rpm (16,000 $\times g$) for 10 min to

remove cell debris. The fluorescence intensity of cell lysates was measured using a Fluorolog-3 (Jobin Yvon Horiba) spectrofluorometer at $\lambda_{\text{ex}} = 496 \text{ nm}$, $\lambda_{\text{em}} = 592 \text{ nm}$ for DOX in order to determine the uptake of IDNP and AIDNP by the cells. DOX calibration was previously created in a mixture of ICG-DOX dissolved in DMSO along with untreated cells. The protein content in the lysates was measured using a BCA assay kit. The uptake data was obtained by normalizing DOX content to the cellular protein level for three different experiments (three wells each for each treatment per cell line). A paired t-test was used to compare the 24-hr uptake of DOX between the treatments corresponding to the same equivalent concentration.

3.2.11. Cytotoxicity and hyperthermia assessment:

Cell viability after drug treatment was measured using a Sulforhodamine B (SRB) assay (Invitrogen, Carlsbad, CA, USA), which measures cellular protein (Monks A 1991). In this study, AIDNP and IDNP (equivalent to 0.1, 1 and 10 μM DOX) were incubated with MES-SA, Dx5 and SKOV-3 cells for 24 h to evaluate their toxicity. The detailed procedure for toxicity assessment has already been discussed in our previous report (Tang 2009). Average absorbance value was measured from four wells corresponding to the same treatment and cell line. An average ($\pm\text{S.D.}$) was determined from three independent experiments and was plotted against the respective DOX concentration. Cell growth fractions were determined by normalizing the data corresponding to treatment to control.

The methods for delivering hyperthermia and measuring its synergistic effect with DOX have also been published previously (Tang 2009). ICG at a dose of 5 μM during 3-min exposure to an 808-nm laser at energy density of 1,440 J/cm^2 (6.7 W/cm^2 , power density) increases the temperature of incubated cells from a baseline of 37 $^{\circ}\text{C}$ to 43 $^{\circ}\text{C}$ for selective hyperthermia. In this study, the cytotoxicity of four different treatments (IDNP w/o laser treatment, AIDNP w/o laser treatment, IDNP w/ laser treatment and AIDNP w/ laser treatment) were investigated. Cells were seeded into a 96-well plate on the first day, and after overnight incubation they were exposed to different treatments (each corresponding to equivalent DOX concentration of 10 μM and ICG concentration of 6.2 μM). The cytotoxicity was measured with the SRB assay 24 h post treatment in each case. Average cell growth fractions were obtained from four wells for each treatment (n=3). The data were acquired and represented in the same manner as the toxicity of the formulations discussed in the previous paragraph.

3.2.12. Statistical significance:

Statistical significance was identified by one-way ANOVA (SPSS, Chicago, IL, USA) for the difference among treatment groups at the same DOX concentration. A p-value <0.05 was considered to be statistically significant.

3.3. Results and Discussion

3.3.1. Characterization of NPs

Size, size distribution, zeta potential and drug loading:

The size and size distribution, charge and drug loading of non-targeted ICG and DOX loaded PLGA nanoparticles (IDNP) and anti-HER-2 targeted ICG and DOX PLGA nanoparticles (AIDNP) are listed in Table 1.

This shows that IDNP exhibited a size of 167 ± 5 nm with polydispersity of 0.06 ± 0.03 . An increase in size and size distribution of IDNP is observed after antibody conjugation. Similar results were observed in several studies (Mo 2005; Yang 2007; Lei 2010). IDNP and AIDNP both have the potential to accumulate in tumors due to passive targeting, however, AIDNP could further enhance drug accumulation inside tumor cells through enhanced internalization (Kirpotin 2006; Lei 2010). Because anti-HER-2 has an isoelectric point (pI) around 8.5, it has a positive charge at pH 7 (Barua S 2013) which masks the negative charge of the IDNP, creating a reduced nanoparticle surface charge. After antibody conjugation, the drug loading efficiency of IDNP is significantly reduced ($p < 0.05$) because of drug leaching during the conjugation process. This observation is consistent with literature reports (Mo 2005; Chittasupho 2009; Lei 2010).

	Size (nm)	PDI	Zeta potential (mV)	Loading % (w/w)	Antibody conjugation efficiency (μg per mg nanoparticles)
IDNP	167 ± 5	0.06 ± 0.03	-11.3 ± 1.6	1.79 ± 0.02 % ICG	
				2.32 ± 0.01 % DOX	
AIDNP	210 ± 7	0.16 ± 0.01	-1.0 ± 0.5	1.39 ± 0.01 % ICG	8.99 ± 0.66
				1.91 ± 0.02 % DOX	

Table 1. Mean size, PDI, zeta potential, percent drug loading and antibody conjugation efficiency for IDNPs and AIDNPs (n =3).

3.3.2 *In vitro* release kinetics profile:

Cumulative percentage of DOX released from AIDNPs is shown in Figure 2. Approximately $41 \pm 2.2\%$ DOX is released in 8 hours, reaching a sustained release of $48 \pm 2\%$ in 48 hrs. This release kinetic profile is similar to that of IDNP previously reported by our group (Manchanda 2009), as well as to the biphasic DOX release profile reported by Zheng et al. from lipid-PLGA nanoparticles, which showed 39.13 % DOX release by 8 hours.

Figure 2. DOX release kinetic profile of AIDNPs. (error bars denote a mean and SD of n=3 experiments)

The slow sustained release of DOX from targeted AIDNP and the biphasic behavior of the release profile observed in both AIDNP and IDNP are consistent with other literature reports as well (Yang 2007; Chittasupho 2009; Lei 2010). The two phases of DOX release correspond to an initial burst release, followed by a sustained slow release. The first phase results from the release of drug that was adsorbed onto nanoparticle surfaces during formulation, whereas the second phase is due to nanoparticle degradation and drug diffusion from within the polymer carrier. In our nanoparticles, the high molecular weight and hydrophobicity of PLGA result in a slow release of the drug from the polymer. Polymers with higher molecular weight undergo slower degradation in comparison to lower molecular weight polymers, because a longer chain of interlinked monomers takes longer to degrade once sequestered in acidic cellular compartments

such as lysosomes. The hydrophobicity of PLGA can be easily tuned by changing the ratio of lactic acid to glycolic acid. High hydrophobicity of the polymer results in higher DOX-polymer interactions, which slows down the release of drug from the carrier. Additionally, high polymer hydrophobicity also results in lower water absorption and slower degradation and drug release (Dinarvand 2011).

3.3.3. Antibody conjugation efficiency:

The antibody conjugation efficiency of AIDNP was measured to be 8.90 ± 0.66 μg antibodies per milligram of NPs, which is consistent with literature reports (Kocbek 2007; Lei 2010).

3.3.4. Cell culture studies

3.3.4.1. Subcellular localization studies

Figures 3, 4, and 5 show fluorescence microscopy images which illustrate the subcellular localization of IDNP and AIDNP in SKOV-3, MES-SA and Dx5 cell lines, respectively. The image exposure times were kept constant for each drug in the respective cell line for comparison purposes, and concentrations of IDNP and AIDNP used were, as previously described, determined by their corresponding equivalent ICG and DOX concentrations. The qualitative results show that AIDNP exhibited higher fluorescence for DOX and ICG in SKOV-3, when compared to the non-targeted formulation. AIDNP and IDNP showed similar fluorescence in both MES-SA and MES-SA/Dx5 cells.

Figure 3. Subcellular localization of DOX and ICG in SKOV-3 cells; 24 hours post-incubation; the exposure time for DOX and ICG channel were 500 ms and 2000 ms respectively. a. DOX fluorescence of IDNP; b ICG fluorescence of IDNP; c merged picture of a & b; d DOX fluorescence of AIDNPs; e ICG fluorescence of AIDNPs; f merged picture of d & e.

Figure 4. Subcellular localization of DOX and ICG in MES-SA cells; 24 hours post-incubation; the exposure time for DOX and ICG channel were 500 ms and 2000 ms respectively. a. DOX fluorescence of IDNP; b ICG fluorescence of IDNP; c merged picture of a & b; d DOX fluorescence of AIDNPs; e ICG fluorescence of AIDNPs; f merged picture of d & e.

Figure 5. Subcellular localization of DOX and ICG in Dx5 cells; 24 hours post-incubation; the exposure time for DOX and ICG channel were 500 ms and 2000 ms respectively. a. DOX fluorescence of IDNP; b. ICG fluorescence of IDNP; c. merged picture of a. & b.; d. DOX fluorescence of AIDNPs; e. ICG fluorescence of AIDNPs; f. merged picture of d. & e.

This is consistent with our expectations for increased uptake in SKOV-3 cells, given that this cell line over expresses HER-2 receptors (approximately 10^5 receptor/cell) (Xu 1999). Thus, high internalization rate of the receptor along with high specificity of the antibody to the receptor explains the higher uptake of AIDNP compared to IDNP in SKOV3 cells (Rudnick S 2011). MES-SA and Dx5 cells, used as negative control cells, do not over express the receptor and as a result we do not see any differences in fluorescence intensity between the IDNP and AIDNP groups.

IDNP and AIDNP exhibited similar intracellular localization in all three cell lines, and this localization was consistent with previous literature reports which have established that PLGA nanoparticles are internalized through endocytosis (Lei 2010; Tang 2010). The intracellular release of DOX from PLGA nanoparticles is due to hydrolysis of ester bonds in the PLGA polymer and to the increased solubility of DOX in mildly acidic intercellular components such as lysosomes (Tewes F 2007). Whereas DOX fluorescence in the cytoplasm might be the combined result of the internalized drug-entrapped nanoparticles as well as drug released from the nanoparticles, DOX fluorescence in the nucleus must be from drug released from the nanoparticles, given that nanoparticles cannot enter the nuclear pores due to size limitations (Tang 2010).

3.3.4.2. Cellular uptake experiments:

The 24 h uptake of IDNP and AIDNP in MES-SA, Dx5, and SKOV-3 cells was quantified by measuring the DOX fluorescence in cells normalized to cellular protein content.

Figure 6. 24-hour intracellular DOX uptake data in SKOV-3, MES-SA, and Dx5 cells, n = 3 experiments, 3 wells per treatment. * P < 0.05 (by ANOVA) between free drug and NPs formulation for each cell line, indicating significant differences due to loading of DOX into PLGA NPs.

Figure 6 shows that AIDNP significantly enhanced DOX uptake in SKOV-3 cells in comparison to IDNP, with an approximate two- fold increase in the uptake of AIDNP in SKOV-3 in comparison to IDNP. This can be attributed to HER-2 receptor mediated endocytosis in SKOV3 cells, given that AIDNP and IDNP exhibited similar uptake in the negative control cell lines MES-SA and Dx5. These results are consistent with our previous report and other group reports on targeted drug delivery PLGA nanoparticle systems (Mo 2005; Lei 2010). The zeta potential results of IDNP showed that they are negatively charged (-11 mV). Harush-Frenkel et al. reported that negatively charged PLGA nanoparticles are taken up by cancer cells through adsorptive endocytosis (Harush-Frenkel, Debotton et al. 2007). Receptor mediated endocytosis, however, is more efficient and specific in comparison to adsorptive endocytosis (Ogris M 2001).

The involvement of efficient receptor-mediated endocytosis is likely to be the mechanism that contributes to the higher uptake of AIDNP compared to IDNP in SKOV3 (HER-2 positive cell line).

3.3.4.3. Cytotoxicity and effect of hyperthermia experiments:

PLGA is a widely used polymer approved by the FDA for various applications (Danhier F 2012). Our previous reports have suggested that PLGA nanoparticles do not have toxic effects in the tested cancer cell lines up to 1 mg/ml, which is much higher than the concentration used for toxicity and uptake experiments (Vasir JK and Labhasetwar V 2005). This is consistent with the safety profile of PLGA nanoparticles reported by Liu et al. (1.02 ± 0.37 mg/ml) (Liu 2012).

Our cytotoxicity studies showed a dose-dependent increase in cytotoxicity for both IDNP and AIDNP in all three cell lines (Figures 7, 8 and 9). A 10 μ M concentration of DOX was chosen due to its clinical relevance (Coldwell 2008), and our previous reports have shown synergistic cancer cell killing using 10 μ M DOX and 6.2 μ M ICG based chemo-laser phototherapy (Tang 2009).

Figure 7. SKOV-3 cell growth for different drug formulations (without laser hyperthermia), $n=3$ experiments, 4 wells per treatment. No statistical significance seen between treatment groups in all cell lines.

Figure 8. MES-SA cell growth for different drug formulations (without laser hyperthermia), $n=3$ experiments, 4 wells per treatment. No statistical significance seen between treatment groups in all cell lines.

Figure 9. Dx5 cell growth for different drug formulations (without laser hyperthermia), n=3 experiments, 4 wells per treatment. No statistical significance seen between treatment groups in all cell lines.

The results of the present work show a small enhancement of cytotoxicity in SKOV-3 cells (without laser treatment) when AIDNP was used compared to IDNP. The difference is likely due to enhanced uptake of the targeted formulation by receptor-mediated endocytosis, although the difference in cytotoxicity did not reach statistical significance. These results are consistent with our previous report of comparable toxicity of antibody-conjugated DOX-loaded PLGA nanoparticles (ADNP) and DOX-loaded PLGA nanoparticles (DNP) (Lei 2010). Chittasupho et al also reported similar results, wherein c-LABL peptide conjugated DOX loaded nanoparticles exhibited comparable

toxicity to the non-conjugated nanoparticles (Chittasupho 2009). The comparable toxicity of AIDNP and IDNP (without laser treatment) that we observed despite the difference in uptake could be due to the aggressive nature of SKOV-3 cell lines. These cells have genetic mutations in caspase-3 and in apoptotic protease factor 1 expression, which make them resistant to DOX and in general to varied environmental stresses (Sood 2004). (Wolf BB 2001). For example, SKOV-3 cells have also been reported to be inherently thermotolerant, which could be attributed to their p53 mediated drug resistance (Van de Broek 2011; Manchanda 2012). The genetic characteristics of SKOV-3 may counteract the potential effects of enhanced uptake of DOX through AIDNP (in the absence of laser treatment). Our previous work suggested that combined laser hyperthermia and chemotherapy delivered through PLGA nanoparticles (IDNP) has the ability to significantly reduce the cell viability of this aggressive cell line and can partially overcome its ability to withstand chemical and thermal insults (Tang 2010). This observation was also confirmed by the present work, as AIDNP showed significantly enhanced toxicity (three-fold toxicity levels) in SKOV-3 after laser exposure when compared to the same treatment with IDNP (Figure 10). The negative controls (MES-SA and Dx5) exhibited comparable cytotoxicity after exposure to laser. These results are consistent with our expectations because these cells do not overexpress HER-2 receptors, and in turn this supports the theory that targeting ability and the resulting enhanced uptake are related to the large increase in therapeutic effect that was observed in SKOV-3 when a combinational approach was used. Thus, targeted PLGA nanoparticles with simultaneously entrapped ICG and DOX could selectively enhance

cell killing in SKOV-3 cells through enhanced uptake and combinational therapy, creating opportunities for efficacious treatments against this aggressive cell line.

