A Ternary Drug Delivery Complex to Target CD44 Over Expressing Cancerous Cell Lines

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the Kent State University Honors College
in partial fulfillment of
the requirements for University Honors

by
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Chapter I

Introduction

The purpose of this research was to develop a 3-part nanoparticle drug delivery system for the delivery of a small molecule drug to target cancer cell lines that over express the cell surface receptor CD44; a common trait seen in many tumor types. This strategy could potentially lower the number of harmful side effects seen in various chemotherapeutic agents that specifically target cellular division and growth. Such agents are also known to affect healthy, non-cancerous cells and organs that also rapidly divide such as: bone marrow, the digestive tract, and hair follicles. The agent’s attack on the normal cells result in the commonly known side effects of cancer therapy, which includes: hair loss, a suppressed immune system, and inflammation of the digestive tract. To prevent these side effects, the use of nanocarriers to deliver drug molecules to targeted tissue types is currently being investigated.

Due to recent advancements in the field of nanotechnology, the integration of nanoparticles and other nanocarriers with cancer research has been under close investigation. The proposed use of nanocarriers for cancer studies is to manipulate said nanocarriers to target and selectively deliver therapeutic drug molecules to cancerous cell lines while avoiding uptake of the drug by non-cancerous cell lines, thereby decreasing harmful side effects. Cell specific targeting would increase the accumulation
of a drug molecule in tumors over normal cells and potentially improve a previously toxic drug’s therapeutic index$^{1-3}$. Presently, there are already several FDA approved nanoparticle based cancer therapies on the market and in clinical trials. This list includes the clinically approved PEGylated liposome delivery system Doxil used to treat breast and ovarian cancer$^4$.

Generally, there are two mechanisms through which a drug can accumulate in a tumor through the use of a nanocarrier. The first is termed ‘passive targeting’; a process which allows the drug to be taken into a tumor through the enhanced permeability and retention (EPR) effect seen in the leaky vasculature of cancer cells$^{1,5}$. Leaky vasculature is a common hallmark seen in tumors and is thought to be caused by many contributing factors such as: a lack of barrier functionality caused by a missing endothelial monolayer, gaps within the tumor caused by loose interconnections and focal intracellular openings, and the general disorganization and shape irregularity that is exhibited in tumors$^{5-7}$. The second method of drug accumulation is known as ‘active targeting’. Here, nanocarriers are directed towards a target by the surface attachment of ligands that will target and bind to specific receptors expressed on the cell surface. Active targeting allows for delivery of the nanosystem to the targeted receptors that are over expressed on the cell line of interest$^{8,1}$. The research of this thesis aims to utilize
the latter form of drug accumulation in a tumor to effectively deliver a small drug molecule to a targeted cell line that over-expresses a particular cell surface receptor.

Many different aspects of a nanocarrier system could potentially affect both the stability of the nanosystem, its targeting efficiency, and biodistribution\textsuperscript{9,10}. Among other factors, variables such as nanocarrier’s size and charge, hydrophobicity of the system, type of polymer coating, targeting ligand type, and the ratio of targeting ligand to therapeutic drug per nanocarrier, could greatly affect the targeting efficiency and overall effectiveness of the system. However, it has been determined that the amount targeting ligand on a nanoparticle surface does not necessarily increase the targeting efficiency of the system due to possible steric hindrance between the multiple ligands which can inhibit binding with the specific receptor present on the targeted cell\textsuperscript{1,8,11}. Clearly, there are many complexities that arise when designing an active tumor targeting system. Many different methods examining the use of different targets, ligands, nanocarriers, and drug molecules are currently under investigation.

There are many different classes of proposed nanocarriers systems that could deliver a drug to a targeted tissue type; each possess their own benefits and concerns for use. These types of carriers include but are not limited to: elemental metallic nanoparticles including gold nanoparticles (GNPs), quantum dots, nanoshells, nanospheres, paramagnetic nanoparticles, micelles, polymers, liposomes, and carbon
nanotubes\textsuperscript{12}. This thesis investigates the use of spherical GNP as the carrier component for the targeting therapy for the large number of benefits that GNP exhibits over most other nanocarriers; these points will be discussed in full later.

Recent advancements in nanotechnology diagnosis have increased the study and interest of both super paramagnetic nanoparticles and quantum dots. Super paramagnetic nanoparticles are currently being investigated to utilize magnetic separation and magnetic targeting in order to act as nanocarriers for biomolecules to a target for imaging. These particles are made by either the precipitation of Iron Oxide or coating Iron Oxide with polymers\textsuperscript{13}. According to a recent study, super paramagnetic nanoparticles were used for hepatic tumor imaging since they were rapidly introduced into the cell after intravenous administration. These particles can then be imaged in the cell by MRI\textsuperscript{12,14}.

In a paper by Apopa et al, the use of Iron nanoparticles as a possible cancer-targeting tool was investigated. In this paper, super paramagnetic nanoparticles (Fe\textsubscript{2}O\textsubscript{3}) were used to induce an increase in cell permeability in human microvascular endothelial cells through the production of ROS and the stabilization of microtubules\textsuperscript{15}. In this report, 100-700nm Iron nanoparticles with a mean diameter of 298 nm were used to measure the cellular uptake and increasing endothelial cell permeability of particles by the use of confocal microscopy. Studies showed that 60\% of the nanoparticles were
taken up within 30 minutes and expelled out of the cells in one hour with only 10% of the nanoparticles remaining inside. The iron nanoparticle treatment was shown to induce cell permeability without inducing cytotoxicity by causing intracellular gaps within the epithelial microvasculature and by inducing microtubule remodeling caused by ROS. These effects functioned to cause the cell lines treated with iron nanoparticles to become permeable to therapeutic drug molecules.\(^\text{15}\)

The treatment with iron nanoparticles caused a long term disruption of the epithelial monolayer; however, it was discovered that exceedingly high dosages were needed to reach deep compartments in body.\(^\text{16}\) In comparison, GNPs do not induce a long time disruption of the cells.

