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EXPLOITING BIOMARKERS ASSISTS IN DEGRADATION OF BREAST CANCER CELLS AND REDUCES EXTRAVASATION

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ABSTRACT

Cancer is the second leading cause of death in the United States. Conventional chemotherapeutics kill 5% of patients and outcomes are very poor for stage 3 and stage 4 patients. Most current treatment methods, based on the conventional paradigm that rapidly replicating cells have the most uptake of the chemotherapeutic agent, harm benign, rapidly replicating cells in addition to cancerous cells. Therefore, a novel approach involves targeting cancer cells specifically and preventing them from growing and metastasizing. Healthy cells should be minimally harmed if not completely unharmed during the treatment process. Ceramide nanoliposomes are one possible treatment that could specifically target cancerous cells. The ceramide nanoliposomes inhibit primary breast, pancreatic, and melanoma tumors that express CD44 while having no effect on benign epithelial cells. The effects of ceramide nanoliposomes on the migration of MDA-MB-231 (human breast adenocarcinoma) cells were tested since a treatment for metastasized cancer would be especially efficacious. Survival rates decrease as cancer cells migrate in a body and metastasize. The five-year survival rate for breast cancer patients decreases from 100% for stage 1 (located only in primary tissue) to 22% for stage 4 (located in multiple remote organs). Ceramide nanoliposomes were shown to use lysosomal degradation of the membrane protein, CD44, to mediate anoikis and reduce extravasation. Similar results were observed when siRNA was used to downregulate CD44. The data collected indicates that ceramide nanoliposomes could be used to prevent and treat CD44-dependent tumor metastasis.

While ceramide nanoliposomes have yet to be used on humans, docetaxel is one of the most common drugs currently being used to treat breast cancer. However, docetaxel does not specifically target malignant cells. Since docetaxel disrupts the assembly of the mitotic spindle, it can inhibit various cells from dividing, not just cancerous ones. Delivery via calcium phosphosilicate nanoparticles (CPSNPs) is currently being tested as a proper technique for specific targeting of cancerous cells. CPSNPs encapsulate the drug and provide intracellular release. In addition, the surface of the CPSNPs can be bioconjugated

with molecules that bind to cell receptors. In order to potentially maximize the exploitation of cell markers, receptor proteins on cancerous cells other than CD44 were investigated. CPSNPs were conjugated with anti-CD71, which allows the nanoparticles to bind specifically to cells that express CD71 on their membrane. MDA-MB-231 breast cancer cells were used because they are one type of cell line that overexpresses the membrane protein CD71. The docetaxel CPSNPs are further being studied for their effects on cell growth and extravasation.

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Chapter 1

Introduction

1.1.1 Cancer prevalence

Cancer continues to be one of the top five leading causes of death in the world.[1] It kills indiscriminately without regard to age, race, sex, demographics, or social and economic positions. In the United States, it is the second leading cause of death, constituting one-fourth of all deaths in the United States.[2] Cancer, a disease that affects millions of people each year, requires more attention and research so that the mortality rate can be improved.

1.1.2 Pancreatic Cancer

People diagnosed with pancreatic cancer have one of the highest mortality rates. According to the American Cancer Society, most people die within 1 year of diagnosis of pancreatic cancer and only 6% live for at least 5 years after diagnosis.[3] Furthermore, the death rates of pancreatic cancer patients have recently been slowly increasing whereas death rates of most major cancers have decreased.[3] It is expected that 19,850 women and 20,710 men will die because of this disease in 2015.[2] Indeed, research in pancreatic cancer prevention, detection, and treatment deserves more attention. Contemporary treatments for pancreatic cancer are surgery, chemotherapy, radiation therapy, chemoradiation therapy, and targeted therapy.[3] Surgery involves removing part of or the entire pancreas and some tissue from surrounding organs (intestines and liver). If the disease is detected too late, as many cases are, surgery is not a viable option for the patient. The common chemotherapeutic agent for pancreatic cancer is gemcitabine and it is usually the physician's first recommendation.[3] Gemcitabine is usually followed by capecitabine or 5-fluorouracil as part of chemoradiation, which is a combination of chemotherapy and radiation therapy. Radiation therapy involves delivering radiation through an external machine or an implanted drug.

Chemoradiation leads to an augmented treatment method but with more severe side effects. Targeted therapy uses other drugs to interfere with tumor progression.

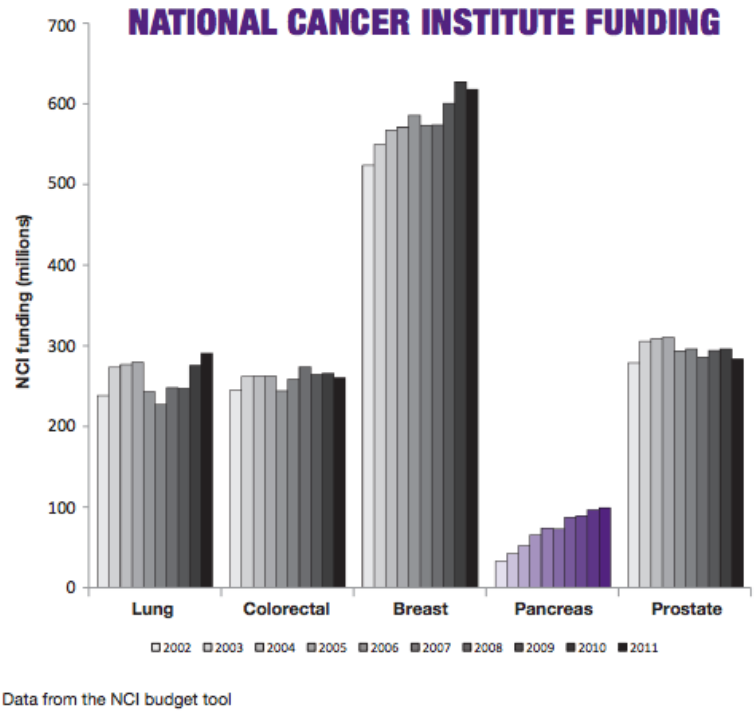


Figure 1. Compared to funding for other cancers, research for pancreatic cancer receives a small amount of funding.[4]

1.1.3 Breast Cancer

Breast cancer is the second most prevalent cancer to affect women.[2] Yet, it does not only affect women. The American Cancer Society estimated that 40,290 women and 440 men will die in 2015 due to breast cancer.[2] Women and men who express the BRCA1 and BRCA2 genes are both at risk for breast cancer.[3] On a positive note, breast cancer can be effectively treated with early detection times. Surgery can be combined with chemotherapy or radiation therapy. Hormonal and targeted therapies are other options as well. However, the options just mentioned are significantly less effective if the cancer is discovered late. Thus, alternative methods need to be studied.

1.2 Cancer Physiology

Cancer originates at a specific point in the body. Through angiogenesis, the formation of new blood vessels, these cancerous cells travel into the main blood vessels.[5] The process of cells entering the blood system is called intravasation. The cells flow through the blood vessels and can associate with leukocytes and platelets to make adhesion more probable.[5] Once the cancer cells adhere to the blood vessel wall, they can migrate through the wall and settle in tissue other than their origin.[5] This step of cell migration is called extravasation. Metastasized cancer is when a tumor develops in a location other than that of the primary tumor. Cancer cells may not only migrate in the cardiovascular system, but also in the lymphatic system and colonize in lymph nodes. The level of cancer metastasis increases with the number cancer cells present in lymph nodes and the further they are located from the primary tumor.[3]

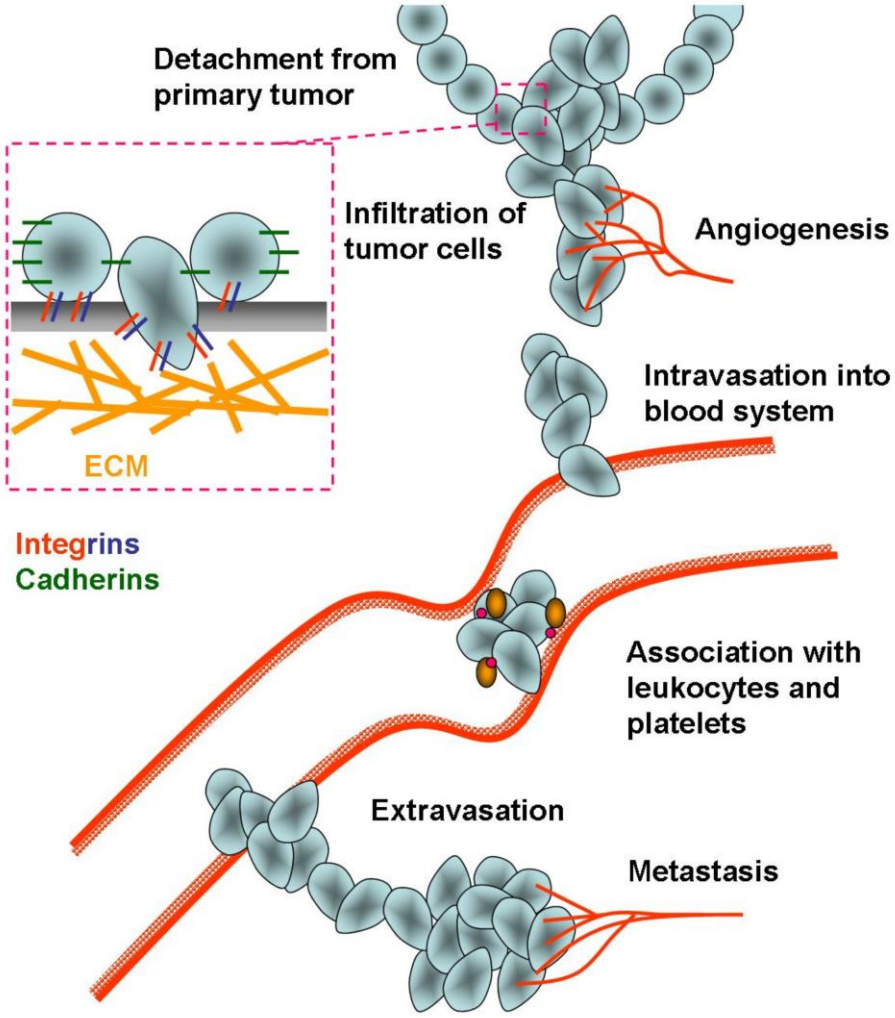


Figure 2. The major steps of cancer metastasis are shown. Cells initially detach from the primary tumor and make it into major blood vessels through newly made vessels that were produced as a result of angiogenesis. Then they flow through the vessels associating with leukocytes and platelets until they adhere to the endothelial wall and extravasate out of the blood vessel. Once a colonization begins at site other than the primary tumor, the cancer is considered metastasic.[5]