Figure 10. Cytotoxicity of IDNP and AIDNP when excited by NIR laser. n=3 experiments, 4 wells per treatment. The concentration of IDNP and AIDNP was around 0.25 mg/ml, which contains 10 μ M DOX and 6.2 μ M ICG. * = significant difference ($p < 0.05$) versus non-laser group, = ** significant difference ($p < 0.05$) between laser groups.

IDNP and AIDNP exhibited similar toxicity in the negative control cells MES-SA and Dx5, and 3-min laser hyperthermia exposure to IDNP and AIDNP did not provide any advantage over the free drug forms (ICG and DOX) in either cell line. Because the main difference between the two formulations is related to targeting capability, and these cells do not over express HER-2, it was to be expected that IDNP or AIDNP after laser

treatment could not provide a competitive cell killing advantage over their free drug forms. Additionally, the results obtained in Dx5 cells are consistent with our previous report where IDNP with 3 min laser exposure did not enhance cell killing. Extended laser exposure of IDNP to 5 min, however, was able to significantly increase toxicity to Dx5 cells, indicating that longer exposures to hyperthermia which create higher thermal doses may be effective in treating these DOX-resistant cells (Tang 2010). Zheng et al. also conducted a study by incorporating ICG and DOX into lipid-PLGA (IDLPNP) nanoparticles to test their effect on the drug sensitive and drug resistant cancer cells lines MCF-7 and MCF-7/Adr respectively. They reported that combined photo-chemotherapy through IDLPNP (with equivalent drug concentrations of 5.5 $\mu\text{g/ml}$ ICG, 6.0 $\mu\text{g/ml}$ DOX) upon exposure to NIR laser with irradiance of 1.6 W/cm^2 irradiance for 5 min resulted in only 5% cell growth inhibition of the MCF-7/Adr cells 72 hours after laser exposure (Zheng 2013).

Multi-drug resistance (MDR) in cancer cells is exhibited through over expression of multi-drug resistance proteins (MRP1). The level of expression of these proteins (or transcripts for expression of this protein; i.e., mRNA) can be correlated to their degree of drug resistance (Loe DW 1996). MCF-7/Adr cells are a drug resistant variant of MCF-7 cells exhibiting 6,000 multi-resistant protein 1(MRP1) mRNA copies. Dx5 (the drug resistant variant of MES-SA cells) exhibited four times more MRP1 mRNA copies than MCF-7/ Adr cells (Yang 2002). Thus, Dx5 cells are expected to be more drug resistant than MCF-7/Adr cells. Kampinga et al. reported that combinatorial heat and drug administration causes damage to MDR proteins; thereby sensitizing the cells to the

effect of drugs (Kampinga HH 2006). Protein denaturation due to hyperthermia treatment is highly dependent on the thermal dose provided, and at least 5% protein denaturation is required to bring about detectable cell toxicity (Lepock JR 2003). The above reason suggests the requirement of a high thermal dose to bring about appreciable protein denaturation and hence higher cell killing, and is consistent with our observations in Dx5 which support an effect at longer treatment durations (and thus higher thermal doses). Franke et al. have described additional mechanisms of cytotoxicity through combined chemotherapy and hyperthermia in MDR cell lines. They reported that there was a reduced expression of MRP1 in cell membranes of MDR cells after the combinational treatment, and showed that the mechanism through which this phenomenon occurs is that hyperthermia impairs the translocation of MRP1 to the membrane through increased ROS production at the protein level (Franke K 2013). Therefore, there can be a complex interplay of different mechanisms which mediate toxicity during photo-chemotherapy treatment in MDR cell lines. All the mechanisms however, depend on the thermal dose provided during combined chemo-phototherapy.

Conclusions:

AIDNP can specifically target cancer cells (SKOV-3) which over express the receptor corresponding to the conjugated ligand (anti- HER-2 monoclonal antibody). AIDNPs also show great promise for combinatorial treatment employing both chemotherapy and hyperthermia, as an enhanced killing effect can be obtained from combinational treatment using the targeted formulation. In our next chapter, we will investigate the

effect of IDNP and AIDNP in combination with localized vs. generalized hyperthermia, in order to better understand the molecular mechanisms behind the therapeutic response mediated by these nanoparticles and their effect on apoptotic pathways.

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CHAPTER 4: UNDERSTANDING THE CELLULAR RESPONSE OF COMBINATORIAL CHEMOTHERAPY AND HYPERTHERMIA IN FREE DRUG FORM AND NANOPARTICLE FORM

The purpose of specific aim #2 was to understand the cellular response of combined chemo-hyperthermia when administered in free drug form and in the form of nanoparticles. In order to understand the cellular effects of different modes of hyperthermia when applied in combination with the chemotherapy drug doxorubicin (DOX), we used two different hyperthermia types to emulate those administered in clinical scenarios: laser-based rapid rate hyperthermia (which mimics localized hyperthermia) and incubator hyperthermia (which mimics whole-body hyperthermia). We performed studies to estimate the expression of heat shock protein 70 (HSP70) and caspase-3. These are two major proteins that play an important role in apoptosis and would help us understand treatment efficacy.

4.1. Background:

Hyperthermia involves the application of heat as a cancer therapeutic modality. The idea of utilizing heat to kill cancer cells is not novel, however, a practical implementation of this approach has been hampered by the lack of technologies that can deliver effective and homogeneous heat to deep tumors, as well as a lack of non-invasive technologies to monitor temperatures obtained *in vivo*. Recent developments involving nanotechnology, computer modeling, and non-invasive thermometry have once more stimulated an

interest in hyperthermia-based cancer therapy. The temperatures commonly used for hyperthermia therapy in clinical scenarios are 41-43 °C, because this range of temperatures can selectively kill tumor cells without affecting normal cells *in vivo* (Cavaliere 1967). This thermal toxicity is selective towards tumors due to the inherent properties of tumor tissue, such as low pH and hypoxia, in comparison to normal tissues (Overgaard 1977). The artificially-created temperature rise at the tumor site can elicit cytotoxicity by creating irreversible biological damage, or can be used synergistically along with other modalities such as radiotherapy and/ or chemotherapy (Kampinga 2006).

Hyperthermia has been reported to eliminate radiation-resistant tumor cells, thus serving as a potential radiosensitizer (Horsman 2007). It also has the ability to kill cells under conditions of hypoxia and low pH, which in turn are reasons for developing resistance to radiation and chemotherapy (Kampinga 2001). Hyperthermic temperatures of 43 °C have been shown to have more than an additive or synergistic cytotoxic effect when employed with several anticancer drugs. One mechanism by which hyperthermia helps to enhance drug uptake is through an increase in blood flow and vascular permeability at tumor sites (Jain 2005). Hyperthermia has been shown to enhance toxicity when administered concurrently with drugs due to an increased intracellular uptake of drugs, and increased rate of reactions with cellular targets (e.g. increased DNA damage), as well as MDR reversion (Averill and Su 1999; Issels 2008).

Hyperthermia is administered clinically through conducting heat sources, or non-ionizing radiation such as electromagnetic fields in an invasive or non-invasive manner. Different techniques for clinical hyperthermia include *whole body hyperthermia*, *perfusion hyperthermia* of organs, limbs or body cavities, and *localized hyperthermia* (by means of external or internal energy sources), as seen in Figure 2.1 (van der Zee 2008). *Whole body hyperthermia* is widely used to treat metastatic cancer by heating the patient to a temperature of 43 °C. This form of mild hyperthermia is often used in combination with cytotoxic drugs. However, a major limitation of this mode of heat administration is its inability to heat deep tumors (Bull 1984). *Perfusion hyperthermia* is used to regionally heat limbs, organs (liver, pelvis, stomach), or body cavities using heated fluids. Perfusion hyperthermia is performed by removing blood, heating it and circulating it back (Petrovich 1989). *Localized hyperthermia* involves elevating the temperatures of either superficial or deep tumors without affecting the surrounding normal tissues by employing external heating modalities. Non-invasive techniques such as high-frequency (e.g. electromagnetic or ultrasound) waves can be employed to heat a target region (Marmor 1979 ; Mendecki 1980). This technique is more appropriate to treat solid tumors (Storm 1979), and has shown great promise when used with chemotherapy for the treatment of prostate carcinoma (Tilly 2005) and bladder cancer (Rampersaud 2010).

Figure11: Methods for clinical application of hyperthermia (van der Zee 2008)

We have investigated two different thermo-chemotherapy modalities employing DOX as a common chemotherapeutic agent, combined with either (1) rapid rate indocyanine green (ICG)-mediated laser photo-chemotherapy or (2) incubator-based slow rate heating. The ICG-laser-based hyperthermia approach emulates localized hyperthermia, whereas incubator-based hyperthermia emulates a whole-body hyperthermia method. Our results showed that rapid-rate hyperthermia triggered by exposure of ICG to NIR laser (808 nm) showed a synergistic effect with DOX when used in combination with this chemotherapeutic drug, whereas slow-rate incubator hyperthermia showed a sub-

additive effect with DOX (Tang 2009). ICG is a tricarboyanine dye with near-infrared absorption properties (peak absorption around 800 nm), and its ability to generate heat upon exposure to NIR illumination was initially investigated several years ago (Chen 1995). Because body tissues are transparent to the near-infrared portion of the electromagnetic spectrum, deeper tissue penetration occurs in this region of the spectrum (Anderson and Parrish 1981). This allows for ICG to be used as an *in vivo* NIR-based imaging agent for blood volume determination, cardiac output measurement, liver function measurements, and visualization of retinal and choroidal vasculature (Benya 1989). A single treatment with ICG and laser alone was not found to be effective in treating tumors, highlighting the need to combine this approach with other treatment modalities such as chemotherapy.

When excited by near-infrared (NIR) laser, 5 μ M ICG is capable of raising cell temperature to 43 °C from 37 °C after 3-minute exposure. Incubator induced hyperthermia can also increase *in vitro* temperature to 43 °C, but requiring a longer time to reach the temperature. The prolonged duration and slow rate induction of heat can induce thermotolerance and reduce the effect of treatment.

Thermotolerance is conferred due to induction of proteins such as heat shock protein 70 (HSP70) within a time frame of several hours. HSP70 is a class of chaperone protein whose role is to help repair misfolded proteins. Therefore, the presence of denatured and/or misfolded proteins inside cells provides an input signal for generation of heat

shock proteins (Li 1995). The synergy between chemotherapy (DOX) and hyperthermia can be decreased if heating is done prior to chemotherapy, because heat exposure prior to chemotherapy treatment causes the induction of HSP70 which confers resistance to subsequent therapy (Hahn 1976; Herman 1982). A potential approach could be to deliver hyperthermia and chemotherapy simultaneously, which is made possible by using multifunctional nanoparticles. Moreover, drug release from the nanoparticles can be controlled to maximize cytotoxic effects. There are a few smart nanoparticle designs which employ thermoresponsive polymers to deliver the chemotherapeutic drug upon heat exposure, leading to 100% drug release (Ponce 2006; Yarmolenko 2010). In terms of optimal timing of hyperthermia and other therapies, Overgaard et al. showed that application of hyperthermia at 42-43 °C three to four hours after radiation was optimal for radiosensitization of cells (Overgaard 1982). Radiosensitization and chemosensitization to DNA-intercalating agents such as DOX both result in cytotoxic effects by preventing DNA damage repair mechanisms (Kampinga 2001; Blasiak, Widera et al. 2003). Therefore, the optimal timing between chemotherapy and hyperthermia can be expected to be similar to the timing between radiotherapy and hyperthermia. It has also been shown that the rate of heating is a critical factor in determining the synergistic effect of hyperthermia and chemotherapy (Herman 1982; Tang 2013).

The purpose of this dissertation was to investigate protein expression kinetics in response to rapid or slow-rate heating, and provide a rationale for hyperthermia

delivered through intracellularly-localized excitable molecules. The proteins under investigation were HSP70, to study thermotolerance profiles in cells; and caspase-3 (an end-effector apoptotic protein), to understand cellular response to different heating rates used with chemotherapy and delivered to cells in free or nanoparticulate form. Some previous studies by our laboratory group were focused on the cellular effects of rapid-rate and slow-rate heating after 24 h exposure, but not on protein expression profile during heat exposure (Tang 2009; Tang 2013; Lei 2014). Understanding the kinetics of protein expression, on the other hand, would be more useful in understanding cellular responses, and would provide a rational approach to choosing appropriate targets and planning combinational treatment with other modalities such as radiation or hormonal therapy. Previous work in our laboratory has studied HSP70 -induction after rapid-rate hyperthermia and slow-rate hyperthermia. However, this work involved the study of HSP and caspase-3 expression upon exposure to a single modality (hyperthermia or chemotherapy), rather than combinatorial therapy. Additionally, Tang's work only looked at protein expression 24h after exposure. A deeper understanding of protein expression kinetics requires investigating earlier time points in order to obtain time-based expression profiles of HSP70 and caspase-3. In this dissertation, it is hypothesized that rapid-rate hyperthermia would induce a faster induction of HSP70 in comparison to slow-rate hyperthermia, due to faster denaturation of cellular proteins. However, once cell damage becomes too large for the cells to overcome, there would be a down-regulation of thermotolerance proteins (HSP70 in our case), and this would pave the way for cells to undergo apoptosis or necrosis based on the degree and mode of cellular

damage. Hence, our study further enhances understanding the cellular events taking place upon combinatorial treatment via rapid-rate or slow-rate hyperthermia-chemotherapy.

4.2. Materials and Methods:

4.2.1. Drugs and chemicals

Poly (DL-lactide-co-glycolide) (PLGA, L:G 50:50; MW: 40– 75 kDa; glass transition temperature (T_g): 45–50 °C), dimethylsulfoxide (DMSO > 99.9% reagent grade), and polyvinyl alcohol (PVA, 87–89% hydrolyzed; 13–23 KDa) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dichloromethane (DCM) was purchased from Burdick & Jackson (Muskegon, MI, USA). Free-drug formulations were prepared by making DOX and ICG solutions as follows: Doxorubicin hydrochloride or DOX (Sigma-Aldrich, Saint Louis, MI) solution was prepared by dissolving 10mg DOX powder in 1mL of DMSO (Sigma-Aldrich). The desired concentration of DOX was obtained by further diluting the stock solution using Dulbecco's phosphate buffered saline (DPBS). ICG (Acros Organic) was stable in solution only up to 8h. Therefore, it was desirable to prepare fresh batches before every experiment. Briefly, stock ICG solution was prepared by dissolving 1mg ICG in 1mL DI water, and further dilutions were obtained using DPBS (Tang 2013). PLGA nanoparticles simultaneously entrapping ICG and DOX (designated as IDNP) were prepared using an oil-in-water emulsion solvent evaporation technique. The IDNPs were conjugated with anti-HER-2 by means

of carbodiimide chemistry and the resulting formulation was denoted as AIDNP (Srinivasan 2014). Nanoparticle preparation and characterization have been described in great detail in Chapter 1.