Quantum dots have also been used for targeting specific cell types for imaging. Quantum dots are nanocrystals that are composed of an inorganic elemental core and a surrounding metal shell.\(^\text{12}\) Recent studies have shown that these coated nanocrystals can target specific tissues based on the type of antibody or nucleic acid present on the surface of the nanocarriers. In addition to targeting tissues, they can act as a fluorescent probe and biomolecule carrier (with potentiality for drug loading). The use of Quantum dots in humans, however, will require more investigation after recent reports of cytotoxicity due to the cadmium metal shell covering the nanocrystal when cadmium was
used\textsuperscript{12,17}. GNPs, in comparison, do not cause cytotoxicity and are in fact, biocompatible \textit{in vivo}\textsuperscript{(28)}.

The therapeutic value of nanometer sized mesoporous silica nanoparticles is also currently being investigated. These nanoparticles are comprised of a rigid assembly of silica channels, or pores, that are capable of absorbing small molecules, potentially small molecules of therapeutic drug agents\textsuperscript{18}. It was determined that different functionalized groups could be attached through grafting procedures and that the resulting nanocarriers could potentially deliver a hydrophobic anti-cancer drug to targeted tumorigenic cell lines. Research by Di Pasqua et al concluded that the functionalized silica particles did induce cytotoxicity however; the silica caused pulmonary toxicity and apoptosis, which was possibly generated by ROS. Again, research shows that GNPs do not elicit such a response when used in a system and could prove to be beneficial as a nanocarriers system\textsuperscript{(28)}.

Carbon based nanoparticles are another proposed group of nanocarriers; these particles treat cancer cell growth by absorbing different molecules to their surface and enter the cell by endocytosis. Cytotoxicity studies, with the use of various forms of carbon based nanocarriers such as carbon nanotubes, are currently under investigation which so far has shown that the carbon based nanomaterials inhibits cellular growth and cell death on treated tissue. This effect is increased when there are chemically
active functional groups attached to the carbon based nanocarriers. In a 2006 paper by Magrez et al, the cellular toxicity of carbon-based nanomaterials was studied due to the concern that carbon nanotube structure is closely related to asbestos, which causes lung cancer when inhaled.

In vivo studies were performed on lung tumor cells and showed that carbon based nanoparticles did indeed induce cytotoxic effects. These effects were seen by the morphological alteration of treated cells that possessed shrunken nuclei, cytoplasm restriction, and loss of anchorage to growth flask. These cellular responses cause irreversible cell injury, eventually resulting in cellular death. These studies also produced results that showed chemical modification of the carbon nanotubes caused an increase in cytotoxicity and concluded that further steps are needed to prevent cytotoxicity of the carbon based nanoparticles before they could be safe to use in clinical trials.

Liposomes have also been widely investigated to determine their potential to specifically target and deliver a drug to cancerous cell lines. Liposomes are artificial phospholipid vesicles made up of an aqueous interior surrounded by a lipid bilayer. Most do not elicit a biological response and are biocompatible and are rapidly removed from circulation. The use of liposomes is currently under heavy investigation for the use as a nanocarrier to deliver a therapeutic drug to tumors; the FDA has clinically approved
the use of such agent known as Doxil. However, due to localization of the liposomes in skin capillaries, many patients suffered from mucositis (painful inflammation and ulceration of the mucous membranes lining the digestive tract) in clinical trials of Doxil and further investigation of targeting needs to be investigated.

Gold Nanorods are a potential therapeutic nanoparticle that is more closely related to this research project. Gold nanorods are rod shaped GNPs that have a strong absorbance near infrared light and possess unique optical properties. In a paper by Atsushi et al, gold nanorods that naturally accumulated in the tumor were used as a therapeutic treatment, and the photothermal effect was used as a treatment by exhibiting the conversion of absorbed light energy into heat using near-infrared laser irradiation. This procedure was investigated as a therapeutic treatment. It was further discovered that hydrophobicity changes in response to the temperature change could be used for targeted drug delivery and release. The side effect to this type of treatment was that larger amounts of gold nanorods were found in non-targeted organs like the lungs, liver and spleen than in the targeted laser irradiated tumors.

Another group of nanocarriers that is currently under investigation is spherical GNPs. The four major uses for these GNP include labeling, molecule delivery, heating, and sensing. Typically, GNPs utilize passive targeting for labeling and imaging mechanisms. As previously mentioned, this mechanism does not alter any part of the
GNP and will accumulate in the tumor due to the EPR effect, characteristic of tumors. However, this project utilizes the active targeting capabilities of GNPs when attached to a ligand to actively target cancerous cell lines over non-cancerous cell lines.

For this project, spherical GNPs were selected as a suitable nanocarrier for a drug transportation system; they have been found to have many beneficial properties that make them suitable for this project. GNPs have low production costs and are relatively easy to synthesize and characterize due to their Surface Plasmon Resonance (SPR)\(^ {24} \). GNPs are amenable to simple surface chemistry, which allows for easy modification and functionalization of the surface\(^ {1} \). GNPs have been found to be internalized by cells via an endocytosis mechanism,\(^ {1,25-27} \) and currently, the most readily internalized size without targeting mechanisms is 50nm\(^ {28} \). Most importantly, GNPs are chemically inert and have proven to be non-toxic because of their biocompatibility and non-immunogenicity properties\(^ {28} \).

Synthesis of GNPs can include a large range of diameters from single nanometer sizes up to hundreds of nanometers. In order to control the size of the GNPs during synthesis, we will be using trisodium citrate in the citrate reduction method\(^ {29} \). In this method, the gold salt HAuCl\(_4\) was reduced by the reducing agent trisodium citrate; leading to the formation of gold ions into nanoparticles. Additionally, the size of the
synthesized GNP with this procedure was found to be inversely proportional to the concentration of trisodium citrate in the reaction.

