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Exploration of a
doxorubicin-polymer conjugate in lipid-polymer hybrid nanoparticle drug delivery

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EXPLORATION OF A DOXORUBICIN-POLYMER CONJUGATE IN
LIPID-POLYMER HYBRID NANOPARTICLE DRUG DELIVERY

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EXPLORATION OF A DOXORUBICIN-POLYMER CONJUGATE IN LIPID-POLYMER HYBRID NANOPARTICLE DRUG DELIVERY

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ABSTRACT

Nanoparticle (NP) drug delivery is a major focus in the research community because of its potential to use existing drugs in safer and more effective ways. Chemotherapy encapsulation in NPs shields the drug from the rest of the body while it is within the NP, with less systemic exposure leading to fewer off-target effects of the drug. However, passive loading of drugs into NPs is a suboptimal method, often leading to burst release upon administration. This work explores the impact of incorporating the drug-polymer conjugate doxorubicin-poly (lactic-co-glycolic) acid (Dox-PLGA) into a lipid-polymer hybrid nanoparticle (LPN).

The primary difference in using a drug-polymer conjugate for NP drug delivery is the drug’s release kinetics. Dox-PLGA LPNs showed a more sustained and prolonged release profile over 28 days compared to LPNs with passively loaded, unconjugated doxorubicin. This sustained release translates to cytotoxicity; when systemic circulation was simulated using dialysis, Dox-PLGA LPNs retained their cytotoxicity at a higher level than the passively loaded LPNs. The in vivo implication of preserving cytotoxic potency through a slower release profile is that the majority of Dox delivered via Dox-
PLGA LPNs will be kept within the LPN until it reaches the tumor. This will result in fewer systemic side effects and more effective treatments given the higher drug concentration at the tumor site.

An intriguing clinical application of this drug delivery approach lies in using Dox-PLGA LPNs to cross the blood-brain barrier (BBB). The incorporation of Dox-PLGA is hypothesized to have a protective effect on the BBB as its slow release profile will prevent drug from harming the BBB. Using induced pluripotent stem cells differentiated to human brain microvascular endothelial cells that comprise the BBB, the Dox-PLGA LPNs were shown to be less destructive to the BBB than their passively loaded counterparts. Dox-PLGA LPNs showed superior cytotoxicity against plated tumor cells than the passively loaded Dox LPNs after passing through an *in vitro* transwell BBB model. Dox-PLGA LPNs and drug-polymer conjugates are exciting alternatives to passively loaded NPs and show strong clinical promise of a treatment that is more potent with fewer side effects and less frequent administration.
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LIST OF ABBREVIATIONS

ANG.................................................................angiopep-2 targeting peptide
BBB .................................................................blood-brain barrier
bEND3 ..............................................................murine brain endothelial cells
BMEC .................................................................brain microvascular endothelial cells
CHCL₃ ...............................................................chloroform
DCM .................................................................dichloromethane
DLS .................................................................dynamic light scattering
DMEM ...............................................................Dulbecco’s Modification of Eagle’s Medium
DMF .................................................................dimethyl formamide
Dox LPN .........................................................LPN loaded with unconjugated doxorubicin
Dox-PLGA .........................................................doxorubicin-PLGA conjugate
Dox-PLGA LPN .................................................LPN loaded with Dox-PLGA conjugate
DSPE-PEG(2k)..................1,2-diastearoyl-sn-glycero-3-phosphoethanolamine-N- [amino(polyethylene glycol)-2000]
DSPE-PEG(3.4k)-mal........1,2-diastearoyl-sn-glycero-3-phosphoethanolamine-N- [maleimide(polyethylene glycol)-3400]
EDTA ...............................................................ethylenediaminetetraacetic acid
IC₅₀ .................................................................concentration at which half the cell population is nonviable
iPSC .................................................................induced pluripotent stem cells
KB .................................................................HeLa contaminant cells
LPN .................................................................lipid-polymer hybrid nanoparticle
MDA-MB-231 ................................................................. triple negative breast cancer cells

MTT ............................................................. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide

NP ........................................................................................... nanoparticle

PBS .......................................................................................... phosphate-buffered saline

PLGA ......................................................................................... poly(lactic-co-glycolic) acid

TEER .................................................................................. transendothelial electrical resistance

TEM ........................................................................................ transmission electron microscopy

TLC ........................................................................................ thin layer chromatography
1. Background

1.1 Introduction to Chemotherapy

Chemotherapy treatment of cancer has made great strides following the World War II discovery and use of nitrogen mustard as an alkylating agent to combat lymphoma\(^1\). In the decades since, our arsenal of chemotherapy drugs has expanded beyond alkylating agents to include antimetabolites, topoisomerase inhibitors, cytotoxic antibiotics, and others\(^2\) in an attempt to solve the ultimate quandary of how to rid the body of cancer without irreparably harming the self. A steady arrival of new chemotherapy drugs has kept the treatments feeling innovative, and the primary research focus has been on discovering or synthetically creating a perfect drug. Additionally, the increasing number of drug options has caused combination chemotherapy to move to the forefront of clinical treatment in an attempt to optimize the therapy’s success by attacking the cancer via multiple mechanisms simultaneously\(^3,4,5\). These efforts have demonstrated clinical advancement through outcome measures such as enhanced tumor response and prolonged progression-free survival; however, a significant increase in overall survival remains elusive in many cancers.

1.2 Treating Brain Cancer and the Blood-Brain Barrier

One of the types of cancer that has shown very little improvement in chemotherapy administration is cancer located in the brain\(^6\). The main obstacle in accessing the brain from systemic circulation is the blood-brain barrier (BBB), whose very purpose is to prevent most molecules from entering the brain in order to maintain
the brain’s delicate homeostasis. The BBB is comprised of a layer of endothelial cells connected by tight junctions that surround the vasculature in the brain\textsuperscript{7,8}, as shown in Figure 1.

**Figure 1:** Diagram illustrating the biological structures of a non-brain capillary (left) and a brain capillary (right). Note that the brain capillary has less permeable tight junctions and a lack of fenestrations and other accessibility channels when compared to the non-brain capillary. Image from Papademetriou & Porter.\textsuperscript{9}

These cells closely monitor and regulate transport between the systemic vasculature and brain parenchyma. The BBB is so strong that an estimated 98% of small molecules and likely 100% of biologics are incapable of entering the brain unaided, with the remaining 2% consisting of small, lipophilic molecules. The majority of transport that does occur is protein-mediated active transport. The brain imports nutrients, hormones, nucleosides, and other items necessary for healthy function while expelling neurotoxins and metabolites no longer wanted or needed\textsuperscript{9}. Unfortunately, both primary
and metastasized tumors have managed to expand into the brain. Systemic cancers metastasize to the brain by crossing the BBB and seeding in the brain, while primary brain tumors both begin and develop within the brain.

Brain metastases are fairly common in certain types of cancers, with breast, lung, and melanoma responsible for up to 80% of all metastatic cancer in the brain\textsuperscript{10}. Although the exact incidence rates of brain metastases are difficult to quantify\textsuperscript{11}, a general incidence of 9–17% of all cancers is thought to be fairly accurate\textsuperscript{10} with approximately 150,000 annual cases of brain metastases in the United States\textsuperscript{12}. These numbers have been steadily increasing in the past decade; however, that is likely due to the availability of improved imaging techniques that aid in early detection. The increasing incidence rate is also due to the fact that improved chemotherapeutic treatments for systemic cancers extend survival, and that increased survival raises the likelihood of the cancer metastasizing to the brain\textsuperscript{10}. Prognosis for metastatic brain disease is difficult to quantify in a general sense, as the primary cancer type strongly influences the length of survival. An average survival time for people with metastatic brain disease is reported to be a mere 6 months\textsuperscript{11}, though many groups have much longer average survivals.

As metastatic tumor morphology mirrors that of primary tumor morphology, the same chemotherapeutics used to treat primary tumors should theoretically work to treat metastatic cancer. The chemotherapeutic doxorubicin has been used since its discovery in 1967\textsuperscript{13} as powerful anticancer drug for a wide variety of cancers, including breast cancer\textsuperscript{14}. Doxorubicin works by intercalating into the tumor cell’s DNA when the DNA strands are separated, therefore preventing the DNA from replication and transcription.
The tumor cell recognizes its inability to maintain its basic functions, resulting in apoptosis\textsuperscript{15}. Although doxorubicin has long been used for successful treatment of breast cancer, the functionality of it and other chemotherapeutics sharply decreases when the tumor is located in the brain. The driving force behind these poor results is the lack of accessibility of systemically administered chemotherapeutics to the brain. In typical intravenous administration, <1% of chemotherapy reaches the brain\textsuperscript{16}. This delivery problem highlights the obligation for researchers to account for the BBB in designing treatments for tumors in the brain.

The discouraging statistics surrounding metastatic brain cancers demonstrate the power of the BBB in preventing treatment from accessing the tumor. Although the integrity of the BBB in brain tumors is more heterogeneous than in healthy brain tissue\textsuperscript{17}, its increase in permeability is lower than that in systemic tumors\textsuperscript{5} and still renders intravenous chemotherapy inadequate. While certain treatments may be able to be given in sufficiently high doses that they might overcome this impediment, chemotherapy is so toxic to the healthy cells in the body that the doses necessary to treat the cancer are simply unfeasible. Therefore, in order to successfully treat this cancer using chemotherapy, researchers and clinicians have to address the issue of either crossing or circumnavigating the BBB to increase the chemotherapy’s concentration in the brain. This issue is entirely nontrivial; though many approaches have been made, few have been effective clinically. The three general methods for treating tumors located in the brain are as follows: direct modification of the drug, encapsulation of the drug in a carrier, and local administration of the drug\textsuperscript{9,18}. 
Direct modification of the drug involves altering its chemical properties to make it an apt substrate for diffusion across the BBB. In order to do so, the drug must be small in size, have low hydrogen bonding capabilities, and be highly lipid soluble\textsuperscript{18}. The second method for transporting drugs across the BBB is to utilize a nanocarrier that is typically combined with a biomolecule to facilitate either carrier-mediated transport or receptor-mediated transport\textsuperscript{9,18}. This technique is the most commonly used as there are many types of nanocarriers as well as biomolecules capable of being synthesized in the laboratory. Each of these first two methods are implemented through systemic administration of the treatment. The final method for accessing tumors in the brain is to circumvent the BBB locally. There have been three approaches designed for bypassing the BBB: implantable devices, convection-enhanced delivery, and focused ultrasound-mediated disruption of the BBB. The first two require a craniotomy to access the tumor directly and are therefore highly invasive. The third strategy leverages focused ultrasound transmitted through the skull and thus is noninvasive while remaining highly localized\textsuperscript{9}. There are clinical trials in place that use each of the three methods described; while some show promise, none are currently past phase II. As such, the gold standard for cancer in the brain remains a combination of surgery to debulk the tumor followed by whole brain radiotherapy or systemically administered chemotherapy in an attempt to kill any remaining malignant cells in the surgical margins or other micrometastases located in the brain\textsuperscript{16}.

As the aforementioned examples illuminate, the focus in developing the most effective treatment strategy is not to synthesize a new chemotherapy compound but to
utilize the drugs that already exist in delivery vehicles that optimize their safety and
efficacy. The chemotherapy drugs in the clinic are incredibly effective at killing cells\textsuperscript{1,2}
but their remarkable potency often results in a dangerously narrow therapeutic window\textsuperscript{19}. Increasing the size of a drug’s therapeutic window by designing a vehicle capable of
delivering the agent while both retaining its cytotoxicity and mitigating its adverse effects
is the cornerstone of drug delivery. Due to the typical size of chemotherapy agents and
the work done to determine the optimal size for tumor penetration, the majority of cancer
drug delivery research is centered around nano-sized particles\textsuperscript{20}, which is why the focus
of the remainder of this work is on nanoparticle (NP) chemotherapy delivery.