1.3 Current Treatments

Although there are many drugs to combat cancer cells, an efficient targeting method is yet to be available. Healthy, quickly replicating cells tend to suffer along with malignant ones. In addition, late diagnosis causes treatments to be less effective or even non-viable if the cancer spread throughout the body. Cancer treatment has vast potential for improvement with nanoscience applied. This research

focuses on two potential treatments for metastatic carcinoma cells, including breast cancer cells. The first is liposomal delivery of ceramide, and the second uses nanoparticles for targeted delivery of docetaxel, one of the most commonly used therapeutic drugs for breast cancer. These two potential treatments are described below.

1.4 Ceramide Nanoliposomes (CNLs)

Ceramides are long hydrocarbon chains bonded to a fatty acid.[6] They are naturally found in cell membranes and are thought to function in signal transduction and transport across the membrane.[6] One of the major processes that ceramides initiate is apoptosis, or programmed cell death.[7] C_6-ceramide can selectively induce apoptosis in cancerous cells, but it has two major limitations. Firstly, the long hydrophobic chain in ceramide (as shown in Figure 3) makes it difficult to dissolve in aqueous solutions and would likely lead to the formation of micelles.[7] Secondly, the ceramide can be metabolized by enzymes in the body and used for other purposes than treating cancer.[7] To solve these issues, liposomal delivery has been used to release short-chain ceramides and successfully induced apoptosis in pancreatic cancer, breast cancer, melanoma, and hepatocellular carcinoma cells.[6] Figure 4 shows the basic structure of liposomes (80 ± 15 nm), which deliver drugs like C_6-ceramide through the cellular membrane. Although, CNLs have been shown to be efficacious in causing apoptosis, their mechanism in regulating metastatic cancer was still not fully understood. CNLs were previously shown to block neurotensin-induced breast cancer cell migration and matrix metallopeptidase (MMP) secretion so it was hypothesized that CNLs would induce anoikis, or programmed cell death that involves detachment from the surrounding extracellular matrix, and inhibit extravasation in metastatic carcinoma cells, such as pancreatic cancer, breast cancer, and melanoma cells.[8,9]

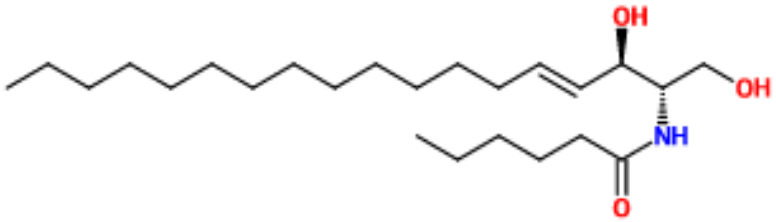


Figure 3. The structure of C_6-Ceramide is shown to depict its hydrophobic long chain and polar head. This structure was drawn using eMolecules software. The C_6 designation describes the short chain composed of six carbon atoms.

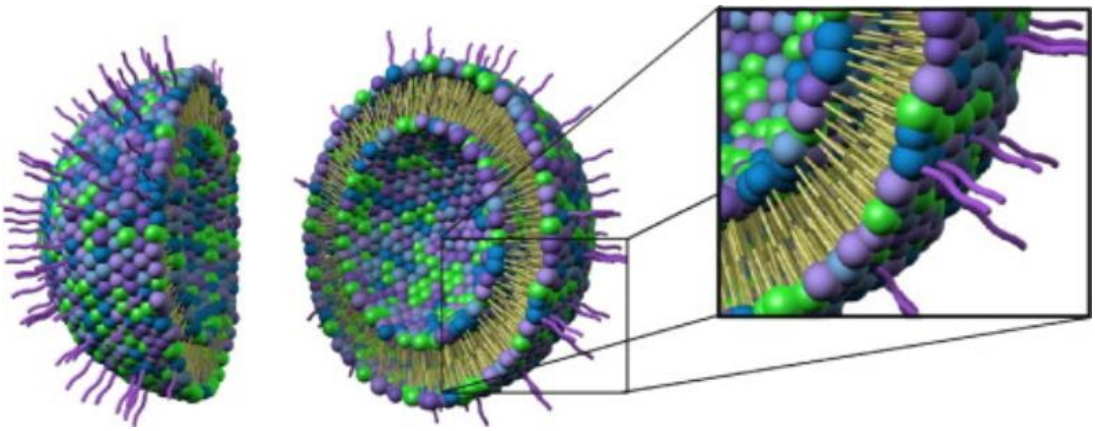


Figure 4. Liposomes are double-walled spheres that consist of phospholipid molecules. They can be used to encapsulate drugs such as C_6-ceramide for delivery in experiments-in vitro or in vivo.[10]

1.5 SiRNA

Small-interfering RNA (siRNA) is used to temporarily negate the effects of certain genes in a cell. In breast cancer cells, siRNA was used to downregulate a pro-migratory gene, CD44.[11] CD44 is a transmembrane adhesion molecule that has been implicated in metastasis. It is found at high levels in breast cancer, melanoma, and pancreatic cancer.[11,12] Extracellularly, CD44 can bind endothelial cell

surface molecules, such as E-selectin, as well as components of the extracellular matrix, most notably hyaluronic acid.[11,13] Intracellularly, CD44 binds ezrin, facilitating cancer cell migration and metastasis.[14] Mammalian cells can be exposed to siRNA through a process called electroporation (Figure 5). SiCD44 describes siRNA that diminishes CD44 levels, and siScr describes scrambled RNA, which acts as a negative control and does not affect gene expression. Downregulation of the gene encoding CD44 was studied to determine the effect on cancer cell migration of breast cancer cells. The results of downregulating CD44 were expected to be similar to the effects of CNLs.

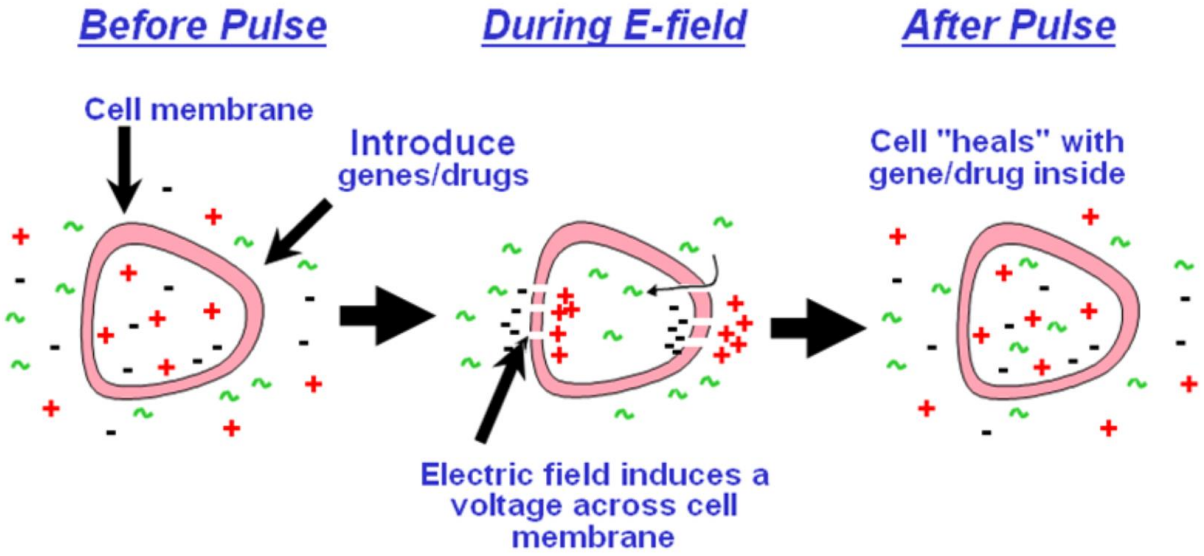


Figure 5. Electroporation opens up pores in the plasma membrane for substances, such as siRNA, to enter into a cell.[15]

1.6 Docetaxel

Docetaxel is one of the most common drugs used to treat breast cancer cells.[16] It induces death in MDA-MB-231 cell mainly by mitotic catastrophe.[17] In other words, the docetaxel inhibits microtubule formation causing missegregation of chromosomes prior to cell division.[17] Unlike ceramide, docetaxel has

many polar subgroups (Figure 6). It will not form micelles in blood, but is still negatively kills benign cells in addition to malignant ones. The overarching aim now is to adapt the delivery method of docetaxel so that it only targets cancerous cells. Prior to that point, the effects of docetaxel on extravasation need to be analyzed. If docetaxel can significantly reduce extravasation as expected, the nanoparticles could be studied for their efficiency. Bioconjugated nanoparticles can potentially provide the solution to passive targeting by acting as delivery vehicles for chemotherapeutic drugs.