10nm GNPs were used as the selected diameter for the nanocarrier system. This particular size was carefully chosen due to synthesizing issues that arose during characterization of the various nanoparticle sizes. It was found that GNPs ~1.4 nm in diameter readily entered the nucleus of a cell and bound to the DNA double helix with high affinity; this would disrupt normal cellular function, eventually leading to apoptosis. In a study by Pan et al, GNP with a diameter of ~1.4 nm triggered necrosis, mitochondrial damage, and oxidative stress on many normal cell lines. GNP would need to be at least 10 nm in diameter to avoid first pass elimination through kidneys, which would render the treatment to be ineffective. On the other hand, GNPs would also have to be smaller than 200 nm to avoid sequestration by sinusoids in the spleen and by the liver.

The citrate reduction methods also allows for the GNP to be able to be functionalized with thiolated molecules because of the strong interactions between the thiols and metallic gold surfaces. The addition of trisodium citrate effectively acts as a coating agent for the GNPs, providing colloidal stability because of its negative surface charge. The negative charge present on the nanoparticle due to the citrate coating
allows for the GNPs in solution to repel each other so that they can remain in suspension, avoiding particle aggregation.

GNPs are able to remain in suspension by their electrostatic repulsions that are created by the negative charge (δ⁻) on each particle’s surface. However, once these particles are in the presence of monovalent cations (such as that of a salt solution), the charge repulsion exhibited by the citrate coated GNPs is negated, causing the particles to aggregate and precipitate out of solution. Additionally, coating the GNPs with a hydrophilic polymer such as polyethylene glycol (PEG) or hyaluronic acid could be used to protect the GNP from aggregating.

Hyaluronic acid is a naturally occurring glycosaminoglycan; its structure is composed of an unbranched linear chains of repeating D-glucuronic acid and N-acetyl-D-glucosamine disaccharide units (Fig. 1). It is found in both the extracellular matrix, in synovial fluid of joints, and is biocompatible. Hyaluronic acid was used to deliver the chemotherapeutic drug molecule Taxol to cancer cells that expressed CD44 cell surface receptors.

In order to selectively deliver the drug to the cancerous cell lines over the non-cancerous one, the cell surface glycoprotein CD44 was targeted utilizing its function as a
receptor for hyaluronic acid. CD44 is an integral transmembrane cell-surface glycoprotein that is involved in cell-cell, cell-matrix interactions and regulating cell movement and transport of extracellular components. It also facilitates cell differentiation and seems to be associated with the malignant progression and metastasis of cancer. Certain tumors including the ovarian cell cancer lines A2008 and C-13 over-express the CD44 cell surface receptor, while it is found at low levels on epithelial, hematopoietic, and neuronal cell lines. CD44 receptors are also present on certain normal cell lines however, these cell types either do not have direct contact with the blood or require activation before they can sense a particular ligand, such as Hyaluronic Acid. In a report by Tzicis et al, the chemotaxis of a cell towards hyaluronan was investigated based on the cell’s CD44 expression. It was shown that certain cancer cell lines that over-expressed the CD44 receptor migrated in a directional movement towards the site of introduction of a hyaluronic acid molecule. However, the NIH-3T3 mouse fibroblasts did not migrate towards the hyaluronic acid stimulus, even when the cells were transfected with genes that encoded for CD44 receptors.

With the hyaluronic acid acting as a targeting ligand for the CD44 receptors, it is possible to specifically target cancerous cell lines that over-express the cell surface receptor while avoiding drug delivery to the normal, healthy tissues. In this present
study, hyaluronic acid will be used as the targeting ligand since the CD44 receptors readily bind to it.

According to Choi et al, hyaluronic acid nanoparticles were used as a drug carrier in an \textit{in vivo} study for cancer cells that over-expressed the CD44 receptor. In this study, the cell lines that over-expressed CD44 were found to efficiently take up the self-assembled hyaluronic nanoparticles, due to the fact that CD44 is a receptor for hyaluronic acid and that hyaluronic acid has a known binding affinity to the CD44 cell surface receptor\textsuperscript{33}. It was also found that the hyaluronic nanoparticles where endocytosed by and had the highest accumulation in the targeted tumor, with weak signals at both the heart and muscle cells\textsuperscript{35}. It was also found that the hyaluronic acid was slowly excreted from the body\textsuperscript{33}.

For a nanoparticle drug delivery system to be efficient, it must deliver a drug to a selected tissue type in adequate concentrations and also deliver that drug in its active state to cause cytotoxicity. This requires adequate amounts of the selected drug molecule present on the GNP, while still possessing necessary amounts of the targeting and particle protecting ligand, hyaluronic acid. The question of drug loading ability compared to targeting efficiency was addressed in a recent publication where it was determined that there was a limit on the amount of drug molecules present on a nanocarrier until the targeting of the CD44 receptors was greatly diminished\textsuperscript{29}. Thus it is
important to establish an optimum ratio of drug to ligand. For this project, the small therapeutic drug molecule, Doxorubicin (Dox), would be used as the therapeutic agent in accordance with the targeting ligand and GNP to efficiently deliver a drug to a targeted cell type.

For this project, we used a thiolated derivative of Dox, an Anthracycline antibiotic\textsuperscript{36} (Fig. 2). Dox is one of the most widely used anticancer drugs; however, the use of this drug is limited to a lifetime dosage amount that, if exceeded, will induce cardio toxic effects due to oxidative stress that could eventually result in death\textsuperscript{37}. In a paper by Pointon et al, it was determined through \textit{in vivo} studies that the major mechanism of Dox cardiotoxicity was because of damage to or the inhibition of the electron transport chain and with ATP loss. Anthracyclines also cause lipid peroxidation in heart muscle tissues, which leads to membrane damage. Transcriptional regulatory proteins that aid in regulation of cardiac specific genes are also susceptible to anthracyclines\textsuperscript{36}. These mechanisms of Dox induced cardiotoxicity lead to myofilament damage, apoptosis and necrosis of myocytes\textsuperscript{38}. In a 2003 paper, a study was performed...
on the cardiotoxicity of Dox which found that patients who received doses up to 700mg/m² of Dox had a 48% risk of congestive heart failure. The use of a targeted drug delivery system to deliver the Dox to the cancerous cells could prevent the cardiotoxicity of the free drug.

