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DEPARTMENT OF BIOLOGY

ESTABLISHMENT OF SUBCULTURE PROTOCOL FOR THE MAINTENANCE OF BxPC3 CELL HEALTH IN PREPARATION FOR IN VITRO CYTOXICOLOGY SCREENING OF CALCIUM PHOSPHOSILICATE NANOPARTICLES

DANIELLE N. KUMPF
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Reviewed and approved* by the following:

James H. Adair
Professor of Materials Science and Engineering, Biomedical Engineering and Pharmacology
Thesis Supervisor

Richard Cyr
Professor of Biology
Honors Adviser

* Signatures are on file in the Schreyer Honors College.

ABSTRACT

Current treatment methods utilized for the treatment of pancreatic cancer have historically been largely unsuccessful, leading to a 5-year survival rate of <4%. A major obstacle contributing to past failures in treating pancreatic cancer is an inability to deliver high dosages of chemotherapeutic drugs to the tumor site. This project seeks to investigate calcium phosphosilicate nanoparticles targeted to the pancreas for specialized drug delivery. A novel cell culture protocol is described to afford healthy pancreatic tumor cells from the primary cell line BxPC3 for in vitro testing. Current studies are investigating the use of variations of the gastrin peptide as a pancreas-targeting agent for tumor specific delivery of chemotherapeutic drugs. Future studies hope calcium phosphosilicate nanoparticles will allow for specialized delivery of chemotherapeutics to tumor sites, thereby treating cancer with greater efficiency and minimizing harmful drug side effects throughout healthy tissue.

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Chapter 1

Pancreatic Cancer

Although pancreatic cancer occurs at a low rate compared to cancers of other types, it remains the fourth leading cause of cancer-related deaths in the United States.[1,2] Labeled an epithelial neoplasm, ductal adenocarcinoma, the most common form of pancreatic cancer, results in the rapid and faulty production of glandular tissue.[3] As early detection of pancreatic cancer is difficult and invasive to the patient, diagnosis does not occur for 91% of cases until the tumor has already metastasized throughout the body, leading to poor prognosis.[1,4] Additionally, pancreatic tumors exhibit high degrees of drug-resistance, as it is difficult to force therapeutic agents to accumulate at the tumor site. Despite experimentation with multiple permutations of surgical and chemoradiation therapies, the 5 year survival rate of pancreatic ductal adenocarcinoma remains at <4%.[5] A plethora of unique features for pancreatic cancer contribute to the poor prognosis, as current treatment methods are rarely curative.

When the Cancer Chemotherapy National Service Center was founded in 1955, the theoretical idea of utilizing chemical drugs to treat cancer began to evolve as a visionary, ideal treatment. As previous treatment plans focused on surgical and radiation therapies, the concept of chemotherapy offered the possibility of an eventual cure for cancer.[6] Although research into the field proved beneficial for a number of cancer types, with remission rates increasing exponentially in the later half of the 20th century, such chemotherapy development techniques have been unsuccessful in improving pancreatic cancer prognosis.[1,7]

Despite advances in the treatment of many cancer types over the past 50 years, modern approaches to the treatment of pancreatic cancer are ineffective. Research into the mechanisms and biology of pancreatic cancer itself has provided explanation to standard therapy failures and insight into those obstacles that must be overcome to achieve functional disease treatment. Key characteristics of

pancreatic cancer that contribute to its resistance to modern chemotherapy include highly desmoplastic stromal layers, reduced symptom expression, early metastases, and decreased immune responses at tumor sites.[4,7,8]

The complex desmoplastic stroma of pancreatic tumors plays a multi-faceted role in tumor resistance to drugs, as it creates a strong barrier between circulatory vessels and solid tumor tissue. Traditionally, chemotherapeutic drugs travel through the blood stream and accumulate at tumor sites due to the enhanced permeability and retention (EPR) effect. The EPR effect describes that the rapid and uncontrolled growth of tumor cells requires an accelerated production of blood vessels to provide blood and oxygen to tumor cells. This accelerated vasculature production leads tumor vessels to be widely fenestrated, creating a foundation through which drugs can diffuse from blood plasma to tumor tissue (Figure 1).[9–11] Additionally, solid tumors often lack proper lymphatic clearing vessel networks as a defense against the immune system's clearing of foreign cancer cells; a lack of lymphatic vessels also delays the clearing of drugs and contributes to their accumulation at tumor sites.[9,10]

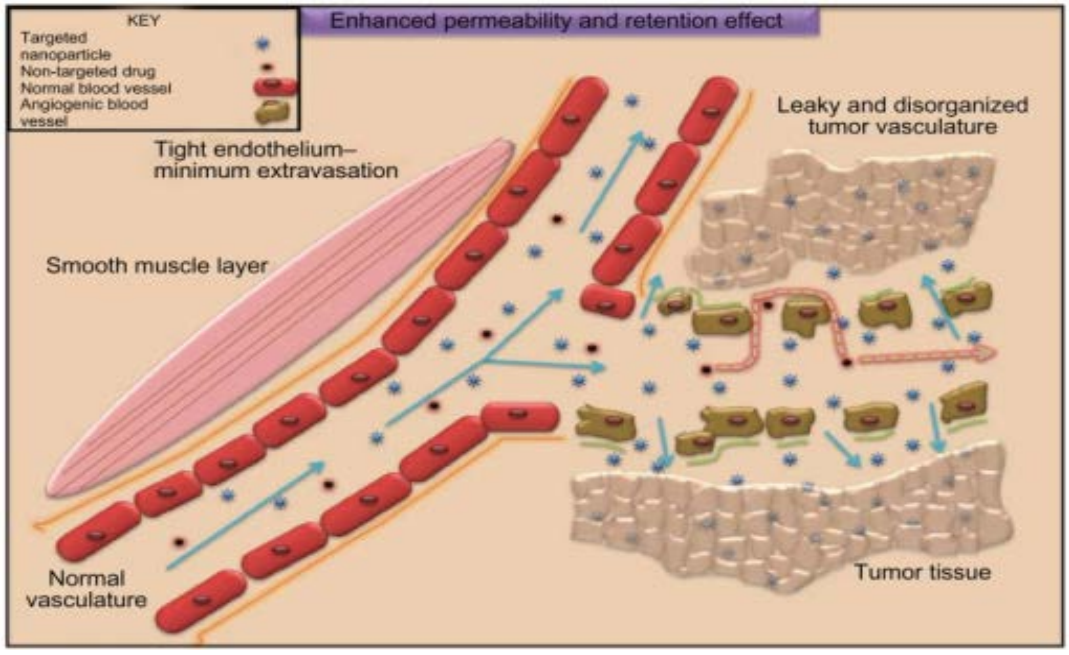

Figure 1 Enhanced Permeability and Retention Effect.
The enhanced permeability and retention effect shows the tendencies of tumors to show highly fenestrated vasculature as a consequence of rapid angiogenesis. Consequently, particles in the blood stream tend to leak out of arteries and veins and into the surrounding tumor tissue.[12]

Pancreatic cancer characteristically fails to display high levels of the EPR effect, due to extensive desmoplastic stromal layers. Such fibrous stromal layers form dense barriers surrounding the pancreatic tumor cells and provide an obstacle to drug infiltration.[7] As pancreatic tumors display low levels of angiogenesis and a lack of vascular growth throughout, delivery of drugs directly to the tumor cells remains difficult.

Current Treatment Options

As with most cancers, the treatment protocol for pancreatic cancer is highly dependent on the extent to which cancer cells have spread throughout the body.[13] As chemotherapeutic drugs have historically shown limited benefit in the treatment of pancreatic tumors, surgical resection is the only known curative measure to counteract disease.[13,14] If the cancer is diagnosed prior to metastasis, complete tumor resection is the recommended treatment.[15–17] Total tumor resection minimizes the likelihood of cancer recurrence and elevates the probability of long-term remission.[7,15,18]

Tumor resection method is dependent on generalized tumor location and proximity to critical arteries. Commonly referred to as a Whipple Procedure, a pancreaticoduodenectomy is the primary intervention against tumors located in or near the pancreas head and involves removal of the intestinal duodenum (Figure 2).[18,19] Tumors originating from the body or tail of the pancreas often require removal of the spleen, left kidney, and parts of the liver and intestine along with a distal pancreatectomy.[19]