1.3 Nanoparticles for Chemotherapy Delivery

There are a number of different types of NPs utilized for delivery of
chemotherapeutics, each with its own strengths and weaknesses. Although each type of
NP could be optimal for a particular application, two of the most widely used NPs are
liposomes and polymeric NPs, and it is these two types we will continue to explore. A
rendering of both liposomes and polymeric NP can be found in Figure 2.
The incidence of cancer has been increasing in recent decades, and eradication of the major types of the disease remains an elusive clinical goal, largely due to the heterogeneous and idiopathic nature of individual cancers, and the inability to target tumors to neoplastic areas without damaging normal tissue. This wide spread biodistribution of chemotherapeutics results in both anticancer effects and off-target adverse effects. Thus far, a validated therapeutic protocol is to administer chemotherapeutics to neoplastic areas without preferential localization to cancer tissue. This wide biodistribution with minimal recognition and elimination by the reticuloendothelial system, great effort should be devoted to optimizing the physicochemical profile of nanoparticles. Therefore, to achieve a favorable blood half-life and clearing of nanoparticles and/or their metabolites from the body.

Emerging and current methods: focus on cancer nanomedicines and targeting controlled drug delivery.

1.3.1 Liposomes

Liposomes are composed of a phospholipid bilayer surrounding an aqueous core. Liposomes were first described in 1965 by Alec Bangham and were first used as models for cell membrane systems. Due to the large aqueous core of the liposomes, there is significant potential for loading of hydrophilic drugs. Techniques such as a freeze-thaw cycle and the use of pH gradients have been used to enhance loading of hydrophilic drugs. Liposomes are also capable of carrying a hydrophobic payload trapped within the hydrophobic tails of the phospholipid bilayer. However, the small volume of hydrophobic space in the phospholipid bilayer severely limits its loading potential and methods such as the purposeful creation of multilamellar vesicles have been implemented in order to increase the hydrophobic carrying space.

**Figure 2:** Graphic renderings of a liposome (left) and polymeric nanoparticle (right). The liposomes are comprised of a phospholipid bilayer with an aqueous core while the polymeric nanoparticles are comprised of a polymeric core coated with a layer of polyethylene glycol. Image from Sechi et al. 21
Apart from drug loading potential, liposomes have several positive attributes that contribute to their widespread use. The use of a lipid shell allows for precise control of the surface of the NP that is exposed to the body upon administration. Use of lipids with varying parameters, such as pH-sensitivity, can be used to alter the behavior of the NP upon entrance into varying microenvironments, which subsequently affects the structural integrity of the liposome and release of its contents. One near-universal addition to liposomes is that of polyethylene glycol (PEG), a hydrophilic polyether chain compound first used in the clinic in the early 1990s. By adding PEG to the surface of the liposome, often in a form bound to one of the lipids, a hydrophilic brush is created that prevents absorption of surrounding compounds to the surface of the NP. This is often most important in the case of immune recognition, which is one of the primary causes of NP clearance in an in vivo environment. By decorating the liposome with a PEG layer and therefore evading immune recognition, the NP is able to circulate in the patient’s bloodstream for a several hours as opposed to several minutes, thereby allowing the NP a longer period of time in which to accumulate in the tumor space and deliver its contents.

The use of a customizable lipid shell also allows for the addition of a targeting moiety to the surface of the liposome. Adding a targeting ligand creates a more directed NP that should preferentially enter cells containing the ligand’s matching receptor. This ability to target specific cells of interest can be a huge advantage in the effort to maximize drug delivery. While the addition of PEG allows liposomes to essentially solve the problem of rapid clearance, a drawback that remains is a distinct lack of
structural integrity of liposomes due to the fact that the interior of liposomes is simply an amorphous aqueous compartment. That liposomes rely only on a phospholipid bilayer for support leads to leakage of the interior contents and general instability that must be addressed through addition of a stabilizing agent such as cholesterol in the membrane\textsuperscript{30}.

1.3.2 Polymeric Nanoparticles

Polymeric NPs are the second category that is ubiquitous in the drug delivery community. Polymers had been identified as having potential for drug delivery in the 1960s\textsuperscript{31}, but initial reports of polymeric NP used nonbiodegradable polymers\textsuperscript{32}. After chronic problems such as toxicities and inflammatory reactions were frequently reported, the field switched to primarily biodegradable polymers\textsuperscript{33}. The first controlled release polymeric system was reported in 1976\textsuperscript{34}. The polymers now used to make these NP are typically biodegradable and biocompatible. Though there are many types of polymeric NP, the one most closely related to this work is the polymeric micelle, which will be the focus of the remainder of the discussion regarding polymeric NP.

Polymeric micelles typically contain multiple polymer blocks of varying hydrophilicities. Through a self-assembly synthesis process, the most hydrophobic polymer chains are oriented in the core of the micelle while the most hydrophilic chains are oriented outward so as to be thermodynamically stable\textsuperscript{20} as well as creating a brush-like effect comparable to the PEG coating on a liposome\textsuperscript{35}. Once the polymer components have been selected, the self-assembly process is quite simple and is considered a positive attribute of this type of micelle\textsuperscript{33}.
Another positive characteristic of polymeric micelles is the high loading capacity for hydrophobic contents when compared to liposomes. As the entire inner volume of the micelle is typically comprised of hydrophobic polymer, hydrophobic drugs can associate with the core of the micelle. Because the hydrophobic drugs have unfavorable interactions with the hydrophilic polymer chains, the contents remain loaded into the core of the micelle while the micelle remains intact. Depending on the composition of the polymeric components of the micelle, it is possible to include a PEG chain or a targeting moiety on the end of a polymer segment. However, the components must be conjugated together prior to assembly as the polymeric micelle system is not as modular as the lipid shell of a liposome.

1.3.3 Lipid-Polymer Hybrid Nanoparticles

Both liposomes and polymeric micelles have had significant successes in the chemotherapy delivery space; however, each has its strengths and weaknesses. In an attempt to combine the positive aspects of each, a novel type of NP was introduced in 2008 called a lipid-polymer hybrid NP (LPN). The LPN is composed of a hydrophobic polymeric core surrounded by a phospholipid monolayer shell. By using a polymeric core, the LPN exploits the high hydrophobic loading capacity, long-term stability, and ease of synthesis associated with polymeric NP. The lipid monolayer shell then adds modularity of a customizable lipid membrane, superior biocompatibility, and long circulation times associated with liposomes. A diagram illustrating the structure of the LPN can be found in Figure 3.
LPNs are synthesized through a self-assembly process called nanoprecipitation. The lipid shell components are dissolved in an aqueous solution and the core components (typically the polymer and the drug cargo) are dissolved in a smaller volume of organic solution. By adding the organic core solution dropwise to the aqueous shell solution and then evaporating the organic solvents, the phospholipids orient themselves around the polymeric nanostructures such that the hydrophobic tails are oriented in towards the core and the hydrophilic heads are oriented outward toward the aqueous medium. This creates a thermodynamically stable suspension of NPs that have high hydrophobic loading capacity, similar to polymeric NPs, due to the polymeric core and the customizable, modular design and longer circulation time of liposomes due to the phospholipid shell.

Figure 3: Schematic of a lipid-polymer hybrid nanoparticle (LPN). The core is composed of the polymer PLGA and loaded with hydrophobic drug. The shell is composed of a phospholipid monolayer containing a molar percentage of lipid-PEG to extend circulation time and prevent immune recognition.
1.3.4 The Challenge of Drug Release

Given the unenviable task of accounting for the myriad variables necessary to design the ideal NP delivery system for a particular application, there have been published reviews exploring how to optimize the success of NPs by changing features such as their size, shape, and surface chemistry\textsuperscript{40–42}. These have led to some general conclusions such as NP between 20 and 200nm are most likely to have long circulation potential\textsuperscript{33}, non-spherical particles can exhibit higher internalization rates than spherical particles\textsuperscript{40}, and surface hydrophobicity helps determine the organ through which the NP is ultimately cleared\textsuperscript{43}. However, the characteristic that most closely dictates the cytotoxicity of the NP is one that is much more difficult to control: its drug release kinetics\textsuperscript{44}. Unless the drug contained in the NP gains access to the interior of the cancer cells it is completely ineffective as a chemotherapeutic agent. Most chemotherapeutics have particular structures that interact with the cells in very specific ways that require the drug to be unencumbered by its delivery vehicle, indicating that the drug must find its way out of the NP and into the surrounding tissue in order to serve as a cytotoxic threat to the tumor. Release kinetics are influenced by every aspect of the NP design, but the most significant variable is the way in which the drug is loaded into and contained within the NP. As it rationally follows, the weaker the association between the drug and the NP and the fewer barriers to the drug’s escape, the faster the drug will elute out of the NP and into its surrounding environment.

With countless specific indications for NP drug delivery, each application has its own optimal release kinetics. In the cases of polymeric micelles and liposomes, passive
drug loading is the simplest method for encapsulating cargo. With both of these types of NP, passive loading consists of simply dissolving the drug into the solvent that will comprise the core of the NP during synthesis. The drug is then retained in the NP due to the hydrophobic or hydrophilic interactions between the drug and its compartment within the NP. While thermodynamically it is most advantageous for the drug to remain in the interior of the NP once it is passively loaded, exposure to an aqueous environment and biomolecular solutes \textit{(i.e.} plasma proteins\textit{)} can destabilize the NP, leading to drug leakage. The more drug that leaks out, the more compromised the integrity of the NP becomes until it eventually falls apart altogether. When a drug is passively loaded in this manner and leaks out after a short time post-administration, the drug experiences burst release. Burst release does typically have a short-term positive effect on the size of a tumor; however, it is generally accepted in the drug delivery community that a more sustained release profile typically leads to better long-term outcomes\textsuperscript{33,44}.

\subsection*{1.3.5 Drug-Polymer Conjugates}

The primary method for more closely controlling, and in particular slowing, a drug’s release from the NP carrier is to increase the chemical affinity between the drug and its loading compartment within the NP beyond the simple hydrophobic or hydrophilic interactions associated with passive drug loading. To further increase the drug’s affinity for the NP, the drug can be chemically conjugated to part of the NP, thus creating a bond that would prevent the drug from rapidly diffusing out of the NP. The intention of this conjugation is to keep the drug with the NP long enough for it to travel
through the systemic vasculature, accumulate within the tumor space, and then slowly leak out over time as the bond or NP dissolves. Drug-polymer conjugates have been investigated for free delivery (i.e. unencapsulated, outside of NPs) to tumors\textsuperscript{45,46}, but the focus of this section will be on the more applicable use within NPs. Use of drug-polymer conjugates within liposomes is sadly ineffective; as there is no solid content in the aqueous core, there is nothing to which the drug can be bound. Chemotherapy drugs have been bound to lipids to form prodrugs\textsuperscript{47}, but unfortunately the bulky drugs prevent the formation of liposomes.

The drug-NP conjugation method is widely used in the case of polymeric NPs. With polymeric micelles, the drug can be directly conjugated to the end of the polymer chain that already contains both the hydrophobic and hydrophilic blocks of polymer. This strategy is appealing because it allows for the ultimate use of a single synthesized polymer chain and a simple self-assembly process to create uniform micelles of known drug content. Mahmud et al. utilized this strategy by forming micelles with poly(ethylene oxide) (PEO) and poly(ε-caprolactone) (PCL) block copolymers with doxorubicin bound to the PCL side groups\textsuperscript{48}. Similarly, along with the primary block hydrophobic-hydrophilic polymer chain, a secondary polymer conjugated to the drug of interest can be incorporated into the polymeric NP. Provided the secondary polymer is hydrophobic, the drug-polymer conjugate will remain in the core of the NP. Kolishetti et al. demonstrated this with their use of a cisplatin prodrug bound to a polylactide polymer incorporated into a NP formed with a PLGA-PEG copolymer\textsuperscript{49}. In addition to the cisplatin-poly lactide conjugate, this group included unconjugated, passively loaded
docetaxel into the core of their NPs. They illustrated the power of the conjugation’s effect on release kinetics by reporting a faster release rate of the unconjugated docetaxel compared to the conjugated cisplatin.