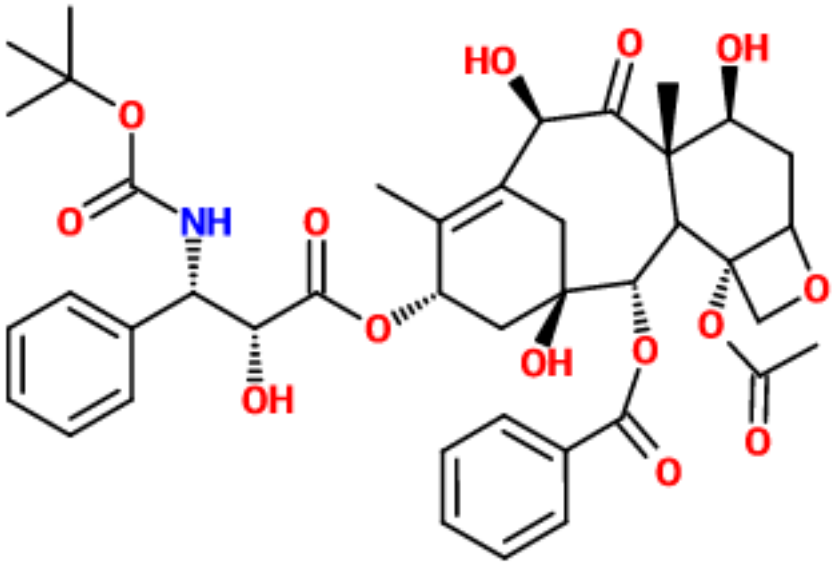


Figure 6. Docetaxel has many polar groups and little hydrocarbon branching, which makes it a polar compound. This structure was drawn using eMolecules software.

1.8.1 Calcium Phosphosilicate Nanoparticles (CPSNPs)

In addition to nanoliposomes, CPSNPs also provide a method of delivering drugs to cells. Although ceramide was used in nanoliposome delivery, ceramide was shown to have a maximum of 0.019% yield encapsulation in CPSNPs.[18] This low encapsulation efficiency was a factor in deciding to encapsulate docetaxel in the CPSNPs in addition to it already being a common therapeutic drug for humans with breast cancer. One benefit of CPSNPS is that they are made of biocompatible materials such

as calcium and phosphate. They can pass through the cell wall and release the drugs in the late endolysosome.[19] The drugs are released at that time because the low pH in the lysosome transforms the calcium and phosphate to soluble ions.[19] As long as the CPSNPs are not exposed to low pH, the encapsulated drug will not leak like it sometimes does in liposomes.[20] The second benefit of CPSNPs is their ability to bind to ligands, which allow the CPSNPs to bind directly with compatible cellular receptors.[19] If a receptor is exclusively or overly expressed on a cancer cell, the CPSNPs can be conjugated with those complementary ligands to greatly increase the specific effect on cancer cells.

CPSNPs were recently used to encapsulate and deliver docetaxel to MDA-MB-231 cells in static shear experiments. The results of these experiments showed an increase in uptake of RWT-CPSNPs when the particles were conjugated with anti-CD71.[21] Therefore, it is expected that docetaxel encapsulated anti-CD71 CPSNPs would cause a greater reduction in extravasation than docetaxel encapsulated methoxy-PEG CPSNPs. Before these particles can be tested, it is important to assess the effects of methoxy-PEG CPSNPs on toxicity and extravasation of the breast cancer cells. In vivo, pegylation makes passive targeting more feasible and assists in the evasion of renal filtration.[22] Although methoxy-PEG particles rely on the enhanced permeability and retention (EPR) effect to reach tumors via leaky channels, a more specific targeting method that exploits malignant cells' biomarkers would do less harm to the patient.[22]

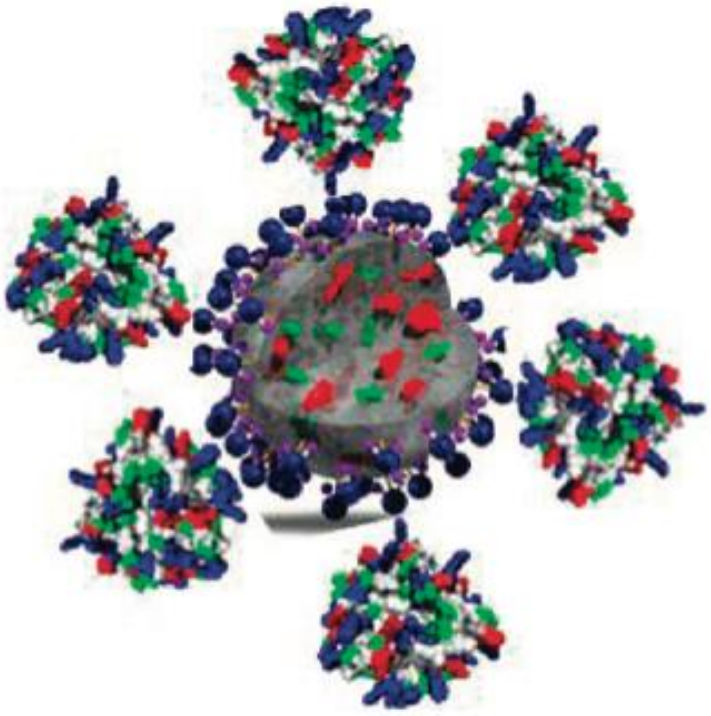


Figure 7. A CPSNP model is shown encapsulating a drug and bioconjugated to six molecules of a ligand that would interact with a specific cell receptor.[23]

1.8.2 CPSNP Characterization

The particle number concentration and particle diameter distribution of CPSNPs are currently difficult to accurately determine. Dynamic light scattering (DLS) is available to measure the particle diameter distribution but it has two major issues for inaccuracies to occur. Firstly, DLS measures the hydrodynamic radius, which is larger than the radius of the solid nanoparticle core. Polymer chains on the surface of the CPSNP increase the measured diameter in a colloidal suspension using DLS. The transmission electron microscope (TEM) however, indicates a smaller diameter because it only measures the solid particle. Secondly, particle number concentration and diameter distribution measurements by DLS can be skewed because of multiple scatterings of a photon. Also, the laser (630.6 nm) used in DLS excites encapsulated fluorophores such as Rhodamine-WT (RWT) and Cyanine 3 (Cy3).

Recently, a new instrument, an Izon qNano, was acquired by Dr. Adair's lab to more accurately measure the particle number concentration particle size distribution. The qNano was used on batches of nanoparticles so the effects of changing particle number concentration and distribution size could be compared. The Izon qNano technology utilizes tunable resistive pulse sensing, or TRPS, with pores on a nanoscale to enable particle-by-particle characterization.[24] This non-optical technology allows quantification of various aspects of the nanoparticle, including size, concentration, charge, and dynamics. The Izon system consists of a voltage that is applied across a fluid cell via silver/silver chloride (Ag/AgCl) electrodes.[24] The voltage allows ions to pass between the electrodes and produce a current across the aperture opening. Attached to the electrodes is a voltage-clamping signal detector, which recognizes the temporary changes in current due to particle movement through the aperture. Fluctuations in the current allows for volume detection and counting of nanoparticles in an electrolyte solution.

The nanoscale sized aperture through which the particles pass is called the nanopore. Made of flexible thermoplastic polyurethane, the nanopore has an adjustable conical opening in the center.[24] In order to be analyzed, the sample particles must be between 50-10,000 nm in diameter and have concentrations between 10^5 and 10^{12} particles/mL.[24] The allowable concentration detection range varies according to the size of the nanoparticles in the sample. The benefit of using an Izon q-nano is that it measures a wide range of synthetic and biological particle types, such as viruses, bacteria, polymers, exosomes, and both charged and uncharged particles.[24]

Chapter 2

Materials and Methods

2.1 Cell Culture

EI cells, which are Fibroblast L-cells transfected to express ICAM-1 in addition to the already present E-selectin, were cultured in RPMI medium. The RPMI medium was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin streptomycin (PenStrep). Human MDA MB-231 breast cancer cells were cultured in Dulbecco's Modified Eagle Medium (DMEM). DMEM was also supplemented with 10% FBS and 1% PenStrep. The cell media ratios mentioned are all volume percentages. Cell cultures were maintained at 37^{o}C and 5% CO_2. Cells were harvested with 0.25% trypsin/EDTA when confluent. All materials were purchased from Life Technologies Corporation.

2.2 Nanoparticle Fabrication

Two separate microemulsions were made. Each one had 14 mL of 29 volume % Igepal CO-520 in cyclohexane. A CO_2-free solution (650 µL) of $CaCl_2$ was added to one of the emulsions. The second emulsion received 65 µL Na_2HPO_4, 65 µL Na_2SiO_3, and 520 µL degassed water. The second emulsion was stirred slowly with Na_2HCit for a retention time of either 2 or 30 minutes. The two emulsions were combined and all micellular exchange was allowed to reach equilibrium. 222 µL of Na_2HCit was injected into the new emulsion and stirred for 15 minutes at a slow rate. Na_2HCit EtOH (50 mL) was then added and stirred for 1.5 minutes at a slow rate to disperse the CPSNPs and disrupt the microemulsion. In order to encapsulate docetaxel or RWT, the process is performed with the respective substance (docetaxel or RWT) being added to the second initial emulsion. The produced CPSNPs were laundered twice using a

modified version of high performance liquid chromatography called (HPLC). The Van der Waals HPLC laundering concentrates particles and removes excess reagents to be used on cells. The samples were then filtered through a 0.2 μm regenerated cellulose filter and stored at 4^{o} C. When the nanoparticles were to be used, the solution was dried with argon gas and resuspended in Dulbecco's phosphate-buffered saline (DPBS).

2.3 Nanoparticle Conjugation

To conjugate polyethyene glycol (PEG) to ghost-CPSNPs, fabricated CPSNPs were first treated with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) and then with sulfo-N-hydroxysuccinimide (sulfo-NHS) as shown in Figure 8. PEG molecules were then added to these particles. The particles were washed using centrifugation, filtered, and dried with argon gas. To make the anti-CD71 CPSNPs, EDAC and sulfo-NHS would be added to non-conjugated CPSNPs. The resulting CPSNPs would then be PEGylated and reacted with the anti-CD71 to form CPSNPs that can target the breast cancer (MDA-MB-231) cells being studied. The resulting particles were also washed through centrifugation, filtered, and dried with argon gas. Welley Loc, a graduate student in Dr. Adair's laboratory, made the particles used in an MTS assay with a 2-minute retention time.