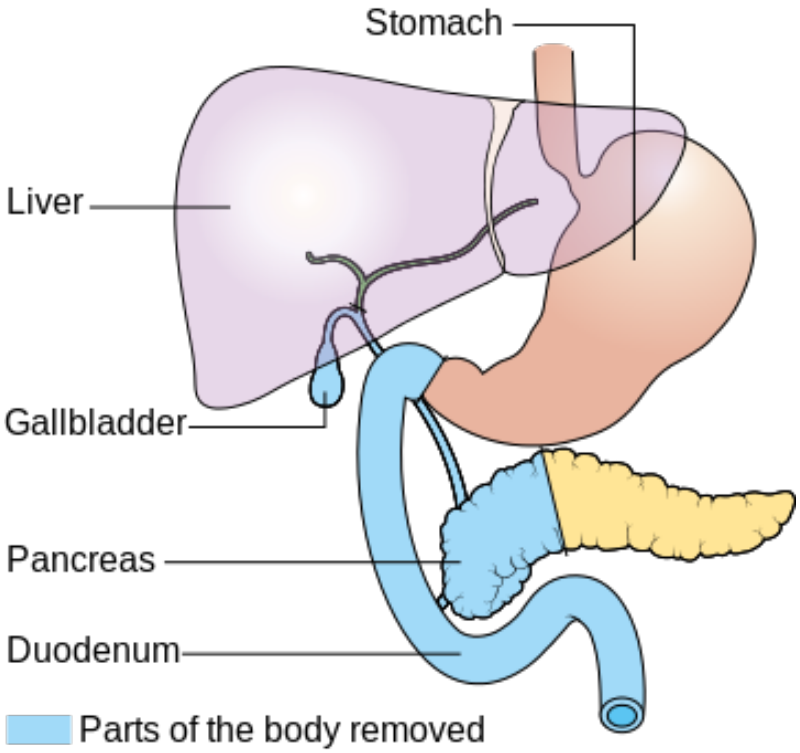

Figure 2 Standard Whipple Procedure.
Reprinted from a Cancer Research UK open knowledge project, the schematic indicates the regions of various organs often removed during a standard Whipple procedure (blue), including the duodenum, gallbladder and pancreatic head.[20]

Despite its status as the preferred method of treatment, surgical resection of pancreatic tumors is not without high risks. Pancreaticoduodenectomy is only characterized as curative if the tumor has negative margins and can be entirely resected.[14,21] Even when complete resection is achieved, long term survival is rare, with reports indicating low 5-year remission and survival rates of 3%-16%.[18] Post-operative complications aside, roughly 29% of patients having undergone a pancreaticoduodenectomy are readmitted into the hospital between 31 days and 90days post surgery. The majority of these patients are readmitted as a result of disease prognosis, indicating that even when complete tumor resection is possible, it is often not curative.[22]

Though surgical resection is the only treatment with an intent to cure, top cancer treatment centers such as the Memorial Sloan Kettering Cancer Center often recommend chemoradiation in addition to surgical procedures, particularly when complete resection is not possible.[23] Gemcitabine is one of the

most commonly used chemotherapeutics used in the treatment of pancreatic cancer. Gemcitabine is close in structure to the nucleoside deoxycytidine and can therefore be incorporated into replicating cells during DNA synthesis. Such incorporation ultimately interferes with the ability of polymerase to continue DNA elongation, thereby disrupting cell division and causing cell death.[24] Although gemcitabine requires activation within a cell, it does not explicitly target cancer cells and therefore plays a role in the death of a patient's healthy, systemic cells. Gemcitabine must be delivered intravenously and relies on transport via the blood stream to reach the pancreas. Unfortunately, pancreatic tumors characteristically display reduced vascular density, thereby hindering the delivery of gemcitabine to inner tumor cells.[7,11] Studies indicate that gemcitabine treatment after resection prolongs remission length if complete surgical resection (R_0) is achieved prior to tumor metastasis. However, gemcitabine treatment provides little benefit to patients whose cancer has progressed to include metastases or cannot be entirely removed surgically (R_1 and R_2).[23,24]

Top cancer centers attempt to treat late stage pancreatic cancer cases with a cocktail of chemotherapeutic drugs. The treatment regimen known as FOLFIRINOX is composed of four cytotoxic agents that each counteract cancer cells by a unique mechanism.[25] Combinations of folinic acid, 5-fluorouracil, irinotecan, and oxaliplatin are administered via IV drip; the exact radio of drugs is patient dependant.[25,26] Though the FOLFIRINOX regimen appears to benefit patients with advanced cases of pancreatic cancer, it is only recommended for patients with highly advanced cases who are otherwise in good health, as the side effects are severe. When compared with patients receiving traditional chemotherapy treatment with gemcitabine, those treated with FOLFIRINOX displayed increased development of febrile neutropenia, a life-threatening fever characterized by dangerously low neutrophil levels that often lead to hospitalization.[26,27] Patients treated with FOLFIRINOX also display an increased risk of developing a plethora of neural side effects, with neurotoxicity harming both the central and peripheral nervous systems. Oxaliplatin, an antineoplastic platinum analog used in the FOLFIRINOX treatment regimen, has been shown to induce episodes of dysarthria, a speech disorder caused by a loss of

control of the motor muscles used to produce language.[28,29] When combined with the other drugs prescribed in the regimen, 5-Fluorouracil (5-FU) can induce neurotoxic effects, as it crosses the blood-brain barrier and induces seizures.[28] Consequently, FOLFIRINOX is only indicated in high-risk cases in which surgical resection and/or treatment with gemcitabine are unsuccessful.

When chemotherapy is administered via IV, the cytotoxic agents travel systemically through the body, harming even healthy cells as they travel to the target site. Such systemic circulation also influences the recommended dosages of various anticancer agents. To have an active effect, drugs must encounter the targeted tissue at a minimum therapeutic concentration.[11] Unfortunately, circulatory drug distribution does not permit delivery of the entire dose to the tissue site, as some of the medication accumulates in health tissue sites and some is removed by the body's natural clearance mechanisms. Therefore, an elevated dose must be administered to account for that lost before the treatment reaches the target site. However, such increases in treatment administration subsequently increase side effects and the risk of secondary disease development and cytotoxicity.

Chapter 2

Current Targeted Drug Delivery Systems

The concept of specialized delivery of drugs to specific target site has circulated within the scientific community since the 1960s, as outlined in Table 1.[30] A number of drug delivery systems have been investigated but traditionally fail to combine targeted delivery with controlled drug release and particle clearance.[30–32] Under ideal circumstances, a drug delivery system would allow complete encapsulation of a drug, preventing it from having an effect on systemic tissues as it travels to the targeted tissue site. As the need for an efficient delivery system becomes more apparent, a plethora of studies have investigated the use of various nanoparticles formulations in targeted drug delivery, such as dendrimers, quantum dots and gold nanoshells.[11] Extended analysis of materials suggests the promising use of nanoliposomes in drug delivery techniques.

Table 1 Proposed Nanoparticle Systems for Drug Delivery.
Reprinted from Tabakovic et al., previously investigated potential nanoparticle drug delivery systems are compared in terms of size, delivered agent, advantages and limitations.[33]

Nanoparticle System	Size (nm)	Therapeutic Agent(s) Carried	Advantages	Limitations
Biodegradable Polymers	10-100	Plasmid DNA, proteins, peptides, low molecular weight organic compounds	Sustained localized drug delivery for weeks	Exocytosis of undissolved nanoparticles. Fixed functionality after synthesis may require new synthetic pathways for alternate surface functionalities
Ceramic	<100	Proteins, DNA, chemotherapeutic agents, high molecular weight organic compounds	Easily prepared, water dispersible, stable in biological environments	Toxicity of materials, exocytosis of undissolved nanoparticles, time consuming synthesis, surface decoration instead of encapsulation
Metals	<50	Proteins, DNA, chemotherapeutic agents	Small particles present a large surface area for surface decoration delivery	Toxicity of materials, exocytosis of undissolved nanoparticles, time consuming synthesis, surface decoration instead of encapsulation
Polymeric Micelles	<100	Proteins, DNA, chemotherapeutic agents	Suitable for water-insoluble drugs because of hydrophobic core	Toxicity of materials, fixed functionality after synthesis
Dendrimers	<10	Chemotherapeutic agents, anti-bacterial, anti-viral agents, DNA, high molecular weight organic compounds	Suitable for hydrophobic or hydrophilic drugs	May use toxic materials, time consuming synthesis, fixed functionality after synthesis may require new synthetic pathways for alternate surface functionalities
Liposomes	50-100	Chemotherapeutic agents, proteins, DNA	Reduced systemic toxicity, increased circulation time	Fixed functionality after synthesis, some leakage of encapsulated agent, lack of colloidal stability
PRINT	20-2000	Chemotherapeutic agents, proteins, DNA, imaging agents	Precise control over size, shape and surface functionalization	Toxicity of materials depending on material
Calcium Phosphosilicate	20-60	Chemotherapeutic agents, RNA, high and low MW organic compounds, imaging agents	Simple preparation, suitable for hydrophilic or hydrophobic drugs, colloidal stability in physiological environments, pH-dependent dissolution results in intracellular delivery of drugs, composed of bioresorbable material	Encapsulated materials limited to solubility in water or organic solvent

There are a number of criteria that a successful drug delivery system must possess, as described in Table 2. Ideally, a targeted delivery system is composed of easy to synthesize bioparticles, not themselves toxic in the human body. This places significant limitations on the materials that can be utilized in particle synthesis. Particles must be easily produced in mass quantities and conjugated with a variety of agents for site-specific delivery. Such bioconjugation of the particles permits specialized delivery to any target tissue. Within the context of pancreatic cancer treatment, nanoparticles would ideally present as non-toxic and colloidally stable. Particles must efficiently encapsulate the desired agent (chemotherapeutic) and prevent leakage of the agent during transport throughout the body. The particles should not be recognized as foreign by the immune system, thereby preventing elimination by immune cells. They must be small and colloidally stable in physiological conditions. It is essential that the particles involved in an ideal delivery system retain the encapsulated agent and avoid clearance until they reach the target tissue. However, once this target site is met, the particles must be stimulated to degrade, releasing the active agent in the target tissue in high concentrations. After degradation, the free particles should have an efficient clearance mechanism to avoid accumulation in the body.[11,34,35] Finding a delivery system that meets all desired criteria has, up to this point, been largely unsuccessful.