Unlike the ability of polymeric micelles to incorporate a drug-polymer conjugate with a single polymer chain, use of drug-polymer conjugates within LPNs always requires multiple components. The drug-polymer conjugate sits in the core of the NP and can be used either as the sole core component or with the addition of a secondary, unconjugated polymer. The phospholipid shell is formed around the core and is unchanged regardless of the use of the drug-polymer conjugate. There are few examples of drug-polymer conjugates used in LPNs in the literature; interestingly, most examples are of combinatorial delivery systems where multiple drugs or treatments are delivered in the same LPN. One such report was published by Aryal et al.\textsuperscript{50} where both doxorubicin and camptothecin were conjugated separately to polylactide polymers before they were incorporated into the core of LPNs. The cytotoxicity of their LPN formulation was higher than the unencapsulated drug-polymer conjugates, which the authors describe is because when the unencapsulated conjugates passively diffused into the cells they became substrates for the efflux pumps located in the cell membrane. The drug-polymer conjugates located in the LPNs managed to evade the efflux pumps because the drug did not leave the LPN until it was well within the cell and no longer in close proximity to the efflux pumps\textsuperscript{50}. An example more closely comparable to the work presented here is reported by Agrawal et al. in which a cyclo-[Arg-Gly-Asp-d-Phe-Lys] (cRGDfK) modified paclitaxel was encapsulated in a PLGA-based LPN and its release kinetics
observed. This group’s work indicated a slightly more prolonged paclitaxel release of the conjugated paclitaxel compared to the unconjugated paclitaxel\textsuperscript{51}. As a final example, Sengupta et al. showed that the use of a drug-polymer conjugate in a slightly modified version of an LPN has been shown to release its drug much more slowly when compared to passively loaded drug\textsuperscript{52}, demonstrating the power of a drug-polymer conjugate in controlling release kinetics in NP drug delivery.

1.4 Using Nanoparticles for Drug Delivery Across the Blood-Brain Barrier

Nanoparticles have been critical in developing novel techniques for delivering chemotherapy agents across the BBB. As was mentioned previously, nanocarrier-based approaches typically involve use of either the carrier-mediated transport (CMT) or receptor-mediated transport (RMT) systems already in place to cross the BBB. The CMT system works via active or facilitated transport proteins on both the blood and brain sides of the BBB cells with simple diffusion within the cells. Because of the specific nature of the substrates needed to cross the BBB via these transporters, CMT is primarily used for small molecules, many related to energy production\textsuperscript{9}. Due to the fact that the substrates are fairly limited, very little NP drug delivery has focused on utilizing the CMT pathway. However, two different formulations of liposomes have recently been made to target a glucose transporter\textsuperscript{53} and a glutathione transporter (2B3-101)\textsuperscript{54} to enhance delivery of daunorubicin and doxorubicin, respectively. These liposomes have had success by showing increased drug concentration across the BBB by targeting the CMT transporters, with 2B3-101 in clinical trials.
The large majority of NP-based methods for crossing the BBB involve exploitation of the RMT pathways. The RMT pathways are generally used for macromolecular substrates and are thus more aptly adapted for NP-sized transport. A typical RMT transport involves binding to a surface receptor on the blood side of the BBB, endocytosis into and vesicle transport across the cell, followed by exocytosis of the vesicle and delivery of its contents into the brain parenchyma\(^9\). Unlike the CMT pathway, the RMT transport substrates are never exposed to the interior of the BBB endothelial cells as they are always surrounded by a secondary vesicular membrane. There are several receptors expressed on the BBB endothelial cells that serve as targets to initiate RMT. The transferrin receptor is one of the most heavily researched receptors; in addition to being expressed on the BBB cells it has high levels of expression on the liver and is overexpressed in brain cancer\(^{55}\). The LRP1 receptor is part of the LDLR family of receptors; in addition to BBB endothelial cells it is expressed on malignant gliomas\(^{56}\). A ligand that is often used to target the LRP1 receptor is angiopep-2\(^{57}\) (referred to hereafter as “angiopep” or ANG), which enables targeting to and transcytosis across BBB endothelial cells\(^{58}\).

Both liposomes and polymeric NP have reportedly been used to deliver chemotherapeutics across the BBB via the RMT system. Two transferrin-targeted liposomal formulations loaded with doxorubicin\(^{59}\) and epirubicin\(^{60}\) have shown enhanced BBB penetration and increased uptake by glioma cells. A third liposome containing docetaxel and targeted to LRP1 by the addition of angiopep-2 and a tumor homing peptide successfully inhibited tumor growth when compared to the nontargeted
alternative\textsuperscript{61}. Polymeric NPs with 3 different targeting moieties also had demonstrated antitumor activity. The first, targeted using angiopep-2, enhanced paclitaxel delivery and antitumor activity over both free paclitaxel and nontargeted polymeric NPs\textsuperscript{62}. A polymeric NP using peptide-22 to target an LDLR receptor on the BBB increased both transport across the BBB and glioma death compared to the nontargeted NPs. The third polymeric NP formulation was a micelle using a CDX peptide to target the nicotinic acetylcholine receptor; this NP successfully crossed the BBB, accumulated in the brain, inhibited tumor growth, and prolonged survival of mouse gliomas\textsuperscript{63}.

1.5 Motivation for Project

It is clear that the field of NP drug delivery to the brain is multifaceted, intricate, and incredibly complex. There is intense focus on brain cancer due to its poor prognosis and dearth of treatment options, but successes are few and infrequent. It is the goal of this work to combine one of the more recent NP formulations with a drug-polymer conjugate to highlight the importance of constructive drug release kinetics in order to maximize drug concentration at the site of the tumor and minimize off-target toxicities. Accomplishing this task would lead to the ability for larger concentrations of chemotherapy to be used and therefore more effective treatments and more positive outcome measures.
2. Proposed Drug Delivery System and Specific Aims

2.1 Proposed Drug Delivery System

This dissertation introduces a lipid-polymer hybrid nanoparticle (LPN). While there are many variations of the LPN within this dissertation, the final version consists of a core with a 75/25 mass ratio of doxorubicin-poly(lactic-co-glycolic) acid conjugate (Dox-PLGA) and unconjugated poly(lactic-co-glycolic) acid (PLGA). The shell is composed of a 75/24/1 molar ratio of L-α-phosphatidylcholine, hydrogenated (lecithin), 1,2-diastearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG2k), and 1,2-diastearoyl-sn-glycero-3-phosphoethanolamine-N-(polyethylene glycol)-3400-angiopep-2 (DSPE-PEG(3.4k)-ANG). The Dox-PLGA conjugate in the core of the LPN creates a sustained doxorubicin release profile and the angiopep-2 ligand on the outer PEG layer serves as a targeting moiety for blood-brain barrier (BBB) endothelial cells.

Using this LPN doxorubicin delivery system, my general hypothesis is as follows:

A Dox-PLGA conjugate-based LPN is more effective at controlling drug release, maintaining cytotoxicity, and preventing BBB damage than a NP with passively loaded Dox.
2.2 Specific Aims

To test and evaluate the aforementioned hypothesis, the following specific aims will be addressed:

**Specific Aim 1: Synthesize Dox-PLGA conjugate and create a conjugate-loaded LPN**

The primary focus of Aim 1 is to create the components necessary for the LPN delivery system and assemble those components into a stable LPN. This Aim will consist of establishing the creation and stability of the LPN, the chemistry of synthesizing the Dox-PLGA conjugate, incorporating the conjugate into the LPN, evaluating the Dox content both in the conjugate and in the LPN, and assessing the cytotoxicity of the Dox-PLGA LPNs.

**Specific Aim 2: Evaluate the impact of the Dox-PLGA conjugation on Dox release kinetics from LPNs and investigate the cytotoxicity of LPNs containing the Dox-PLGA conjugate.**

Aim 2 is designed to test the effectiveness of the Dox-PLGA conjugation on two variables: Dox release kinetics and LPN cytotoxicity. The hypothesis of this aim is that the incorporation of the Dox-PLGA conjugate into the LPN will result in a more sustained release profile than LPNs loaded with unconjugated Dox. Due to the longer Dox retention in the LPN, the cytotoxicity of the LPN will be maintained for a longer period of time when compared to LPNs loaded with unconjugated Dox.
Specific Aim 3: Decorate LPNs containing DOX-PLGA with angiopep and evaluate toxicity against cancer cells after BBB penetration and effect on BBB function.

In Aim 3 the Dox release and cytotoxicity data will be applied to more complex experiments that more closely mirror the BBB. The guiding hypothesis in this aim is that incorporation of the Dox-PLGA conjugate into the LPN and the sustained release profile that it exhibits will have a protective effect on the BBB, preventing the drug from leaking out of the LPN while it crosses the BBB. Additionally, decorating the LPN outer shell with angiopep will enable transcytosis across the BBB and drug delivery to malignant cells protected by the BBB. A transwell model will be used in 12-well plates so that brain endothelial cells can be cultured on the insert membrane and tumor cells can be cultured in the bottom well. Transendothelial electrical resistance (TEER) will be used to assess the integrity of the BBB model. A lipid-angiopep-2 conjugate will be synthesized to test the targeting capacity of the LPN across the BBB and the cytotoxicity of both the BBB layer and the tumor layer will be observed.
3. Synthesize Dox-PLGA Conjugate & Incorporate into Nanoparticle

The primary focus of Chapter 3 is to fulfill aim 1 by creating the components necessary for the LPN delivery system and assembling those components into a stable LPN. This Aim will consist of establishing the creation and stability of the LPN, the chemistry of synthesizing the Dox-PLGA conjugate, incorporating the conjugate into the LPN, evaluating the Dox content both in the conjugate and in the LPN, and assessing the cytotoxicity of the Dox-PLGA LPNs.

3.1 Materials & Methods

3.1.1 Materials

Doxorubicin hydrochloride was purchased from LC laboratories. Poly (lactic-co-glycolic) acid in both ester-terminated and carboxylic acid-terminated forms (both 50:50 lactide:glycolide ratio, inherent viscosity 0.26 – 0.54 dL/g) was purchased from Durect Lactel Absorbable Polymers. L-α-phosphatidylcholine, hydrogenated (HSPC, lecithin), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and cholesterol hemisuccinate (CHEMS) was purchased from Avanti Polar Lipids. 1,2-diastearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG2K) was purchased from Lipoid. Amicon Ultra-4 centrifugal filters with 10kDa cutoff were purchased from EMD Millipore. Argon gas was purchased from Airgas. KB cells, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and MTT detergent were purchased from American Type Culture Collection. Dulbecco’s
Modification of Eagle’s Medium (DMEM) was purchased from Thermo Fisher Scientific. Dichloromethane (DCM), dimethyl formamide (DMF), triethylamine, and p-nitrophenyl chloroformate were purchased from Sigma Aldrich. Ethanol, acetonitrile, and diethyl ether were purchased from Fisher Scientific.

3.1.2 Preparation of Lipid-Polymer Hybrid Nanoparticles (LPNs)

LPNs were prepared using a single-step nanoprecipitation method\(^{37}\). Lecithin and DSPE-PEG(2k) were each dissolved at 0.25mg/mL 4% ethanol in water and heated to 70°C to ensure lipids were dissolved. PLGA was dissolved in acetonitrile at 5mg/mL. The PLGA solution was added to the aqueous solution containing the lipid shell components dropwise and vortexed on high speed for 3 minutes. The organic solvents were evaporated using a rotary evaporator, which commenced self-assembly of LPNs. Three centrifugal washes using Amicon centrifugal filters (10kDa MWCO) and DI water were performed for 10 minutes each at 4,000rpm to ensure removal of any residual organic solvent. LPNs were stored in DI water for storage at 4°C. Molar ratio of materials used for the lipid shell was 75/25 lecithin to DSPE-PEG(2k) and the shell mass totaled 20% of the core mass.

3.1.3 LPN Characterization: Dynamic Light Scattering & Zeta Potential

Samples for DLS were prepared by adding 3mL of 10mM NaCl to a cuvette and then adding 100uL of the LPN solution. Cuvette was placed in the DLS machine and 5 runs of 30 seconds each were performed to gain information on the hydrodynamic
diameter and polydispersity of the sample. Using a separate program and the same LPN sample, zeta potential data was gathered with settings of 10 runs and 10 cycles per run.

To test the stability of this LPN formulation over time, DLS measurements of hydrodynamic diameter and polydispersity were taken daily beginning on the day the LPNs were made (day 1) through day 7. Three measurements were made each day and their standard deviations incorporated into the analysis.

3.1.4 LPN Characterization: Transmission Electron Microscopy

LPNs with a PLGA core and a 75/25 molar ratio of lecithin/DSPE-PEG(2k) as the shell were synthesized for TEM analysis. To prepare the LPN sample for TEM imaging, the LPN sample was wetted to a metal mesh grid and stained with a 1.5% uranyl acetate stain. The sample was then rinsed several times in DI water before being loaded into the microscope.