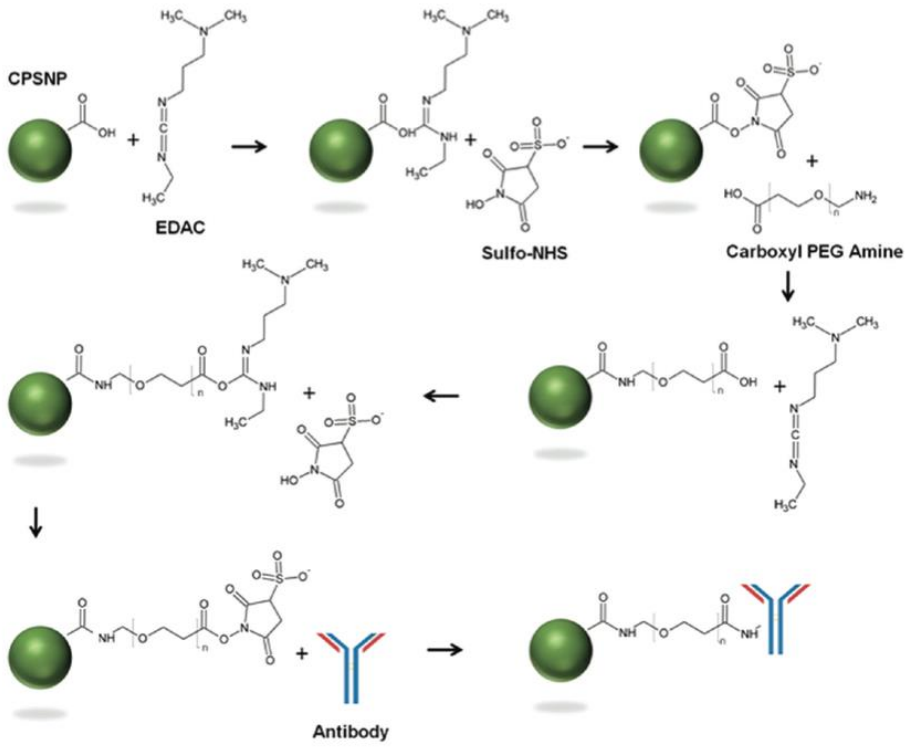


Figure 8. The conjugation of the CPSNPs with PEG is shown. The conjugation of anti-CD71 to CPSNPs is accomplished by first PEGylating the CPSNPs and then reacting the nanoparticles with anti-CD71.[26]

2.4 Izon qNano

The Izon qNano instrument and software were first turned on. An electrolyte buffer solution DPBS (75 µL), was added to the lower cell. Any observed bubbles were removed from the solution. The nanopore was fixed onto the lower cell and stretched to the appropriate aperture size. A caliper was used to measure the aperture size. The upper cell was attached to the lower one and DPBS (40 µL) was added to the cell. The voltage and aperture stretch were adjusted to establish a baseline current, ideally above 70 amps with a noise range or amplitude below 10 amps. The buffer solution was then removed from the upper cell and replaced with nanoparticles or breast cancer cells dispersed in DPBS (40 µL). At least 500 particles or breast cancer cells were recorded to achieve a statistically good data set. The upper cell was

rinsed by pipetting DPBS (40 µL) three times after the data was collected. A calibration solution (40 µL) with a known concentration and particle diameter was added in to the upper cell. At least 500 particles were also recorded in the calibration data set. The voltage and aperture stretch were not changed when the calibration solution was measured. The qNano determined particle number per milliliter and particle size distribution.

2.5 MTS Assay

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assays were conducted to measure the proliferation of MDA-MB-231 cells. About 5,000 cells in 100 µL were added to each well under study. The well plate was incubated for 24 hours and cells were washed with DPBS (50 µL). DMEM+10%FBS medium (100 µL) was added to all rows in one column. Media (50 µL) and diluted samples (50 µL) were added to the rows in other columns. Thus, samples were diluted by a factor of 2. All the media was removed after 48 hours, and replaced with a solution of media (100 µL) and MTS (20 µL). After incubation (3 hrs), absorbance was recorded at 490 nm with a reference of 680 nm. MTS assay demonstrated the toxicity levels of ghost nanoparticles and free docetaxel as absorbance was directly related to cell viability.

2.6 Flow Migration Chamber

An in vitro micro-fluidic device was used to simulate cellular extravasation under dynamic conditions. A motor pumped the cellular solution through a continuous loop (Figure 9). The device consists of a modified chemotactic Boyden chamber (Figure 10). The bottom piece of the chamber is a 48-well acrylic plate (Neuro Probe, Gaithersburg, MD) and the top piece is an acrylic plate with an inlet and an outlet for flow media. The two plates are separated by a 0.02-inch thick silicone gasket

(PharmElast, Trelleborg, Hudson, MA). A 7 cm x 2 cm opening was cut in the center of the gasket to form the flow field. A monolayer of EI cells was grown to confluence on the side of a sterilized polyvinylpyrrolidone-free polycarbonate filter (8 μm pore size; Neuro Probe, Gaithersburg, MD) that was coated with fibronectin (30 μg/mL, 3 h) (BD Discovery Labware, Bedford, MA). The other side of the filter was scraped before placing it in the chamber to remove EI cells than may have grown on the bottom side of the filter. The center 12 wells of the bottom plate were filled with soluble type IV collagen (100 μg/mL in DMEM/0.1 % BSA) (BD Discovery Labware, Bedford, MA). The rest of the wells were filled with medium (DMEM/0.1 % BSA).

The cells being studied (untreated, siScr treated, or siCD44 treated) (500,000 cells) were mixed in the flow medium (DMEM/0.1 % BSA). The chamber was placed in an incubator (37 C, 4h). The media was circulated through the chamber at the desired flow rate (50 or 100 s^{-1}). Other cases (untreated, CPSNPs, or free docetaxel) (2 million cells) were also conducted (50 s^{-1}). The volumetric flow rate (Q) was related to the wall shear stress (τ_W) by:

$$\tau_W = 6\mu Q/wh^2$$

In this relationship, μ is the fluid viscosity, *h* is height, and *w* is width of the flow field. The filter was removed from the chamber and stained with HEMA-3 (Fisher Scientific, Pittsburgh, PA). The filter was attached to a microscope slide and the top side was scraped to remove the EI cell monolayer. The migrated cells were quantified in 5 different locations (mm^2) and averaged for each slide. At least 3 slides were analyzed for each case.

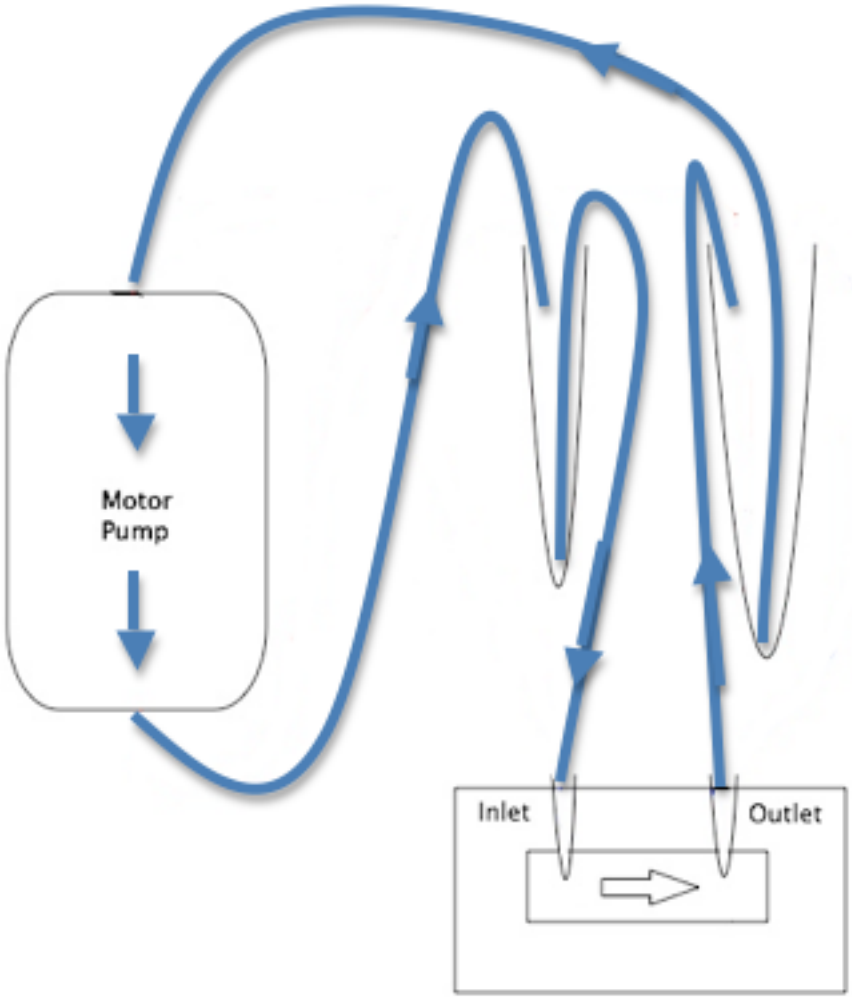


Figure 9. A schematic of the flow loop of the dynamic in vitro extravasation studies is shown.