When Dox is introduced to a cell, it causes several necessary biochemical pathways in the cell to halt causing cellular death. The intercalation of DNA, leading to the inhibition of both DNA replication and RNA transcription is one of the majorly investigated aspects of cellular death by Dox. Other mechanisms for the Dox induced cytotoxicity in cancer cells include: generation of free radicals, DNA binding and alkylation, DNA cross linking, an interference with DNA unwinding, strand separation and helicase activity, membrane damage, and inhibition of topoisomerase II.

In a paper that is closely related to this project by Eliaz et al, liposome-encapsulated Dox was used to target tumors that over-expressed the cell surface receptor CD44. In this project, hyaluronic acid molecules were incorporated in the lipid bilayer of a phosphatidylethanolamine lipid that encapsulated Dox in order to target the B16F10 murine melanoma cell line which over-expressed CD44 receptors effectively delivered the Dox to the targeted cells. They concluded that their system was effective due to the fact that the HA-liposome delivered the drug to the targeted cancer cells at higher levels than it did to the CV-1 kidney control cell line.
Through the guidance from previous studies, the aim of this study is to create a 3-part nanoconjugate system that selectively targets and delivers a small drug molecule to cancerous cell lines that over-express CD44 receptors while simultaneously lowering the uptake by normal cell lines. This will potentially lower the amount of harmful side-effects seen in treatment with the free drug that is not tissue specific. Another major barrier of efficient targeting needs to be overcome, to avoid natural uptake and deposit of the GNPs into non-targeted tissues such as the liver and spleen; this could be alleviated by the targeting ligand hyaluronic acid.


Chapter II

Experimental Materials and Methods

Instrumentation

- Carey UV-Vis Spectroscopy for all UV read outs
- FV500 Confocal Microscope was used for confocal studies
- Synergy 2 Multi-Mode Microplate Reader was used for MTS assays and plate reading experiments.

II.A. Synthesis of GNP, HASH, and MPDOX

II.A.i GNP Size Dependency

The first step in this project was to synthesize and determine the various sizes of GNPs (3 nm, 10 nm, 20 nm, 30 nm, 75 nm, and 100 nm) that would be used for this project. In order to control the size of the GNPs during synthesis, trisodium citrate was used in the citrate reduction method. The 3nm GNP used a slightly modified procedure discovered in a previously cited paper\(^{40}\). GNP size was confirmed using UV-VIS spectroscopy to measure the peak in absorbance due to the plasmon resonance effect exhibited by the particles. Eventually, it was decided that 10 nm GNP would be used for all experiments.
II.A.ii **10 nm Gold Nanoparticle Synthesis**

The 10 nm GNP used in this project were synthesized by a previously established trisodium citrate reduction method using HAuCl₄ as the gold salt to be reduced. 800 mL of water and 20 mL of 10 nM HAuCl₄ were slowly heated in an oil bath to 115°C. Once the sample had reached a constant 115°C, 22.5 mL of 1% trisodium citrate was added. The solution was then allowed to reflux for an additional 20 minutes after the solution had transitioned from the once light purple color to a deep red hue. The product was then left to stand at room temperature overnight. For all experiments, the GNP size was confirmed using UV-VIS spectroscopy with an absorbance peak around 520 nm.

II.A.iii **Synthesis of 3.5-30kDa Hyaluronic Acid**

Two grams of hyaluronic acid (HA) was dissolved in 500 mL of phosphate buffered saline with a pH of 6.5. This was then treated with hyaluronidase (10 units/mg HA) for enzymatic digestion of the HA. The digestion was carried out at 37°C for 24 hours. The solution was heated to 95°C for 20 minutes to denature hyaluronidase. The reaction mixture was filtered through a nitrocellulose membrane and then purified by dialysis against water using a 3.5 kDa membrane for 96 hours. The dialyzed solution was
filtered using an amicon filter tube with a molecular weight cut off of 30 kDa.

After filtration, the solution was lyophilized for approximately 48 hours to provide a final product of hyaluronic acid with an approximate molecular weight of 3.5-30 kDa.

II.A.iv Synthesis of Thiolated Hyaluronic Acid

Synthesis of thiolated hyaluronic acid was performed using a modification of a previously published procedure\(^2\); the modified ligand that was used possessed a thiol group attached to the reducing end of the hyaluronic strand. In order to end thiolate the low molecular weight HA, 45 mg of 3.5-30 kDa HA was added to 60 mg (266 µmol) of cystamine. 30 mL of Borate buffered saline (PBS) with a pH of 8.5 was added and stirred for two hours at room temperature. 0.38 g of NaBH\(_3\)CN was added to the solution before it was incubated at 37˚C overnight. In order to break the disulfide bonds present from the cystamine, a 10 molar equivalent of DTT with respect to the amount of cystamine was added and allowed to incubate for 24 hours at 37˚C (Synthetic Scheme Appendix Fig. 1). This was then lyophilized for 48 hours and stored at -20˚C.
II.A.v Synthesis of MPDOX

Synthesis of the thiolated Dox derivative, MPDOX, was performed by Eric Soehnlen with my assistance. $9.15 \times 10^{-4}$ Moles of DOX was dissolved in one mL of water. The pH was adjusted to 8.5 and then 1 µL of the 3-methyl-mercapto-propionaldehyde linker was added. Within five minutes a solid precipitate began to form in conjunction with a noted drop to 7.2 pH. The reaction was returned to a pH of 8.5 with the addition of NaOH and was allowed to run for one hour. The precipitate was then centrifuged out, washed twice with water, dried and then stored at -20°C. The purified product was then analyzed by electrospray ionization mass spectroscopy that demonstrated the expected product peak at 628.3 g/mol (performed by Eric Soehnlen) and visualized in Synthetic Scheme Appendix Figure 2. Concentration of the MPDOX was determined using Beer’s law using the absorbance of the stock MPDOX obtained by UV-VIS spectroscopy.
II.B Assembly, Visualization, and Stability of the 3-part Nanoconjugate System

II.B.i Stability of the GNP in the Presence of a Salt Solution with the Protection of HASH

Starting with a concentration of 0.5 mg/mL of HASH dissolved in water, 13 two-fold dilutions of HASH were made. 100 µL of the GNP solution was then added to 50 µL of each dilution. Each sample was allowed to sit for 20 minutes before 50 µL of a 4 M NaCl was added, yielding a final NaCl concentration of 1 M NaCl. In order to determine the percentage of particle protection seen in each sample, a UV absorbance spectrum was taken for each sample after 1 hour and again after 24 hours. Changes in the absorbance maxima at 520 nm (the absorbance maxima for 10 nm GNP) were indicative of a change in particle protection and stability.