4.2.2. Cell culture:

Human uterine sarcoma MES-SA cells, and their drug resistant counterpart (P-gP over-expressing derivative) MES-SA/Dx5 (Dx5) cells, human ovarian carcinoma SKOV-3 cancer cells, McCoy's 5A medium, and fetal bovine serum were purchased from American Type Culture Collection (Manassas, VA). All three cell lines were cultured with McCoy's 5A media (ATCC) supplemented with 10% fetal bovine serum (ATCC) and 1% penicillin-streptomycin (GIBCO, Carlsbad, CA) and maintained in a Heracell (Thermo Scientific, Waltham, MA) cell culture incubator containing 5% carbon dioxide at 37 °C. Cells were harvested using 0.25% trypsin-EDTA (GIBCO) and sub-cultivated at a 1:10 ratio upon confluence.

4.2.3. Incubator hyperthermia (HT) delivery system and temperature increase profile:

Previously reported work by Tang et al served as the basis for this experiment (Tang 2009; Tang 2013). A Hera incubator (Thermo Scientific, Waltham, MA) was used as a heat energy source to mimic whole body hyperthermia as mentioned in the introduction section. The temperature of the incubator was set to 43 °C. 96-well plates were incubated at 37 °C in a separate incubator and later transferred to the 43 °C incubator. A temperature calibration study was done to determine the temperature profile of the

plates. The temperature calibration was also done for samples containing nanoparticles to account for the effect of the nanoparticle solution on the temperature profile.

4.2.4. Laser- HT delivery system and temperature increase profile:

For rapid-rate hyperthermia delivery, ICG was taken up by cells and activated by NIR laser. A laser heating system was designed for this purpose. The system is comprised of a laser module RLDH808-1200-5, Roithner Lasertechnik, Austria), a laser holder, a heated stage insert (WPI Heated Stage Insert, World Precision Instruments Inc, Sarasota) and a mobile stage with an extension arm. The whole laser system was encased within a box for operator safety and minimization of air currents effect. The heating stage insert was placed in a mobile stage below the laser and powered by an external source. The plate was used to maintain a temperature of 37 °C. The NIR laser source emits light at 808 nm with an output power of 1.2 W. The spot size was 15 mm² and the energy density was calculated to be 1440 J/cm². The small spot size ensured that laser power would deliver only to a single well at a time. The laser beam was focused onto the designated ICG-containing wells by moving the stage with the help of the extension arm outside the box. This extended arm can be used to focus the NIR laser beam onto each well one at a time. The experimental setup does not use any optical filters or optical lenses to focus the square beam (see Figure 2.2). The temperature profile of ICG, IDNP and AIDNP (at a final concentration of 6.5 µM ICG) was

measured. Exposures longer than 3 minutes resulted in photobleaching of ICG, and a subsequent reduction in heat production.

Figure 12. NIR- Laser system (a) and incubator system (b) used for hyperthermia delivery (Tang 2009).

4.2.5. Thermal dose calculation

The isoeffective dose was used to compare two hyperthermia treatments of different temperature-time modes producing a similar cytotoxic effect. Our previous reports suggested that 43 °C incubator hyperthermia showed similar cytotoxicity to 3-minute laser-ICG hyperthermia (Tang 2009). The relationship between temperature and time can be described using the isoeffective dose equation

$$t_1 = t_2 * R^{T_1 - T_2}$$

where t_1 and t_2 are the durations of treatment at temperatures T_1 and T_2 respectively. The value R is a constant that was calculated by the method described by Sapareto et al., (1984). The value of R is assumed to be 0.5 for a temperature of 43 °C. With a slight modification of the equation, the cumulative equivalent minutes at 43 °C (CEM43) can be measured. The temperature T_1 is adjusted to 43 °C, and T_2 is varied to obtain an integral equation where the upper limit of t is the endpoint time of the experiment (3 minutes for laser hyperthermia, or 60 minutes for incubator hyperthermia).

$$CEM_{43} = \int_0^t R^{43-T(t)} dt$$

4.2.6. Thermotolerance detection

Heat shock protein 70 kDa (HSP70) was used to study the expression of induction of thermotolerance upon exposure to ICG and DOX in free form or in nanoparticle form (IDNP, AIDNP; (corresponding to final ICG and DOX concentrations of 6.5 μ M and 10 μ M respectively in the cell culture media). ELISA was used to measure HSP70 induction after post-treatment times of 4, 6, 8, 12, 16, and 24 h. The protein levels were normalized to their respective controls (those which received no treatment) and cell growth fractions were used to report cell viability after each treatment.

4.2.7. Mode of cell death

Caspase-3 is an end-effector protein which is responsible for the initiation of intracellular events in apoptosis. A caspase-3 activity assay kit (Roche Applied Science,

Indianapolis, IN) was used to measure caspase-3 levels after the different combinatorial therapies. This is a fluorometric immunosorbent enzyme assay (FIENA) for the quantitative determination of caspase-3 activity in 96-well plates pre-coated with anti-caspase-3 agent. Caspase-3 levels were quantified 4, 6, 8, 12, 16 and 24 h after the hyperthermia treatment and normalized to their respective control, which is the caspase-3 value from cells that did not receive any treatment; and to SRB values to take into consideration cell viability upon each of the treatment conditions.

4.3. Results and Discussion:

Hyperthermia is gaining popularity in its application as an adjuvant therapy administered with chemotherapy, and has shown a synergistic effect along with some cancer drugs (Kampinga 2006; Ko 2006). Its efficacy when used alone or in combinatorial therapy depends on temperature achieved, duration of treatment, and rate of heating (Sapareto 1984; Tang 2009).

The thermal dose in the form of CEM43 °C (or CEM₄₃, the cumulative equivalent minutes at 43 °C) was calculated using method by Sapareto et al. (Sapareto 1984). The calculations were performed for both rapid-rate ICG laser hyperthermia and incubator hyperthermia in the presence of nanoparticles (IDNP and AIDNP). Laser-ICG hyperthermia at 3 min exposure produced a much lower thermal dose compared to the 43 °C incubator hyperthermia treatment, when delivered through nanoparticles. These

results are consistent with our previous report on similar experiments done with free-form ICG or nanoparticle-form IR820 (Tang 2009; Lei 2014). IR820 is an ICG derivative with a chloro-substituted cyclohexene group). ICG and IR820 are both near-infrared dyes which can generate hyperthermia. At 5 μ M concentration and exposure to 3min laser (λ = 808 nm; energy 1440 J/cm²), these dyes produce a temperature increase within the desired range required to kill cancer cells, i.e., 41-45 °C (Tang 2009; Fernandez-Fernandez 2012; Tang 2013; Lei 2014; Srinivasan 2014). A CEM₄₃-based thermal dose model is used to express the thermal load onto the living cells or tissues by inducing the given thermal stress in specific time duration. For example, the CEM₄₃ for skin tissues corresponds to 16 min, which produces similar cell growth as 64 min at 42 °C, or 1 min at 47 °C (Murbach 2013). The higher CEM₄₃ and slower rate of heat generation observed in the temperature-time profile of incubator hyperthermia, in comparison to laser-ICG hyperthermia, suggests that thermotolerance may be induced during incubator hyperthermia. Figure 13 shows that the temperature profile during free ICG-laser hyperthermia reaches a higher peak temperature than when using nanoparticles (i.e., IDNP and AIDNP). This could be due to the fact that the nanoparticle shell prevented the diffusion of heat from the nanoparticle core to the surrounding media. The overall thermal dose required is dependent on the area under the curve of the temperature-time profile, and the heating time using the laser (808 nm; 6.7 W/cm² fluence rate) is dependent on providing an isoeffective dose in comparison to that provided in incubator hyperthermia. There is a temperature reduction seen at the 180 sec time point, which could be caused by possible degradation of ICG due to heat or laser.

The laser irradiation duration of 3 min was chosen because it helped potentiate maximum combinatorial therapy in all the three cell lines MES-SA, MES-SA/Dx5 and SKOV3 cell lines (Tang 2010)

Figure 13: Temperature profile during hyperthermia treatment. (a) Temperature generation as a function of ICG in free form and nanoparticles form each at a concentration of 6.5 μM ($n = 3$). Data are presented as mean $\pm$ SD; (b) temperature elevation profile during 43°C incubator hyperthermia (done in presence of ICG and ICG-loaded nanoparticles to know their effect ($n= 1$)).

Exposure of cells to thermal stress causes either cell killing or induction of thermotolerance, depending on the thermal dose provided. The direct effect of heat which leads to either cell killing or thermotolerance involves protein denaturation. Thermodynamic arguments (Johnson 1974) and the requirement of high activation energy (Westra 1971) suggest a correlation between protein denaturation and cell killing. Similar correlations also exist between protein denaturation and heat-shock protein induction, which is widely involved in cellular thermotolerance. This theory has been proved by the fact that injection of denatured proteins and immediate induction of proteolytic signals has served as a trigger for induction of heat shock protein expression.

Thus, there seems to exist a common pathway connecting cell killing, thermotolerance, and heat shock protein induction, all of which are a consequence of cellular protein denaturation (Hahn 1990). Cellular proteins retain their flexibility and plasticity at the optimal temperature T required for their proper functioning. Exposure to heat (at temperatures above this optimal temperature T) makes proteins thermolabile and susceptible to causing stress in their host cells. Spectroscopic and differential scanning calorimetric studies have suggested that protein denaturation is the cause of thermal damage to cell membranes and organelles (Lepock 1983; Lepock, Cheng et al. 1987; Lepock 1989). Therefore, there exists a level of protein denaturation which either causes hyperthermic cell killing or heat shock protein-based thermotolerance (Lepock, Frey et al. 1993). The level of thermal damage decides the level of protein denaturation, which in turn decides its fate as (1) being processed by molecular chaperones through HSP induction or (2) when the repair is irreversible, damaged proteins are degraded by means of the ubiquitin–proteasome pathway which plays a critical role in apoptosis (Murata 2003). This phenomenon is also important in the case of combined thermo-chemotherapy. A description of the two pathways is provided in the following paragraphs.

Thermo-chemosensitization of cells in combined therapy can either cause enhanced or reduced effects due to induced thermotolerance. Thermotolerance, also known as thermal preconditioning, is an effect by which cells can tolerate sub-lethal heat stress after they have been previously exposed to heat. Thermotolerance is often achieved

when cells are exposed to mild heat treatment that involves reversible damage to labile proteins, as well as acceleration of cellular metabolic process by expression of anti-apoptotic proteins such as heat shock proteins (Kregel 2002.). Heat treatment of cells causes proteins to unfold from their native state, preventing them from performing their functions inside the cell. The presence of these malformed proteins inside cells causes the induction of molecular chaperones, which help refold these proteins into their native folded state (Frydman 2001). Heat shock proteins belong to this class of molecular chaperones that help with proper folding of newly synthesized proteins and/or refolding misfolded proteins (Baler 1992; Lepock 2005.). However, heat shock protein induction is dependent on several factors including temperature, duration of exposure, and cell type. Protein denaturation is the rate-limiting step of heat shock protein generation. Most mammalian cells respond to a temperature increase of 5-6 °C if their ambient growth temperature condition is at 37 °C (Morimoto 1996.). The signaling of heat shock protein is driven by heat shock transcription factors (HSF), as shown in Figure 2.3. HSFs are stress-induced and do not contribute to the constitutive levels of HSP (Huang 2001). In the absence of stressful conditions, HSPs are in their inactive form where HSPs are bound to HSFs. When cells undergo stress, HSFs are dissociated from HSPs and translocate to the nucleus. Inside the nucleus, HSFs bind to the promoter region of the HSP operon known as the heat shock element (HSE) which transcribes and overexpresses HSPs (Kiang 1998; Huang 2001; Trinklein 2004). The proteins that are irreversibly damaged during thermal stress undergo degradation via the ubiquitin-proteasome complex in the lysosome (Bond 1988). However, when the level of thermal

damage is much higher than the activation energy for cell killing, the damaged or denatured proteins accumulate and lead to cell killing (Lepock 2003).

Figure 14: Schematic of Hsp70 response pathway in cells (Morimoto 1996).

Our protein expression data after combinatorial therapy suggests that there is a transient and rapid overexpression of HSP70 (peaking at 6h) in rapid-rate laser hyperthermia-chemotherapy, in contrast to a slower rate of induction of HSP70 expression (peaking at 8h) in slow-rate incubator hyperthermia-chemotherapy. The transient overexpression of HSP70 suggests the presence of denatured proteins upon heat stress. These results are consistent with Beckham and coworkers (Beckham 2004). Differences between the two modes of combinatorial therapy are to be expected, because the rapid-rate heating induced by laser therapy is likely to induce faster cellular protein denaturation in comparison to slow-rate incubator hyperthermia, and protein denaturation is the rate-limiting step for HSP generation. Chen and coworkers also suggested a similar effect in gold nanorod-mediated photothermal therapy. Laser photothermal therapy causes heat generation in cells at a rapid rate, resulting in sudden local heating and thermal explosions which induce rapid protein denaturation and damage in cellular structures.

This damage can compromise cell membrane integrity and hence increase drug permeability in combinational therapy (Chen 2010). Beckam and coworkers also showed that HSP70 expression upon laser irradiation and incubator hyperthermia conditions peak around 4-8 h post-treatment, and then decrease after 12 h incubation. This delayed response could be because there needs to be sufficient time for the HSP70 gene promoter to receive the signal to activate transcription from HSF1 to the required mRNA, and for further production of the functional protein to occur, as suggested in the schematic of HSP70 response in Figure 3. The earliest time of HSP70 response has been reported as 2-3 hours post heat stress. The up-regulation of mRNA levels itself takes about 90 minutes (Amici 1992). Yang and coworkers observed transient expression of HSP70 upon exposure of MCF-7 breast cancer cells to Apt-Au-GO nanocomposites at 1.74 $\mu\text{g/mL}$ concentration using a NIR laser ($\lambda = 808 \text{ nm}$, 5 min exposure time) for photothermal therapy. They reported that there was a transient expression of HSP70 at 3 h, followed by a down-regulation of these proteins at 12 and 24 h. They explained their results by suggesting that cells undergo two stages upon heat exposure: a *compensatory stage*, as the cell tries to overcome the presence of damaged proteins by overexpressing HSP70; and a *decompensatory stage*, where induced HSP70 is unable to repair heat-damaged proteins due to irreversible damage. In this decompensatory stage, HSP70 induction is down-regulated and the cell is allowed to switch to cell death mechanisms through apoptosis or necrosis (Yang 2015).