3.1.5 LPN Synthesis Optimization

One of the first LPN experiments conducted was to assess the LPNs obtained by adding the organic polymer solution to the aqueous lipid solution dropwise or by adding it all at once followed immediately by sonication using a probe sonicator. The core was composed solely of PLGA initially dissolved in water-miscible acetonitrile while the lipid shell consisted of a 60:20:20 molar ratio of DOPE:CHEMS:DSPE-PEG(2k). Other than the technique of organic solvent addition, the preparation protocol was identical for the two formulations.
There were other variables tested throughout several months while conducting initial experiments with the LPNs that were not formal experiments but still resulted in conclusions based on slight intentional variations and observations during synthesis. Those variables included method of evaporation, organic-to-aqueous solvent ratio, and polymer concentration in organic solvent. More detail of these experiments can be found in the corresponding results section of this chapter.

3.1.6 Dox-PLGA Conjugate Synthesis

To synthesize the Dox-PLGA conjugate, the following protocol was used (Figure 6), which is based on a compilation of those previously published\(^{65,66}\). To activate the PLGA, 1g PLGA-CO\(_2\)H was dissolved in 10mL DCM. 26.7mg p-nitrophenyl chloroformate and 21mg pyridine were added dropwise at 0°C. The reaction was run for 3 hours at room temperature under argon atmosphere. The activated PLGA was then precipitated out of the solution in ice-cold diethyl ether. Suspension was centrifuged and supernatant was removed to isolate activated polymer, with the final solvent remnants removed via lyophilization.

To conjugate the activated PLGA to doxorubicin, 250mg activated polymer was dissolved in 7.5mL DMF. 10mg of doxorubicin hydrochloride and 6.75mg triethylamine were added and stirred for 24 hours at room temperature under argon atmosphere. The final product was precipitated in ice-cold diethyl ether and centrifuged to collect it. Supernatant was removed and product was lyophilized to remove any remaining solvent.
3.1.7 Dox-PLGA Conjugation Characterization

To calibrate the spectrofluorophotometer to doxorubicin concentration, a 1mg/mL doxorubicin solution in methanol was made. From that solution, serial dilutions were made to create doxorubicin concentrations from 0.0078125µg/mL to 5µg/mL. Beginning at the largest concentration of 5 µg/mL the spectrofluorophotometer was set to 2D excitation in order to determine the optimal excitation frequency for doxorubicin. The emission wavelength was set to 585nm and the excitation was measured from 440 – 600nm. The fluorescence peaked at 498nm. Using that 498nm wavelength as the excitation wavelength, the setting was switched to 2D emission and the emission was measured from 510 – 630nm. The slit widths were optimized to produce the highest fluorescence without saturating the detector; the final slit width settings were 3nm excitation slit width and 5nm emission slit width. These 2D emission settings were used for all future doxorubicin concentration measurements. The 7 dilutions of doxorubicin were measured 3 times each and their fluorescence measurements recorded. A graph of doxorubicin concentration vs. fluorescence was created and a linear trendline added for future use. This calibration curve was then used to compare the fluorescence from a known mass of Dox-PLGA conjugate dissolved in organic solvent to determine the drug content in the conjugate.

3.1.8 Preparation of LPNs containing Dox-PLGA Conjugate

LPNs were prepared using a variation on the nanoprecipitation method previously presented. Lecithin and DSPE-PEG(2k) were each dissolved at 0.25mg/mL 4% ethanol
in water and heated to 70°C to ensure lipids were dissolved. Both PLGA and Dox-PLGA conjugate were dissolved separately in DCM at 5mg/mL. While nanoprecipitation is typically done with water-miscible organic solvents, Dox-PLGA was insoluble in all water-miscible organic solvents tested and thus DCM was used. Instead, to properly mix the immiscible solvents, the LPN solution was sonicated at 20% intensity for 1 minute. This sonication injected sufficient energy into the system to blend the solvents and expose the contents of the core to those of the shell. The LPN solution was then placed on the rotary evaporator to facilitate evaporation of organic solvents and self-assembly of NP. Three centrifugal washes using Amicon centrifugal filters were performed for 10 minutes each at 4,000 rpm to ensure removal of any residual organic solvent. LPNs were stored in DI water at 4°C. Molar ratio of shell was 75/25 lecithin to DSPE-PEG(2k) and the shell mass totaled 20% of the core mass. Three different mass ratios of Dox-PLGA conjugate to unconjugated PLGA were used in three separate preparations: 100% Dox-PLGA, 75% Dox-PLGA to 25% PLGA, and 50% Dox-PLGA to 50% PLGA. Sizes of the three different formulations of LPNs were measured using DLS as previously described.

Once the ratio of Dox-PLGA to PLGA was established, the encapsulation efficiency of doxorubicin was measured using the spectrofluorophotometer. The same settings were used as previously described; a sample of the Dox-PLGA LPNs was added to 1mL of acetonitrile to disrupt the LPNs and the fluorescence of doxorubicin was measured in the spectrofluorophotometer. That fluorescence measurement was applied to the calibration graph previously shown to obtain the concentration of doxorubicin and
then back-calculated based on the known amount of Dox-PLGA conjugate used and the doxorubicin content in the conjugate to determine the amount of the conjugate that had been successfully encapsulated in the LPNs.

3.1.9 Exploration of Cytotoxicity of Dox-PLGA LPNs

3.1.9.1 Cell Culture Conditions

KB cells were cultured in 75 cm$^2$ flasks using Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were stored in an incubator maintained at 37°C with a 5% CO$_2$ atmosphere.

3.1.9.2 Determination of Optimal Seeding Density

KB cell solutions were made in dilutions ranging from $10^3$ – $10^7$ cells/mL. These dilutions were added to a flat-bottomed 96-well plate in 100µL aliquots, thus ranging from 0.1 – 100 thousand cells/well. Each dilution was present in 6 wells for statistical purposes. Cells were grown for 48 hours, at which point the MTT assay was performed. Briefly, this consists of adding the MTT salt to each well, incubating the cells with the salt for 2–4 hours, and then adding detergent to lyse the cells. Reading the absorbance of the purple crystal at 570 nm in a plate reader assigned each well an absorbance value. The negative controls in this experiment were wells that contained media but no cells or other treatment. The average absorbance value of the negative control wells was subtracted from each of the wells containing cells to obtain the true viability number. These values were graphed to show absorbance as a function of cell density in the wells.
3.1.9.3 MTT Assay

KB cells were seeded on a 96-well plate at a density of 8,000 cells per well and let rest overnight. Two sets of LPNs were prepared: one containing a core of 75/25 mass ratio of Dox-PLGA to PLGA and the other containing a core of unconjugated PLGA. The shell composition of each formulation was 75mol/25mol lecithin/DSPE-PEG(2k). The Dox-PLGA LPNs were diluted to contain doxorubicin concentrations of between 1 and 100 µM and the PLGA LPNs were diluted to have the same polymer content in each dilution as the Dox-PLGA LPNs. Free doxorubicin hydrochloride was dissolved in media at the same drug concentrations as the Dox-PLGA LPNs. Media was removed from the wells and 100µL of treatment was added to each well, with each treatment at each concentration placed in 4 wells. After 24 hours, the treatments were replaced with untreated cell media and left for 12 hours to recover. MTT reagent was added to each well and placed in incubator for 4 hours. Detergent was then added to each well to lyse the cells and homogenize the distribution of the purple precipitate. Plates were left in the dark overnight. Absorbance was measured using a plate reader at 570nm the following morning.

The negative controls in this experiment were wells that contained media but no cells and no treatment. The positive controls were wells that contained cultured cells but no treatments, meaning the positive control represents the healthiest version of the cells in the plate. To interpret the absorbance data, the average negative control value was subtracted from the average positive control value (termed “new positive control” for ease of reference). The negative control was then subtracted from the average value for
each treatment concentration and the result was divided by the new positive control to obtain a fraction that represents cell viability, with a value of 1 considered fully viable. These general negative and positive controls as well as the method of data analysis was used for all MTT assays presented in this dissertation.

3.2 Results & Discussion

3.2.1 LPN Preparation & Characterization

Dynamic light scattering (DLS) and zeta potential are two of the most common methods to characterize NPs. DLS provides the hydrodynamic diameter of a NP solution. Brownian motion of a suspension of NPs causes laser light to scatter, and analysis of the scattering patterns produces the size and size distribution of the NPs. Zeta potential is the magnitude of the charge between a NP and the bulk solution and it can have a significant impact on NP stability. The zeta potential of a NP is an important parameter in its in vivo applicability, as positively-charged NP often suffer from nonspecific uptake into cells due to the slightly negative charge of cell membranes. Similarly, a highly negatively charged NP might be repelled by all cells due to the like-like charge repulsion. Optimally charged NP are therefore thought to be neutral to slightly negative, in the -10mV to 0mV range.\textsuperscript{40}

LPNs were successfully synthesized with a PLGA core and a 75/25 molar ratio of lecithin/DSPE-PEG(2k) as the shell. LPN samples of this formulation measured a mean hydrodynamic diameter of 110nm; further iterations of this LPN synthesis produced LPNs that consistently measured just over 100nm on average. Polydispersity measured
0.121 and consistently ranged between 0.08 and 0.16. The diameter and polydispersity values are within the range of those previously published on LPNs\textsuperscript{37,64}.

To analyze the stability of the LPNs, the hydrodynamic diameters and polydispersity indices were graphed for the 7-day experiment. This graph is shown in Figure 4, with hydrodynamic diameter on the left y-axis and polydispersity index on the right y-axis. Standard deviation data are represented by error bars on the diameter data. Throughout the 7 days, the hydrodynamic diameter changed negligibly, ranging from 106.0nm to 110.1nm. The polydispersity had a slightly higher range, that of 0.036 to 0.165. The minimal variation of both of these variables over the 7-day period shows excellent stability of the LPNs.

![Figure 4: Size and Polydispersity data for PLGA LPNs taken each day for 7 days. Error bars represent standard deviation based on triplicate samples.](image-url)
The zeta potential of the LPNs was measured to be -10.8 mV. While this is at the end of the semi-neutral spectrum, the author believes it is more advantageous to have a slightly negative LPN compared to a slightly positive one. With a slightly negative LPN, there is likely to be less nonspecific cell internalization, and cell- or tissue-specific uptake can be better controlled through the addition of a targeting moiety on the outer shell of the LPN.

Transmission electron microscopy (TEM) is an imaging technique that uses an electron beam shone through a thin specimen to visualize the specimen. It is helpful with NP solutions to see the actual composition of the NP as confirmation of the NP synthesis in addition to less visual sizing techniques. A representative TEM image of the LPN sample can be seen in Figure 5. The white core in the center is the PLGA and the gray ring around the white core is the lipid shell. The black scale bar at the bottom of the image is 100nm, indicating that the LPNs are slightly larger than 100nm. The image also shows what seem to be less well-defined objects, which could be interpreted to be extra lipids floating in solution. The size characterization via TEM is consistent with the DLS data previously presented and further confirms the composition of the LPN as having a PLGA core and a lipid shell.
Figure 5: Transmission electron microscopy (TEM) image of PLGA LPNs. The white centers show the PLGA core and the gray rings show the lipid shells. Sample was stained with 1.5% uranyl acetate for imaging.

3.2.2 LPN Synthesis Optimization

Prior to finalizing the LPN synthesis protocol with the methods previously described, a series of investigational experiments were conducted to learn more about the LPN preparation process and to optimize the protocol. These experiments were carried out informally and thus do not contain statistical significance, but are worthwhile to those wishing to learn more about LPN synthesis or to continue this work.

The experiment testing the method of organic solvent addition to the aqueous solution resulted in similar diameter and polydispersity values that slightly favored the LPNs made using the dropwise addition process. The LPNs made using sonication had a
mean hydrodynamic diameter of 208.6nm and a polydispersity value of 0.201, while the LPNs made using dropwise addition had a mean hydrodynamic diameter of 181.9nm and a polydispersity of 0.112. The smaller diameter and PDI values indicated that the dropwise addition was the preferable method for LPN synthesis; however, as was discovered at a later date, using a core component that is insoluble in a water-miscible organic solvent necessitates the use of sonication to ensure proper dispersion of the water-immiscible organic solvent in the aqueous lipid solution.