II.B.ii Preparation of the Alexa$_{633}$ Labeled HASH

HASH was labeled with the dye Alexa$_{633}$ to ensure when the UV spectrum was taken of the sample; an absorbance maximum would be present that would be outside the spectrum of Dox and would indicate the presence of HASH. Alexa$_{633}$ has a molecular weight of 1150 g/mol. 50 mg of dry HASH was added to 15 mL of water giving a final concentration of 3.33 mg/mL. 15mL of the 3.33 mg/mL HASH was stirred at high speed while 400 µL of a 10 mg/mL of EDAC was added to it. 100 µL of the 1 mg/mL Alexa$_{633}$
dye was then added and the reaction was allowed to sit, covered in aluminum foil, at room temperature for 24 hours and was gently stirred. The solution was then dialyzed for 72 hours against a 3.5 kDa membrane to remove excess dye and other unwanted reagents.

II.B.iii Stability of the Alexa<sub>633</sub> Labeled HASH coated GNP in the Presence of a Salt Solution

A 3 mg/mL starting concentration of Alexa<sub>633</sub> labeled HASH was made by dissolving 9 mg of the Alexa<sub>633</sub> labeled HASH in 3 mL of water. After an initial concentration of 3 mg/mL was made, 13 two-fold dilutions of the labeled HASH were made. 100 µL of the GNP solution was then added to 50 µL of each dilution. Each sample was allowed to sit for 20 minutes before 50 µL of a 4M NaCl was added, yielding a final NaCl concentration of 1 M NaCl. In order to determine the percentage of particle protection seen in each sample, a UV absorbance spectrum was taken of each sample after one hour and again after 24 hours. Changes in the absorbance maxima at 520 nm (the absorbance maxima for 10 nm GNP) were indicative of a change in particle protection and stability. The level of stability seen with the labeled HASH was then compared to the level of stability seen in the unlabeled HASH.
II.B.iv Assembly of the Nanoconjugate System

The nanoconjugate system was prepared with the addition of both the thiolated ligands, HASH and MPDOX, bound to the GNP. Since both ligands were found to compete for a binding location on the GNP surface, 250 µl of both the HASH and MPDOX were added to a microcentrifuge tube before the GNPs were added to ensure proper coating of each ligand. Following the addition of each ligand, 1000 µL of the four-fold concentrated 10 nm GNP and 15 µl of 0.1%PEG was added and remained untouched for 30 minutes. Prior to any cell treatment procedures, the samples were vortexed and washed with 25% DMSO twice to remove any unbound ligand. A cartoon of the 3-part nanoconjugate system is depicted in Fig. 3.

![Diagram of the 3-part nanoconjugate system (MPDOX, HASH, and the GNP core)](image)}

**Fig 3:** The 3-part nanoconjugate system (MPDOX, HASH, and the GNP core)
Before the nanoconjugate system was used to treat the various cell lines, a proper washing technique was needed in order to remove any excess, unbound ligands in the solution. Too little of a wash would allow unbound ligands to remain in solution; too vigorous of a wash would cause the nanoparticles to aggregate and no longer exhibit targeting nor possess drug loading capabilities. In order to determine the proper washing techniques, the speed of centrifugation, the spinning time, and the amount of DMSO used, were varied until a stable washing technique was established.

II.B.v Determination of Optimal Ligand Concentration in the 3-Part System

Optimal concentrations of both the targeting ligand and the thiolated drug (MPDOX) in the complete nanoconjugate system needed to be determined in order to efficiently deliver the maximal amount of drug to the targeted cancerous cell line. The concentrations of both ligands were varied in a 96 well plate with decreasing MPDOX concentrations from left to right and HASH concentrations decreasing from top to bottom. The first row of the plate was used as a HASH control and was thereby free of any HASH; it only contained GNPs with bound MPDOX molecules. For the control, GNPs were in a solution that did not contain DMSO or MPDOX, but contained the same volume of water instead. Spectra read-outs of each well were taken after one hour and again after 24 hours. Color variations seen in the plate were visually compared to the control and the spectrum of each well was compared to the control spectra.
Concentrations and amounts of each ligand for the individual samples are shown in table 1 in the appendix.

II.B.vi Confirmation of the assembly of the 3-Part Nanoconjugate System

In order to determine that there was indeed a 3-part nanoconjugate system with both ligands bound to the GNP, an experiment was performed to create a visualization of bound ligands assisted by UV absorbance spectrum. First, the entire nanoconjugate system was synthesized; each sample consisted of 50 μL of both the MPDOX and the HASH, and 100 μL of the four fold GNP solution. The particles were then washed three times to remove any unbound ligands. 100 μL of β-mercaptoethanol was then added to displace GNP bound ligands into the solvent. The particles were then centrifuged and a UV absorbance spectrum was taken of the supernatant where the previously bound ligands were present.
II.C Determination of Targeting Efficiency and Drug Uptake

II.C.i Cell Culture Conditions for A-2008, C-13, and NIH-3T3

The C-13 and A-2008 cancer cell lines required an RMPI media that contained 1% antibiotic and 10% fetal bovine serum by volume. The NIH-3T3 control cell line used a DMEM high media that contained 0.9% antibiotic and 9% fetal bovine serum by volume. The NIH-3T3, A2008, and C-13 cell lines were passed with a 1/6 dilution of the amount of cells in media every three days; when at least 80% confluency occurred. The cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air. For all of the experiments, cells were harvested from cultures using trypsin and were then re-suspended in fresh media before plating.