Appropriate heat deposition in cancer cells through the careful choice of laser parameters can help in increasing the efficacy of the heat treatment when used alone or

in combined therapy. The goal is to achieve significant temperature elevation, decreased cell viability, and down-regulation of HSP expression, leading to a successful cancer treatment alternative as suggested by Fisher and coworkers (Fisher 2010). They employed MWCNTs for photothermal cancer therapy and reported that lower heat duration (1.5 W/cm^2 , 1.5 min) upregulated HSP and increased cell viability, whereas higher irradiation duration (1.5 W/cm^2 , 5 min) downregulated HSP and lowered cell viability. Successful thermal therapies hence lead to downregulation of HSP70 and induce biological effects such as cell membrane blebbing and necrosis (Diederich 2005). When HSP70 is downregulated, the Bax protein is translocated from the cytosol into mitochondria, resulting in the formation of pores to release cytochrome c and apoptosis-inducing factor (AIF). The released AIF translocates to the nucleus to induce cell death (Jürgensmeier 1998). This suggests that HSP70 is a potential anti-apoptotic protein (Rérole 2011). Transient exposure to mild elevated temperatures is tolerable, since cells can rapidly turn on their repair mechanisms. However, above a given time and temperature threshold, the cell will succumb to death. Therefore, cellular fate ultimately depends on the relative strength of cell death and cell survival signals.

When looking at the HSP70 generation profile in different cell lines for a given mode of hyperthermia, we see that different cell lines show similar profiles, as long as the hyperthermia mode is the same. That is, when looking at rapid-rate hyperthermia-chemotherapy combination therapy, the HSP70 generation profile is similar in cell lines MES-SA, MES-SA/Dx5 and SKOV3. When looking at the HSP70 generation profile of slow-rate hyperthermia-chemotherapy, cell lines also showed similar profiles when

compared to one another. These results indicate that the thermosensitivity of cells with different drug sensitivity or resistance is the same. Similar results have been reported by Asaumi and coworkers, who found similar thermosensitivity in drug-sensitive and multi-drug resistant Ehrlich ascites tumor cells (Asaumi 1996). Wrzal and coworkers also showed that drug-sensitive and multi-drug resistant Chinese Hamster ovarian cells exhibited similar thermosensitivity (Wrzal 2008). In our work, incubator-hyperthermia-chemotherapy exhibited a time-dependent increase in caspase-3 expression in all three cells lines under study, MES-SA, MES-SA/Dx5 and SKOV3, with significant expression starting from 12 h. Laser-hyperthermia-chemotherapy exhibited the same levels of caspase-3 expression at all post-irradiation times and in all three cell lines. These results can be explained on the basis of previous reports by Tang and coworkers in our laboratory, who showed that incubator-hyperthermia-chemotherapy showed a sub-additive effect of therapies and induced apoptosis in cells, and laser-hyperthermia-chemotherapy exhibited a synergistic effect of therapies and induced necrosis (Tang 2013). These results are consistent with literature stating that caspase-3 proteins are the end effectors of apoptosis which trigger a further cascade of apoptosis- executors for DNA fragmentation and chromatin condensation (Cohen 1997). However, they are completely downregulated in the necrosis processes (Denecker 2001).

Our results show that peak HSP70 expression was comparable after exposure to AIDNP, IDNP and ICG- DOX based laser therapy or incubator therapy in MES-SA cells. The same can be said for peak caspase-3 expression in MES-SA cells, which was comparable across treatment groups (See Figure 2.5). In MES-SA/Dx5 cells, both

HSP70 and caspase-3 showed higher generation for AIDNP and IDNP, in contrast with free form ICG-DOX based laser therapy and incubator therapy (See Figure 2.6). Finally, in SKOV-3 cells, AIDNP treatment caused much higher HSP70 and caspase-3 generation than IDNP and ICG-DOX based laser therapy and incubator therapy (See Figure 2.7).

Figure 15 HSP70 and caspase-3 induction after incubator hyperthermia (a&c) and laser hyperthermia treatment (with or without chemotherapy) (b&d) in MES-SA cells (n=3). ■ denotes peak expression of protein. * denotes statistical significance between treatment groups ($p < 0.05$).

Figure 16. HSP70 and caspase-3 induction after incubator hyperthermia (a&c)(e&f) and laser hyperthermia treatment (with or without chemotherapy) (b&d) in MES-SA/Dx5 cells (n=3). ■ denotes peak expression of protein . * denotes statistical significance between treatment groups ($p < 0.05$).

Figure 17. HSP70 and caspase-3 induction after incubator hyperthermia (a & c) and laser hyperthermia treatment (with or without chemotherapy) (b & d) in SKOV-3 cells (n=3). ■ denotes peak expression of protein. * denotes statistical significance between treatment groups (p < 0.05).

These results can be explained based on an understanding of cell characteristics and their drug sensitivity or resistance. MES-SA cells are drug-sensitive uterine cancer cells and hence, uptake of ICG and DOX into these cells occurs both in the free form and in nanoparticles. Therefore, there is no advantage to a nanoparticle formulation of ICG and DOX because the free form can already be delivered to these cells. MES-SA/Dx5 is a drug-resistant variant of MES-SA cells that overexpresses a multidrug-resistance pump, P-glycoprotein. The incorporation of ICG and DOX into nanoparticles in the form of IDNP and AIDNP is able to have a competitive advantage over the agents in free form, because nanoparticles bypass the multidrug-resistant protein overexpressed in these cells

so that entrapped agents can be delivered more efficiently into the cell. SKOV3 is a drug-resistant ovarian cancer cell line with a p53 gene deletion. These cells overexpress HER-2 receptors. AIDNP are nanoparticles containing ICG and DOX, and which have been surface-conjugated with an antibody to HER-2 receptors. Hence, SKOV3 exhibit a higher uptake of ICG and DOX in the form of AIDNP than IDNP or free form (ICG and DOX) (Tang 2010; Srinivasan 2014). The uptake of the hyperthermia agent (ICG) shows differential intracellular availability when delivered through different nanoparticle forms (AIDNP and IDNP) or as free drug in cells with different drug sensitivity or resistance properties. The delivery of different heat modalities to intracellularly-accumulated ICG further causes a differential protein response as hyperthermia exerts its toxic effect. For example, AIDNP showed the highest uptake in SKOV-3 in comparison to ICG and DOX delivered through other forms (IDNP and ICG and DOX). As a result, higher ICG intracellular availability and subsequent heat generation causes more extensive protein denaturation and higher HSP expression in comparison to the same agents delivered through IDNP or free form. The uptake of the hyperthermia agent (ICG) determined intracellular availability, and the mode of hyperthermia (laser or incubator) also affects the extent of intracellular damage and the amount and timing of protein expression (HSP70 and capase-3).

Conclusions:

Our study demonstrated a transient expression of the anti-apoptotic protein HSP70 in rapid-rate ICG-laser-chemotherapy and slow-rate incubator hyperthermia-chemotherapy

applied to cancer cells. Previous work done in our laboratory by Tang showed a differential HSP response of rapid-rate and slow-rate hyperthermia 24 h post-treatment (Tang 2013). The present dissertation focuses on studying the HSP generation profile from the above mentioned hyperthermia modes in combination with chemotherapy (instead of hyperthermia alone), and also includes earlier time points. A faster HSP70 response was seen in rapid-rate ICG-laser-chemotherapy in comparison to slow-rate incubator hyperthermia-chemotherapy. HSP is expected to be dependent on the level of protein denaturation in cells. Because rapid- rate hyperthermia induces a faster and greater percentage of protein denaturation, we can expect to observe faster generation of HSP in comparison to slow rate hyperthermia. Cells with different drug sensitivity or drug resistance showed similar thermosensitivity. A time-dependent increase of caspase-3 was observed in slow-rate incubator hyperthermia-chemotherapy indicating apoptotic cell death; however, a flat response of caspase-3 was induced in rapid-rate ICG-laser-chemotherapy, indicating necrotic cell death in cancer cells. AIDNP, IDNP (ICG and DOX entrapped into nanoparticles) and free ICG and DOX showed different HSP70 and caspase-3 responses in cancer cells with different drug sensitivity or resistance. The observed pattern of HSP70 and caspase-3 generation must be taken into account to understand the efficacy of the combinatorial hyperthermia-chemotherapy treatment rendered through two different hyperthermia methods, and it can assist planning when used in combination with other treatment modalities such as radiation therapy. Understanding the timing of expression of anti-apoptotic and apoptotic proteins can help in making decisions of when to add other treatment modalities to the combinatorial

therapy. For example, additional radiation or hormonal therapy can be provided 12 h after combinatorial hyperthermia-chemotherapy treatment, because both rapid-rate-hyperthermia-chemotherapy and slow-rate-hyperthermia-chemotherapy result in down-regulation of HSP post-treatment. As a result, cells can be more sensitive to other treatment modalities.

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CHAPTER 5: MULTIFUNCTIONAL SILVER NANOPARTICLES FOR TARGETED CANCER THERAPY AND UNDERSTANDING THEIR MECHANISM OF ACTION

Our work described in the previous chapters has shown that polymeric nanoparticles loaded with chemotherapy and hyperthermia agents, and decorated with receptor-specific targeting moieties, can be used to produce enhanced toxicity in cancer cells compared to treatment approaches using a single modality or a non-targeted nanoagent. Furthermore, the application of hyperthermia via an intracellular agent results in enhanced response to the treatment compared to environmental hyperthermia such as that produced by an incubator, which mimics whole body hyperthermia. Localized hyperthermia, when used in combination with chemotherapy, shows faster HSP70 response in comparison to similar treatment using incubator hyperthermia and chemotherapy. The mode of cell death is also different: combined chemo-laser phototherapy causes necrotic cell death, in contrast to apoptotic cell death which is observed in combinatorial chemo-incubator hyperthermia.

These observations support the use of targeted multifunctional agents in the treatment of cancer using combined chemotherapy and hyperthermia. However, there are some concerns with the use of polymeric nanoparticles as therapeutic carriers. Some of their disadvantages include toxicity of preparation solvents, acidity of degradation byproducts, and drug release profiles which are typically biphasic and may be difficult to control (Fernandez-Fernandez 2011).

An additional concern regarding dual agent multifunctional nanoparticles is brought by the shortcomings of both doxorubicin and indocyanine green. Nanoparticle

encapsulation of DOX decreases its off-target toxicity and helps circumvent the development of resistance, but there are still problems in achieving a predictable release profile from a polymeric nanoparticle in terms of timing, as well as in reaching desired concentrations in tumor tissue. One of the major constraints in terms of the effects of different timing of combined chemotherapy and hyperthermia is the possible induction of thermotolerance. Thermotolerance can be induced if the hyperthermia agent is released first and exposed to the laser to generate heat, inducing overexpression of heat shock protein that can confer cell resistance to subsequent chemotherapeutic treatment. This has the potential to decrease the efficacy of the treatment combination. Therefore, we ideally want to avoid premature release of the hyperthermia agent. Thus, a drug delivery system with simultaneous incorporation of hyperthermia agent ICG and chemotherapy agent DOX would be required to have slower release of ICG compared to the release rate of DOX in order to avoid induced tolerance effects.

In practical terms, this means that design optimization is much more challenging as one must simultaneously optimize the release rate of two agents. Selecting and designing a polymeric vehicle that can carry two agents with completely different physicochemical properties, while also providing differential customized timing for their release, becomes quite complicated. For instance, ICG is amphiphilic, whereas DOX is hydrophobic. A hydrophobic polymer will interact more with DOX, thereby entrapping more DOX compared to ICG. Reduced ICG entrapment will result smaller overall amounts of ICG released at the target site. A hydrophilic polymer, on the other hand, will interact more with the amphiphilic agent ICG, thereby entrapping more ICG and less DOX and hence

release smaller amount of DOX at the target site. Neither of the two options will be optimal for combinatorial treatment purposes. Therefore, simultaneous entrapment of therapeutic agents into a polymeric system (with appropriate hydrophobicity to hydrophilicity ratio) with the optimal dose of both agents and with optimal release for effective treatment can be difficult to achieve in practice. In the case of indocyanine green, an additional concern is its poor stability in aqueous solution, which can limit its therapeutic window once it is released from the nanoparticle. This could be circumvented by selecting a more stable dye, although the question of dual entrapment optimization would still remain. Finally, burst release of agents on the surface of polymeric nanoparticles can further alter dosage and timing of agent delivery at the target site.

Another consideration that limits the application of a polymeric multifunctional system is that most polymeric nanoparticles do not possess inherent imaging capabilities, unlike metal nanoparticles, and hence polymeric nanoparticles depend on the imaging capabilities of the agents they entrap.

When considering these disadvantages, it becomes clear that a possible alternative to multifunctional polymeric nanoparticles with dual agent entrapment is the use of metal nanoparticles with inherent multifunctional capabilities for imaging and phototherapy. If we use these nanoparticles to deliver a chemotherapeutic drug, we obtain a multifunctional system capable of image-guided therapy with combinational chemotherapy and phototherapy. In this case, optimizing the release kinetics of the

chemotherapy agent is much easier than if we had to deal with multiple agents. This helps in achieving higher control over the system, and makes it easier to attain clinically relevant dosing and timing for combined therapy at the target site.

Metallic nanoparticles have unique optical properties related to surface plasmon resonance (Levi-Polyachenko 2011). Also, the size and shape of metallic nanoparticles can be more easily controlled compared to polymeric nanoparticles, and this creates the option of tailoring particle size to the specific application as well as for their appropriate phototherapeutic application. Although AgNPs are slightly less chemically stable than gold nanoparticles, they have potential advantages in other applications such as SERS sensing, based on their higher extinction coefficient and large effective scattering cross-section (Caro 2010). Boca et al reported the successful use of chitosan-coated silver nanotriangles (Chit-AgNTs) with resonances in the near-infrared (NIR) as localized hyperthermia agents against a line of human non-small lung cancer cells (NCI-H460) (Boca 2011). Silver nanoparticle formulations have been shown to have cytotoxic effects in several cancer cell lines, including MCF-7 breast cancer cells (Franco-Molina 2010), human epidermoid larynx carcinoma (Satyavani 2012), acute myeloid leukemia (Guo 2014), human epithelial carcinoma cell line HeLa (Sukirtha 2011), Dalton's lymphoma (Sriram 2010). Sriram's group was able to show that mice treated with Ag NPs had survival times increased by 50% with respect to controls, as well as decreased volume of ascites and decreased cancer cell count and white blood cell count within ascitic fluid (Sriram 2010). Sukirtha et al also demonstrated increased lifespan in a lymphoma mouse model after treatment with AgNPs (Sukirtha 2011). Gurunathan's group has reported that

AgNPs can act as an antiangiogenic molecule, which could be a very important weapon to create an unfavorable environment for tumor tissue (Gurunathan 2009). The cytotoxic effect of AgNPs seems to be mediated by generation of reactive oxygen species as well as release of silver ions which interfere with mitochondrial activity (Guo 2013). However, silver appears to be not toxic against healthy cells, and Franco-Molina reports that mice exposed to 50x the recommended manufacturer's dose of colloidal silver for one year showed no negative effects on fertility and tumor development (Franco-Molina 2010).