As the LPN synthesis truly occurs during the organic solvent evaporation step, varying the method of evaporation was a natural question to investigate. Both rotary evaporation and room temperature stirring were used as evaporation techniques during synthesis. In general, both methods produced satisfactory LPNs; however, proper technique while using the rotary evaporator was paramount in ensuring its success. Evaporating too quickly with the rotary evaporator caused much of the sample to be lost and resulted in poor yield. The sample was always spun at its highest speed to maximize the surface area exposed to the vacuum and to prevent sample loss. To use the rotary evaporator with acetonitrile, the water bath was typically set to 40°C and the pressure was started at 500mbar. Once it was observed that the sample was stable (i.e. not violently bubbling or “bumping,” which results in product loss) the vacuum pressure value was slowly decreased (meaning an increase in pressure magnitude) until it fell below 200mbar. Due to the vapor pressure of acetonitrile, this pressure was sufficient to ensure full organic solvent evaporation. When evaporating dichloromethane (necessary for dissolving the Dox-PLGA conjugate), the water bath was kept at room temperature.
There was no need for a higher temperature in the water bath because dichloromethane evaporates very easily and any increase in temperature would cause evaporation to proceed too quickly. The pressure was again started at 500mbar and slowly decreased to 200mbar, at which point the NP suspension was removed from the rotary evaporator. By following these protocols, LPNs were synthesized consistently with suitable size and polydispersity values.

The organic to aqueous solvent ratio was determined to be significant in LPN synthesis. If the organic to aqueous ratio is too high, the lipids will not remain in the aqueous solution and the LPNs will not form properly. Through many trial experiments it was determined that an organic to aqueous solvent ratio of at least 1:5 and ranging up to 1:10 produces well-formed LPNs in the desired size and polydispersity ranges. Depending on the initial lipid concentrations in the aqueous solution, it is often necessary to add additional aqueous solution to the lipid solution prior to addition of the organic polymer solution in order to attain these ratios.

The final variable investigated during initial LPN preparation experiments was that of initial polymer concentration in the organic solvent. The author varied the polymer concentration from 1mg/mL to 10mg/mL and saw minimal changes in size and polydispersity values, leading to the conclusion that any polymer concentration in that range can be used to produce satisfactory LPNs.

3.2.3 Dox-PLGA Conjugate Synthesis & Characterization

Having established an LPN preparation protocol that produces consistently satisfactory LPNs, the doxorubicin-PLGA conjugate was synthesized so it could be
incorporated into the LPNs. The doxorubicin-PLGA conjugate was created by first activating the terminal hydroxyl group of the PLGA and then conjugating it to the primary amine group of doxorubicin. This conjugation scheme is shown in Figure 6.

However, before it was possible to determine the doxorubicin content in the Dox-PLGA conjugate, a calibration curve had to be made to map the concentration of doxorubicin against its resultant fluorescence in the spectrofluorophotometer (Shimadzu RF-5301).

3.2.3.1 Spectrofluorophotometer Calibration

The results of the doxorubicin concentration vs fluorescence calibration are shown in Figure 7. The calibration produced a linear trendline with an excellent fit of 0.99968. The superb fit of this graph combined with the small error bars (present but tiny, so difficult to discern on the graph) provide a high level of confidence in measuring the doxorubicin concentration of an unknown sample within the range of the calibration graph on this spectrofluorophotometer.
Figure 6: Conjugation scheme of doxorubicin to PLGA. Two reactions are taking place: the first (top) is the activation of PLGA and the second (bottom) is the conjugation of activated PLGA to doxorubicin.
3.2.3.2 Determination of Doxorubicin Content in Dox-PLGA Conjugate

Doxorubicin content was measured using the spectrofluorophotometer settings previously described. 0.5 mg of the Dox-PLGA conjugate was dissolved in 1 mL DCM. In order to produce a fluorescence spectrum that did not saturate the spectrofluorophotometer using the necessary settings, this conjugate sample needed to be diluted 20X, which ultimately produced a fluorescence value of 301 a.u. By plugging this number into the trendline produced by the calibration graph, multiplying it by 20X to account for the dilution, and then dividing it by the 500 µg mass of the original Dox-PLGA conjugate sample, it was calculated that the mass percentage of doxorubicin in the Dox-PLGA conjugate was 7.88%.
3.2.4 Preparation of LPNs containing Dox-PLGA Conjugate

In order to best determine what amount of Dox-PLGA conjugate to incorporate into the LPNs, 3 different ratios of Dox-PLGA to unconjugated ester-terminated PLGA were used to create 3 versions of the LPN. The sizes and polydisperosity values of the 3 LPN formulations can be seen in Table 1. The LPNs made with 100% conjugate had the largest average diameter of 305 nm, while the 75/25 formulation was 175 nm and the 50/50 formulation was 159 nm. The polydisperosity value of the LPN made with 100% conjugate was quite high at 0.362, while the 75/25 formulation was 0.164 and the 50/50 formulation was 0.145. As polydisperosity measures the variation in NP size throughout the sample, a lower polydisperosity means a more consistent, monodisperse sample and is more advantageous for a drug delivery application. Optimal NP size varies, but for drug delivery it has been reported that 50–200nm NP are best suited to escape the vasculature and accumulate in the tumor space\textsuperscript{33}. The apparent trend of a higher conjugate content leading to larger LPNs is likely due to a looser core structure and therefore increased diameter of the LPNs containing Dox-PLGA. The presence of the conjugate could cause the core to be unable to compress as tightly as LPNs containing no conjugate. Although the 50/50 formulation had the most optimal (in this case, smallest) values for both diameter and polydisperosity, the values for the 75/25 formulation were only slightly higher and allowed for a greater amount of drug to be incorporated into the LPN. For these reasons, all future LPNs containing Dox-PLGA consisted of a core of 75mass% Dox-PLGA and 25mass% unconjugated PLGA.
<table>
<thead>
<tr>
<th>LPN Formulation</th>
<th>Hydrodynamic Diameter (nm)</th>
<th>Polydispersity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Dox-PLGA</td>
<td>305 ± 20</td>
<td>0.362 ± 0.06</td>
</tr>
<tr>
<td>75% Dox-PLGA/25% PLGA</td>
<td>175 ± 14</td>
<td>0.164 ± 0.03</td>
</tr>
<tr>
<td>50% Dox-PLGA/50% PLGA</td>
<td>159 ± 12</td>
<td>0.145 ± 0.04</td>
</tr>
</tbody>
</table>

Table 1: Size and polydispersity data as obtained from dynamic light scattering (DLS). Three LPN formulations were measured with varied core composition: 100% Dox-PLGA, 75% Dox-PLGA/25% PLGA, and 50% Dox-PLGA/50% PLGA.

Using the spectrofluorophotometer to measure doxorubicin concentration, the encapsulation efficiency of the Dox-PLGA conjugate in the LPNs containing a mass ratio of 75/25 Dox-PLGA to PLGA was 46.9%. A diagram showing a LPN containing both Dox-PLGA and unconjugated PLGA is shown in Figure 8.

![Figure 8: Schematic of the lipid-polymer hybrid nanoparticle used in this dissertation. The core consists of a 75/25 mass ratio of Dox-PLGA conjugate and unconjugated PLGA. The lipid monolayer shell consists of a 75/25 molar ratio of lecithin and DSPE-PEG(2k).](image-url)
3.2.5 Exploration of Cytotoxicity of Dox-PLGA LPNs

An extension to the size and image characterization of the LPNs presented earlier in this chapter is to explore whether the PLGA-loaded LPNs have any inherent toxicity to cells and compare any intrinsic cytotoxicity to that of the LPNs loaded with Dox-PLGA conjugate as well as free doxorubicin (unencapsulated). KB cells are a HeLa contaminant cell line and they were selected as a representative cancer cells line for this experiment. At this stage in the research the application to tumors in the brain had not been selected, which is why a more appropriate cell line was not chosen. Regardless, KB cells are frequently used in the NP drug delivery community to determine cytotoxicity of NP treatments and it therefore remains a valid choice in this analysis.

3.2.5.1 Determination of Optimal Seeding Density

In order to assess the cytotoxicity of a particular treatment, first the optimal cell seeding density needed to be determined for the MTT cytotoxicity assay which would quantify cell viability. MTT in its long form is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, which is a yellow tetrazolium salt that active mitochondrial enzymes convert to a purple crystal. The absorbance of this crystal is measured by a plate reader to quantify the cellular activity of a particular well.

The graph showing cell seeding density vs. absorbance can be seen in Figure 9. The graph has an initial linear portion followed by a decreased slope and ultimate plateau. The linear portion of the graph is enlarged in the second part of the figure to more closely see its linearity. The motivation for performing this assay is to find a cell
seeding density in the linear portion so that the absorbance values have a linear relation to
cell viability in future experiments. As the linear portion of this graph runs from
approximately 0.1 thousand cells/well to slightly past 8 thousand cells per well, 8
thousand cells per well was chosen as the seeding density for future experiments with KB
cells in 96-well plates. Though the density chosen was at the top of the linear range, in
future experiments the untreated cells will serve as the positive control and the majority
of cells tested will be treated with a solution likely to decrease its viability. Therefore, all
of the cells in the experiments should fall within the linear range of the assay.
Figure 9: Top graph is the seeding density MTT assay showing the absorbance of the MTT precipitate as a function of cell seeding density. Bottom graph is an excerpt from the top graph to show the linear portion of the graph from which the optimal seeding density is chosen. Error bars indicate standard deviation.
3.2.6 MTT Assay

The graph showing cell viability of the Dox-PLGA LPNs compared to the PLGA LPNs can be seen in Figure 10. While the PLGA LPNs contain no drug and therefore do not truly apply to the x-axis label, the two formulations contain the same polymer content and are therefore sufficiently comparable to use in this context. The axis label of doxorubicin concentration was selected because it is the more informative variable when compared to polymer content. From this graph, it is apparent that the Dox-PLGA LPNs show significantly more cytotoxicity than the PLGA LPNs. An analysis of variance (ANOVA) was run which confirmed the statistical significance of the PLGA LPN curve compared to both the Dox-PLGA LPN curve and the free Dox curve (p < 0.01 in both cases). The ANOVA (n = 4) showed no statistical difference between the Dox-PLGA LPN curve and the free Dox curve (p > 0.05). The Dox-PLGA LPNs exhibit an IC$_{50}$, which is the concentration at which half the cell population is not viable, of 1.95µM. Conversely, the PLGA LPNs do not have an IC$_{50}$ value because they never induce a cell viability level of below 0.8. It is notable that there is nonzero cell death with the PLGA LPNs and they do seem to have a small amount of cytotoxicity associated with their treatment. It is unclear as to why this is the case as the LPNs only contain biodegradable polymers and phospholipids. However, the cell death of the PLGA LPNs is considerably less than that of the Dox-PLGA LPNs.
**Figure 10:** Cell viability data as obtained by an MTT assay showing the cytotoxicity of Dox-PLGA LPNs compared to unloaded PLGA LPNs and free doxorubicin. Dox-PLGA LPNs show an IC\(_{50}\) of approximately 1.95\(\mu\)M while the PLGA LPNs show minimal cytotoxicity. Error bars indicate standard deviation.

### 3.3 Conclusions

In this chapter, the LPN delivery system was introduced as a viable drug delivery vehicle. The LPNs were shown by both DLS data and TEM images to be just over 100nm in diameter and be stable for at least 7 days when stored at 4°C. A doxorubicin-PLGA conjugate was chemically synthesized and the doxorubicin mass content shown to be 7.88% using spectrofluorophotometry. After 3 different conjugate/polymer ratios were tested the Dox-PLGA conjugate was successfully incorporated into the LPNs using a mass ratio of 75% Dox-PLGA to 25% PLGA. These Dox-PLGA LPNs incorporated
the doxorubicin with an encapsulation efficiency of 46.9% and were measured to be 175 nm in hydrodynamic diameter with a polydispersity of 0.164. Finally, the MTT assay was introduced and the Dox-PLGA LPNs shown to have a high level of cytotoxicity with an IC_{50} of 1.95\mu M while unloaded PLGA LPNs had minimal cell death.
4. Investigate Release and Cytotoxicity of Dox-PLGA LPNs

This chapter explores aim 2, which is designed to test the effectiveness of the Dox-PLGA conjugation on two variables: doxorubicin release kinetics and LPN cytotoxicity. The hypothesis of this aim is that the incorporation of the Dox-PLGA conjugate into the LPN will result in a more sustained release profile than LPN loaded with unconjugated Dox. Due to the longer Dox retention in the LPN, the cytotoxicity of the LPN will be maintained for a longer period of time when compared to LPN loaded with unconjugated Dox.