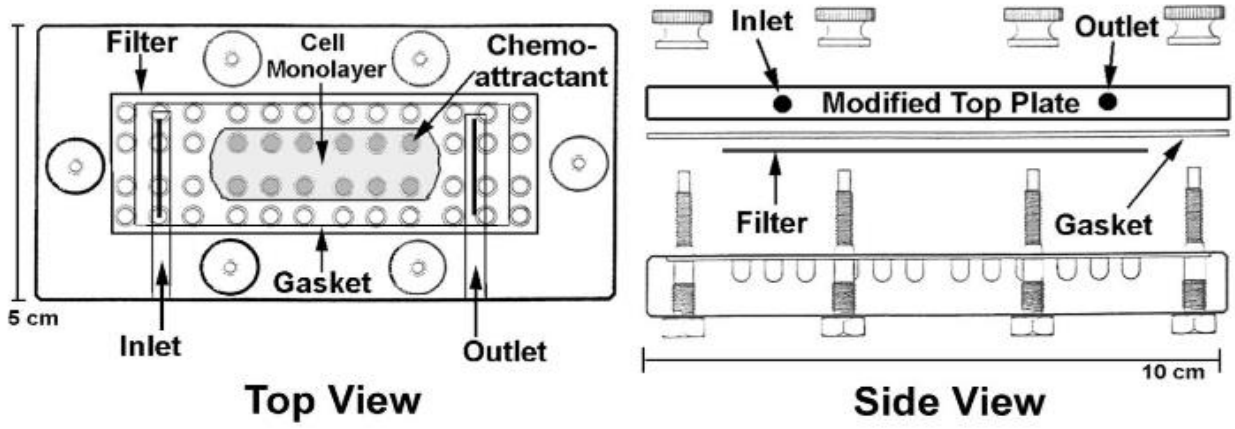


Figure 10. The set-up of a chamber used in flow migration studies is shown in the top and side views. There is a bottom plate with 46 chemotactic wells, a top plate, a silicon gasket with an opening in the center, and a filter with an EI cell monolayer.[27]

2.7 Statistical Analysis

Experimental cases and controls were compared with one-way ANOVA. Two cases were considered significantly different if the p-value was less than 0.05.

Chapter 3

Results

3.1 Ceramide Related Extravasation Results

Three cases (no treatment, siScr, and siCD44) were compared for each shear rate (50 s^{-1} and 100 s^{-1}). A one-way ANOVA test showed that there was a significant decrease in migration of cancer cells when they were treated with siCD44, while no significant difference was shown when they were treated with siScr. In addition, the results showed that an increase in shear rate reduced migration. Treatment of siCD44 apparently also had a greater effect under the low shear rate (50 s^{-1}) than under the high shear rate (100 s^{-1}).

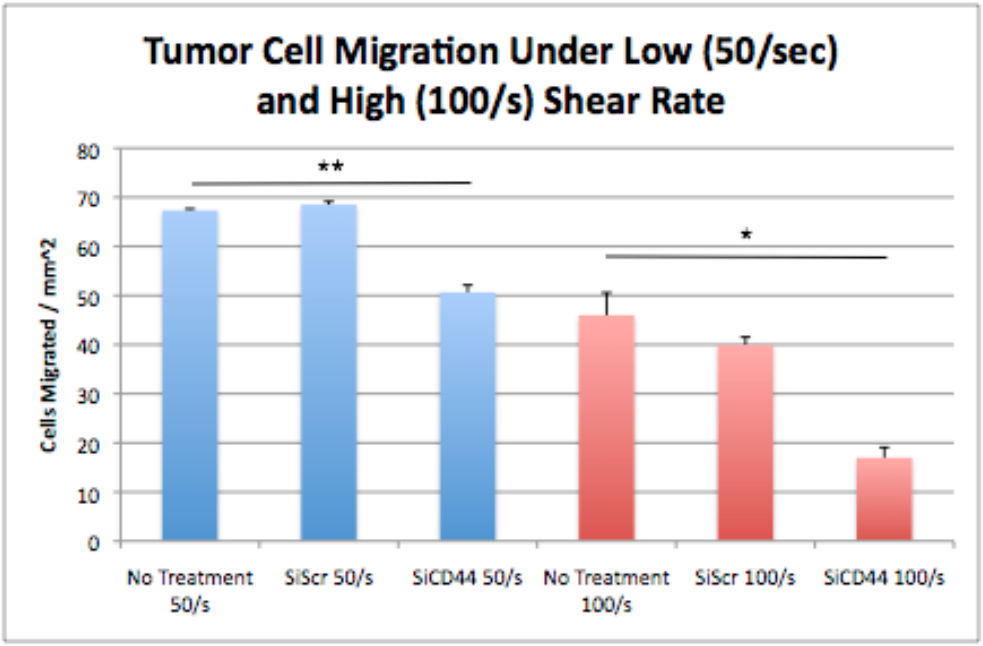


Figure 11. Flow migration results for untreated, siScr treated, and siCD44 treated cells under 50 s^{-1} and 100 s^{-1} shear rates are shown. Extravasation of SiCD44 treated cells is significantly lower than that of untreated cells while those treated with siScr had an insignificant change in extravasation. The p-value of between the siCD44 and no treatment cases under 50 s^{-1} is below 0.01 while the p-value between the two cases under 100 s^{-1} is below 0.05. The error bars are Standard Error (SE) for $n \geq 3$.* P-value < 0.05, ** P-value < 0.01

3.2 Izon Characterization

The qNano's ability to measure the particle number concentration and particle diameter distribution of various samples was tested. A sample of MDA-MB-231 cells was measured to have a mode diameter of 16 μm and a concentration of about 1 million cells per milliliter while cell counting via hemacytometer indicated the concentration was 1.14 million cells per milliliter. The standard deviation of concentration could not be provided for the hemacytometer or Izon counting method as it was not repeated. A sample of citrate ghost-CPSNPs made by Xiaomeng Tang was measured to have a particle number concentration of $2.0*10^{13}$ particles/mL and a mode diameter of 103 nm. The size distribution of the MDA-MB-231 cells is shown in Figure A-1 (Appendix A), and the size distribution of the citrate ghost-CPSNPs is shown in Figure A-2 (Appendix A).

The Welley Loc's 2-minute retention time methoxy-PEG ghost-CPSNPs were measured to be 10^{11} particles/ml and to have an average diameter of 79 nm (n=1403). The qNano was used for measuring the 30-minute retention time methoxy-PEG ghost-CPSNPs as well, but the results were not obtainable. A potential reason for this will be discussed later.

3.3 MTS Assays

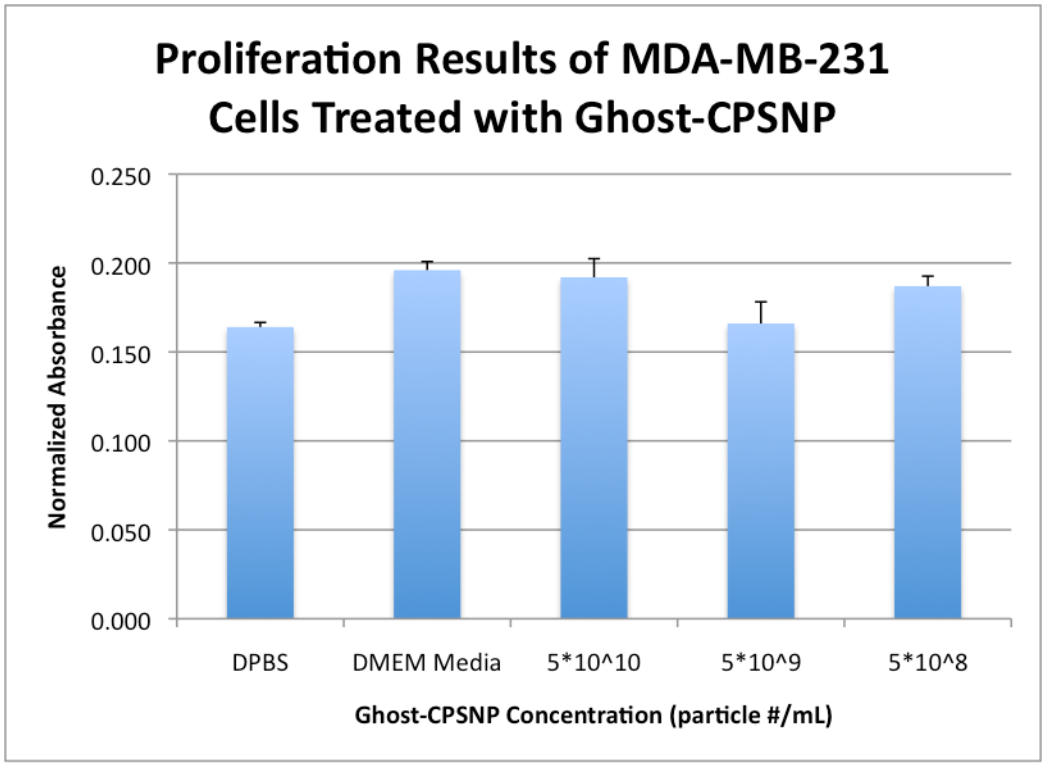


Figure 12. This MTS assay was conducted on MDA-MB-231 cells that were exposed to DPBS, DMEM media only, and methoxy-PEG ghost-CPSNPs ($5*10^{10}$, $5*10^{9}$, and $5*10^{8}$ particles/mL). One-way ANOVA showed that the measured absorbance was insignificant for all cases and the p-value > 0.1. The error bars are Standard Error (SE) for n = 6