Table 2 Criteria for Nanoparticulate Drug Delivery Systems.
Reprinted from Tabaković et al., characteristics required for the development of a viable nanoparticle drug delivery system are outlined and discussed. All criteria must be incorporated to a nanoparticle system for ideal function in vivo.[33]

Characteristic	Comments
Inherent material and degradation product nontoxicity	The initial material selection should be based on nontoxic materials, especially with an aim toward human health care
Small Size (10-200nm)	There is not a particular size that seems most efficacious particularly based on *in vivo* studies. This is the range of particle diameters that have proven most effective for a wide variety of delivery systems. Also of note is the debate around the influence of particle shape
Encapsulation of active agent	To be effective, the active agent must be encapsulated within the nanoparticle vehicle. Surface decoration (i.e., adsorption) will often be effective *in vitro* but falls short for *in vivo* studies because of the reticuloendothelial (RES) system
Colloidal stability in physiological conditions	The nanoparticle vehicle and surface functionalization must be resistant to agglomeration for the solution pH values, ionic strength, macromolecular interactions, and temperature encountered in the physiological environment
Clearance mechanism	The nanoparticle vehicle must have a ready clearance mechanism to avoid the cumulative and/or systemic effects of the drug laden particles
Long clearance times	Resistance to agglomeration and other effects that remove the nanoparticle encapsulated drug from the patient must be avoided to promote long circulation times in the circulatory system for as much of the nanoparticles to find and sequester in the cancer cells as possible
Controlled release of active agent	There should be a trigger mechanism, such as the acidic pH within the tumor or during endosome maturation, designed into the nanoparticle platform to ensure the release of the encapsulated drug into the targeted tissue
Targeted delivery to cell/tissue	The nanoparticle platform needs to be surface bioconjugated to target molecules for a specific cancer and to provide the greatest uptake within the cancer lesions while eliminating side effects to healthy tissues

A number of chemotherapy treatments claim to be targeted drug delivery mechanisms because they rely on a passive mechanism of drug delivery known as the EPR effect, which is based on the physiological characteristics of a generic tumor.[30] Traditionally, solid tumors express elevated levels of angiogenesis, as a complex vascular network is required to maintain the "health" of deep tumor cells.[11] These vessels travel to the inner layers of the developing tumor, providing an access route for the delivery of drugs deep within the tumor. As the rapid formation of such vessels does not allow for optimal vessel development, the vascular network is characterized by highly fenestrated walls, through which drugs traveling through the blood stream can leak out into the tumor tissue.[36] As the EPR effect is a passive process, it cannot be utilized in tissues that do not naturally display the phenomenon, including pancreatic tissue. Thereby, an active targeting system must be developed for specialized delivery to such cells types.

Dendrimers were one of the first nanoparticle delivery systems developed, having been first synthesized in the 1970s and 1980s. Although they are characterized by small size and the ability to be surface functionalized, the delicate synthesis protocol of dendrimers hinders their use in medicinal settings.[37] Similarly, experimentations with quantum dots, gold nanoparticles, fullerenes and titanium dioxide have failed to yield viable delivery systems. Given that the primary components of each of these systems involve elements not found naturally in the human body, it is not entirely surprising that such systems have failed as targeted drug delivery systems.[35] Therefore a successful drug delivery system must be synthesized from molecules of that naturally mimic blood chemistry and successfully encapsulate agents for delivery may provide the key to reduced toxicity and escape from immune response.

Aside from being composed of inorganic elements that the human body can manage in trace amounts, the delivery system particles should fall within specific size parameters. If synthesized to be less than 10 nm, particles are immediately cleared by the renal system. However, the liver and spleen recognize particles above 200nm and target them for phagocytic elimination.[38] Though some particles may fall within this range, they fail to operate as delivery systems because they agglomerate to form overly large particle clumps. Though originally considered one of the top candidates for drug delivery

systems, liposomes fail to display adequate colloidal stability and therefore show elevated levels of agglomeration under physiological conditions.[39]

Agglomeration is negative as it leads to the rapid clearance of particles from the blood stream. Ideally, particles display long clearance time so they can circulate throughout the body and have more opportunities to accumulate in the target tissue.[11,39] However, this long clearance time must be balanced by an efficient clearance mechanism once particles have released their encapsulated agent to the target tissue. Gold nanoparticles failed to satisfy system requirements, as they are not naturally cleared from the body but rather accumulate in tissue.[38] Renal clearance is ideal as it allows physicians to monitor clearance via urine analysis.

Many systems focus on adhering imaging or therapeutic agents to the surface of particles, also known as surface decoration, so they may be guided to target tissues.[33] This poses a problem if the agent of choice is itself toxic, as is the case with chemotherapeutics. In such cases, agents must be encapsulated within a transport system to limit their exposure to healthy tissue. Additionally, once encapsulated, the agents must display limited leakage out of their particle cases, which often occurs in liposomal systems.

Chapter 3

Calcium Phosphosilicate Nanoparticle Drug Delivery System

The development of a calcium phosphosilicate nanoparticle (CPSNP) drug delivery system shows promise as a novel treatment for pancreatic cancer. Found naturally in the body, calcium phosphates play an essential role in mammalian anatomy and physiology in the form of hydroxyapatite.[40] Present in bones, teeth and ligaments hydroxyapatite contributes properties of strength and stress-resistance to force-baring tissues. Within the human body, calcium phosphates exist in multiple states. The hydroxyapatite form develops from the nucleation of amorphous calcium phosphate, resulting in high crystallinity. In the context of the drug delivery system, crystalline particles are undesirable as they fail to remain dispersed in solution, thereby inhibiting colloidal stability needed for optimal drug encapsulation.[41] However, under standard calcium phosphate synthesis protocol, synthesized amorphous calcium phosphate transforms rapidly to hydroxyapatite.[33,41,42] Consequently, calcium phosphates synthesized under standard protocol are not able to maintain the dispersion necessary for agent encapsulation. As encapsulation is not feasible, conventional calcium phosphates rely on surface decoration to carry vehicles to target tissues.[32,43] Recent studies reveal that the incorporation of silicate into the synthesis of calcium phosphate particles via a reverse micelle synthesis maintains calcium phosphate amorphous properties.[35] This synthesis of calcium phosphosilicate presents a novel solution to the problem presented by crystalline hydroxyapatite.

A reverse micelle synthesis approach allowed for drug encapsulation as opposed to surface decoration. Many previously developed nanoparticle delivery systems rely on adherence of molecular agents to nanoparticle carriers.[37] Calcium phosphosilicate nanoparticles show the unique ability to encapsulate drugs of choice due to the reverse micelle exchange involved in particle synthesis (Figure 3).[44] Such encapsulation is preferred over traditional surface decoration as it prevents exposure of potential harmful drug agents to the body until they are deposited at the targeted tissue.

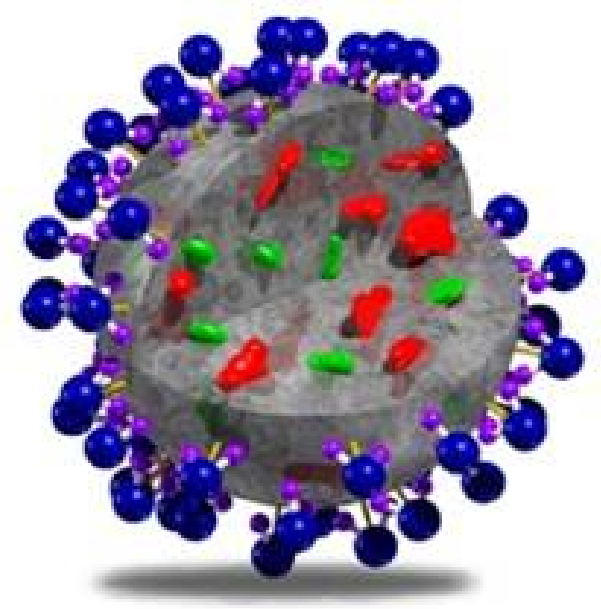

Figure 3 Calcium Phosphosilicate Nanoparticle.
Depiction of calcium phosphosilicate nanoparticle (gray) after encapsulation of therapeutic drug (red) and/or diagnostic agent (green) and bioconjugation with a PEG tether (blue) to exterior carboxylic acid groups (purple).[45]

Much focus is placed on assuring chemotherapeutic agents are tightly encapsulated by the particle system. Leakage of agents from the particles reduces the efficiency of drug delivery to the target tissue. Additionally, if the drug delivery particles allow their agent to leak from encapsulation, the agents will interact with systemic tissue, causing cytotoxicity and harmful physiological responses.[40,45] Of equal importance to tight agent encapsulation is the formation of an efficient release mechanism for particle breakdown once particles have reached their target. Ideally, particles can be constructed to degrade rapidly once inside specific tissues, thereby releasing a complete dose of encapsulated agent to the target tissue. The CPSNP delivery system exploits the pH-dependent changes in particle solubility to control agent release.[33,40,46]

The human body maintains a tightly regulated pH ranging from 7.3 to 7.45 in both intracellular and extracellular environments. Under such conditions, CPSNPs remain in an amorphous state with low solubility, allowing them to maintain tight hold on the encapsulated drug. CPSNPs cannot diffuse across the cell membrane and must therefore rely on uptake into cells via pinocytosis and receptor-mediated

endocytosis (Figure 4). Once within their target tissue, CPSNPs must degrade to release their encapsulated agent. Endosomes conventionally develop as a means of taking up and degrading large molecules to release nutrients into the cytoplasm. Such degradation is achieved by adjusting the endosome environment to a pH between 4 and 5. When the cell takes up the CPSNPs it treats the resulting endosome as an early lysosome, slowly lowering its pH to about 4.5.[45,46] Under such conditions, calcium phosphate particles become increasingly soluble, ultimately dissolving to release their encapsulated agents. Breakdown of capsules and release of agents elevates osmotic pressure within the endosome, forcing the endosome to burst.[46] The bursting of endosomes is essential in drug delivery because it deposits the active agent into the cytoplasm, where it acts to achieve desired therapeutic effects. In doing so, the active agent is freed from the low pH environment and saved from degradation itself because phosphate tends to buffer pH in the late endosome.