4.1 Materials & Methods

4.1.1 Materials

Doxorubicin hydrochloride was purchased from LC laboratories. Poly (lactic-co-glycolic) acid in both ester-terminated and carboxylic acid-terminated forms (both 50:50 lactide:glycolide ratio, inherent viscosity 0.26 – 0.54 dL/g) was purchased from Durect Lactel Absorbable Polymers. L-α-phosphatidylcholine, hydrogenated (HSPC, lecithin) was purchased from Avanti Polar Lipids. 1,2-diastearoyl-sn-glycéro-3-phosphoéthanolamine-N-[amino(polyéthylène glycol)-2000] (DSPE-PEG2K) was purchased from Lipoid. Amicon Ultra-4 centrifugal filters with 10kDa cutoff were purchased from EMD Millipore. Argon gas was purchased from Airgas. KB cells, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and MTT detergent were purchased from American Type Culture Collection. Dulbecco’s
Modification of Eagle’s Medium (DMEM) was purchased from Thermo Fisher Scientific. Dichloromethane, dimethyl formamide, triethylamine, and p-nitrophenyl chloroformate were purchased from Sigma Aldrich. PBS tablets, ethanol, acetonitrile, and diethyl ether were purchased from Fisher Scientific. Slide-A-Lyzer MINI dialysis units were purchased from Thermo Scientific.

4.1.2 Creation of LPNs with Unconjugated Doxorubicin (Dox LPNs)

LPNs were prepared using a single-step nanoprecipitation method similar to that previously presented. Lecithin and DSPE-PEG(2k) were each dissolved at 0.25mg/mL 4% ethanol in water and heated to 70°C to ensure lipids were dissolved. PLGA was dissolved in acetonitrile at 5mg/mL and doxorubicin HCl was dissolved in methanol at 1mg/mL. The PLGA and doxorubicin solutions were added to the aqueous solution containing the lipid shell components dropwise and vortexed on high speed for 3 minutes. The organic solvents were evaporated using a rotary evaporator, which commenced self-assembly of LPNs. Three centrifugal washes using Amicon centrifugal filters and DI water were performed for 10 minutes each at 4,000 rpm to ensure removal of any residual organic solvent. LPNs were stored in DI water at 4°C. Molar ratio of shell was 75/25 lecithin to DSPE-PEG(2k) and the shell mass totaled 20% of the core mass. The doxorubicin content added to the core was 5% of the mass of PLGA used in the core. DLS, zeta potential, and encapsulation efficiency were all calculated using the methods previously outlined.
4.1.3 Doxorubicin Release Kinetics

Two sets of LPNs were prepared for this experiment. One set is the same Dox LPNs containing unconjugated doxorubicin, and the other was a new iteration of LPN that contained a 75/25 mass ratio of Dox-PLGA/PLGA and also contained an amount of unconjugated doxorubicin at 5% of the total PLGA mass in the LPN. 100µL aliquots of each formulation were placed in mini dialysis cassettes with a 2,000Da molecular weight cutoff and suspended in 2L PBS with slow stirring for 28 days. At established time points three 100µL aliquots were removed and each was added to 1mL acetonitrile. Doxorubicin content of each of the three samples was measured using the spectrofluorophotometer as described in the previous chapter to determine the percentage of doxorubicin released from the NP formulations.

4.1.4 Cytotoxicity Pre- and Post- Dialysis

Cell culture conditions were the same as previously outlined. KB cells were seeded at 8,000 cells/well on a 96-well plate and grown to 80% confluence. Two sets of LPNs were prepared: Dox-PLGA LPNs and the Dox LPNs. Post synthesis, each LPN formulation was divided into two even aliquots. One aliquot of each formulation was resuspended in cell media, diluted to predetermined concentrations of doxorubicin, and incubated with KB cells. Each concentration of each treatment was used in four separate wells for statistical analysis. The other aliquot of each formulation was dialyzed in 1L PBS for 1 hour before being resuspended in cell media, diluted to the necessary concentrations, and incubated with KB cells. Treatments were incubated with cells for
24 hours from the time the first aliquot was added to the cells. After 24 hours, the treatments were replaced with untreated cell media and left for 12 hours to recover. MTT reagent was added to each well and placed in incubator for 3 hours. Detergent was then added to each well to lyse the cells and homogenize the distribution of the purple precipitate. Plates were left in the dark at room temperature overnight. Cell viability was measured using a plate reader absorbance at 570nm the following morning.

4.2 Results & Discussion

4.2.1 Preparation of Dox-LPNs

Preparation of Dox LPNs produced Dox LPNs with a hydrodynamic diameter of 157nm and a polydispersity of 0.151. The zeta potential was -13.32mV. Encapsulation efficiency of the doxorubicin hydrochloride passively loaded into LPNs was 39.8%. These Dox LPNs are slightly smaller than Dox-PLGA LPNs, have a comparable polydispersity, and have about a 15% lower encapsulation efficiency.

4.2.2 Doxorubicin Release Kinetics

Figure 11 displays the percentage of doxorubicin released from the two formulations of LPN over a period of 28 days. At each time point, three aliquots were collected and the error bars show the standard deviation for the three measurements. The LPNs loaded with both doxorubicin hydrochloride and Dox-PLGA conjugate show a much faster burst release and quicker ascension to plateau than the LPNs loaded only
with Dox-PLGA conjugate, which show a slower burst release followed by a fairly linear release after the first 7 days. After 1 day, the LPNs loaded with doxorubicin HCl plus Dox-PLGA conjugate released over 40% of the drug, while the NP loaded with only Dox-PLGA conjugate released 19% of the drug.

![Graph showing doxorubicin release kinetics over 28 days from two LPN formulations: Dox-PLGA LPNs and a LPN loaded with both Dox-PLGA and unconjugated doxorubicin hydrochloride. Error bars indicate standard deviation.](image)

**Figure 11:** Doxorubicin release kinetics over 28 days from two LPN formulations: Dox-PLGA LPNs and a LPN loaded with both Dox-PLGA and unconjugated doxorubicin hydrochloride. Error bars indicate standard deviation.

These results confirm the hypothesis that loading doxorubicin via a drug-polymer conjugate compared to an unconjugated passive loading increases drug retention time in the LPN. As intravenous injection of the LPN formulation would take time to accumulate in the tumor microenvironment, the slower release of the Dox-PLGA NP could prevent drug release in the vasculature thus reducing systemic side effects of chemotherapy administration. In addition to the slower burst release of the Dox-PLGA
LPNs over approximately 7 days, this formulation also demonstrates a more linear release over the remaining 21 days of the experiment. The positive slope of the Dox-PLGA LPN release data through the end of the experiment implies that more drug had yet to be released; this slower burst release plus long-term linear release indicates that these LPNs could serve as a drug-eluting depot at the tumor site for several weeks post administration. These observations are in contrast to the Dox HCl + Dox-PLGA LPN formulation, which released 83% of its drug in the first 5 days and only reached 89% of drug released at the conclusion of the experiment, at which point the near-zero slope of the data implies a completion of drug release. This fast burst release and early plateau of the Dox LPNs indicates a general lack of control of release kinetics and would likely suggest that a significant percentage of the drug could be released prior to accumulation at the tumor site.

4.2.3 Cytotoxicity

Given the sustained release profile of Dox-PLGA LPNs, the author chose to investigate what impact it had on *in vitro* cytotoxicity. This experiment was designed to test whether Dox-PLGA LPNs would retain their cytotoxicity post dialysis, with the dialysis representing vascular circulation prior to accumulation at the tumor site. Figure 12 shows the cytotoxicity results of Dox-PLGA LPNs (A, top) compared with Dox LPNs (B, bottom). Each formulation shows results from immediate treatment as well as after 1 hour of dialysis. 1 hour was chosen as a representative time frame that the LPNs might be in circulation prior to accumulation in the tumor space. Each graph also shows the
cytotoxicity of free doxorubicin HCl at the same concentrations as a standard for comparison to the LPN treatments. The Dox-PLGA LPNs show almost identical cytotoxicity to free doxorubicin HCl when used for immediate treatment and have an IC$_{50}$ of approximately 2µM, with a 2.6-fold increase in IC$_{50}$ after 1 hour of dialysis in PBS. The Dox LPNs show both a lower starting cytotoxicity (IC$_{50}$ of 7.3µM) when the NP are used immediately as well as a much higher 6.3-fold increase to 46.1µM in IC$_{50}$ after 1 hour of dialysis in PBS. Using the ANOVA statistical technique with an n = 4 for each treatment concentration, it was determined that the pre-dialysis Dox-PLGA LPN curve was not statistically different from the free Dox treatment (p > 0.05) but it was statistically different from the post-dialysis Dox-PLGA LPN curve (p < 0.01). The pre- and post-dialysis Dox NP treatments were statistically different from each other and from the free Dox treatment (all p values < 0.01).
Figure 12: Cell viability data from MTT assay. Both graphs show cytotoxicity from free doxorubicin hydrochloride and from an LPN formulation immediately after synthesis and after 1 hour of dialysis. Top graph shows Dox-PLGA LPNs and bottom graph shows Dox LPNs. n = 4 for each treatment concentration. Error bars represent standard deviation.
There was a much more drastic decrease in cytotoxicity of the Dox LPNs when comparing their immediate use to application after 1 hour of dialysis than exhibited by the Dox-PLGA LPNs. This indicates that the 1 hour of dialysis caused the Dox LPNs to lose more cytotoxic potency than the Dox-PLGA LPNs. These results are consistent with the release kinetics shown in Figure 11, as those show a faster burst release from LPNs that are passively loaded with doxorubicin compared to Dox-PLGA LPNs. The cytotoxicity data confirm the hypothesis that Dox-PLGA LPNs retain their cytotoxicity better than Dox LPNs.

On an absolute rather than relative scale, Dox-PLGA LPNs are also more cytotoxic even when used immediately when compared to Dox LPNs. The Dox-PLGA LPNs mirrored the cytotoxicity of free doxorubicin when used for immediate treatment. Overall it is a positive attribute that the Dox-PLGA LPNs are as potent as the clinically-used doxorubicin; however, this result is somewhat counterintuitive because according to the release data previously presented, the Dox-PLGA LPNs release less drug than the Dox LPNs in the first 24 hours post-synthesis. As this cytotoxicity data was collected from freshly-synthesized LPNs, it is not immediately apparent why the Dox LPNs would not be more cytotoxic than the Dox-PLGA LPNs at the same concentration of drug. This higher immediate cytotoxicity of Dox-PLGA LPNs compared to Dox LPNs likely confirms that the doxorubicin in the Dox-PLGA conjugate is capable of functioning as a cytotoxic agent despite the chemical bond to the polymer. As Dox-LPNs release drug more quickly, it could be that Dox is being released closer to the cell membrane compared to Dox from Dox-PLGA LPNs. The intracellular location of the drug release
is important because as Dox is a substrate for efflux pumps present in the cell membrane, release near the membrane could cause Dox to be pumped out of the cell and therefore prevent cell death. Alternatively, it is worth noting that the release experiment previously presented used PBS as the dialysis solution for the LPNs and this cytotoxicity experiment takes place in serum-containing media. The addition of the serum proteins is significant as proteins can interfere with the integrity of LPNs in circulation. In this case, it is possible that the serum proteins caused faster release from the LPNs than shown in the release study, thus increasing the immediate cytotoxicity of the LPNs.

### 4.3 Conclusions

The primary conclusion of this aim is to demonstrate the impact on release kinetics and cytotoxicity of using a drug-polymer conjugate to encapsulate chemotherapy in LPNs. The sustained release kinetics obtained through use of the Dox-PLGA conjugate allow for the nanoparticle to serve as a drug-eluting depot over several weeks. Examining the effect of the Dox-PLGA conjugate from a cytotoxicity perspective highlighted the importance of the conjugate in retaining LPN cytotoxicity after 1 hour of dialysis. These results showing a more sustained release profile and greater ability to retain cytotoxicity for a longer period of time confirm the hypothesis posed at the beginning of this chapter.