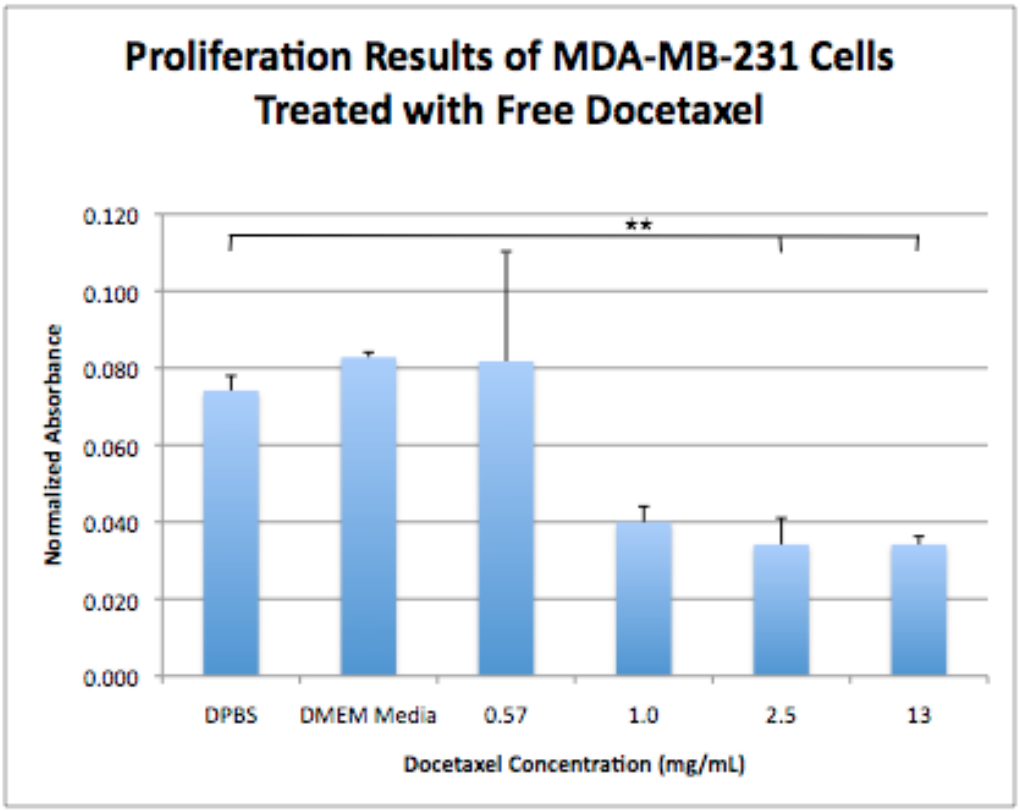


Figure 13. MTS assay results of MDA-MB-231 cells treated with DPBS (15% EtOH), DMEM media only, and free docetaxel (0.57, 1.0, 2.5, and 13 mg/mL). The error bars are SE for $n \geq 4$; ** P-value < 0.01

The two MTS assays provided information for understanding the effects of CPSNPs and docetaxel on cellular proliferation. The first MTS assay showed that the Methoxy-PEG CPSNPs at concentrations of $5*10^{10}$, $5*10^{9}$, and $5*10^{8}$ particles/mL had caused no significant change in the absorbance of the breast cancer cells. The second MTS assay showed that the absorbance was significantly lower for cells treated with a docetaxel concentration of either 2.5 mg/mL or 13 mg/mL than those treated with DPBS (15% EtOH).

3.4 Docetaxel Related Extravasation Results

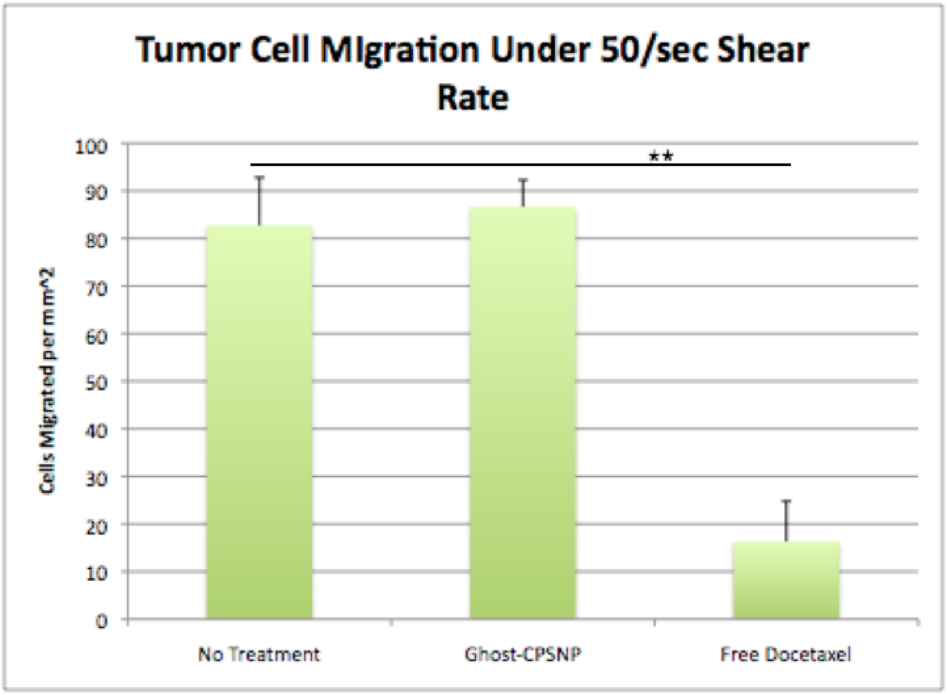


Figure 14. The flow migration results shown above demonstrate the migration of untreated, methoxy-PEG ghost-CPSNP treated, and free docetaxel treated MDA-MB-231 cells. A one-way ANOVA test showed that the cells exposed to free docetaxel had significantly less migration than untreated cells whereas cells treated with methoxy-PEG ghost-CPSNPs had insignificantly different migration results. The error bars are SE for $n \geq 3$, **P-value < 0.01

One result of these flow migration experiments showed that docetaxel at a concentration of 2.5 mg/mL was sufficient to significantly decrease breast cancer cell extravasation. Another result is that treatment with methoxy-PEG ghost-CPSNPs had no significant effect on MDA-MB-231 cell extravasation.

Chapter 4

Discussion

4.1 Analysis of Ceramide Nanoliposomes (CNLs)

The extravasation flow experiments showed that migration decreased for cells with reduced levels of CD44. These results were also compared to extravasation of cells treated with CNLs. Under a physiological flow rate of 50 sec^{-1}, 1 μM of CNL decreased migration by 56%, 5 μM of CNL decreased migration by 80%, and siCD44 decreased migration by 26%.[8] Under a physiological flow rate of 100 sec^{-1}, 5 of μM CNL completely abolished cell migration, while siCD44 decreased migration by 58%.[8] The cited article did not provide uncertainty ranges for the differences in extravasation. Since CNL had a greater effect on flow migration than on static migration, it is likely that ceramide is affecting both cell motility and adhesion, with the greatest effect seen at the highest shear rate and the highest concentration of CNL.[8] The results also showed extravasation decrease when the shear rate was increased. Increased shear forces prevent circulating tumor cells from being able to adhere to endothelial cells, thus preventing extravasation. Other evidence via western blots, immunofluorescence experiments, RT-PCR, MTS assays, anoikis assays, and mammosphere assays was gathered to further understand CNLs' effects on cancer metastasis.[8] In the collective analysis of ceramide nanoliposomes' effects on metastatic carcinoma cells, it was found that CNLs induced anoikis and inhibited extravasation in MDA-MB-231 cells via lysosomal degradation of CD44.[8] Since CD44 has been found to play an essential role in cancer cell migration, drugs that target CD44 should be able to diminish extravasation.[28,29]

4.2 Analysis of Calcium Phosphosilicate Nanoparticles (CPSNPs)

The Izon qNano, a modified flow chamber, and MTS assays were used to study CPSNPs. The qNano revealed information regarding particle number concentration and diameter distribution of the

particles. Prior to being used on the CPSNPs, the qNano was used on breast cancer cells to observe how it compared to another counting tool – a hemacytometer. After analyzing a sample of MDA-MB-231 cells, the qNano reported a concentration of about 1 million cells and a mode diameter of 16 µm. The concentration using a hemacytometer was determined to be 1.14 million cells. The discrepancy in the measured concentration is most likely due to the fact that the average diameter for mammalian cells is 15-25 µm, while the aperture size in the qNano used was only 10 µm. A larger aperture should be used if mammalian cells are going to quantified in the future.

In regards to measuring nanoparticles, a sample of citrate ghost-CPSNPs was studied. The recorded mode diameter was 103 nm and the concentration was $2*10^{13}$ particles/cm^3. The measured concentration is similar to the expected value but the diameter was more than double what was expected.[30] Possibly, the particles agglomerated over time (~6 months) and appeared twice as large as normal. These two tests demonstrated that the qNano is a potentially accurate and novel technique for determining particle number concentration of a sample. However, some other samples of particles were unable to be measured. Either no particles were counted or very few (<< 500) were detected. Since the qNano's measuring range is 50 nm – 10 µm, the particles in those samples must be either too large to pass through the nanopore or too small to disrupt the current. If the particles are too large, it may be due to agglomeration. If that is the case, then adding a small amount of Igepal CO-520 could help emulsify the solution. If the particles are too small to be detected, producing larger particles or improving the detection range of the Izon should be attempted and a smaller aperture used.

When CPSNPs were utilized in MTS Assays, it was found that the CPSNPs were non-toxic to the MDA-MB-231 cells. At concentrations of $5*10^{10}$, $5*10^{9}$, and $5*10^{8}$ particles/mL, the CPSNPs did not significantly change the absorbance values for cells that were only exposed to DMEM media and DPBS. If the CPSNPs had killed a significant number of cells, then NADH would not have been able to reduce MTS into the colored product formazan, and the detected absorbance values would be significantly low. When the cells were treated with docetaxel, however, the absorbance values decreased since the cells died

and were unable to convert MTS. The minimum concentration of free docetaxel observed to cause significant cell death was 2.5 mg/mL. There was a 54% decrease in cell proliferation when either 2.5 mg/mL or 13 mg/mL was administered. The rise in docetaxel concentration from 2.5 mg/mL to 13 mg/mL may have not been large enough to affect more cells or it could be that all the cells were exposed to docetaxel in both cases but simply did not die in the 48 hour exposure time. Docetaxel (2.5 mg/mL) was then used to in an extravasation study to determine if it would cause a significant decrease in extravasation. The result showed that it indeed significantly lowered the extravasation by about 80%. It is important to note that ethanol was used to help dissolve the free docetaxel. Ethanol is also commonly used in docetaxel injections for cancer patients, which unfortunately can lead to symptoms of alcohol intoxication.[31] Docetaxel-CPSNPs could provide an added benefit over free docetaxel delivery by removing the need for ethanol.