II.C.ii MTS Assays to Determine Cell Viability

MTS assays were performed to determine the cell viability and IC₅₀ values of each cell line after treatment with Dox, MPDOX or the entire nanoconjugate system build in above methods. A MTS assay is a colorimetric assay for measuring the cellular activity of a treated cell line. When live cells are present, they metabolize an MTS/PMS reagent that is reduced to a formazan dye, giving a dark purple color which can alter the UV absorbance values of each sample. Cells from each cell line were seeded in separate
96 well plates with a full confluency of 2,000 cells per well. The plates were then incubated for 24 hours. After the 24 hour time period had passed, each cell line was then incubated with varying concentrations of each drug type at 37°C for 48 and 72 hours. The drug was removed and the cells were treated with a MTS/PMS solution with a 2:1 concentration ratio. The solution was allowed to sit on the treated cells for two hours at 37°C before UV absorbance spectra of each well was taken by a Biotech plate reader. A dose dependent cell viability curve was then generated from the data and preliminary IC₅₀ values were calculated.

Another procedure for the determination of cell viability was performed in which the drug treatment time periods were altered. After seeding the cells to a confluency of 2,000 cells per well for 24 hours, each well was treated with either of the free drugs or the entire nanoconjugate system for three hours. After three hours, the cells were washed twice with their corresponding media (RPMI for A2008 and C13; DMEM high for NIH-3T3) to remove the drugs. 200 µl of fresh media was then added to each well. The cells were then incubated at 37°C for 48 or 72 hours. The cells were then read on the plate reader after treatment with the MTS/PMS solution.
II.C.iii Confocal Studies on Cellular Uptake of the Free Drug

Confocal studies allowed for the visualization of the intracellular drug at various time points. Both DOX and MPDOX naturally fluoresce at a wavelength of 477 so a labeling dye was unnecessary. The A-2008 cell line was used for this study and seeded in 8 well plates to full confluency. The plates were then incubated for 24 hours. After the 24 hour time period had passed, the cell line was treated with 300µl of 20µM concentrations of either Dox or MPDOX for various time periods (1, 2, 4, 6, and 12 hours). One hour before the confocal read out, the drug was removed and each well was washed twice with RPMI media. 300 µl of the OPTI-MEM media replaced the RPMI media and the cells were incubated for one hour. Read outs were taken with the Olympus FV500 confocal microscope.
Chapter III

Results and Discussion

III.A Synthesis of Gold Nanoparticles

III.A.i Synthesis and Size Determination of Gold Nanoparticles

One major advantage of spherical GNPs in a targeted drug delivery system is the ease with which they can be synthesized. GNPs of various sizes were synthesized by following an altered version of a previously published protocol\(^29\). GNPs of various sizes (3 nm, 10 nm, 20 nm, 30 nm, and 70 nm) were synthesized using 1% trisodium citrate to reduce gold salt (HAuCl\(_4\)), which allowed them to shape into spherical GNPs. With this method, it was possible to synthesize GNPs with diameters of the following sizes: 10 nm, 20 nm, and 50 nm. The size of each sample of GNPs was determined by the use of UV-VIS spectroscopy. The size of the GNPs can be determined based on the absorbance maxima of each curve (Fig. 4). Synthesis of 70 nm and 100 nm particles proved unsuccessful due to their absorbance maxima (determined by the surface Plasmon resonance effect exhibited for each particle size) not possessing the projected wavelengths at for each nanoparticle size. For all further experiments, 10 nm GNPs were used. The absorbance maxima of the 10nm GNP have a wavelength of around 530 (Fig. 5).
Fig. 4: UV-VIS spectra of 10nm, 50nm, 75nm, and 100nm GNP synthesized via the citrate reduction method. Note that the absorbance values for both 75 nm and 100nm GNP sizes do not correlate with the trend of increasing particle size.
Fig. 5: UV-VIS absorbance spectra of 10nm GNP synthesized by the citrate reduction method.
III.B Assembly, Visualization, and Stability of the 3-part Nanoconjugate System

III.B.i GNP Stability in the Presence of a Salt Solution

Negatively charged citrate coated GNP s remain stable and in suspension due to the nanoparticle’s surface charge repulsion effect. This effect is neutralized in high concentrations of positively charged ions, which leads to particle aggregation. GNP s coated with hydrophilic polymers, such as polyethylene glycol, are protected from aggregation in the presence of salt solutions due to sterric hinderance. Hyaluronic acid, which is also a hydrophilic polymer, could also protect against particle aggregation in the presence of a salt solution. In the presence of a 1 M NaCl solution, it was found that HASH did indeed provide particle protection from aggregation (Fig. 6) and that the optimal concentration of the HASH needed for protection of a 10 nm GNP was 0.0625mg/mL (Fig. 7).
Fig. 6 UV spectra of GNPs with HASH coating vs. citrate coating in the presence of 1M NaCl.
Fig. 7: Percent particle protection from aggregation of 10nm GNP by HASH monitored by the change in the absorption maxima at 520nm
III.B.ii GNP Stability with Alexa\textsubscript{633} Labeled HASH in the Presence of a Salt Solution