Based on these advantages, the next step in our project was to develop a modified approach using targeted AgNPs that could be utilized in combinational chemotherapy and hyperthermia treatments while reducing some of the concerns created by the dual agent - polymeric nanoparticle strategy. In choosing a targeting moiety to use with our AgNPs, we took into account the chemical feasibility and practicality of binding the moiety to AgNPs, as well as biological considerations such as biocompatibility and ability to target cancer tissue. We chose folate because it can be bound to AgNPs via a sulfhydryl link, it is a natural targeting moiety that would not cause immune response in humans, and it is overexpressed in about 90% of ovarian cancers (Figini 2003). The next chapter moves on to describe the development and use of folate-capped AgNPs in combinational cancer therapy.

The purpose of specific aim# 3 is thus to prepare multifunctional silver nanoparticles (AgNPs) for targeted cancer therapy. The AgNPs serve as therapeutic agent as it is seen

to mediate toxicity by generation of reactive oxygen species. The chemotherapeutic agent DOX is attached to AgNPs through a pH-sensitive linkage to allow for pH-driven drug release. The mechanism underlying the action of these nanoparticles is also studied in this chapter.

5.1. Introduction:

Multifunctional agents have the potential to improve diagnosis and treatment of cancer patients by facilitating a holistic approach to patient management. These agents employ combined diagnostic and therapeutic modalities, which help in simultaneous localization and treatment of tumors (Janib 2010; Xie 2010; Fernandez-Fernandez 2011; Lu 2014). The method of combining diagnostic and therapeutic modalities is often referred to as “theranostics” (Caro 2010; Özdemir 2012). An important issue in cancer management is that diagnostic and therapeutic agents often have different biodistribution and selectivity, creating challenges in planning, timing, monitoring, and measuring treatment success. Multifunctional agents confer an advantage by allowing the combination of diagnostic and therapeutic agents onto a single platform. As a result, site localization can be coordinated with therapeutic action so that treatment occurs at the correct location and is also timed appropriately. Additionally, the effect of therapy can be monitored in real time thanks to the diagnostic capabilities of the multifunctional agent.

Nanomedicine is a rapidly progressing area in the field of cancer therapy which provides an effective platform to combine more than one therapeutic and/or diagnostic modality. Nanoscale theranostic systems that have emerged for cancer treatment include organic

polymer based and organic-inorganic nanocomposite based systems (Pregibon 2007; Kim 2008; Neoh 2011; Srinivasan 2014). These systems have several potential advantages over conventional single small molecule-based therapies, some of which include high payload capacity, prolonged blood circulation times, reduced toxicity to healthy tissues, and improved anti-tumor efficacy (Blanco 2009).

Inorganic plasmonic nanoparticles such as gold and silver possess several unique optical properties, and hence have been used in a wide range of biomedical applications such as sensing, imaging and therapy (El-Sayed 2005; Sriram 2010). Metal nanoparticles are prepared by reducing a salt solution of the metal. Commonly used reducing agents used in the synthesis of silver and gold nanoparticles include borohydride, citrate, ascorbic acid, and organometallic compounds such as dimethylformamide and polyols. These reducing agents can sometime be interchangeably used as capping agents as well. Recent developments in metal nanoparticle synthesis involving different capping agents have helped to prevent nanoparticle aggregation as well as to reduce nanoparticle toxicity, thereby making nanoparticles more suitable for application as drug delivery vehicles (Shon 2004; Huynh 2011).

Among the plasmonic nanoparticles, AgNPs are gaining considerable attention due to their favorable physicochemical attributes such as magnetic and optical polarizability, electrical conductivity, catalysis, antimicrobial behavior, and Surface-Enhanced Raman scattering properties (El-Sayed 2005). AgNPs also have very diverse biomedical applications in wound dressings, contraceptive devices, surgical instruments, and bone

prostheses to prevent microbial activity; and have been widely investigated for their antimicrobial and antifungal properties (Sharma 2009; Sau 2010). AgNPs have been employed less in the development of drug delivery vehicles because they exhibit lower chemical stability than gold nanoparticles. However, AgNPs possess some attributes that could confer an advantage over gold nanoparticles, such as higher extinction coefficients, sharper extinction bands, higher ratio of scattering to extinction, and extremely high field enhancement (Carlos Caro 2010). As a result, a lot of interest has arisen recently in the application of AgNPs in cancer therapy. These nanoparticles serve as promising drug carrier candidates thanks to the ability to control their size, facile surface modification, and adequate cell penetration (Sriram 2010; Guo 2013). Careful and thoughtful design of nanoparticle systems can tailor drug release by making formulations responsive to internal or external triggers such as pH change, intracellular glutathione levels, light or temperature changes, etc. (Hong 2006; Aryal 2009; Smeta 2011; Zhang 2012). AgNPs can also be useful in photodynamic applications, as they generate cytotoxic reactive oxygen species when irradiated with suitable light wavelengths (Corato 2012). Several researchers have shown synergistic chemo-photodynamic therapy through combinations of chemotherapeutic and organic dye-based photosensitizers (Penga 2008; Lu 2014). However, nanoparticle based photosensitizers could provide a better alternative to organic dye-based photosensitizers because they do not undergo photobleaching. Gold (AuNPs) and silver nanoparticles (AgNPs) are both potential photosensitizers having the ability to produce reactive

oxygen species upon laser irradiation. The advantages of AgNPs as described above make them an ideal drug delivery vehicle for combined chemo-photodynamic therapy.

Doxorubicin is a chemotherapeutic drug used for the treatment of a wide spectrum of tumors. It belongs to the class of anthracycline antibiotics, which exhibit their cytotoxic effect by intercalating with nuclear DNA and inhibiting topoisomerase II (Belloc 1992; Gieseler 1994). Some of the limitations of this drug include its non-specific cardiotoxicity and the development of drug resistance in cancer cells (Singal 1998; Gottesman 2002). A possible way of enhancing the therapeutic efficacy of DOX is by utilizing prodrug designs that increase drug concentration at target sites while reducing off-site toxicity. Nanoparticle formulations that allow for triggered release of DOX provide a good way to achieve this effect.

With the goal of creating a multifunctional agent with high selectivity, an additional advantage of using nanoparticle formulations is the ability to attach ligands that bind to specific target receptors. The folate receptor (FR) is highly overexpressed in several human malignant tumors, in comparison to normal tissues (Weitman 1992). Folic acid (FA) is a widely-used ligand which possesses high affinity to folate receptors overexpressed in cancer cells. Therefore, FA and its conjugates demonstrate significantly enhanced delivery to FR-positive tumor cells (Zhang 2004; Hilgenbrink 2005). Folic acid conjugates are internalized through folate receptors, dissociate in the intracellular environment of cancer cells ($\text{pH} = 5.0 \sim 5.5$ of endo-lysosomes) and then are released into the cytosol (Antony 1992; Sudimack 2000).

The current manuscript describes a multifunctional folate-targeted silver nanoparticle-based theranostic system that combines the cytotoxic photodynamic properties of AgNPs with the chemotherapeutic properties of DOX, which is tethered to the nanoparticle through a pH-sensitive linker. Upon cargo release inside the cancer cells, a combined chemo-photodynamic effect can be achieved. These multifunctional nanoparticles can also be probed intracellularly through Raman and fluorescence spectroscopy for diagnostic and treatment monitoring purposes. Thus, in addition to active targeting with FA, the selectivity of the nanoparticles is also attributed to activation by near-infrared (NIR) laser once the particles have been confirmed to be localized at the tumor site.

5.2. Materials:

Heterobifunctional polyethylene glycol (PEG), MW 5kDa, was obtained in two forms: one with a sulfhydryl group at one end and a carboxyl group at the other end (SH-PEG-COOH, MW: 5kDa), and another form with a sulfhydryl group at one end and an amine group at the other end (SH-PEG-NH₂, MW: 5kDa). Both forms were purchased from Nanocs, New York, NY. Silver nitrate, hydrazine hydrate, trifluoroacetic acid, N,N'-Dicyclohexylcarbodiimide (DCC) and N-Hydroxysuccinimide (NHS) were obtained from Fluka (Sigma-Aldrich, St. Louis, MO). All chemicals used were analytical grade. HCl and HNO₃ were purchased from Sigma-Aldrich, St. Louis, MO, and used to prepare aqua regia (HCl and HNO₃ in 3:1) to wash all glass apparatus used in the preparation of the colloidal AgNPs. Deionized water used in the experiments had a resistivity of greater

than 18 mega ohm-cm. The cell lines MES-SA (human uterine sarcoma, DOX sensitive cell line), MES-SA/Dx5 (DOX resistant counterpart cell line of MES-SA) and SKOV3 (drug resistant ovarian carcinoma) were obtained from America Type Culture Collection (Manassas, VA, USA).

5.3. Methods:

5.3.1 Preparation and characterization of silver nanoparticles:

Citrate-stabilized AgNPs were produced using a previously described method of reducing a boiling solution of silver nitrate in the presence of trisodium citrate (Lee 1982). Briefly, 100 ml of 1 mM AgNO₃ aqueous solution was boiled to 100 °C, and then 7 ml of 1% trisodium citrate solution were added. The mixture was kept at constant temperature of 100 °C for about 1 hour and then was allowed to cool down to room temperature. The resultant colloidal mixture was of dark grey color. The AgNPs were characterized for their size through UV-visible spectroscopy using a Cary UV Spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Size was confirmed by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK), which was used to measure zeta potential.

5.3.2. Preparation of Folate and DOX conjugates:

5.3.2.1 Folate-PEG conjugate (hereafter referred to as FP conjugate):

Folate-PEG was synthesized via amide bond formation. Briefly, folic acid was reacted with DCC and NHS in anhydrous DMSO in molar ratio 1:1:1.2; Folic acid/ DCC/NHS.

This process helped activate carboxyl groups in folic acid. Subsequently, SH-PEG-NH₂ was added to the reaction mixture and the mixture was incubated overnight at room temperature. This resulted in the synthesis of folate-PEG by formation of an amide bond between folate and SH-PEG-NH₂. The product was dialyzed against water (MWCO: 1kDa) and retrieved through lyophilization.

5.3.2.2 DOX-hyd-PEG conjugate (hereafter referred to as DH conjugate):

Synthesis of DOX-hyd-PEG was done in three steps. In the first step, SH-PEG-COOH was conjugated with hydrazine hydrate to yield SH-PEG-hydrazide as follows: SH-PEG-COOH was dissolved in dichloromethane and reacted with hydrazine monohydrate in a molar ratio of 1:10. The resultant solution was allowed to react for 24h at room temperature and later washed with brine solution. The organic phase was collected and rotavaporized to yield SH-PEG-hydrazide. In the second step of the synthesis, SH-PEG hydrazide and DOX were reacted in an equimolar ratio in anhydrous methanol. Finally, the mixture was reacted in the presence of a drop of trifluoroacetic acid in 60 °C overnight. After solvent removal, the residue was dissolved in water and further dialyzed against water for 24 h (MWCO: 1kDa, Spectrum Labs).

5.3.2.3 DOX-cbm-PEG conjugate (hereafter referred to as DC conjugate):

DOX-cbm-PEG was synthesized via an amide bond and used as a control. Briefly, SH-PEG-COOH was reacted at room temperature overnight with triethylamine, DCC and DOX in dichloromethane. The resultant product was dialyzed against water (MWCO: 1kDa). Later, the purified conjugate was lyophilized.

5.3.2.4 Characterization of Folate and DOX conjugates:

All three conjugates were characterized through UV-visible spectroscopy and ^1H -NMR spectroscopy.

UV-visible absorption data was obtained for each of the polymer conjugates (FP, DH, and DC). Conjugate solutions were prepared in appropriate solvents (DMSO for FP and methanol for the DOX conjugates) and placed in 1.5 mL cuvettes. Samples were diluted to the linear absorption range (< 3 A.U.), and absorption spectra from 200 to 900 nm were recorded using a Cary Win UV spectrophotometer (Varian/Agilent Technologies, Switzerland). Automatic blank correction was performed using the corresponding solvent (DMSO or methanol). An approximate spectrophotometer warm up time of 30 min before measurements was kept constant for all experiments.

^1H -NMR measurements were done on samples of FP, DH and DC all dissolved in deuterated DMSO. The ^1H NMR spectra were recorded on a 400 MHz Avance Bruker NMR spectrometer using 240 scans. Acquired data was processed and analyzed using MestReNova software. Chemical shifts were reported in parts per million (ppm) for ^1H NMR on δ scale based on the dimethyl sulfoxide- d_6 solvent peak ($\delta = 2.5$ ppm) as an internal standard.

5.3.2.5. Preparation of polymer conjugate-self assembled AgNPs:

All the conjugates were solubilized in DMSO (1mg/ml). Equimolar concentrations of FP/DH and FP/DC (corresponding to 1mg total) were respectively added onto 1 mg of citrate-stabilized AgNPs in 10 ml ultrapure water, and allowed to stir for 2h at room temperature in the dark. Thereafter, the nanoparticles were centrifuged at a speed of 12500 rpm for 15 min to remove free polymer conjugates, and washed once with Milli-Q water. The nanoparticles were designated as FHAgNPs (those functionalized with FP/DH) and FCaAgNPs (those functionalized with FP/DC), respectively. The nanoparticles were characterized for size through UV-visible spectroscopy, Dynamic Light Scattering, and Transmission Electron Microscopy; for charge through zeta potential measurements; and for self- assembly of polymer conjugates through Surface-Enhanced Raman Spectroscopy (SERS).

5.3.2.6 Effect of pH on polymer self-assembled AgNPs:

Self-assembled FHAgNPs and FCaAgNPs were incubated with PBS (0.01 M) at pH 7.4 and 5.0 respectively for 2h, 12h and 24h incubation times. The effect of pH on the polymer self-assembled AgNPs was analyzed through SERS.

5.3.2.7. *In vitro* uptake of polymer self-assembled AgNPs:

The concentration of silver in the silver colloids and the uptake of AgNPs in cancer cells was also estimated using Inductively Coupled Plasma- Optical Emission Spectroscopy (ICP-OES). In order to estimate the silver content in cells, cells were deproteinized by a

wet ashing procedure as described by Yong et al (Yong 2006). Briefly, FHAgNPs, FCAgNPs, FAgNPs (AgNPs self-assembled with FP but without DOX) and AgNPs (bare AgNPs) were incubated for 24 h with MES-SA, MES-SA/Dx5, SKOV3 and L1210 cells (at a cell density of 100,000 cells/ ml) at a final concentration of 32.5 ppb. MES-SA and MES-SA/Dx5 are cell lines that do not overexpress folate receptor (i.e., are FR-), and SKOV3 and L1210 are cell lines that overexpress folate receptor (i.e, are FR+) (Spinella 1995; Zhang 2004). Thus, MES-SA and MES-SA/Dx5 serve as negative controls and SKOV3 and L1210 cells serve as positive controls. The cells were then lysed and a portion of the supernatant was utilized to measure the protein content of the cells, whereas the rest was utilized to measure the respective silver content. The *in vitro* uptake of nanoparticles was also assessed through Raman spectroscopy and fluorescence microscopy.