Chapter 5

Conclusions

5.1 Conclusions

Both CNLs and CPSNPs provide an effective way of delivering drugs to cancerous cells. Collectively, CNLs were shown to inhibit cancer cell metastasis by inducing anoikis in cancerous cells and inhibiting extravasation by lysosomal degradation.[8] CNLs do not only remain a possible treatment to selectively treat primary tumors, but also treat metastatic carcinoma cells, including breast cancer, pancreatic cancer, and melanoma cells.[8] Flow migration experiments specifically showed that decreasing levels of CD44 in breast cancer cells, significantly reduced extravasation. This suggests that other treatment methods, such as liposome-encapsulated siCD44, could diminish metastasis of CD44 dependent migratory, cancerous cells. Another option may be to even conjugate CD44 antibodies to liposomes to increase selectivity.

Besides CD44, MDA-MB-231 cells overexpress the transferrin receptor - CD71.[32] Anti-CD71 could be conjugated to potentially increase the effects of CNLs or CPSNPs on extravasation. Ghost-CPSNPs were found to have no significant effect on cell proliferation or extravasation, while free docetaxel was found to diminish both. Bioconjugated CPSNPs that encapsulate docetaxel would provide a targeting method that is more advanced than the current one, which depends on gaps (~200 nm) in leaky vasculature to reach tumor sites.[33] Instead of depending on the EPR effect, anti-CD71 could assist in delivering docetaxel to cancerous cell that overexpress CD71 on their surface. In addition, encapsulating docetaxel in CPSNPs would remove the need to dissolve the drug in ethanol, thereby, preventing alcohol intoxication in patients receiving docetaxel treatment.[31] In summary, delivery methods that exploit cell surface markers, such as CD44 or CD71, could improve the prevention and treatment of solid tumor metastasis.

5.2 Future Directions

To test potential enhancements of CPSNPs, the fluoropore, RWT, was previously encapsulated in both anti-CD71 CPSNPs and methoxy-PEG CPSNPs. At a high shear rate (200 s^{-1}), cells exposed to either anti-CD71 RWT-CPSNPs or methoxy-PEG RWT-CPSNPs, had insignificant differences in fluorescent intensity.[21] Under low shear (62.5 s^{-1}), however, the anti-CD71 RWT-CPSNPs significantly raised the fluorescent intensity of the cell.[21] This indicates that the bioconjugation was more effective at raising the specificity of the CPSNPs at low shear. At high shear, both types of particles are moving at a high speed so there is not much time for the anti-CD71 RWT-CPSNPs to bind to the CD71 receptor on the cell membrane.[21] As a result, increased uptake was not observed under high shear conditions like it was under low shear conditions. In the near future, anti-CD71 docetaxel-CPSNPs should be run in the flow migration and compared to methoxy-PEG docetaxel-CPSNPs. It is expected that at low shear, possibly 50 s^{-1}, there will be a significant reduction in extravasation, while the difference will be less significant under a higher shear condition.

An extravasation run of methoxy-PEG docetaxel-CPSNPs can also be compared to the free docetaxel case. As long as the final concentration of encapsulated docetaxel can be determined, uptake efficiency of encapsulated docetaxel compared to free docetaxel can be understood. The starting concentration of docetaxel for making docetaxel encapsulated CPSNPs is 8.08 mg/mL whereas free docetaxel at a concentration of 2.5 mg/mL was shown to significantly reduce extravasation. The percent yield of encapsulated docetaxel is currently unknown, but if it is determined, then CPSNPs with the same final concentration as the free docetaxel could be delivered. Comparing the two results should indicate if encapsulated docetaxel is more easily uptaken by cells than free docetaxel. In addition, the effects of CPSNP diameter on cell proliferation can be observed after successfully characterizing samples of two different retention times using the qNano.

Two methods of metastatic cancer treatment were researched and discussed (CNLs and CPSNPs). A third treatment could be a combination of both. A combination of C_6-ceramide and docetaxel was recently shown to significantly enhance apoptosis and growth inhibition beyond that caused by C_6-ceramide or docetaxel alone in MDA-MB-231 cells.[34] In the future, CNLs should be combined with anti-CD71 docetaxel-CPSNPs to see if extravasation is further diminished. CNLs can be delivered to cells about 24 hours in advance so CD44 levels can significantly decrease, while anti-CD71 docetaxel-CPSNPs can be introduced at the beginning of the flow migration. After sufficient in vitro testing has been done, docetaxel-CPSNPs should be tested in animal models and eventually in humans if tests continue to be successful.

Appendix A

Izon Particle Distribution Plots

The following images represent the particle distribution plots produced by the qNano.

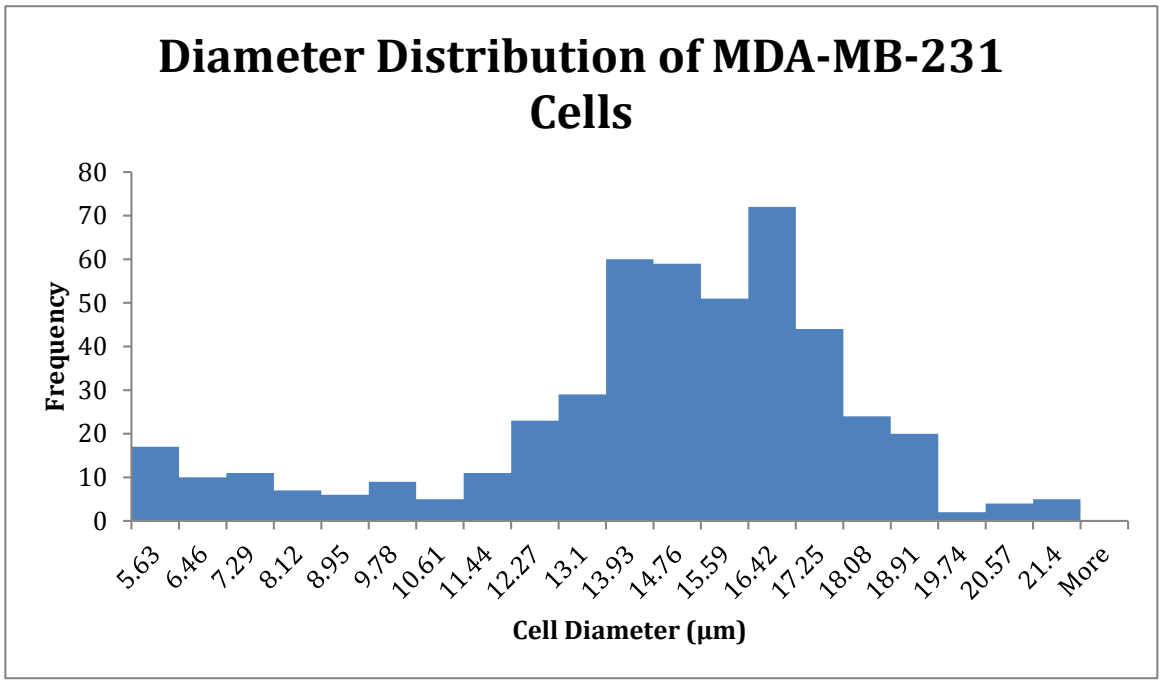


Figure 15. This plot depicts the size and concentration distribution of a sample of MDA-MB-231 cells. The mode diameter of the sample is 16 µm and the concentration was about 1 million cells. The mean diameter of the cell distribution was 14 µm and the standard deviation was 3.4 µm.

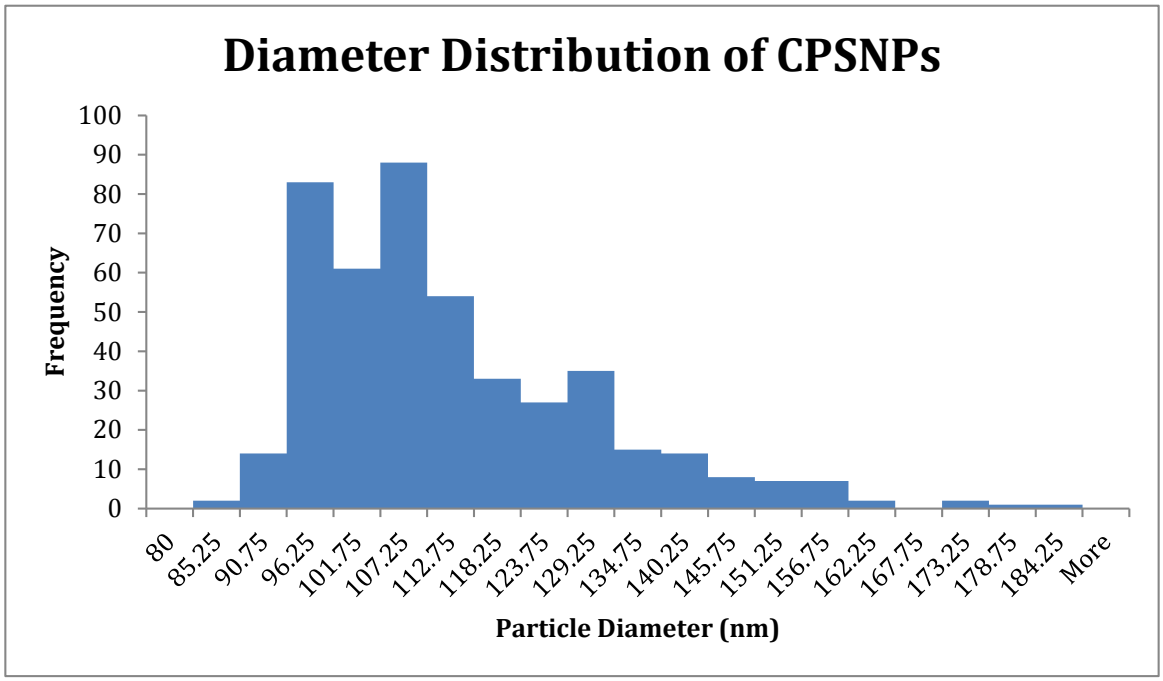


Figure 16. This plot depicts the size and concentration distribution of a sample of citrate ghost-CPSNPs, which was made by Xiaomeng Tang. The mode diameter of the sample is 103 μm and the concentration was about 2.0 *10^13 particles/mL. The mean diameter of the particle distribution was 110 nm and the standard deviation was 17 nm.