These data also have significant potential for clinical application. As nanoparticles take time to accumulate in the tumor space post administration, the prolonged release of Dox-PLGA LPNs ensures minimal drug release during systemic
circulation thus retaining maximum cytotoxicity until the treatment accumulates in the tumor space. These characteristics could lead to improving the safety profile of traditional chemotherapy administration as more drug could be used with greater safety to the patient. Using this doxorubicin-PLGA conjugate encapsulated in a LPN both reduces the negative off-target effects of traditional chemotherapy as well as decreases the necessary frequency of treatment due to the prolonged release profile of the drug.
5. Applications to the Blood-Brain Barrier

This chapter will fulfill aim 3 through application of the doxorubicin release and cytotoxicity data to more complex experiments that more closely mirror the BBB. The guiding hypothesis in this aim is that incorporation of the Dox-PLGA conjugate into the LPN and the sustained release profile that it exhibits will have a protective effect on the BBB, preventing the drug from leaking out of the LPN while it crosses the BBB. Additionally, the use of an angiopep targeting moiety on the outer shell of the LPN will initiate transcytosis across the BBB and thus enhance BBB penetration. In order to test these hypotheses, an in vitro transwell model will be used in 12-well plates so that BBB cells can be cultured on a membrane and tumor cells can be cultured in the bottom well. Transendothelial electrical resistance (TEER) will be used to assess the integrity of the BBB model. A lipid-angiopep conjugate was synthesized and incorporated into the lipid shell coating the LPN. It has been reported in the literature that angiopep (ANG) binds with LRP1 (low density lipoprotein receptor-related protein 1) expressed by brain endothelial cells, which initiates receptor-mediated transcytosis through the cells. A number of studies have shown that ANG-mediated RMT can be exploited for trafficking nanoparticles across the BBB and enabling drug delivery to the brain\textsuperscript{9}. Examples include liposomal delivery of docetaxel\textsuperscript{61} as well as polymeric NP delivery of paclitaxel\textsuperscript{62}, both using ANG-mediated RMT. The capacity of ANG-decorated LPN containing Dox-PLGA conjugate to penetrate a model BBB and kill cancer cells on the opposite side was investigated. Moreover, it was hypothesized that conjugating Dox to the polymeric core
obviated exposure of brain endothelial cells to the cytotoxic drug, thus maintaining the health and functionality of the BBB.

5.1 Materials & Methods

5.1.1 Materials

Doxorubicin hydrochloride was purchased from LC laboratories. Poly (lactic-co-glycolic) acid in both ester-terminated and carboxylic acid-terminated forms (both 50:50 lactide:glycolide ratio, inherent viscosity 0.26 – 0.54 dL/g) was purchased from Durect Lactel Absorbable Polymers. L-α-phosphatidylcholine, hydrogenated (HSPC, lecithin) was purchased from Avanti Polar Lipids. 1,2-diastearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG2K) was purchased from Lipoid. Amicon Ultra-4 centrifugal filters with 10kDa cutoff were purchased from EMD Millipore. Argon gas was purchased from Airgas. KB cells, bEND3 cells, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and MTT detergent were purchased from American Type Culture Collection. Dulbecco’s Modification of Eagle’s Medium (DMEM) was purchased from Thermo Fisher Scientific. Dichloromethane, dimethyl formamide, triethylamine, p-nitrophenyl chloroformate, molybdenum blue, dragendorff’s reagent, Ethylenediaminetetraacetic acid (EDTA), retinoic acid, and Transwell polyester membrane cell culture inserts (12mm with 0.4μm pore size) were purchased from Sigma Aldrich. PBS tablets, ethanol, acetonitrile, and diethyl ether were purchased from Fisher Scientific. Accutase and matrigel were purchased from Thomas Scientific. mTeSR1 was purchased from Stemcell
Technologies. Angiopep peptide was custom ordered from Tufts. MDA-MB-231 cells were a gift from the Seldin lab at Boston University. Induced pluripotent stem cells (iPSCs) were purchased from Wicell. 1,2-diestearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-3400] (DSPE-PEG3.4K-Mal) was purchased from Nanocs.

5.1.2 Differentiation of Human Brain Microvascular Endothelial Cells (BMECs)

The protocol outlined in this section was reported by Stebbins et al.\textsuperscript{67}. Throughout this protocol, cells were cultured in mTeSR1 media with a variety of additives depending on the step in the protocol. To prepare for the differentiation, 6-well plates were coated with Matrigel® on which the iPSCs were plated at 100,000 cells per well. Cells were left for 5 days after the day of plating and media was changed daily. On day 6, media was changed and replaced with a retinoic acid solution (which induces differentiation to BMEC cell type). Media was not changed on day 7. On day 8, transwell membranes on which the cells would be plated were coated with a collagen/fibronectin mixture for 1 hour prior to plating and then the mixture was aspirated and plates were dried in the incubator. Cell media was aspirated and cells were then detached using Accutase for 1 hour in the incubator. Cells were collected via centrifuge and resuspended in the same media containing retinoic acid. 1.1 million cells were plated on each transwell. On day 9, media was switched again to exclude the retinoic acid. TEER was measured beginning on day 9 through day 12 using the EVOM\textsuperscript{2} Epithelial Voltohmmeter purchased from World Precision Instruments. Based on the
published protocol, it was anticipated that the TEER values at day 9 would be approximately 1000 Ω x cm², at day 10 would reach their maximum value of between 2500 and 3000 Ω x cm², and at day 11 would decrease to approximately 1500 Ω x cm².

5.1.3 Transwell Experiment with BMEC BBB

A BMEC BBB was first established according to the protocol just described. Two types of LPNs were used in this experiment: Dox LPNs and Dox-PLGA LPNs. Each was plated in triplicate on the apical chamber of the transwell inserts, incubated for 12 hours and then replaced with fresh media. TEER was measured prior to the addition of the LPNs and at the end of their incubation period.

5.1.4 Synthesis of Lipid-Angiopep Conjugate

A cysteine-modified angiopep peptide was synthesized by the Tufts University Core Facility. The conjugation scheme is shown in Figure 14. To begin, a 50µM solution of the DSPE-PEG(3.4k)-maleimide in water was made to form micelles. As the critical micelle concentration of the DSPE-PEG(3.4k)-maleimide is approximately 10-15µM, a 50µM solution would ensure micelle formation. DLS was performed to confirm micelle formation. ANG was dissolved in PBS with 5mM EDTA at 1mg/mL and then the two solutions were mixed together and stirred at room temperature for 24 hours. Water was removed via lyophilization. Confirmation of conjugation to produce DSPE-PEG(3.4k)-ANG was performed with thin layer chromatography (TLC).
5.1.5 Transwell Cytotoxicity Experiment

Six different LPN formulations were prepared and used in this experiment. The three base LPNs were Dox LPNs, Dox-PLGA LPNs, and PLGA LPNs. Additionally, each of these formulations was made a second time but with the addition of 1mol% DSPE-PEG(3.4k)-ANG in the shell. The molar percentage of DSPE-PEG(2k) was reduced from 25mol% to 24mol% to compensate for the addition of ANG in the targeted LPNs.

Two different cell lines were also used in this experiment: MDA-MB-231 triple negative breast cancer cells were plated at a density of 83,000 cells/well in the basolateral chamber of the 12-well plates (below the transwell inserts) and bEND3 mouse brain endothelial cells were plated at 45,000 cells/well in the apical chamber of the transwell. Schematic of this experiment is shown in Figure 15. Cells were allowed to grow for 48 hours after plating and then TEER data were taken. LPN treatments were added at different doxorubicin concentrations to the apical chamber of the transwells, with each treatment’s concentration placed in three wells. After 4 hours of incubation, the LPN treatments were removed from the apical chamber and replaced with fresh media. Twenty hours later, the media in the basolateral chamber was replaced with fresh media. A second set of TEER data was taken and then the transwell inserts were removed. A MTT cell viability assay was performed on the MDA-MB-231 cells remaining in the 12-well plates. MTT reagent was added to the wells and the plates were incubated for 3.5 hours. Detergent was then added to lyse the cells and they were placed in the dark at room temperature for 2 hours. Absorbance was measured using the plate reader and then
used to calculate cell viability.

5.2 Results & Discussion

One of the difficulties in studying the BBB is that the human BBB is much tighter than \textit{in vitro} models have been able to achieve. As a brief quantitative example, the variable of transendothelial electrical resistance (TEER) measures the resistance between the two sides of the barrier; a higher TEER value indicates a tighter barrier. While \textit{in vivo} measurement of the TEER of the human BBB is impossible due to the irreparable damage it would cause, humans are estimated to have a TEER value in the range of 1000 – 5000 Ω x cm$^2$\textsuperscript{67,68} and many \textit{in vitro} models operate well below that. It was recently shown that human primary brain endothelial cells differentiated from induced pluripotent stem cells (iPSC) could be used to generate an \textit{in vitro} BBB model with TEER values approaching \textit{in vivo} values\textsuperscript{67}. This has great value in the research community; better BBB models will more accurately illustrate a chemotherapy treatment’s ability to cross the BBB as well as its cytotoxic efficacy at the tumor site.

5.2.1 BMEC Differentiation

Differentiation of iPSCs to BMECs went mostly as written the protocol above with one notable exception. On day 8 the cells were incubated with Accutase with the intention of detaching them from the 6-well plate in order to collect, count, and plate on the transwells. However, despite 1.5 hours of incubation with Accutase (when the protocol said detachment should take a maximum of 1 hour) the cells still had not
detached as viewed on a microscope. As the protocol stated that prolonged incubation with Accutase was harmful to cells, the author worried for the health of the cells and did not continue the protocol. Fortunately, as only one of the two plates had been incubated with Accutase as protection against an event such as this, a second round of detachment on day 9 was attempted with the second 6-well plate. In total, the Accutase incubation took 3 hours before the cells had fully detached and were able to be collected, counted and plated on the transwells. The maximum TEER measured once the cells grew to confluence was approximately $1500 \, \Omega \times cm^2$. This value is still significantly higher than reports of models with other cell types, and shows the potential for \textit{in vitro} testing of this BBB model.

5.2.2 Transwell Experiment with BMEC BBB

Continuing with the exploration of BMECs as an \textit{in vitro} model of the BBB, this experiment treats the BMEC BBB with both Dox LPNs and Dox-PLGA LPNs. The data showing TEER as a function of doxorubicin concentration in the LPNs both prior to LPN treatment and post LPN treatment can be found in Figure 13. On each graph, the top line shows the pre-treatment TEER. Note that this top line does not actually have any doxorubicin since the treatment has yet to be applied, but is the well in which the treatment will go. This serves as a direct comparison of the pre- and post-treatment TEER in each well. The pre-treatment TEER on both graphs is above $1000 \, \Omega \times cm^2$. Looking at the Dox LPN graph, the post-treatment data does not change significantly at the lowest doxorubicin concentration of $5\mu M$; however, the larger two doxorubicin
concentrations of 16.7 and 50µM show a TEER of nearly 0. This indicates damage to the BBB layer, which is interpreted to have been induced by the treatment with the Dox LPNs. Comparatively, the post-treatment TEER data for the Dox-PLGA LPNs is somewhat different. Though marginally smaller, the TEER remains above 800 Ω x cm² for doxorubicin concentrations of both 5.6 and 16.7µM and then it drops to near 0 when the doxorubicin concentration reaches 50µM. In comparing these two post-treatment TEER graphs, it appears as though the Dox LPN treatment damaged the BBB layer at a lower doxorubicin concentration than the Dox-PLGA LPN treatment. This conclusion is supported by the release kinetics data already presented, which shows a faster doxorubicin release from LPNs with passively-loaded drug compared to LPNs loaded with polymer-conjugated drug.
Figure 13: TEER data for Dox-LPNs (top) and Dox-PLGA LPNs (bottom) shown both pre- and post-treatment with LPNs. Error bars represent standard deviation.
5.2.3 Synthesis of Lipid-Angiopep Conjugate

As described in Chapter 1 of this manuscript, crossing the BBB is a difficult task. To allow for the greatest chance of success in future experiments designed to test NP translocation across the BBB, it was decided that a ligand should be added to the shell of the LPNs in an attempt to enhance transcytosis into the brain. With success reported using angiopep-2\(^2\)\(^6\) (ANG), a conjugation protocol was developed to conjugate ANG to DSPE-PEG(3.4k) to allow easy incorporation into the lipid shell of the LPNs during synthesis. The schematic for this protocol is found in Figure 14.