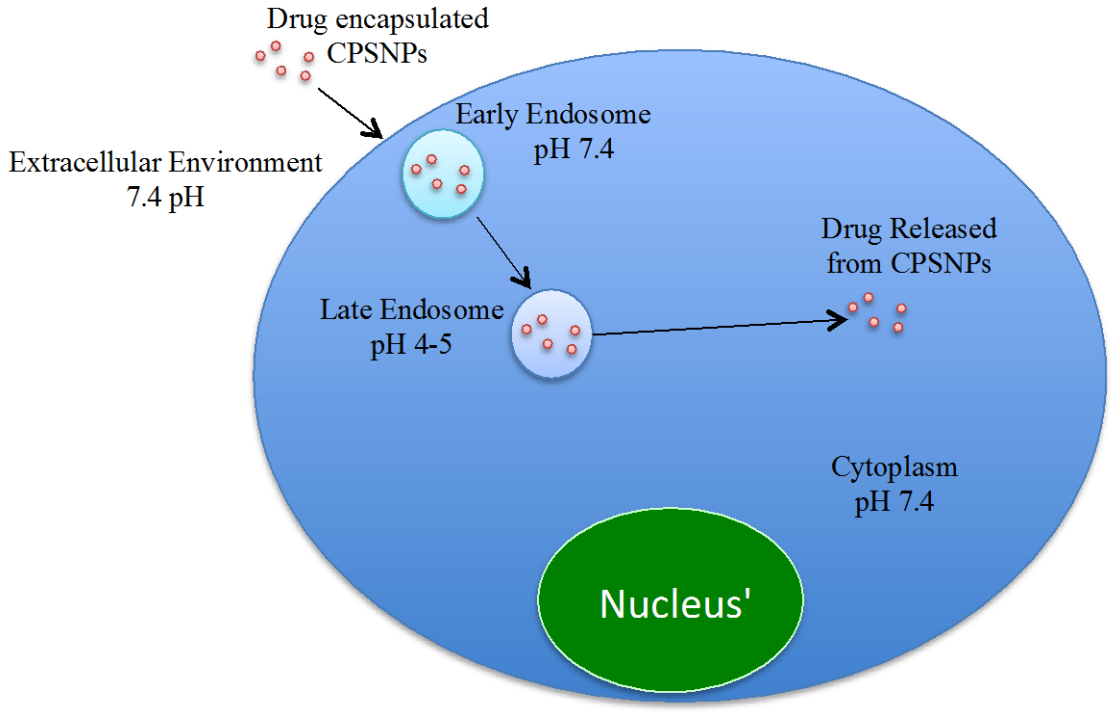

Figure 4 Cellular Uptake of CPSNPs
Taken into the cell in a phagocytic mechanism, CPSNPs originally enter the cell in a newly formed endosome. As the pH of the endosome drops with maturation, the CPSNPs begin to dissolve and release the encapsulated agent. This release alters the osmotic pressure within the endosome and causes it to burst, thereby releasing the newly freed agents into the cytoplasm, where the pH is buffered to 7.4.

Synthesis and Bioconjugation

Calcium phosphosilicate nanoparticles are synthesized via a dual microemulsion process, which allows for efficient drug encapsulation. Both microemulsions are formed in room temperature 29 vol% igepal-cyclohexanes. Microemulsion A included filtered $CaCl_2$ while microemulsion B included filtered Na_2HPO_4, Na_2SO_3. Imaging and therapeutic agents to be encapsulated, such as indocyanine green and 5-FU, are incorporated into either microemulsion A or B depending on the polarities of the agents. After both microemulsions were given time to stir, microemulsion B was poured slowly into A. The pure microemulsions were given minimal time to coalesce and form sodium phosphosilicate reverse micelles. Soon after the microemulsions were mixed, typically several minutes, filtered citrate was added to quench the precipitation of CPSNPs, disperse the CPSNPs, maintain the amorphous phase and prevent the formation of hydroxyapatite. The synthetic mechanism of CPSNPs reduces the risk of leakage, as 5-6 micelles combine to form a capsule around the drug of choice. Once the micelles agglomerate around the agent, calcium bonds to phosphate and silicate ions to form particles. Pores are not present within the particles, thereby reducing the risk of premature leakage of the encapsulated agent during subsequent processing and in vitro and in vivo studies.

Laundering of the CPSNPs proceeds as discussed in Adair group papers.[44,47,48] After citrate quenches the precipitation, the micellular solution is dissolved with neat ethanol to yield a homogeneous solution. The nanoparticles are laundered using the wan der Waals high performance liquid chromatography approach based on a packed bed laundering mechanism. The nanoparticles adhere through van der Waals attractive energy to silica media. The particles are laundered with neat ethanol solution and then eluted with 70:30 vol% ethanol:water. The ethanol:water solution supports charge formation on both the CPSNPs and the silicon dioxide media, resulting in an electrosteric repulsive

energy that permits release of the CPSNPs from the silica media and elutes as a concentrated suspension in 70:30 ethanol:water. Morgan et al. demonstrated that the citrate functionalized CPSNPs from 70:30 ethanol:water could be dried and spontaneously redispersed in the solvent mixture to verify the inherent colloidal stability of the as prepared CPSNPs.[43] The carboxylic acid on the citrate irreversibly bound to the CPSNP surface is used in subsequent bioconjugations.

Throughout the synthesis of CPSNPs, emphasis is placed on maintaining an inherent non-toxic particle on the nano-scale that is colloidally stable. Once achieved, the particles must be further modified to bioconjugate target molecules via surface functionalization. Ultimately, particles should be functionalized to display antibodies, receptors, antigens or aptamers that are characteristic of the target tissue, thus allowing them to accumulate in desired regions of the body. As the exact agent presented on the particle surface is dependent on the area undergoing treatment, various methods of bioconjugation to the particle surface are required. This is problematic as reactions may compromise particle integrity and reintroduce risk of particle agglomeration. Therefore, after the particles are washed, PEGylation is used to attach a tether that can bind desired surface agents that continues to maintain colloidal stability.[43]

PEGylation occurs as a stepwise process to chemically modify particle surfaces for attachment of desired agents (Figure 5). The carboxylic acid groups on the particles' exterior are activated via treatment with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC). Sulfo-NHS is added to stabilize and protect the reactive site, thereby allowing controlled modification of particle surfaces and permitting at least 24 hours of stability for the PEGylation. Activated particles can then be coupled with maleimide-PEG-amine compounds. The amine terminated PEG interacts with the sulfo-NHS protected carboxylic acid via a substitution reaction, resulting in the amide linkage of the PEG tether to particle surfaces. The PEG tether contains a terminal maleimide to which targeting agents can be adhered. Various agents such as receptors, antibodies and aptamers can be added across the maleimide double bond for specific targeting of various tissues. Within the context of this study, a sulfhydral terminated gastrin-10 peptide was bound to maleimide via an addition reaction in an attempt to specifically target pancreatic tumor

cells. Gastrin is a hormonal peptide produced by the gastrointestinal tract in response to the presence of food in the stomach, particularly partially digested peptides.[49] Gastrin-10 was used as a target for pancreatic cancer cells. Gastrin receptors are overexpressed on all human pancreatic cancers, including BxPC3.[45]