5.3.2.8 SERS instrumentation and measurement:

A PerkinElmer Raman microscope (Raman Micro 300) was used to acquire SERS spectra. The system uses a diode laser (785 nm) with an average power of 100 mW at the sample and 100- micron spot size. The spectra obtained were processed using built-in software. All spectra were collected using a total laser exposure of 5-15 sec (4 acquisitions each of 1-3 seconds each). Briefly, a drop of sample was placed on a pre-cleaned glass slide and focused using a 20x objective. The spectra were obtained from different locations of the drop (10 different locations were used to acquire each spectrum). To acquire intracellular SERS data, SKOV3 and MES-SA cells (which

served as positive and negative control respectively for folate targeting) were seeded at a cell density of 100,000 cells/ ml onto quartz coverslips coated with poly-lysine. Different cells were focused on using a 50x objective to acquire the SERS signal, and 22 different cells from the same batch were used to acquire the spectrum. All experiments were repeated three times (n=3).

For fluorescent images, MES-SA and SKOV-3 cells were seeded onto 12 mm coverslips in petri dishes with cell density of 100,000 cells/ml and allowed to adhere for 24 h. Cells were washed twice with PBS, and then incubated with free doxorubicin, FCAgNPs or FHAgNPs at equivalent doxorubicin concentration of 4 μ M in complete RPMI 1640 medium at 37 °C for 24h. Cells were washed twice with ice-cold PBS and fixed with fresh 4% paraformaldehyde for 15 min at room temperature. The cells were counterstained with Hoechst 3322 stain for the cell nucleus following the manufacturer's instructions. The coverslips were mounted on glass microscope slides with a drop of antifade mounting media (Life technologies Co., USA) to reduce fluorescence photobleaching. The intracellular fluorescence localization was visualized under a Delta Vision Deconvolution Microscope (GE Healthcare, Inc., USA). The image ratios of the acquired fluorescence images were calculated by processing them with MATLAB® (The MathWorks Inc., Natick, MA, USA). Briefly, the intensity of each pixel was background subtracted and a region of interest was defined as comprising of pixels with above-background intensity. The ratio was further determined by normalizing the total pixel intensity of this region of interest to its area.

5.3.2.9 *In vitro* toxicity of polymer self-assembled AgNPs:

Cell viability was measured using a Sulforhodamine B (SRB) assay (Invitrogen, Carlsbad, CA, USA). This assay is used to measure cellular protein after drug treatment (Monks 1991). In this study, FC AgNPs, FHAgNPs, and bare AgNPs (corresponding to a final silver concentration of 32.5 ppb and a final concentration of 4 μ M DOX for the conjugates) were incubated with MES-SA, MES-SA/Dx5 and SKOV-3 cells for 24 hours to evaluate their toxicity. The toxicity assessment procedure has already been described in detail in our previous report (Tang 2009). Mean absorbance was calculated from four wells corresponding to the same treatment and cell line. Mean ($\pm$ S.D.) was determined from three different experiments. Cell growth fractions were measured by normalizing data corresponding to each treatment to their respective control.

AgNPs have been shown to absorb NIR radiation and mediate toxicity through induction of reactive oxygen species (ROS) (Corato 2012). In this study, the cytotoxicity of six different treatments (AgNPs with and without laser treatment, FC AgNPs with and without laser treatment, FHAgNPs with and without laser treatment) were investigated. Cells were seeded onto a 96-well plate on the first day, and after overnight incubation were exposed to different treatments (each corresponding to a silver concentration of 32.5 ppb and an equivalent DOX concentration of 4 μ M for self-assembled nanoparticles).

Cell viability 24h post-treatment was measured colorimetrically with an SRB assay which reports total protein. Average cell growth fractions were obtained from four wells

for each treatment (n=3). The data were acquired and represented in the same manner as the toxicity of the formulations discussed in the previous paragraph.

5.3.2.10 Mechanism of toxicity of polymer self-assembled AgNPs

5.3.2.10.1 Ag⁺ release from self-assembled polymer silver conjugate nanoparticles with and without laser:

AgNPs and self-assembled AgNPs (FHAgNPs and FCAgNPs) were centrifuged and the supernatant was collected. To assess silver ion release after laser treatment, the nanoparticles were exposed to an 808-nm laser at fluence rate of 6.7 W/ cm² for 3 min. After laser treatment, the nanoparticles were centrifuged and the supernatant fractions were collected. Silver ion release from each of the groups (AgNPs with and without laser treatment, and self-assembled nanoparticles with and without laser treatment) was measured by assessing the silver content of the respective treatment groups. Silver ion release was reported as dissolution percentage, which is the silver ion released normalized to the initial silver content of the nanoparticles.

5.3.2.10.2. Intracellular ROS:

Oxidative stress has been reported as one of the critical mechanisms of cytotoxicity induced by AgNPs (Foldbjerg 2009). To measure the intracellular generation of reactive oxygen species (ROS), MES-SA, MES-SA/Dx5 and SKOV-3 cells were seeded onto glass-bottom 96-well black well plates. After 24 h growth, the cells were treated with FCAgNPs, FHAgNPs and AgNPs with and without laser treatment. The cells were

further loaded with 10 μ M fluorescent marker 2', 7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA, Sigma, US) in RPMI 1640 for 30 min, then washed with PBS, and immediately used for fluorescence measurements and re-suspended in RPMI 1640 without phenol red. All experiments were conducted in the dark. A 488-nm laser was used for excitation and fluorescence was detected by a 525/30 nm filter (for H₂DCF-DA fluorescence). For each sample the mean fluorescence intensity was determined and further normalized to the respective cell growth fraction measured by SRB assay in order to determine intracellular production of ROS.

5.3.2.10.3. Intracellular GSH and thiol levels:

MES-SA, MES-SA/ Dx5 and SKOV-3 cells were seeded at a density of 100,000 cells/ml. These cells were exposed to AgNPs, FCaAgNPs and FHAgNPs at concentration of 32.5 ppb, and then lysed and assessed for intracellular glutathione (GSH) and thiol concentrations using a GSH assay. Levels were normalized to control cells that were not subjected to any treatment.

5.4 Results and Discussion:

5.4.1. Preparation of AgNPs:

AgNPs were synthesized using a method involving reduction of a silver salt in the presence of a reducing agent, citric acid. The formation of AgNPs was confirmed by UV-visible spectroscopy. These nanoparticles exhibit maximum surface plasmon absorbance at 435 nm (Bhardwaj 2013), and have a size of 70 nm determined through

TEM and charge of -40 mV. The nanoparticle size determined through TEM was consistent with that observed through DLS as seen in Figure 18. A small proportion of rods was seen, which is consistent with the literature (Faulds 2005). The negative charge of the AgNPs could be due to the presence of citrate molecules on the nanoparticle surface, again consistent with literature reports (Wang 2014).

5.4.2. Preparation and characterization of polymer conjugates:

Folic acid and DOX were conjugated to heterobifunctional PEG with sulfhydryl as an end group. Folate-PEG (FP) was synthesized by forming an amide bond between the α -carboxylate group and the amine group of PEG. The α -carbon of folic acid was chosen for the reaction because it does not form a paratope to the folate receptor, and hence could be successfully used for conjugation without harming the targeting potential of the ligand (Chen 2013).

Figure 18. Size of AgNPs, FCAGNPs and FHAGNPs measured through TEM (left; labeled A, C, and E respectively, with slight PEG coating seen in C and E), and DLS (right; labeled B, D, and F] measurements. The scales for TEM images are 100, 100 and 50 nm respectively. The size of FHAGNPs, FCAGNPs and AgNPs estimated by DLS and TEM are consistent with an average size of 70 nm. A slight proportion of rods is seen in the TEM image of AgNPs (A).

Two different DOX-PEG conjugates were synthesized through an amide and hydrazone bond, referred to as DOX-cbm-PEG (DC) and DOX-hyd-PEG (DH) respectively. FP exhibited $\lambda_{\max}$ values of 290 nm and 363 nm corresponding to folic acid in the UV-vis spectrum (Figure 19A), the extent of folate conjugation onto the polymer was detected using UV-visible spectroscopy. DC and DH exhibited $\lambda_{\max}$ of 480 nm in the UV-vis spectrum, corresponding to DOX (Figure 19B), and their fluorescence spectra were consistent with the presence of DOX (Figure 19 C). FP, DC and DH conjugates were also characterized by ^1H -NMR (Figure 20), using a Varian 400 Hz spectrometer. The

¹H- NMR spectrum of DOX-cbm-PEG and DOX-hyd-PEG in DMSO-d⁶ showed characteristic DOX peaks at 7.9, 7.6, 5.3, 4.58, 4.0 ppm and characteristic PEG peak at 3.6 ppm. The ¹H- NMR spectrum of Fol-cbm-PEG in DMSO-d⁶ showed characteristic Folic acid peaks at 8.6, 7.7, 6.6, 4.5 and 4.3 ppm and characteristic PEG peak at 3.6 ppm. Conjugate yield was 72% (FP), 84% (DC), and 72% (DH). The DOX content in DH and DC was estimated by breaking the amide and hydrazone bond in 1N HCl and estimating the amount of drug in the supernatant. DOX content was estimated to be 5.5% w/w in DC and 8% w/w in DH. Huan and coworkers obtained similar DOX content in PEG-hyd-DOX and PEG-cbm-DOX (Huan 2012). The reaction schemes of FP, DC and DH can be seen in Figure 21. The characteristics of FP, DC and DH are tabulated in Table 2.

5.4.3. Preparation and characterization of polymer conjugate-self assembled AgNPs:

Folate-PEG and DOX-PEG conjugates were self-assembled onto AgNPs through the sulfhydryl bond of heterobifunctional PEG. FH and FC nanoparticles had FP/DH and FP/DC conjugates self-assembled onto the nanoparticle surface in equimolar ratios. Self-assembly was allowed so that the nanoparticles would retain their targeting abilities and at the same time have the active agents to exert synergistic chemo-photodynamic therapy.

Figure 19. (A) UV-visible absorption spectra of FA-PEG conjugate (FP) and FA; (B) UV-visible spectra of DOX and DOX conjugates DOX-hyd-PEG (DH) and DOX-cbm-PEG (DC); (C) Fluorescence spectra of DOX and DOX conjugates.

The average sizes of FCaGNPs and FHAgNPs were measured to be 74 and 78 nm from DLS. TEM images are consistent with DLS data. Our results align with those of Shkilnyy and co-workers, who prepared similar PEGylated silver colloids of size 70 nm (Shkilnyy 2009). FH and FC nanoparticles exhibited a slight blue shift of 1 nm in comparison to that of citrate stabilized silver colloids. This blue shift in the spectrum suggests no self-aggregation of FH and FC nanoparticles, and indicates successful

conjugation of FP/DH and FP/DC to the surface of AgNPs (Prabaharan 2009,; Patra 2010). The self-assembly of polymer conjugates was further confirmed using Surface-Enhanced Raman Spectroscopy as can be seen in Figures 22A and 22B and their respective band assignments in Tables 3 and 4.

Figure 20. (A) ^1H -NMR of Fol-cbm-PEG, FP. (B) ^1H -NMR of DOX-cbm-PEG, DC. (C) ^1H -NMR of DOX-hyd-PEG, DH

Figure 21. Reaction schemes for preparation of (A) Folate-cbm-PEG, FP; (B) DOX-cbm-PEG, DC; and (C) DOX-hyd-PEG, DH.

Polymer-conjugate	UV-vis characterization	¹ H-NMR Characterization	Fluorescence
Fol-cbm-PEG (FP)	Absmax@ λ =280 nm, consistent with that of folic acid	8.65 , 7.62 , 6.64 , 4.49 and 4.29 ppm correspond to FA, 3.6 ppm to PEG	N/A
DOX-cbm-PEG (DC)	DOX	7.61, 5.42 and 4.51 ppm correspond to DOX, 3.6 ppm correspond to PEG	λ_{ex} (480–490 nm), λ_{em} ($\geq$ 515 nm)
DOX-hyd-PEG (DH)	Absmax@ λ =480 nm, consistent with that of DOX	7.61 ,5.42 and 4.51 ppm corresponds to DOX, 3.6 ppm corresponds to PEG	λ_{ex} (480–490 nm), λ_{em} ($\geq$ 515 nm)

Table 2: Characterization of Fol-cbm-PEG (FP), DOX-cbm-PEG (DC) and DOX-hyd-PEG (DH)

Figure 22. SERS spectra. (A): Comparison of SERS spectra of FAgNPs (Folate-PEG self-assembled AgNPs), FC AgNPs (Folate-PEG and DOX-cbm-PEG self-assembled AgNPs) and DC AgNPs (DOX-cbm-PEG self-assembled AgNPs). SERS band assignments are in Table 2. (B): Comparison of SERS spectra of FAgNPs (Folate-PEG self-assembled AgNPs), FH AgNPs (Folate-PEG and DOX-hyd-PEG self-assembled AgNPs) and DH AgNPs (DOX-hyd-PEG self-assembled AgNPs). SERS band assignments are in Table 3. (C) and (D): SERS spectra of FC self-assembled AgNPs (C) or FH self-assembled AgNPs (D) incubated with PBS (0.01M, pH 5.0) incubated for different time intervals - 2h, 12h and 24 h. (E) and (F): Comparison of FC self-assembled AgNPs (E) or FH self-assembled silver nanoparticles (F) at different pH with intracellular data (incubation time: 24 h).

The SERS features of FHAgNPs were compared with those of FAgNPs and DHAgNPs (self-assembled with DH); and the SERS features of FCAgNPs were compared with those of FAgNPs and DCAgNPs (AgNPs self-assembled with DC). The presence of a band at 240 cm^{-1} corresponds to the formation of Ag-S via cleavage of thiol bonds, further affirming successful conjugation of polymer conjugates onto AgNPs (Joo 1987). Thus, it can be concluded that there is successful self-assembly of the polymer conjugates onto the nanoparticle surface. This feature also allows us to probe the intracellular fate of these nanoparticles in a label-free manner.

5.4.4. Effect of pH on polymer self-assembled AgNPs:

FH AgNPs and FC AgNPs were incubated in two buffer solutions with different pH (PBS buffer with pH 7.4 and pH 5.0). A buffer with pH 5.0 was used because it emulates the pH of acidic endo-lysosomal compartments inside the cell. FH exhibited a time-dependent reduction in the peak corresponding to DOX at 1452 cm^{-1} when exposed to low pH 5.0 (Yan 1997). FC, on the other hand, retained the DOX peak over time (See Figures 22C and 22D). The supernatant obtained after exposing FHAgNPs to pH 5.0 in a time-dependent manner showed increasing concentrations of DOX. This difference in behavior could be attributed to the way the DOX is tethered to the two types of nanoparticles. In FCAgNPs, DOX is bonded to the nanoparticles through a strong amide bond, whereas in FHAgNPs, DOX is tethered through an acid-labile hydrazone bond. This method of pH-triggered release of drug from nanoparticles has been reported by several other researchers (Wang 2011 ; Huan 2012). An amide bond is a relatively

strong bond which requires extreme pH environments (pH 1-2) to break. A hydrazone bond, on the other hand, takes up the free H^+ ions in media having low pH, and in the process it releases free DOX, converting DOX-hydrazone-PEG back to its original PEG-hydrazide form (Yoona 2010).