Appendix B

Flow Migration and MTS Assay Data

For each extravasation run ($n \geq 3$), migrated cells per mm^2 were counted in 5 different locations and averaged to the closest integer.

Table 1. Average number of migrated cells is shown for those treated with siCD44 under low shear (50 sec^{-1})

No Treatment	SiScr	SiCD44
67	70	51
68	67	53
67	68	48
	69	

Table 2. Average number of migrated cells is shown for those treated with siCD44 under high shear (100 sec^{-1})

No Treatment	SiScr	SiCD44
40	41	16
43	42	14
55	37	21

Table 3. The absorbance values of cells treated with CPSNPs are shown.

DPBS	DMEM Media	CPSNPs 5*10^10 particles/mL	CPSNPs 5*10^9 particles/mL	CPSNPs 5*10^8 particles/mL
0.181	0.164	0.184	0.236	0.173
0.166	0.189	0.151	0.179	0.162
0.176	0.182	0.197	0.164	0.177
0.171	0.175	0.174	0.185	0.198
0.169	0.172	0.228	0.221	0.195
0.164	0.196	0.192	0.166	0.187

Table 4. The absorbance values of cells treated with free docetaxel are shown.

DPBS	DMEM Media	Docetaxel 0.57 mg/mL	Docetaxel 1.0 mg/mL	Docetaxel 2.5 mg/mL	Docetaxel 13 mg/mL
0.096	0.079	0.067	0.008	0.034	0.031
0.057	0.080	0.071	0.040	0.032	0.033
0.076	0.083	0.094	0.037	0.038	0.038
0.082	0.086	0.095	0.035	0.027	0.034
0.081	0.084		0.056	0.030	0.040
0.053	0.085		0.064	0.044	0.029

Table 5. Average number of migrated cells is shown for those treated with CPSNPs or free docetaxel under low shear (50 sec^{-1})

No Treatment	Ghost-CPSNP	Free Docetaxel
63	95	8
98	89	9
102	76	33
68		

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Bachelor of Science: Biomedical Engineering - Chemical Concentration

HONORS AND AWARDS

- Dean's List (6 semesters)
- People to People Student Ambassador
- National Student Leadership Conference

PUBLICATIONS

- Penny, H.L., M. Rifkah, A. Weaver, P. Zaki, A. Young, G. Meloy, and R. Flores. "Dehydrated Human Amnion/Chorion Tissue in Difficult-to-heal Diabetic Wounds: Case Series." Journal of Wound Care 24.3 (2015) 104-111.

- Haakenson, Jeremy K., Khokhlatchev, Andrei V., Choi, Younhee J., Linton, Samuel S., Zhang, Pu, Zaki, Peter M., Fu, Changliang, Cooper, Timothy K., Manni, Andrea, Zhu, Junjia, Fox, Todd E., Dong, Cheng, and Kester, Mark. "Lysosomal Degradation of CD44 Mediates Ceramide Nanoliposome-induced Anoikis and Diminished Extravasation in Metastatic Carcinoma Cells." Journal of Biological Chemistry (2015): jbc-M114.609677.

- Penny, H.L., J. Spinazzola, A. Green, M. Rifkah, M. Faretta, and D. Youshaw, A. Weaver, and P. Zaki. "Negative pressure wound therapy with Bio-Dome dressing technology in the treatment of complex wounds: A case series." Journal of Wound Care 23.4 (2014): S4-S9.

PRESENTATIONS

- Tang, X., O. Pinto, K. Hughes, V. Gonzalez, P. Zaki, C. Dong, and J. Adair. Docetaxel-Doped Calcium Phosphosilicate Nanoparticles in Treatment of Breast Cancer. American Chemical Society, Jun. 2014.

- Penny, H., M. Rifkah, and P. Zaki. EpiFix Micronized in the Non-Complient Diabetic Patient. Academy of Physicians in Wound Healing, Mar. 2014.

- Penny, H.L., M. Rifkah, and J. Spinazzola, A. Weaver, M. Faretta, and P. Zaki. Selected Case Studies Describing the use of Human Fibroblast-Derived Dermal Substitute in Difficult-to-Heal Diabetic Foot Ulcer. Academy of Physicians in Wound Healing, Mar. 2014.

- Zaki, P., A. Green, M. Mathew, H. Penny, and I. Youssef. Incidental Benign Musculoskeletal Findings Often Seen on Positron Emission Tomography-Computed Tomography (PET-CT), Recognition and Appropriate Management. Radiological Society of North America, Nov. 2012.

GRANTS

- **Schreyer Honor College Research Grant** *University Park, PA*
  Summer 2014

RELEVANT EXPERIENCE

Penn State Department of Biomedical Engineering *University Park, PA*
Research Assistant *Fall 2012 – Present*

Conducted an experiment on flow migration to test cancer drugs' impact on breast cancer cell migration; utilized cell culturing, Flow Cytometry, and cone-plate viscometers, dedicating 10 hours a week while balancing a full course load. Presented experimental findings and analyses at formal bi-weekly meetings to the department head, Dr. Cheng Dong. Started a separate initiative to convert all hand-written data into electronic files, streamlining easy data accessibility. Researched scholarly articles discussing cancer treatment to better comprehend experiments, analyze results, and incorporate relevant information to write an undergraduate Schreyer's honors thesis on cancer cell metastases. Submitted results for publication in the Journal of Biological Chemistry showing diminished extravasation of metastatic cancer cells when treated with liposomes.

Cardiology Associates of Altoona *Altoona, PA*
Physician Assistant/Receptionist *May 2013 – Aug 2013*

Collaborated with business representatives from biomedical companies to test working conditions of pacemakers to ensure zero malfunctions when used on patients. Crosschecked over 100 bill invoices of patients and identified and fixed discrepancies, cleaned and properly maintained over 30 medical instruments and learned about their contemporary uses, and filed patient records. Attended one-on-one seminars with clinicians and an electrocardiograph technician to learn about irregular heartbeats, various heart diseases, as well as how to correctly make a diagnosis.

Southern Alleghenies Elite Orthopedics *Hollidaysburg, PA*
Shadowing Assistant *May 2013 – Aug 2013*

Observed and communicated with physicians, physician assistants, nurses, medical assistants, managers, and receptionists to understand the logistics of a medical clinic. Witnessed the use of Subjective, Objective, Assessment, and Plan (SOAP) note and humor in clinical visits and surgical procedures such as arthroscopies and metal plate fixations.

Altoona Regional Hospital/UPMC Altoona *Altoona, PA*
Shadowing Assistant/Volunteer *May 2012 – Aug 2012*

Perceived and gained familiarity with medical procedures such as biopsies, CT scans, X-ray imaging, and ultrasounds, while interacting with patients to improve patient treatment and communication skills. Assisted and gained guidance from multiple interventional and diagnostic radiologists, a physician's assistant, and a radiology practitioner's assistant over a span of 85 hours. Volunteered over 150 hours by discharging approximately five patients an hour, and transporting dietary trays and medical equipment in a timely manner.

LEADERSHIP EXPERIENCE

Student Red Cross Club *University Park, PA*
Current Executive Board – President; Past - Treasurer, Sponsorship Officer *Jan 2009 – Present*

Communicate with other service organization leaders on the Council of LionHearts to further volunteerism on campus and in surrounding communities. Oversee and successfully manage an annual budget of over $28,000, which is distributed amongst over 70 active members to cover expenses relating to blood drives, CPR training, disaster preparation seminars, and youth education programs. Collaborated with the University Park Allocations Committee to receive $17,527 in funding per year, and held formal presentations advocating for additional funding, fundraising approval, and also explaining the role of non-monetary sponsors. Contacted hundreds of student organizations and corporate sponsors and formally presented for funding of the Annual Blood Challenge against Michigan State, resulting in a cumulative donation amount of $12,200 within one semester.

Intramural Soccer *University Park, PA*
Team Manager *Jan 2012 – Apr 2014*

Led and coached a team of eleven players by facilitating two practices a week, researching plays, assigning positions, and maintaining team chemistry, resulting in a winning season within two different semesters. Created personalized improvement plans for each of the eleven players, as well as held one-on-one meetings to discuss individual progress and strategy.

Global Medical Brigades *University Park, PA*
Panama Brigade Participant *Jan 2013 – Aug 2013*

Provided sustainable health care and medical relief in under-served rural Panama communities through medical education, medicine distribution, and latrine construction; served over 200 patients and built 2 latrines in a one-week span. Collaborated with 41 other brigade members to plan and execute three fundraising events to help cover the comprehensive cost of the trip of the Penn State chapter, $40,000. Teamed with licensed medical professionals for one week, shadowed doctors in medical consultations, and assisted in a pharmacy and a dental clinic under the direction of experienced pharmacists and dentists to improve over 200 local citizens' access to healthcare and overall health.

SCHOLARLY OR PROFESSIONAL MEMBERSHIPS

- Alpha Epsilon Delta – The Premedical Honor Society
- National Society of Collegiate Scholars
- National Society of High School Scholars