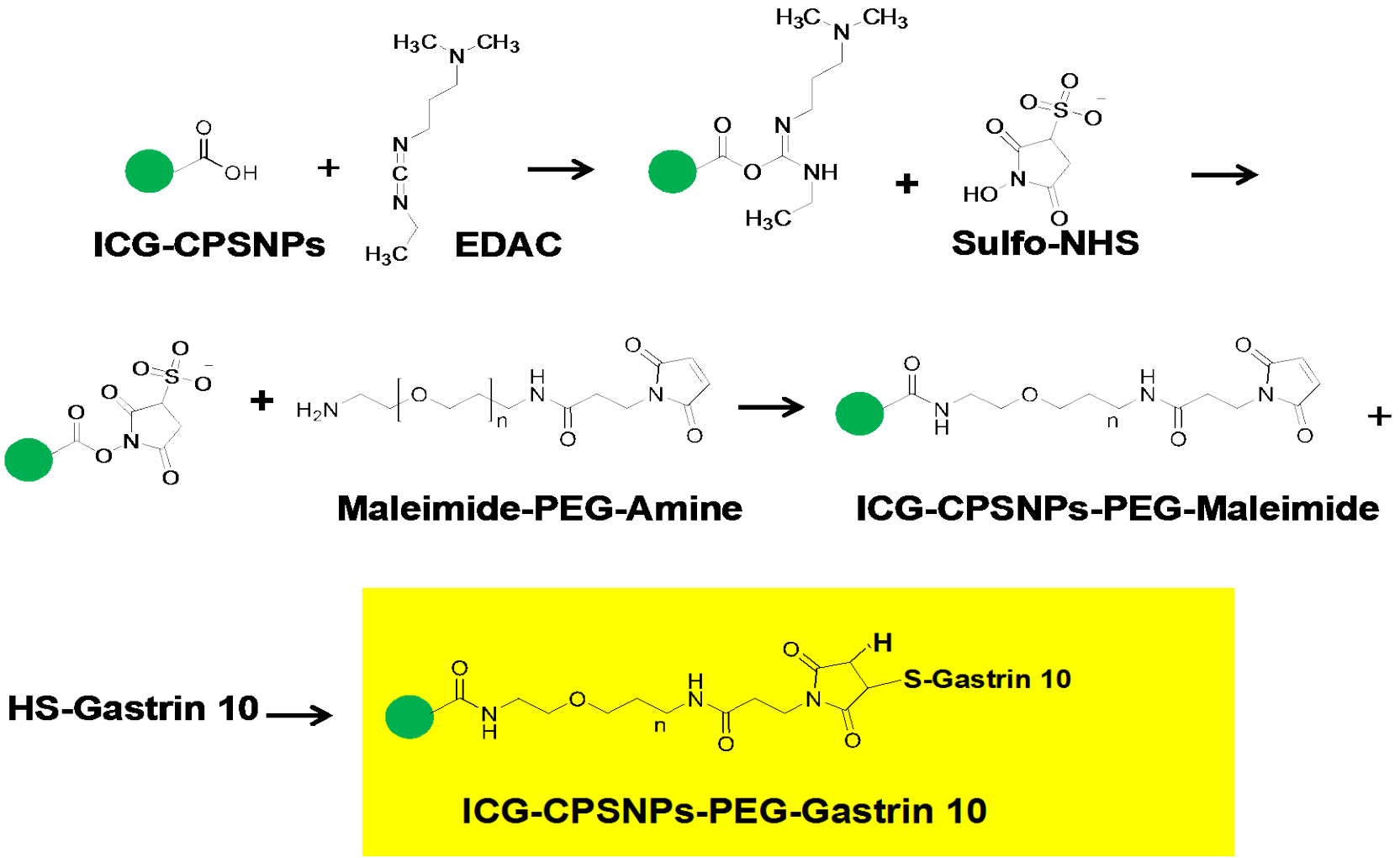

Figure 5 Bioconjugation of CPSNPs with Maleimide PEG and Gastrin 10.
CPSNPs are bioconjugated to attach tumor-specific agents to particle surfaces for targeted delivery. The carboxylic acids of particles are activated with EDAC and sulfo-NHS. PEG is tethered to the particles via an amide bond, while the maleimide end is functionalized with SH-Gastrin 10 for pancreatic targeting.[45]

Chapter 4

In Vitro Testing and Cell Culture Standards

In vitro tests are utilized in research settings as initial screening mechanisms to test the cellular impact of novel medicinal protocol. Within the context of this study, in vitro analysis was used to examine potential cytotoxicity of the synthesized particles. Such studies also allowed for extensive analysis of particle uptake by cancer cells and help confirm the mechanism of controlled agent release from encapsulating particles into targeted cells as well as verifying that at least in vitro targeting was in place.

In vitro testing during initial system analysis provides an environmentally controlled system that can be maintained over selected environmental conditions. As all cultures are maintained in the same conditions, variations among cultures are a direct response to the experimental treatment, thereby promoting proof-of-concept. Tight control of factors such as temperature, culture pH, pressure, and gas composition allows for increased confidence of potential cause and effect relationships.[50] Within the calcium phosphosilicate system, variations in pH among systemic environmental factors and cancer cells play a vital role in the induction of capsule breakdown and agent release. Additionally, in vitro studies tested particle uptake by cancer cells without stromal tissue influence, thereby showing that the dense stromal network was the primary obstacle in drug delivery to a targeted tumor. The in vitro studies afforded proof of concept regarding calcium phosphosilicate encapsulating efficiency, cellular non-toxicity, cellular uptake and release of encapsulated agent. It was essential to establish these characteristics before testing the drug delivery system in vivo.

Cell culture is a protocol utilized by many research groups so they can perform in vitro tests. Although standard protocol for generalized cell culture exists in the literature, various cell lines require unique modifications to maintain optimal cell health.[50] Pancreatic cancer cells show characteristic

resistance to drug uptake in both the physiological and laboratory settings. In the context of applicable cancer treatments, such resistance is problematic in the delivery of therapeutic drugs to the cancer cells. However, in the context of in vitro studies, such resistance also presents obstacles, as cultured cells resist uptake of agents used to maintain cell health while in culture. When the BxPC3 pancreatic cancer cell line was thawed and prepped for cultures to be utilized to test particle cytotoxicity and general uptake, novel adjustments needed to be applied to traditional cell culture techniques, particularly in the subculture and splitting of cells.

The BxPC3 cell line was utilized in this study, as such cells descend directly from a primary pancreatic adenocarcinoma of a 61-year-old female. Although healthy pancreatic cells can be experimentally transformed into cancerous cells via exposure to various mutagens, it is best to use cells from a primary human cancer cell line. While mutated cells may express some of the basic properties of cancerous tissue, there is no guarantee that they possess all the genetic requirements for the formation of an invasive tumor in vivo. Primary cell lines, although often more difficult to work with, allow researchers to conduct studies in cellular environments more practical to in vivo applications. Specifically, investigations focusing on primary human cell lines permit simplified transitions from in vitro to in vivo investigations utilizing athymic nude mice. Past studies indicate that experimental tumor growth from such primary cell lines result in the development of a “human” tumor within the murine test subject.[51]

Comparative investigations suggest that the BxPC3 cell line shows high degrees of binding affinity and adherence in vitro, explaining their resistance to undergo subculture in vitro. Therefore, BxPC3 cells show reduced mobility in cultures, tending to remain tightly bound to surrounding surfaces and neighboring cells. However, the BxPC3 cell line is also known as a highly invasive cell line and is therefore ideal for the study of therapeutic treatments.

Chapter 5

Methods: Establishment of Cell Culture Protocols

To test the uptake and cytotoxicity of particles on pancreatic tumor cells, a frozen stock of BxPC3 cells were thawed and prepared for cell culture. An initial culture protocol was adopted from a procedure previously utilized for AsPC1 cells.[52] Frozen BxPC3 cells were stored in a liquid Nitrogen container. Cell media was prepped to contain 89 vol% RPMI, 10 vol% fetal bovine serum (FBS) and 1 vol% penicillin. Media was warmed in a sterile water bath to 37 ºC for 30 minutes prior to use. A vial of frozen cells was removed from the liquid nitrogen container and thawed in a sterile 37 ºC water bath for 2 minutes. Under a sterile hood, the cells (1mL) were transferred to a 15mL centrifuge tube containing 8mL of previously aliquoted media. The tube was slowly inverted to provide gentle mixing of cells with media. Once adequately mixed, the cell/media mix was centrifuged for 10 minutes at 100rpm. The resulting supernatant was discarded and the cells were resuspended in 5 mL of fresh media. The contents were then transferred to a 1L flask containing 10mL of fresh media (labeled DNK1-81). The cells were incubated under sterile conditions at 37 ºC with 5% CO_2. After 24 hours, the cells were examined for overall health status.

Upon examination, the cells were firmly adhered to the culture flask, with no free-floating cells visible. The cell number showed minimal change compared to cell number upon initial cell plating. This was not surprising, as initial cell division is rarely seen immediately after cell thawing because metabolic and molecular processes require time to restart. As the cells did not appear overcrowded, the cell media was changed without splitting or disrupting cells. Under sterile conditions, the old media was pipetted out of the flask. Roughly 5mL of PBS was added to the flask to coat the cells. Cells were then checked under a microscope to assure that they had not been removed with the media but rather remained adhered to the

flask. After such adherence was confirmed, the PBS was removed and discarded, and 15mL of warmed fresh media was added to the flask. The cells were then returned to the standard incubation conditions.

The cells were examined every 12 hours to observe relative cell abundance and health. Relative cell abundance was judged based on visual observation as opposed to cell counting so as not to disrupt cells as they reached equilibrium. General cell health was based on cell shape and appearance. Cells were regarded generally healthy if they maintained round, slightly spindle-like shapes with the appearance of a glowing-halo surrounding the cell. The change to a sickle shape and absence of such halos indicate cell distress. Cell growth and proliferation initially began at a very slow rate. Observation of cell growth did not indicate that cell subculture was necessary until day 9.

Initial Cell Subculture Trials

Once the flask surface appeared saturated with cells, the culture was prepared for subculture. A generalized procedure was utilized as an initial protocol for subculture. Cell media was warmed in a 37 ^{o}C water bath for 30 minutes. At time 15 minutes, a frozen vial of 0.25 vol% trypsin was added to the water bath to thaw. While waiting for the remaining 15 minutes, old media was removed from the culture flask and PBS was applied to coat the flask surface. The flask was gently rocked to allow complete washing of media from cell surface. After the PBS was removed, 5mL of the newly thawed 0.25 vol% trypsin was transferred to the flask. Trypsin is utilized in traditional cell culture protocols as it gently breaks cell-substrate bonds, thereby freeing cells from the flask surface. The flask was rocked gently to assure all cells were coated with trypsin. After the cells were exposed to trypsin for 5 minutes, microscope analysis showed that cells were still adhered to the flask surface. Therefore, an additional 5 minutes were allowed to pass before the trypsin was deactivated through the addition of 7 mL of warmed media to the flask. As prepared, the media is 10% FBS, which contains α1-antitrypsin, inhibits the cleaving function of trypsin to prevent further action on cells.