FAGNPs	DCAgNPs	FCAgNPs	Band Assignment	Molecule
1620		1620	C=C stretch, C=O stretch	Folic acid (FA)
1582		1582	Ring stretch (aromatic ring)	Folic acid (FA)
1495		1495	NH ₂ bend and wagging	Folic acid (FA)
1471		1471	ν_{ring}	Folic acid (FA)
1452	1453	1455	CH ₂ deformation	Doxorubicin (DOX) and Folic acid (FA)
	1428	1428	($\delta_{as}(CH_3)$)	Doxorubicin (DOX)
1396	1394	1396	CH rocking vibration, CH ₂ deformation	Polyethylene glycol (PEG)
	1316	1316	CH ₃ bending	Doxorubicin (DOX)
1234	1234	1234	C-O stretch, $\nu(C-C)$	Polyethylene glycol (PEG)
1175		1175	CH ₂ rocking vibrations	Folic acid (FA)
1130	1129	1126	Trans C-C stretch	Polyethylene glycol (PEG)
1034	1034	1034	$\delta(C-H)$, $\nu(C-O)$ stretch	Polyethylene glycol (PEG)
855	855	855	$\nu(C-C)$ stretch	Polyethylene glycol (PEG)
690		690	C-H vibrations	Folic acid (FA)
560	560	560	C-H torsion	Polyethylene glycol (PEG)

Table 3. Band assignment of spectra of FAGNPs, FCAgNPs and DCAgNPs

FAGNPs	DHAGNPs	FHAGNPs	Band Assignment	Molecule
1620		1620	C=C stretch, C=O stretch	Folic acid (FA)
1582		1582	Ring stretch (aromatic ring)	Folic acid (FA)
1495		1495	NH ₂ bend and wagging	Folic acid (FA)
1471		1471	ν_{ring}	Folic acid (FA)
1452	1453	1455	CH ₂ deformation	Doxorubicin (DOX) and Folic acid (FA)
	1428	1428	($\delta_{\text{as}}(\text{CH}_3)$)	Doxorubicin (DOX)
1396	1394	1396	CH rocking vibration, CH ₂ deformation	Polyethylene glycol (PEG)
	1316	1316	CH ₃ bending	Doxorubicin (DOX)
1234	1234	1234	C-O stretch, $\nu(\text{C-C})$	Polyethylene glycol (PEG)
1175		1175	CH ₂ rocking vibrations	Folic acid (FA)
1130	1129	1126	Trans C-C stretch	Polyethylene glycol (PEG)
1034	1034	1034	$\delta(\text{C-H})$, $\nu(\text{C-O})$ stretch	Polyethylene glycol (PEG)
855	855	855	$\nu(\text{C-C})$ stretch	Polyethylene glycol (PEG)
690		690	C-H vibrations	Folic acid (FA)
560	560	560	C-H torsion	Polyethylene glycol (PEG)

Table 4: Band assignment of spectra of FAGNPs, FHAGNPs, and DHAGNPs

5.4.5. *In vitro* uptake of polymer self-assembled AgNPs:

The internalization of FHAgNPs, FCAgNPs and bare AgNPs was assessed by measuring the silver content inside the cells through ICP-OES. The amount of AgNPs internalized was further normalized to protein content of the cells. The results are shown in Figure 23.

Figure 23. 24-hour intracellular uptake of AgNPs and folate-targeted AgNPs (FHAgNPs and FCAgNPs) in MES-SA, MES-SA/ Dx5, SKOV-3 and L1210 cells; n=3 experiments, 3 wells per treatment. * p <0.05 (by t-test) between folate-targeted AgNPs and non-targeted AgNPs in positive control cells SKOV-3 and L1210 cells, indicating significant differences due to folate conjugation onto AgNPs.

The folate-targeted nanoparticles (FHAgNPs and FCAgNPs) demonstrated significantly higher cellular uptake than their non-targeted counterparts in SKOV3 and L1210 cells (Folate Receptor positive, FR+) compared to MES-SA and MES-SA/ Dx5 cells (FR-). This significant increase in uptake of folate-targeted nanoparticles could be attributed to receptor-mediated endocytosis in FR+ cell lines (Sabharanjak 2004). However, there was no significant difference in the internalization of FHAgNPs and FCAgNPs, suggesting a similar endocytosis-based internalization of both types of nanoparticles. Thus, the presence of DH and DC along with FP on FHAgNPs and FCAgNPs respectively did not

alter the receptor-mediated endocytosis of the nanoparticles. These results are consistent with Wang et al (Wang 2011).

The similar uptake of both FH and FC nanoparticles in FR+ cells after 24 h incubation suggests different mechanisms for their different intracellular fates. SERS spectra (Figure 22F) and fluorescence images (Figure 24) demonstrate that DOX can be released from FHAgNPs in response to the intracellular acidic micro-environments after 24 h incubation. Cellular entry through the acidic endo-lysosome compartment is the prerequisite for the cleavage of the hydrazone bond, which results in DOX release. Figure 22F shows SERS spectra acquired from FHAgNPs incubated with SKOV-3 for 24 h. The SERS spectra demonstrate an absence of a DOX peak at 1452 cm^{-1} , consistent with SERS spectra of FHAgNPs incubated in PBS at pH 5.0. The SERS spectral results are consistent with the fluorescence images wherein FH nanoparticles exhibit higher intracellular fluorescence than FC nanoparticles. Further, the SERS spectra of FC nanoparticles in SKOV-3 incubated for 24 h retained the DOX peaks (Figure 22E), and these results are again consistent with the fluorescence images of FC in SKOV-3 (Figure 24). The reductive environment of cells (i.e. high intracellular thiol concentration) may mediate release of the self-assembled polymers from nanoparticles, and the Ag-S bond linking the polymer conjugate to AgNPs is more stable than the hydrazone linkage. Further, the reductive environment of the cells is predominant only in the cytoplasm and not in the acidic compartments, thus asserting the fact that the predominant factor affecting drug release from FH nanoparticles was intracellular pH. As a result, intracellular fluorescence was found to be higher in SKOV-3 cells incubated with FH

nanoparticles than in those incubated with FC nanoparticles. Intracellular fluorescence was predominantly located in the perinuclear region of the cells. DOX mediates its toxicity by intercalating with DNA, and hence, intracellular release is a prerequisite for its cytotoxic action.

Figure 24. Subcellular localization of DOX in three different forms: FC AgNps, FH AgNps and free DOX. All images are merged pictures of the DOX channel (green stain) and Hoechst channel (blue nuclear stain). Images A, B and C show MES-SA cells exposed to FC AgNps, FH AgNps and free DOX respectively. (Their image ratios (R) corresponding to DOX fluorescence are 1.14, 1.39, and 5.98. Images D, E, and F show SKOV-3 cells exposed to FC AgNps, FH AgNps and free DOX respectively. Their image ratios (R) corresponding to DOX fluorescence are 1.29, 2.87, and 2.86.

SERS spectra can also be utilized to understand the biochemical changes that occur in the cells upon exposure to FH nanoparticles. The absence of bands around 1300 and 1060 cm^{-1} corresponding to phospholipid (Brauchle and 2013) and the appearance of protein bands around 1110 cm^{-1} in comparison to AgNPs (Bergholt 2011) in Figure 22F shows positive signs of apoptosis in SKOV-3 cells when incubated with FH nanoparticles. Band assignments for intracellular SERS data are tabulated in Table 5. The uptake of FC and

FH nanoparticles and free DOX in MES-SA (FR-) and SKOV3 (FR+) cells by fluorescence images is shown in Figure 24. The images show higher intracellular fluorescence from FH nanoparticles than from FC nanoparticles in both MES-SA and SKOV-3 cells (FR- and FR+ cells, respectively). The higher intracellular fluorescence from FH nanoparticles could be due to the release of DOX from FH through acid-labile hydrazone bonds in the acidic endo-lysosomal compartment in the cells. Fluorescence is mainly seen in the perinuclear space and less in the nucleus. These results are consistent with Wang et al, who reported a similar perinuclear distribution of DOX released from DOX-tethered gold nanoparticles through hydrazone bond after 24 h incubation (Wang 2011).

5.4.6 *In vitro* toxicity of polymer self-assembled AgNPs:

The above data demonstrated enhanced cellular drug accumulation of acid-responsive FH nanoparticles due to rapid release of tethered drug. FHAgNPs exhibited significantly higher toxicity than FCAgNPs and AgNPs in FR+ SKOV-3 and L1210 cells in comparison to their FR- counterparts (MES-SA and MES-SA/Dx5 cells). This could be due to higher uptake of FH nanoparticles through receptor-mediated endocytosis and respective higher release of DOX in FR+ cells. FCAgNPs, on the other hand, are not acid responsive and did not generate significantly different toxicity levels in comparison to their non-targeted counterparts in either FR+ or FR- cells (See Figure 25A).

Raman band (cm ⁻¹)	Band assignment	Depiction	Meaning
1580	Ring stretch (aromatic ring)	Corresponds to FH AgNPs and FC AgNPs outside and inside cells	
1453	($\delta_{as}(\text{CH}_3)$)	DOX peak absent in FH AgNPs and present in FC AgNPs inside cells	Depicts intracellular DOX release in case of FH AgNPs
1130	Trans C-C stretch	Corresponds to FH AgNPs and FC AgNPs outside and inside cells	Positive sign of uptake
850	$\nu(\text{C-C})$ stretch	Corresponds to FH AgNPs and FC AgNPs outside and inside cells	Positive sign of uptake
1300	Phospholipid band	Decreased in spectra of FH AgNPs and FC AgNPs compared to that of AgNPs inside cells	Shows positive signs of apoptosis
1160	DNA band	Decreased in spectra of FH AgNPs and FC AgNPs compared to that of AgNPs inside cells	Shows positive signs of apoptosis
1060	Phospholipid band	Decreased in spectra of FH AgNPs and FC AgNPs compared to that of AgNPs inside cells	Shows positive signs of apoptosis
1110	Amide III	Increased in spectra of FH AgNPs compared to that of AgNPs inside cells	Shows positive signs of apoptosis

Table 5: Band assignment of intracellular SERS data of FH AgNPs (or FH) and FC AgNPs (or FC) in SKOV-3 cells

These results are consistent with Guo et al., who reported similar enhanced toxicity of FA-PECL-hyd-DOX nanoparticles in folate-overexpressing cancer cells in comparison to folate receptor negative cells (Guo 2013). After incubation with FHAgNPs, FCAgNPs, and AgNPs, cells were subjected to NIR laser to examine the photodynamic effect of nanoparticles on cells. FHAgNPs, when exposed to NIR laser (808 nm, fluence rate 6.7 W/cm²), enhanced the toxicity by a factor of two in comparison to bare AgNPs in L1210 and SKOV-3 (FR+) cell lines.

The negative controls exhibited comparable cytotoxicity after exposure to laser. The maximum plasmon absorption of AgNPs is at 435 nm; however, there is sufficient light absorption at much higher wavelengths (as in our case at 808 nm). This observation of light at higher wavelengths could be due to the close distance between individual nanoparticles that results in coupling of plasmons of adjoining nanoparticles.

Corato and coworkers reported similar toxicity of AgNPs embedded in magnetic beads upon NIR laser illumination (830 nm, fluence rate of 7.7 mW/μm², 5min) (Corato 2012). The fluence rate used in our experiment is several folds less than that used by Corato and coworkers. This helps in generation of cytotoxic levels of silver ions that can be synergistic along with the application of chemotherapy. Additionally, the NIR laser treatment alone at the fluence rate used in our experiments has shown no adverse effect on cells (Srinivasan 2014). Guo and coworkers also reported significantly lesser toxicity of FA-PECL-cbm-DOX nanoparticles in comparison to FA-PECL-hyd-DOX nanoparticles in both folate receptor overexpressing and receptor negative cell lines (Guo 2013).

Figure 25. (A) Cytotoxicity of nanoparticles (FCAgNPs, FHAgNPs, and AgNPs) with NIR laser exposure of 808 nm at a fluence rate of 6.7 W/cm². The total concentration of silver was 32.5 ppb. n = 3 experiments, 4 wells per treatment. * p < 0.05 (by ANOVA) between targeted and non-targeted nanoparticles, indicating significant cytotoxicity difference with and without laser exposure. (B) Silver ion dissolution of AgNPs and FHAgNPs with and without laser. *p < 0.05 indicates significant reduction of silver ion release from surface of FHAgNPs in comparison to AgNPs due to thiol capping. The thiol capping however, is not able to hinder the silver ion release from FHAgNPs upon exposure to NIR laser (6.7 W/cm², 3min). (C) and (D) Intracellular thiol levels (C) and GSH levels (D) after treatment with AgNPs, FCAgNPs, and FHAgNPs. *p < 0.05 indicates significant reduction in intracellular thiol or GSH levels between treatment groups FHAgNPs and AgNPs; and FHAgNPs and FCAgNPs. (E) Intracellular ROS production estimated using fluorescent dye CM-H₂DCFDA assay from MES-SA, Dx5 and SKOV-3 cells after different treatments (AgNPs, FCAgNPs (FC) and FHAgNPs (FH) each with and without laser treatment). *p < 0.05 indicates significantly different ROS production in SKOV-3 cells between treatment groups AgNPs and FHAgNPs with and without laser treatment.

Little intracellular release of free DOX is seen in FC incubated cells, which could possibly be through cleavage of amide bonds through intracellular enzymes. However, most of the DOX was intact in the nanoparticle form. Thus, the reduced release of DOX in cells incubated with FCAgNPs could be the possible reason for their reduced toxicity in comparison to FHAgNPs.

Chemotherapeutic drugs such as DOX are mostly limited in their application due to non-specificity to tumors. Thus, these molecules require a suitable delivery vehicle with unique properties such as small size, low toxicity, and ease of surface functionalization. Based on these desirable characteristics, AgNPs can serve as potential candidates for delivery of DOX. Further, employing pH-mediated release helps enhance intracellular delivery of the chemotherapeutic agent. It is worth noting that the PEG spacer could potentially prolong circulation and eventually increase tumor accumulation through the enhanced permeation and retention effect *in vivo*.

In summary, the acid-responsive behavior of both FH and FC nanoparticles was assessed both extracellularly and intracellularly through Raman and fluorescence spectroscopy. Further, we observed that pH-sensitive FH nanoparticles exhibit enhanced toxicity upon laser exposure, thus showing a synergistic chemo-photodynamic effect.

The mechanism behind the toxicity of FH and FC nanoparticles with and without laser exposure was assessed through three different methods as described below.