Hyaluronic acid does not have a known absorbance peak detectable by UV-Vis spectroscopy. In order to determine the existence of a 3-part nanoconjugate system, HASH was covalently linked to a labeling dye, Alexa\textsubscript{633}. This conjugation allowed for the HASH to be detected by the presence of an absorbance maximum of 633 nm. HASH is essential to the nanoconjugate system for prevention of particle aggregation in the presence of a salt solution. It is important for nanoconjugates to remain stable under increased ionic strength. In a cell culture media, the system will encounter greater than 100 mM salt; the nanoconjugates must maintain their integrity even in such conditions. A salt stability assay was performed in order to ensure that the addition of the dye did not negate the protection of the particles. In the presence of a 1 M NaCl solution, it was found that HASH\textsubscript{633} provided particle protection from aggregation. However, it was noted that the concentration of the HASH\textsubscript{633} needed for particle stability was much higher than the unlabeled HASH. That the optimal concentration of the HASH\textsubscript{633} needed for protection of a 10 nm GNP was between 2.3 mg/ml and 3 mg/ml (Fig. 8). Since this seemed to effect the stability of the particle, it was decided that the HASH\textsubscript{633} would only be used for labeling purposes, and that the unlabeled HASH would be used for all cell and particle assays.
Fig. 8: Percent particle protection of 10nm GNP by Alexa$_{633}$ HASH from aggregation monitored by the change in the absorption maxima at 520nm
III.B.iii Investigation of Proper Washing Procedure for the Nanoconjugate System

Before the nanoconjugate system was used to treat the various cell lines, a proper washing technique was needed in order to remove any excess and unbound ligands in the solution. Too little of a wash would allow unbound ligands to remain in solution; too vigorous of a wash would cause the nanoparticles to aggregate and no longer exhibit targeting or possess drug loading capabilities. After varying the spin speed, and types of washes for the washing procedure, it was found that the most efficient washing technique for concentrated and stable particles was achieved by using the following protocol: 25 mL of the nanoconjugate solution was spun at 16,000 RPM for 30 minutes before the removal and disposal of the supernatant. The system was then resuspended in 25 mL of 25% DMSO and spun at 16,000 RPM for an additional 30 minutes. The supernatant of this spin cycle was also removed and disposed. 25 mL of deionized water was then added to the sample and re-spun at 16,000 RPM for 30 minutes. The supernatant was removed and the remaining super concentrated sample was pipetted into a small test tube, where deionized water was added until the final volume reached 300 µL.
III.B.iv **Determination of Optimal Ligand Concentration in the 3-Part System**

In order to determine the optimal nanoconjugate composition, thereby giving the system stability and drug loading, an assay was performed in which the concentrations for both ligands were varied. 25 µL of each ligand at various concentrations was added to a 96 well plate (Table 1). To each of these samples, 100 µL of GNP was added. The assay also had two different controls, DMSO (-) wells which showed complete particle protection, and DMSO (+), which exhibited particle aggregation in the absence of HASH. Because MPDOX is not soluble in water, each well (with the exception of the DMSO (-) lane 12 contained 15% DMSO by volume. In the first observation of the assay, any color variation from the DMSO (-) control lane was indicative of particle aggregation. Qualitatively, protected particles exhibited an orange color. Thus, wells that appeared orange were presumably more protected than those with varying color, particularly the ones that veered towards purple (Fig.9). The UV spectrum of each well from the assay was also taken to obtain a quantitative result. A spectra shift to the right, or a change in absorbance maxima from the DMSO (-) control, was indicative of particle aggregation (Fig. 10). By comparing each sample’s spectrum to the DMSO (-) that showed no particle aggregation, relative particle stability was able to be determined. In the first row without any HASH and in the presence of 15% DMSO, the particles were completely aggregated; this was indicated by the extreme color
change and the apparent change in the absorbance spectrum. However, when the particles were in the presence of 15% DMSO and the HASH was included in the system, the particles were protected from aggregation. It was also observed that as the concentration of MPDOX increased, particle aggregation increased, which indicates that MPDOX and HASH are competitive for GNP surface binding.

The nanoconjugate competition assay confirmed that the optimal composition and concentration for each part of the nanoconjugate system as seen in row B with a concentration of 0.5mg/mL of HASH and anywhere from 0.5 mg/mL to 0.0625 mg/mL of MPDOX. This series of nanoconjugate compositions provided both stable particle protection and adequate drug loading properties. For the remainder of this study row B in figures 9 and 10 was selected for use due to its high levels of particle stability.
Fig. 9: Thiolated Hyaluronic acid/MPDOX competition assay. Columns from left to right are numbered from 1-11, while rows from top to bottom are designated A-G. Protected particles exhibited an orange color. Thus, wells that appeared orange were presumably more protected than those with purple or blue colors.
**Fig. 10:** Thiolated Hyaluronic acid/MPDOX competition assay. Columns from left to right are numbered from 1-11, while rows from top to bottom are designated A-G. A spectra shift to the right, or a change in absorbance maxima from the DMSO (-) control, was indicative of particle aggregation. The spectra were obtained by measuring UV-absorbance of each well of the plate in figure 9.
III.B.v Conformation of the 3-part Nanoconjugate System

In order to demonstrate that both ligands (HASH and MPDOX) were bound to the GNP and not just free in the solution, the UV spectrum was taken of the nanoconjugate system with the MPDOX and the Alexa₆₃₃ labeled HASH present in the sample. A sample was made by adding 50 µL of both ligands and 200 µL of the GNP solution; which was then washed several times to remove unbound ligands. 100 µL of β-mercaptoethanol was added so that the ligands bound to the GNP would be displaced into the solvent. The UV absorbance spectrum of the supernatant was recorded; it exhibited an absorbance peak of 633 nm, which is indicative of the presence of Alexa₆₃₃ labeled HASH. A peak was also noticed at 477 nm, due to the presence of MPDOX in the system (Fig. 11). This result indicates the presence of both ligands (MPDOX and HASH) bound to the GNP surface.
**Fig. 11:** UV absorbance spectra displaying absorbance peaks at 477 nm and 633 nm, indicating the presence of both ligands (MPDOX and HASH) on the same GNP surface.
II.C **Determination of Targeting Efficiency and Drug Uptake**

II.C.i **Cell Viability and IC\textsubscript{50} Values of the Nanoconjugate vs Free Drug**

MTS assays were performed in order to generate dose dependent cell viability for treatment of two ovarian cancer cell lines (A-2008 and C-13) with Dox, MPDOX, and the established nanoconjugate drug delivery system at 48 and 72-hour post-treatment respectively. Cell viability curves were constructed with the resulting data and the IC\textsubscript{50} values of each assay were generated using the cell viability curve. The IC\textsubscript{50} values were then compared to conclude which type of treatment was most efficient. However, was shown that the nanoconjugate system and both free drugs appeared to have similar cell viability curves and IC\textsubscript{50} values at the 72 hour time period for the C-13 cell line (Fig. 12).