After the flask was rocked to assure adequate mixing of media to completely inactivate trypsin, the flask contents were transferred to a 15mL centrifuge tube and spun for 10 minutes at 100 rpm. While the cells were spinning, the original flask was analyzed under the microscope to assure that cells had indeed been moved from the flask surface to the centrifuge tube. However such analysis revealed that the flask surface still appeared to be coated with adhered cells, indicating that many cells failed to react with trypsin and undergo subculture. To prevent cell loss, 10mL of fresh media was applied to sample DNK1-81 and the flask was returned to the incubator. Little to no cell pellet was observed after cell spinning was complete so the tube was spun for an additional 10 minutes at 100rpm to determine if any cells had been successfully transferred from the original flask. After the second spin cycle, a minimal pellet was seen. The supernatant was removed and the pellet resuspended in 5mL of fresh media. The tube contents were then transferred to a fresh flask containing 10mL of fresh media (DNK1-86). The flask was analyzed under the microscope to reveal a very low concentration of cells. The sample was placed in the incubator. Six hours after subculture attempt, both samples were examined for general cell concentration and health. DNK1-86 contained a very low concentration of cells, which had adhered to the flask surface. Very high concentrations of cells were still adhered to the DNK1-81 sample, indicating that the trypsin treatment had failed to dissociate the cell-substrate bonds to free cells for subculture. Consequently, a study was initiated to develop a novel cell culture protocol that allowed for optimal subculture efficiency.

Subculture Efficiency Optimization

A number of changes were made to the subculture protocol to improve subculture efficiency. The ability to efficiently subculture cells is essential in the maintenance of healthy cultures. As the BxPC3 cell line grows in a monolayer manner, once cells cover the entirety of the flask, a portion must be removed and either discarded or transferred to another flask, providing the cells with more room to grow. If cells are not allotted such room to expand, they will begin to smother each other, ultimately resulting in cell

death. All future subcultures were performed with PBS warmed to 37 ºC rather than room temperature. Additionally, all trypsin to be used in subcultures was allowed to thaw slowly in the fridge overnight, with brief warming in the warm water bath prior to use as opposed to the transfer of a frozen trypsin vial directly to the warm water bath. This rapid temperature may have resulted in a heat shock to the trypsin, consequently inactivating the agent.

Upon examination, sample DNK1-81 appeared extremely concentrated, with cells beginning to grow atop each other as masses. Therefore if the cells were to be salvaged, it was essential that they be split. Highly concentrated trypsin agents are rarely used in cell culture, as trypsin is known to damage the cell membrane and cause cell death when applied to cells at high concentrations. Trypsin frees cells from the flask surface by cleaving the adhesive bonds. At high concentration, trypsin can begin to inactivate cellular surface proteins via cleavage of extracellular exposed proteins.[53] However, the pressing need to subculture cells indicated the need to use 2.5 vol% trypsin to assure cells would be successful.

As there was a large quantity of cells in DNK1-81, the next subculture trial was attempted on DNK1-81. Immediately before subculture began, the thawed vial of trypsin was removed from the fridge and transferred to the warm water bath for a brief warm-up prior to use. Media was removed from the flask and discarded. PBS warmed to 37 ºC was added to the flask to coat the cells. The flask was rocked to assure all media was washed away from cells. The PBS was removed and 2.5 vol% trypsin (7mL) was added to the flask. After a brief rocking to assure the trypsin was adequately distributed, the flask was placed in the incubator for 20 minutes. Upon examination, large clumps of cells had visibly lifted from the flask surface, indicating successful cleaving of the bonds between the flask surface and cells. After 7mL of media were added to the flask to quench the trypsin activity, the contents of the flask were transferred in equal volumes to two 15mL vials. The cells were spun at 100rpm for 10 minutes. Large pellets were visible in both vials. The supernatant was removed from each vial and the pellets were both resuspended in 10mL of fresh media. Given the large quantity of cells, the cells were added to three separate flasks, labeled DNK1-95, DNK1-96, and DNK1-97. As they all came from DNK1-81, the cells

residing in the new flasks were theoretically all of equal overall health. Flasks DNK1-95, DNK1-96 and DNK1-97 were examined 6 hours after subculture. All cells appeared in good health and at low concentrations within their flasks. Three days later, the four flasks were used to conduct a trial to determine the minimum concentration of trypsin that could be used to efficiently subculture, so as to minimize the negative impact of trypsin on cell membranes.

Determination of Minimum Trypsin Concentration

Samples DNK1-86, DNK1-95, DNK1-96 and DNK1-97 were utilized in the trypsin concentration trials. DNK1-97 served as a negative control and was not subcultured; the media was merely exchanged for fresh media and the sample placed back in the incubator. DNK1-86, DNK1-95 and DNK1-96 underwent subculture. Old media was removed from the flasks and cells were washed with warm PBS warmed to 37 ^{o}C. Once the PBS was removed, 6mL of trypsin was added to each flask. DNK1-86 received 2.125 vol% trypsin to provide a positive control, DNK1-95 received 1.375 vol% trypsin and DNK1-96 received 1 vol% trypsin. All subculture flasks were incubated with their assigned trypsin for 20 minutes. All trypsin reactions were quenched via addition of 6 mL of fresh media to each flask. After the samples were transferred to an appropriately labeled 15mL test tube, they were spun at 100rpm for 10 minutes. The resulting pellets all seemed to be roughly the same size. The supernatants were removed and each pellet was resuspended in 5mL fresh RPMI. Half of each sample (2.5mL) was then transferred to its correspondingly labeled new flask containing 10mL fresh media. Samples were stored in the incubator. Old flasks were discarded after microscope analysis indicated that all cells had been removed.

Cells were examined 6 hours after subculture for general health analysis. DNK1-97 cells appeared healthy with a relatively high concentration, though did not appear at risk of becoming overcrowded overnight. Those samples that underwent subculture showed similar cell concentrations, with cells occupying roughly 1/3 of the flask surface. Similar concentrations indicated that efficient

subculture of the BxPC3 cell line could be achieved using the lowest trypsin concentration tested, 1 vol% trypsin, under the given protocol. Cells in all samples presented as healthy at this time, as judged by morphology analysis.

The samples were examined every 12 hours after subculture. Within 24 hours, DNK1-86 began to develop a dull appearance, an initial indicator of cell stress. However since their shape had not yet changed and slight halo-rings were still visible, the sample was placed back in the incubator for further analysis. Thirty-six hours after the subculture trials, DNK1-86 cells showed excessive cell growth in a spotted manner. In terms of growth, rather than spreading to cover the flask surface, cells grew atop each other, forming colonies of cell masses. Additionally, the halo-like glow of cells had disappeared and cells had altered to an elongated shape with jagged edges. Evidence of cell death was also present in the flask, with cell fragments visible under microscope analysis. These results suggest that extended exposure to relatively highly concentrated trypsin (2.5 vol%) did indeed harm cells, thereby nullifying its potential use in cell culture. Samples DNK1-95, DNK1-96 and DNK1-97 all appeared in good condition, indicating that subculture could be efficiently carried out using 1 vol% trypsin if the previously mentioned modifications were adopted. Subculture of BxPC3 cells required washing with PBS warmed to 37^{oC} as opposed to room temperature PBS prior to trypsin application. Trypsin was transferred from the freezer to fridge the day before subculture to allow samples to thaw slowly, thereby reducing the impact of freeze-thaw cycles. Finally, cells were incubated when interacting with trypsin, again to reduce affects of temperature changes.

Minimal Efficient Trypsin Concentration

Given the damage inferred on cells from high trypsin concentrations, it became apparent a minimum concentration of trypsin should be used to subculture cells that would be used to conduct the cytotoxicity screens to ensure that any observed cell distress was a direct result of particle application as

opposed to delayed effects from trypsin damage. After washing with warmed PBS, the three samples were subcultured using 1 vol% trypsin so their cell concentrations were equivalent at the start of the study. When the samples appeared to have proliferated to fill their respective flasks, DNK1-95, DNK1-96, and DNK1-97 were all subcultured with 6mL of 0.75 vol% trypsin for 20 in the incubator. After the reactions were quenched, cells were spun at 100rpm for 10 minutes and pellets were resuspended in 8mL of fresh RPMI. New flasks were labeled appropriately, filled with 9mL of fresh media and plated with 3mL of their respective resuspended cells. A second set of fresh flasks, labeled DNK1-95_B, DNK1-96_B, and DNK1-97_B were also filled with 9mL of fresh media and plated with 3mL of the appropriate cell suspension; these would be used for additional examination of the influence of trypsin concentration and exposure time on cell health.