5.4.7. Ag^+ release from self-assembled polymer silver conjugate nanoparticles with and without laser:

The most widely reported cytotoxicity mechanism of AgNPs is the release of Ag^+ ions from its surface which results in the generation of reactive oxygen species (ROS) and depletion of intracellular thiol and glutathione (GSH) concentrations (Mukherjee 2014). We studied the release of Ag^+ ions from the surface of these nanoparticles under different conditions, in order to understand the toxicity mechanisms of self-assembled

nanoparticles (FHAgNPs) and citrate-stabilized (AgNPs) AgNPs. The release of metallic ions from the surface of metal nanoparticles depends on different factors such as capping agent, medium pH, incubation time, and incubation temperature, which further aid in induction of reactive oxygen species (ROS) (Liu 2010). AgNPs exhibited a silver ion release of 11% in cell-culture medium after 24 h incubation. Corato and coworkers showed similar levels of silver ion release (16%) from AgNPs in cell culture medium (Corato 2012). FHAgNPs exhibited a significant reduction in silver ion release in cell culture medium with the same incubation time. This reduction in silver ion release from the nanoparticle surface could be due to the presence of a thiol-ended polymer conjugate self-assembled onto the nanoparticle surface. Binding of silver to thiol compounds drive free silver ion (Ag^+) to very low concentrations (Pokhrel 2013). FHAgNPs and AgNPs exhibited similar silver ion release after NIR laser exposure (See Figure 25B). This can be explained if the laser at the given fluence would either dislodge the thiol-capping from the nanoparticle surface, or if silver ions were released through the pores of the polymer self-assembled onto the AgNPs. SERS-based experiments were conducted where the presence of thiol-capping on AgNPs was assessed before and after laser irradiation. The results showed that laser irradiation did not disrupt the Ag-S bond, as there was no change in Surface Enhanced Raman spectroscopy data after laser irradiation. Thus, pore-mediated release as discussed above seems to be a more feasible explanation, although it would need to be explored in further detail in the future.

5.4.8. Intracellular GSH and thiol levels:

Glutathione (GSH) and intracellular thiol molecules serve as major intracellular

antioxidant scavengers that protect cells from oxidative stress through binding with ROS. Thus, reduction of GSH-mediated antioxidant defense systems is critical for cell death (Sies 1999.). Better outcomes can be obtained by using therapeutic modalities that can enhance ROS production and deplete intracellular antioxidant defenses, rather than using just one of the methods (intracellular ROS generation or intracellular GSH depletion). Our AgNPs and silver conjugates have shown significant reduction of GSH and intracellular thiol concentrations in comparison to control cells with no treatment (see Figures 25C and 25D). FHAgNPs also exhibited a significant reduction of GSH and intracellular thiol concentrations in comparison to FCgNPs and AgNPs in FR+ cancer cells. The negative controls exhibited similar reductions of GSH and thiol concentration. These results are consistent with Piao et al and Mukherjee et al, who also reported similar intracellular glutathione depletion in cells subjected to silver nanoparticle treatment (Piao 2011; Mukherjee 2014).

5.4.9. Intracellular ROS:

Reactive oxygen species (ROS) are regularly generated in small amounts in all biological systems, and they are constantly eliminated from the system through anti-oxidant defense mechanisms. When generated in excessive amounts, ROS can induce DNA mutations that may result in the initiation of apoptosis leading to cell death (Mate 2000). Our experimental results shown in Figure 25E indicate that FHAgNPs exhibit significant ROS generation in comparison to both FCgNPs and AgNPs before and after laser irradiation in FR+ SKOV-3 cells. These results indicate the possibility of ROS being generated by DOX released from nanoparticles, as DOX has been investigated for its ROS generation

capacity (Gouaze 2001). The enhanced uptake of FHAgNPs and enhanced release of DOX in these cells could provide an explanation for the enhanced ROS production in FR+ cells. All AgNPs and conjugates exhibited higher ROS production after laser irradiation, due to the possible release of silver ions as discussed in section 5.3.7. The negative controls MES-SA and MES-SA/Dx5 did not exhibit significant ROS production from FHAgNPs, FCAgNPs and AgNPs before and after laser irradiation. The intracellular ROS data is hence consistent with the cytotoxicity and silver ion release data.

Conclusions:

We successfully prepared the polymer conjugates FP (folate-PEG conjugate by means of an amide bond), DH (DOX-PEG conjugate by means of an acid-labile hydrazine bond) and DC (DOX-PEG conjugated by means of an amide bond), and self-assembled these polymer conjugates onto AgNPs to form FHAgNPs FP and DH self-assembled onto AgNPs), and FCAgNPs (FP and DC self-assembled onto AgNPs). FHAgNPs and FCAgNPs exhibited specific targeting potential in SKOV-3 and L1210 cells that overexpress folate receptors. FHAgNPs exhibited pH-triggered drug release inside cells. These nanoparticles also exhibited enhanced toxicity upon laser exposure, thus showing great promise for combinatorial treatment employing both chemotherapy and photodynamic therapy. Future work will involve exploring their potential for simultaneous photodynamic and chemotherapy treatment *in vivo*.

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CHAPTER 6: OVERALL CONCLUSION, LIMITATIONS AND SCOPE FOR FUTURE WORK:

There is an increasing interest in the development of multifunctional agents for cancer management with the intent of improving diagnostics and therapy for cancer patients. Nanotechnology serves as a unique platform where both diagnostic and therapeutic agents can be combined into a single platform, in what has been termed a theranostic approach. Recent advances in the field of nanotechnology have led to increased interest in research and development of these theranostic agents, and on making them suitable for personalized medicine by modifying their size, charge, and other physicochemical properties. This method of delivering diagnostic and therapeutic agents simultaneously, including the subfield of image-guided therapy, can help to better diagnose and treat a heterogeneous disease such as cancer, with the potential to detect and eradicate tumor tissue in its earlier stages. These agents can be further tailored to specifically target cancer cells that overexpress particular receptors, thus allowing for targeted therapy.

It has been found that no one treatment modality can be utilized to treat cancer, and in a clinical scenario it is most common for two or more modalities to be used to completely eradicate a tumor. Phototherapy has been used as a mode of cancer therapy, serving as an adjuvant treatment to conventional treatment modalities such as chemotherapy and radiotherapy.

In this project, we chose to study two multifunctional nanoparticle systems for targeted combined chemo-phototherapy, and we also tried to understand the cellular response to these nanoparticles. The overarching goal of the project was to prepare multifunctional

nanoparticles for combined theranostics, as well as to understand the different mechanisms and effects that derive from utilization of different multifunctional designs. We also evaluated drug delivery strategies with different co-therapy types, by designing two nanoparticle formulations that contained DOX as a chemotherapy agent but where one formulation used photothermal therapy, and the other used photodynamic therapy as the combinatorial therapy. A complete understanding of how to apply these multifunctional systems in vivo requires the exploration of their cellular response, which would throw light on appropriate choices of treatment strategies, such as optimal timing for therapy.

In the chemotherapy-photothermal therapy approach, we prepared a multifunctional nanoparticle system by the simultaneous entrapment of a near-infrared light-activatable agent (ICG) and a chemotherapeutic drug (DOX) into polymeric nanoparticles. Several researchers have investigated the potential of multifunctional inorganic nanoparticles for targeted combined chemo-photothermal therapy, such as mesoporous silica encapsulated gold nanorods (Shen 2013), folic acid conjugated graphene oxide for chemo-photothermal therapy (Qin 2013), and hollow gold nanospheres entrapped with DOX (You 2010). However, polymeric biomaterials are preferable for drug delivery due to their biodegradability and ease of manipulation for efficient and controlled drug release (Nair 2007). PLGA is an FDA-approved polymer for drug delivery applications with several positive attributes such as ease of manipulation of monomer ratio or polymer weight, as well as biologically harmless metabolites (Danhier 2012), and hence was chosen for the study. These PLGA nanoparticles with entrapped ICG and DOX were

surface-decorated with anti-Human Epithelial Receptor-2 in order to specifically target the corresponding receptors overexpressed on cancer cells. Indeed, we observed an enhanced uptake of these nanoparticles in SKOV-3, which are cancer cells that overexpress these receptors. We also saw an enhanced cytotoxic effect of these nanoparticles upon laser irradiation (as ICG showed hyperthermia capabilities upon laser irradiation), which indicated that they have potential for targeted chemo-hyperthermia applications.

We further investigated the cellular response upon exposure of these nanoparticles to two different heat modalities: laser therapy and incubator hyperthermia. We chose the two different hyperthermia modes to mimic the approaches used in a clinical setting, i.e., localized and whole body hyperthermia. HSP70 (an anti-apoptotic protein) and caspase-3 (pro-apoptotic protein) were chosen to study their expression kinetics upon nanoparticle administration. These two proteins were used for the study because combinational therapy based on photothermal therapy has been shown to cause aggregation and denaturation of cellular proteins by affecting their folding. Intracellular protein synthesis is decreased in a dose-dependent manner upon exposure to temperatures between 42 and 45 °C. When cells undergo heat stress, there is an increase in expression of heat shock proteins. These are molecular chaperone proteins that increase cell survival by preventing the disaggregation of folded proteins and degrading damaged proteins (Hildebrandt 2002). However, when cellular damage goes over a threshold limit, heat shock protein expression becomes downregulated, and cells will undergo death by means of apoptosis or necrosis depending on the degree of thermal insult. Understanding the expression kinetics of apoptotic and anti-apoptotic proteins helps elucidate possible cellular fate and

mechanisms of death, and may also provide insights into future treatment targets that focus on key players in death pathways. .

In our study, we found that cells showed a faster heat shock response when exposed to nanoparticles and laser irradiation in comparison to exposure to nanoparticles and incubator hyperthermia. Based on our caspase-3 expression results, we also showed that cells underwent necrotic death upon exposure to nanoparticles and laser irradiation, versus apoptotic death upon exposure to nanoparticles and incubator hyperthermia. .

One of our overarching goals was to gain an insight into the differences between combinatorial chemotherapy-photothermal therapy and chemotherapy-photodynamic therapy. Therefore, we formulated nanoparticles that could exert a photodynamic effect as well as entrap DOX. For combinatorial chemotherapy-photodynamic therapy, metal nanoparticles such as gold and silver were an appropriate choice due to their unique optical properties and their ability to be utilized as drug delivery vehicles. We selected silver because it exhibited several positive attributes over gold such as higher extinction coefficients, sharper extinction bands, higher ratio of scattering to extinction, and extremely high field enhancements (Carlos Caro 2010). Gold nanoparticles have also been investigated for application in combined chemo-photodynamic therapy (Li 2015), however, silver nanoparticles have better photodynamic activity than gold nanoparticles (Vankayala 2013). Several researchers have also investigated the potential application of organic dyes as photosensitizers when used in conjunction with chemotherapy. For instance, Peng and coworkers investigated the potential of chlorin-core micelles with simultaneously entrapped hydrophobic drugs and photosensitizer SN-38 for combined

chemo-photodynamic therapy in a HT-29 human colon cancer xenograft model (Peng 2009), and Nahabedian and coworkers studied the interaction of the PDT agent hematoporphyrin derivative and DOX in a murine tumor model (Nahabedian 1988). However, organic dye-based photosensitizers suffer from the limitation of potential photobleaching.

Therefore, in the chemotherapy-photodynamic therapy approach, we created a different set of targeted multifunctional silver nanoparticles where the nanoparticle itself can serve as a phototherapeutic agent and would entrap the same chemotherapy agent, DOX. We functionalized the surface of AgNPs by attaching folic acid and DOX via a PEG spacer. Folic acid was attached to the PEG spacer through an amide bond, and DOX through a pH-sensitive bond. This design allowed for pH-triggered release of DOX. Silver nanoparticles exhibited a photodynamic effect upon laser exposure and showed enhanced cytotoxicity upon laser exposure, thus demonstrating potential for targeted chemo-photodynamic therapy applications. Silver nanoparticles are photosensitizers which affect the cellular redox balance. Loss of redox homeostasis has been implicated in tumorigenesis, with an imbalance between the cell's antioxidant defense system (such as glutathione or thiol) and the production of reactive oxygen species (Acharya 2010). Potentially, targeting redox status can provide a pathway to destroy cancer cells. Therefore, we also studied the cellular response of our multifunctional nanoparticles, which includes enhanced ROS production and reduction of intracellular thiol levels upon nanoparticle exposure. The rationale for investigating these markers was that combinational therapy using photodynamic therapy will affect the redox status of the cell.

Both of the formulations we created are novel approaches to combinatorial therapy which have potential for targeted cytotoxic effects, enhanced therapeutic effect brought about by the simultaneous application of chemotherapy and phototherapy on cancer cells, and theranostic capabilities that can be exploited for treatment monitoring. These nanoparticles serve as unique multifunctional platforms where more than one therapeutic modality can be used to treat tumors. This is certainly consistent with current clinical approaches to cancer management, which typically combine more than one treatment modality. The therapeutic combination of chemotherapy and phototherapy has been shown to possess synergistic effect (Tang 2009), which justifies the creation of multifunctional platforms where both these modalities can be delivered simultaneously to enhance outcomes. Our work can also help inform future researchers when developing new nanoparticle formulations or refining existing designs. Studying two different multifunctional nanoparticle formulations with different forms of combinational therapy enabled us to better understand cellular response to co-therapy, including the combined effects of chemotherapy and photothermal or photodynamic therapy. We were also able to investigate the different mechanisms underlying each of the two approaches in term of cell response to therapy, as well as their sensing capabilities for theranostic applications, and the potential advantages and limitations of each of the nanoformulations. These studies will also help in progressing towards *in vivo* biodistribution and efficacy testing, and also help researchers to understand the fundamentals of these novel platforms for further usage in conjunction with other treatment modalities

Every study suffers from some limitations and hence further research is generally required. Similarly, our study also has certain limitations that need to be kept in mind and improved by further research. The following limitations were observed:

- 1) In specific aim #1: PLGA nanoparticles were utilized to simultaneously entrap hydrophilic ICG and hydrophobic DOX. However, the resulting nanoparticle formulation resulted in faster release of ICG than DOX. Therefore, a formulation that can result in slower release of ICG (probably conjugating ICG through a labile bond to the polymeric matrix) will be more helpful and further research needs to be done in this regard.
- 2) In specific aim #2: Anti-apoptotic protein HSP70 and apoptotic protein caspase-3 expression kinetics were studied during laser-phototherapy-chemotherapy and incubator-phototherapy-chemotherapy. In future studies, molecular profiling using many more apoptotic and anti-apoptotic proteins (such as Bax or Bcl-2) should be employed to achieve a better understanding of the efficacy of the treatment. Further, the free drugs and nanoparticles should be investigated on *in vivo* tumor models, and the anti-apoptotic and apoptotic proteins should be retrieved from tissue sections after exposure to accurately measure protein expression *in vivo*.
- 3) In specific aim #3: The method of purification of polymer conjugates was dialysis. A more extensive purification of the polymer-conjugates needs to be done in order to obtain the active extract. The system also needs to be evaluated *in vivo* to assess its *in vivo* efficacy.

4) In general: Additional chemical modifications in the nanoparticle formulations could be used to facilitate endo-lysosomal escape, and to incorporate specific intracellular targeting potential based on the desired target organelle.

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APPENDICES

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PUBLICATIONS AND PRESENTATIONS (ABRIDGED LIST)

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