Since MPDOX has similar values as its parent compound, Dox, it can be determined that modification of the drug (in order to enhance binding affinity to the GNP) did not affect the toxicity of the drug. Table 2 in the appendix lists the IC\textsubscript{50} values for all cell lines, treatment times, and drug type. At the close of this project, it was determined that more research is needed to increase the cytotoxicity of the cell line by the nanoconjugate system. When observing the cell plates treated with the nanoconjugate system, particle aggregation was observed, possibly due to the washing system; this may have altered the drug delivery and cytotoxicity of the system.
Fig. 12: Dose dependent cell viability curve comparison between the nanoconjugate system and the free drugs for the C-13 cell line at 72 hour treatment times.
III.C.ii Visualization of Cellular Uptake of the Free Drugs DOX and MPDOX

One way to visualize intracellular uptake and localization of the free drugs and the nanoconjugate system is through the use of confocal microscopy. Dox and its modified counterpart, MPDOX naturally fluoresce to emit a vibrant green color and can be seen inside of a cell following cellular internalization. The amount of the drug inside of the cell can be qualitatively compared based upon the intensity of the fluorescence signal observed in the cell.

In order to visualize the free drugs inside of the cell at various time points, the A2008 cell line was treated with 20 µM and 10 µM concentrations of both free drugs (Dox and MPDOX) for 1, 2, 4, 6, 12, and 24 hours. Confocal microscopy studies showed that for both the Dox and MPDOX, the two longest time periods (12 hour and 24 hour) produced the greatest intensity of fluorescence and uptake of the drugs. The highest intensity was shown with the 20 µM concentrations for both the Dox and the MPDOX (figures 13-16). Similar levels of intensity were seen in cells treated by both Dox and MPDOX, indicating that the alteration of the drug did not affect drug uptake by the cells. Further investigation would need to be performed on this system to increase the targeting efficacy of the system.
Fig. 13: A2008 cell lines treated with 10μM DOX at: A) 1 hour, B) 2 hour, C) 4 hour, D) 6 hour, E) 12 hour, F) 24 hour treatment times.

Fig. 14: A2008 cell lines treated with 20μM DOX at: A) 1 hour, B) 2 hour, C) 4 hour, D) 6 hour, E) 12 hour, F) 24 hour treatment times.

Fig. 15: A2008 cell lines treated with 10μM MPDOX at: A) 1 hour, B) 2 hour, C) 4 hour, D) 6 hour, E) 12 hour, F) 24 hour treatment times.

Fig. 16: A2008 cell lines treated with 20μM MPDOX at: A) 1 hour, B) 2 hour, C) 4 hour, D) 6 hour, E) 12 hour, F) 24 hour treatment times.
Chapter IV

Conclusions

A 3-part nanoconjugate system was designed to selectively deliver a small molecule drug to targeted cancerous cell lines over non-cancerous cell lines. Each component of this system (the GNP core, the HASH targeting ligand, and the small drug molecule MPDOX) was successfully synthesized and characterized. Thiolated hyaluronic acid was utilized to act as a particle protectant to prevent aggregation. It was determined that hyaluronic acid provided protection from particle aggregation in the presence of a 1M salt solution. Optimal concentrations of both the HASH and MPDOX were determined to produce a stable nanoconjugate system that possessed adequate drug loading. MTS assays for two different ovarian cancer cell lines (A2008 and C-13) and the control cell line (NIH-3T3) were performed to generate dose dependent cell viability curves and IC\textsubscript{50} values for each cell line and treatment type. Data from the two cancerous cell lines showed that the cell viability curves and the IC\textsubscript{50} values were highly similar between the nanoconjugate system and both of the free drugs. These results indicate that the efficacy of the MPDOX and the nanoconjugate system was not lowered by the alteration of the parent drug, Dox. At the present moment, the IC\textsubscript{50} values of the nanoconjugate system for the NIH-3T3 control cell line do not indicate successful targeting \textit{in vitro}. Confocal microscopy was also performed in the A2008 cell line with
both free drugs (Dox and MPDOX) in 10 μM and 20 μM concentrations to visualize the location of the free drug in the cell at various time points. From this data, it can be further concluded that the alteration of Dox to MPDOX did not have an effect of cellular internalization. However, at present time, receptor specific drug targeting of the cancerous cell lines over non-cancerous cell lines has not been observed. Further studies will need to be performed in order to enhance cell-targeting efficiency.
**Appendix**

**Synthetic Scheme Appendix Figure 1:** End thiolation of Hyaluronic acid.

**Synthetic Scheme Appendix Figure 2:** End thiolation of Doxorubicin (MPDOX).
Table 1: Sample components for MPDOX/HASH binding competition assay.
Table 2: IC<sub>50</sub> Values for the Cell viability MTS assays

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Drug Name</th>
<th>Treatment time</th>
<th>IC-50 Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-2008</td>
<td>Doxorubicin</td>
<td>72 hr</td>
<td>102 nM</td>
</tr>
<tr>
<td>NIH-3T3</td>
<td>Doxorubicin</td>
<td>72 hr</td>
<td>133 nM</td>
</tr>
<tr>
<td>C-13</td>
<td>Doxorubicin</td>
<td>72 hr</td>
<td>128 nM</td>
</tr>
<tr>
<td>A-2008</td>
<td>MPDOX</td>
<td>72 hr</td>
<td>101 nM</td>
</tr>
<tr>
<td>NIH-3T3</td>
<td>MPDOX</td>
<td>72 hr</td>
<td>204 nM</td>
</tr>
<tr>
<td>C-13</td>
<td>MPDOX</td>
<td>72 hr</td>
<td>214 nM</td>
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<tr>
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</tr>
<tr>
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<td>72 hr</td>
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