Microscopic examination of old flasks revealed that cells had been successfully removed from the flask surfaces with 0.75 vol% trypsin in the stated protocol. When the cells were analyzed 6 hours after subculture, all samples appeared in good health, as judged by cell morphology and growth spacing. After four days, both sets of samples were subcultured under two different protocols. Samples DNK1-95, DNK1-96 and DNK1-97 were subcultured using 0.25 vol% trypsin for incubated for 20 minutes while samples DNK1-95_B, DNK1-96_B, and DNK1-97_B were incubated with 2.5 vol% trypsin for 10 minutes; other aspects of the modified protocol remained the same.

The trials utilizing 2.5 vol% trypsin for 10 minutes (samples subscripted B) were used to determine if a highly concentrated trypsin could be used without harming cells if it were applied for shorter periods of time. When these subcultures were analyzed 12 hours after the 2.5 vol% trypsin exposure, all three samples showed signs of cellular distress. The halo-like rings around cells were not apparent and large cell fragments could be seen freely floating in flask media. After 24 hours, those cells that had adhered to a flask surface appeared disfigured, with many displaying signs of cell death. This indicated that subculture should not be performed on BxPC3 using high concentrations of trypsin,

particularly when cells are not overgrown within their flask and therefore experience greater amounts of interactions with applied agents.

Trials performed on the DNK1-95, DNK1-96 and DNK1-97 samples utilized 0.25 vol% trypsin for 20 minutes as a means of determining if the developed protocol allowed for the use of very low trypsin concentration in BxPC3 subculture. The use of low concentration trypsin is useful when trying to conserve resources and limit cell damage during subculture. When the cells were analyzed for general health 12 hours after subculture with 0.25 vol% all samples appeared in good health, as measured by cell morphology and proliferation spacing.

Final Protocol Conclusions

An extended analysis of subculture protocol for BxPC3 cells was performed to assure cells used throughout in vitro trials were solely influenced by particle application and the actions of therapeutic agents. Previous studies utilizing the cell line struggled with extensive cell death, causing uncertainty regarding the cause of death. This investigation established a subculture protocol to assure maximum health of cells prior to the application of experimental treatments. Any subsequently observed changes in cell health could then be confidently associated with the applied treatment.

Extended trial analysis of various protocols for the subculture of BxPC3 cells indicate the negative consequences associated with the use of high concentrations of trypsin. As an adhesive cell line, BxPC3 cells must be subcultured with strong agents to break the association between cells and growth substrate. Previously utilized lab group protocol relied on extended exposure to highly concentrated trypsin to lift cells from flask surface for subculture. Trials investigating the use of 2.5 vol% trypsin afforded the visible separation of cells and flask but also compromised the integrity of cellular membrane and overall cell health. Though the cells initially underwent cell culture and appeared viable, analysis

after 12 and 24 hours showed rapid deterioration of cell health, with many displaying signs of considerable cell death.

Though initial trials with low concentration trypsin failed to efficiently subculture the cells, a final protocol was developed that appears to yield acceptable cell subculture without compromising cell health. BxPC3 cells respond positively to incubation with low concentration trypsin for 20 minutes. Under the established protocol modifications, low concentrations of trypsin allow for successful cell subculture, leading to healthy cells that can be utilized in cytotoxicity screenings.

Chapter 6

In Vitro and In Vivo Studies

After the appropriate cell culture protocol was established, group members successfully conducted a series of cytotoxicology screenings on the prepared particles. Furthermore, studies revealed that BxPC3 cells cultured in vitro showed increased uptake of CPSNPs targeted with a Gastrin 10 peptide as opposed to untargeted CPSNPs.[45] The use of a pH sensitive fluorescent (fluorescein) probe within the nanoparticles revealed targeted particle uptake five minutes after particle application, which is not seen in the untargeted groups (Figure 6a). At time 60 minutes post particle application, a significantly larger proportion of particles had undergone cellular uptake in the targeted group compared to the untargeted group. The bright blue-green fluorescence seen with targeted particles at 60 minutes indicates that the particles had undergone cellular uptake and dissolved to release encapsulated agents. Cell analysis via flow cytometry confirmed results indicated by fluorescence analysis (Figure 6b).

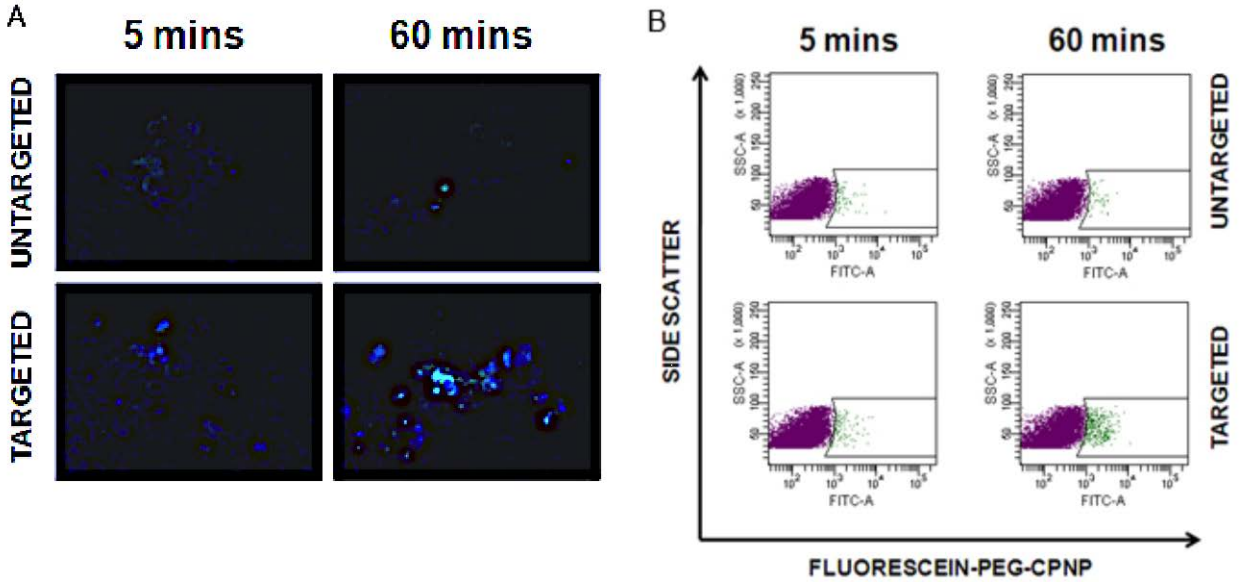

Figure 6 In Vitro Uptake of Targeted and Untargeted CPSNPs.
In vitro studies of BxPC3 cells utilized (A) fluorescent probes and (B) flow cytometry to investigate variations in cell uptake of CPSNPs that were either targeted with Gastrin 10 or untargeted. Both forms of analysis show enhanced uptake of targeted particles compared to untargeted particles.

Subsequent in vivo studies utilized the immune-deficient athymic nude mouse, which lack a thymus, making them unable to produce T-cells and prone to development of foreign tissues, specifically human cancer cells. Studies that investigate murine tumors can provide useful information regarding therapeutic agent toxicity and cellular factors that contribute to tumor formation.[54] However, it is difficult to predict how a human tumor will respond to a novel treatment based on the reaction of a mouse tumor. As athymic nude mice have compromised immune systems, they are unable to recognize and attack injected human cells as foreign agents. Therefore, they can be injected with cancerous human cells extracted from a viable tumor, allowing a human tumor to grow within the murine model.[54] Researchers can then interpret the efficiency with which novel agents infiltrate and counteract genuine human tumors. Furthermore, orthotopic mouse models have been developed for the study of pancreatic cancer to stimulate more precisely the physiological obstacles of delivering drugs to the pancreas.

After orthotopic pancreatic cancer tumors were established within the experimental athymic mice, treatments were administered systemically via tail vein injections. All particles encapsulated NIR fluorescent probes to monitor particle uptake throughout various regions of the body. Mice were injected with untargeted PEG-NIR-CPSNPs, Gastrin10-NIR-CPSNPs, or Gastrin 5-NIR-CPSNPs. As gastrin receptors show a characteristically high expression in the pancreas, truncated gastrin peptides composed of either 10 (Gastrin 10) or 5 (Gastrin 5) amino acids were investigated for their value as pancreas targeting agents.[55] Mice were imaged at regular time intervals to monitor the partitioning trends of untargeted verses targeted particles.

Imaging of mice 24 hours after particle injection indicated enhanced tumor uptake of particles targeted with Gastrin-10 compared to untargeted particles relying on accumulation via the EPR effect (Figure 7). Tumor uptake of Gastrin 5 targeted particles did not differ from that of untargeted particles, indicating that Gastrin 5 does not show elevated specificity for pancreatic cells and is a poor targeting agent. It is likely that some of the key amino acids that bind to pancreatic gastrin receptors are found after the fifth amino acid in the gastrin peptide. Therefore, a peptide fragment that extends beyond the first five

amino acids in the gastrin protein must be utilized to target the pancreas. Gastrin-10 likely contains a greater portion of the receptor binding sequence as Gastrin-10 displays targeted uptake in the pancreatic cancer. Therefore initial imaging at 24 hours presented gastrin as a promising agent for the development of a pancreas specific drug delivery system.

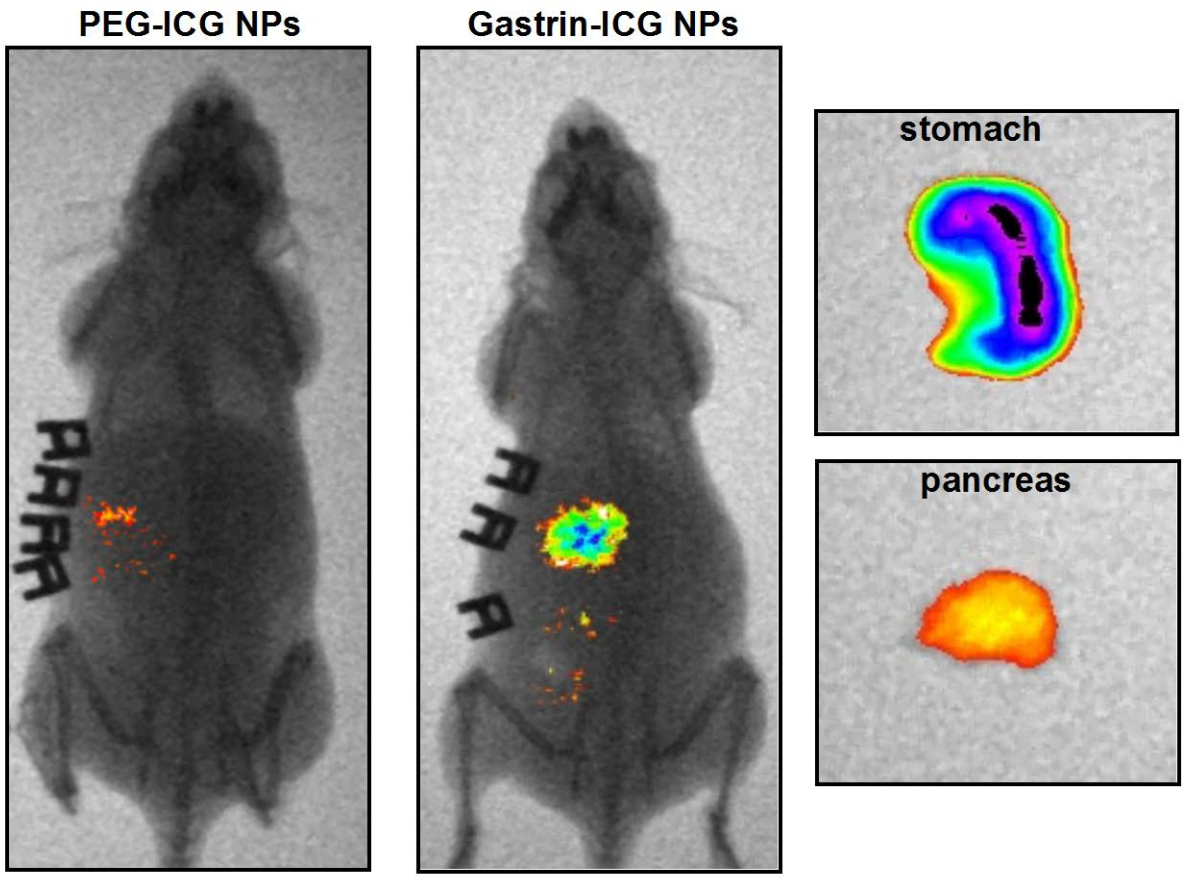

Figure 7 In Vivo Imaging 24 Hours After Tail Vein Injection of Targeted and Untargeted CPSNPs. Athymic mice were injected via tail vein injection with ICG encapsulated CPSNPs that were either untargeted, Gastrin 5 targeted or Gastrin 10 targeted. When subjects were scanned 24 hours after particle injections, neither the untargeted nor the Gastrin 5 targeted particles showed significant accumulation in the pancreas, as measured by fluorescence intensity. Particles targeted with Gastrin 10 showed high levels of accumulation in the pancreas.[45]

Imaging of mice 96 hours after particle injection indicated that although the gastrin 10 targeted particles showed enhanced accumulation at the tumor site, they also crossed the blood brain barrier and accumulated in the brain (Figure 8). Gastrin interacts with G-coupled protein receptors (CCKRs) that are prominent in both the GI track and central nervous system.[56] As unintentional delivery of chemotherapeutics to the brain poses a plethora of negative side effects, Gastrin 10 does not provide an

efficient target agent for the specialized delivery to the pancreas. This is unfortunate as the Gastrin 10 has enhanced accumulation in the pancreas, as well as the brain. It is hypothesized that if a slightly more hydrophobic version of gastrin is employed, it will not cross the blood-brain-barrier.

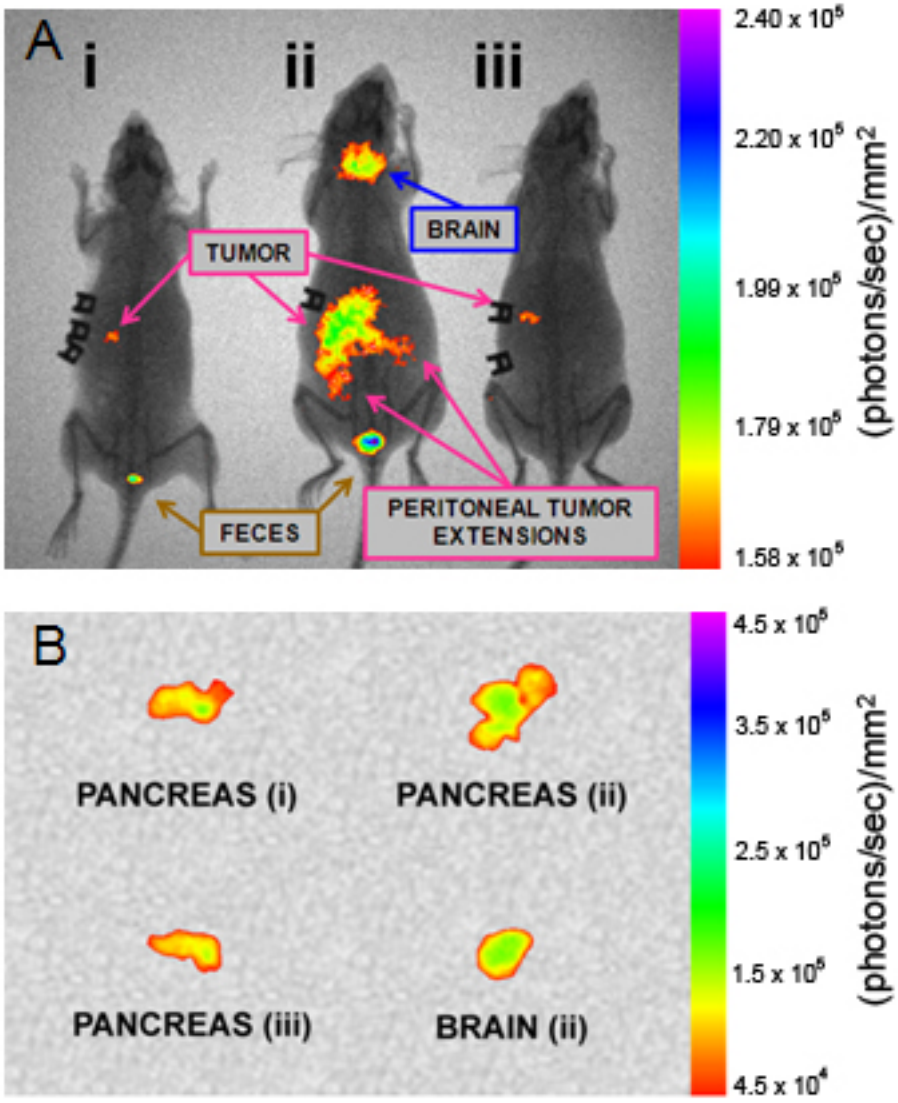

Figure 8 In Vivo Imaging 96 Hours After Tail Vein Injection of Targeted and Untargeted CPSNPs. Subjects treated with tail vein injects of either untargeted particles (mouse i), Gastrin 5 targeted particles (mouse iii) or Gastrin 10 targeted particles (mouse ii) were scanned 96 hours after treatment. Those treated with Gastrin 10 targeted particles showed increased particle accumulation in the pancreas, confirming results seen at 24 hours after injection. However after 96 hours, Gastrin 10 targeted particles also targeted gastrin receptors in the brain.[45]

Current studies are investigating the use of a longer gastrin target as well as aptamer targets for more discriminate delivery to the pancreas without accumulation in the central nervous system. Once an

appropriate target is identified, particles encapsulating chemotherapeutic drugs such as 5-FU can be tested in vivo to determine their function in treating pancreatic tumors. In the future, lab researchers hope targeted drug delivery via calcium phosphosilicate nanoparticles will allow for specialized delivery of chemotherapeutics to tumor sites, thereby treating cancer with greater efficiency and minimizing harmful drug side effects throughout healthy tissue.

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ACADEMIC VITA

Danielle N. Kumpf
dnkumpf@gmail.com

Education

The Pennsylvania State University
Class of 2015
Schreyer Honors College
Bachelor of Science in Biology
Vertebrate Physiology Option
Eberly College of Science

Honors, Awards and Memberships

- Dean's List 2011-2015
- Marine Corp Academic Excellence Award Recipient
- Phi Kappa Phi National Honors Society

Professional Experience

- Undergraduate Research, Penn State Materials Science and Engineering and Biomedical Engineering Lab
- Emergency Medical Technician certified through the Center for Emergency Medicine of Western Pennsylvania
- Research Assistant, University of Pittsburgh Cancer Institute
- High School Biology and Math Tutor