![Figure 14: Conjugation scheme for angiopep ligand to DSPE-PEG(3.4k)-maleimide](image)

Figure 14: Conjugation scheme for angiopep ligand to DSPE-PEG(3.4k)-maleimide

The initial step of creating micelles with the DSPE-PEG(3.4k)-maleimide dissolved in water was successful, with a hydrodynamic diameter of 35nm. The conjugation proceeded as intended and was confirmed using TLC. Three solutions were spotted: unconjugated angiopep, unconjugated DSPE-PEG(3.4k)-maleimide, and the post-conjugation DSPE-PEG(3.4k)-ANG product. The three stains used for TLC analysis were UV light, which shows proteins, molybdenum blue, which stains phospholipids, and dragendorff’s reagent to stain for amino acids. Multiple ratios of
chloroform and methanol ranging from 75\text{CHCl}_3/25\text{MeOH} to 90\text{CHCl}_3/10\text{MeOH} were used in the attempt to isolate the structures on the TLC plate. Ultimately, the most successful solution was an 85/15 chloroform/methanol mixture and all 3 stains were used to confirm visualization that the conjugation occurred.

5.2.4 Transwell Cytotoxicity Experiment

The final experiment in this chapter aimed to use the ANG conjugate synthesized in the previous section to test translocation across the BBB and cytotoxicity of breast cancer cells plated on the basolateral side of the transwell after transcytosis. The primary goal of this experiment was to test whether decorating the LPN surface with ANG enhanced transcytosis across the BBB and affected the cytotoxicity of the LPNs. While translocation was not directly measured, the cytotoxicity of the cells plated on the basolateral side of the transwell is indicative of LPN translocation. Two cell types were used in this experiment: bEND3 murine brain endothelial cells were used to form the BBB and MDA-MB-231 human breast cancer cells were plated on the basolateral side of the BBB. bEND3 cells were used instead of BMEC cells as a representation of a leaky BBB and MDA-MB-231 cells were used to represent a breast cancer metastasis in the brain. A schematic for this experimental model is shown in Figure 15.
Figure 15: Schematic of transwell experiment with bEND3 cells plated on apical chamber of the transwells to form the BBB and MDA-MB-231 cells plated on basolateral chamber of transwells to represent breast cancer metastasis.

The TEER values obtained during this experiment were very low, ranging from the 10s to the 30s with no discernable pattern when analyzing the data. These low values are incomparable to those previously presented from the BMEC experiment due to the fact that they were obtained from murine brain endothelial cells instead of human brain endothelial cells. However, these data are considered low even by bEND3 standards, which often report TEER values of ~100 Ω x cm². A possible reason for these low values was lack of formation of a full BBB; longer incubation time could have led to a tighter barrier with higher TEER values. Higher seeding density in future experiments could also lead to formation of a tighter barrier.

The cell viability data obtained via the MTT assay is shown in Figure 16. In addition to the positive and negative controls of the assay, the PLGA LPNs with no doxorubicin loaded also serve as a type of control, to verify the lack of cytotoxicity of these unloaded LPNs. Indeed, the top right of the graph shows the two PLGA LPN formulations used in this experiment and they show little to no cytotoxicity. It is notable that, as in previous experiments, the axis label of doxorubicin concentration does not
apply to the PLGA LPNs since they do not contain doxorubicin. However, the polymer and lipid content of the PLGA LPNs used in this experiment are the same as those of the other LPN formulations at the highest doxorubicin concentration of 600µM. The other four LPN formulations have very comparable cytotoxicities at the doxorubicin concentrations of 66.7µM and 200µM; an ANOVA (n=3) confirmed that the differences were insignificant at these concentrations (p > 0.05). However, the four data curves were significant at the 600µM doxorubicin concentration (p < 0.01). At the 600µM doxorubicin concentration, the four doxorubicin-loaded formulations all showed a decrease in cell viability, with the ANG-modified Dox-PLGA LPNs (A:Dox-PLGA LPNs) as the most cytotoxic formulation. The ANG-modified vs. unmodified Dox LPN formulations at 600µM were not significantly different, though the ANG-modified vs unmodified Dox-PLGA LPNs were statistically significant (p < 0.05). The ANG-modified Dox-PLGA LPNs were also statistically different from both the modified and unmodified Dox LPNs (p < 0.05).
**Figure 16**: Cell viability data from MTT assay using MDA-MB-231 breast cancer cells plated on the basolateral chamber of transwell inserts. Six LPN formulations were used as shown in legend. Error bars represent standard deviation.

Although the general trend of the cytotoxicity data is aligned with the doxorubicin concentrations, the ANG targeting moiety seems to have improved the cytotoxicity of the Dox-PLGA LPNs while having no statistically significant effect on the Dox LPNs. Two possible reasons for this inconsistent impact include an insufficient amount of ANG incorporated into the LPNs; it is also possible that the sheer number of LPNs added to each transwell overwhelmed and saturated the ANG’s LRP1 receptors so there was no real impact on transcytosis by the addition of ANG. Alternatively, the low TEER obtained from the bEND3 BBB could have meant that the LPNs did not have a true BBB across which to transcytose and thus could have simply diffused through the 400nm holes.
in the transwell membrane. At 600µM doxorubicin concentration both of the Dox-PLGA LPN formulations had higher cytotoxicity compared to the Dox LPNs. The differences are approximately 5% for the Dox LPNs and 10% for the Dox-PLGA LPNs. While not incredibly large differences, these differences could indicate that this experiment is showing the same trend as the cytotoxicity data previously presented, where the Dox-PLGA LPNs showed a higher cytotoxicity when used immediately compared to the Dox LPNs. The two most divergent values at 600µM doxorubicin concentration were the ANG-modified Dox-PLGA LPNs and ANG-modified Dox LPNs. With no change in ANG usage in these two formulations, the difference in cytotoxicity stems solely from the conjugated vs. unconjugated state of doxorubicin. The A:Dox-PLGA LPNs were more cytotoxic than the A:Dox LPNs (p < 0.05), which could be due to doxorubicin release from the A:Dox LPNs on the apical chamber of the transwell, before the LPNs moved to the basolateral chamber where the MDA-MB-231 cells were located.

The absolute cytotoxicities of all four doxorubicin-loaded formulations never reached lower than 40% cell viability. As the previous cytotoxicity data presented showed nearly 100% cell death at just over 30µM and this experiment reaches 600µM, these numbers do not seem to make sense given the LPN formulations appear essentially identical in terms of potential cytotoxicity. However, the experimental design accounts for this discrepancy. The doxorubicin concentration numbers are created for the apical chamber of the transwell, which has a working volume of 0.5mL. The basolateral chamber, however, has a working volume of 1.5mL. Even assuming every LPN in the 0.5mL LPN sample crossed the transwell membrane, the doxorubicin concentration
actually exposed to the cells in the basolateral chamber is now diluted by a factor of 3. In addition to this dilution factor, it is much more likely that a small fraction of the LPNs added to the apical chamber will translocate to the basolateral chamber as opposed to the theoretical 100%. In fact, an unpublished observation in the author’s laboratory is that even ANG-modified LPNs typically show only a 1% transcytosis efficiency. Assuming that number is accurate, taking 1% of the already-3x-diluted doxorubicin concentrations puts the IC$_{50}$ values of the LPN formulations right in line with what was observed and reported earlier in this dissertation. The IC$_{50}$ of the A:Dox-PLGA LPN formulation shown is approximately 500µM; taking 3% of that is about 167µM and 1% of that is 1.67µM, which is comparable to the 1.95µM value previously shown. A final factor that should be considered in this experimental analysis is treatment time. Increasing the treatment time from the 4 hours used for this experiment would likely allow for a greater amount of LPNs to cross the transwell membrane and in vitro BBB, resulting in a higher level of cytotoxicity for the cells in the basolateral chamber. By implementing these changes, it is entirely possible to achieve a higher level of cell death by focusing on improved experimental design, without changing the LPNs formulations.

5.3 Conclusions

The chief conclusions from this aim are that iPSC differentiation into BMEC cells and subsequent plating on transwell membranes creates a viable and realistic in vitro model for the BBB. Treatment of a BMEC BBB with Dox LPNs resulted in deterioration of the integrity of the BBB at a lower concentration of doxorubicin compared to
treatment with Dox-PLGA LPNs. This observation is consistent with the release data previously presented that shows a faster release of doxorubicin from passively loaded LPNs. It is also consistent with the hypothesis presented at the beginning of this chapter that posited use of the Dox-PLGA conjugate in LPNs would have a protective effect on the BBB when compared to unconjugated Dox LPNs. In addition, this chapter explored the use of an angiopep ligand in the LPN shell to expedite transcytosis across a murine-model BBB. While the impact of the angiopep modification was inconsistent, possibly due to the low TEER values of the BBB, cytotoxicity of the angiopep-modified Dox-PLGA LPNs was on par with those previously presented and significantly higher than angiopep-modified Dox LPNs. This result again shows the impact of incorporating doxorubicin via a drug-polymer conjugate compared to passively loaded drug.
6. Conclusions & Future Directions

The work presented in this dissertation focuses on the impact of using a doxorubicin-polymer conjugate in a lipid polymer hybrid nanoparticle, specifically with the intention of using this NP for delivery to tumors located across the blood-brain barrier. Though nanoparticle drug delivery of chemotherapeutics is fairly commonplace in the research community, there is a dearth of data regarding the potential for drug-polymer conjugates’ use in NPs to reach a brain tumor while maintaining an intact BBB. The primary aim of this work was to highlight the advantages of using a drug-polymer conjugate in creating a NP with a sustained release profile that remained favorably cytotoxic in an in vitro model of the BBB in order to take steps toward elucidating the pathway toward more frequent use of drug-polymer conjugates in NP drug delivery to the brain.

In the first aim of this dissertation, the Dox-PLGA conjugate was synthesized and characterized, as was the LPN itself. The PLGA LPNs proved to be stable in size for at least 7 days and the conjugate had a doxorubicin content of 7.9%. The Dox-PLGA conjugate was incorporated into the LPN with an encapsulation efficiency of 46.9% and the resultant cytotoxicity was confirmed with an IC\textsubscript{50} of 1.95\textmu M. Each of these steps were required to establish the foundation for the LPN delivery system so that further exploration could be done as to more details characterization and specific applications in the remainder of this work.

The second aim examined the doxorubicin release kinetics and cytotoxicity
implications of the Dox-PLGA LPNs compared to LPNs with passively loaded doxorubicin. The hypothesis for these experiments was that the Dox-PLGA LPNs will exhibit a more sustained release profile than the passively loaded LPNs and that sustained release profile will result in the Dox-PLGA LPNs retaining their cytotoxicity for a longer period of time when compared to the passively loaded LPNs. The addition of unconjugated doxorubicin had a significant impact on the drug’s release kinetics; the drug eluted much more quickly when it was passively loaded compared to the slower, more sustained release profile of the Dox-PLGA LPNs. The impact of the Dox-PLGA conjugation in cytotoxicity retention was established by dialyzing freshly-made Dox-PLGA LPNs and Dox LPNs. The Dox-PLGA LPNs preserved their cytotoxicity much more effectively than the Dox LPNs, which is consistent with the hypothesis of this aim.

The final aim of this dissertation focused on application of the Dox-PLGA LPN to an in vitro model of the BBB, the hypothesis for which posited that the sustained release profile of the conjugate-loaded LPNs will have a protective effect on the BBB. The hypothesis additionally claimed that incorporation of the targeting moiety angiopep will facilitate and expedite transcytosis across the BBB. The protective effect due to the use of the Dox-PLGA conjugate was demonstrated using iPSC cells differentiated to human brain microvascular endothelial cells and plated on transwells. The Dox-PLGA LPNs were able to reach a higher drug concentration before causing damage to the BBB layer compared to Dox LPNs, which is consistent with the first hypothesis in this aim. Angiopep incorporation intended to enhance transcytosis was inconsistent in its effects due to the poor integrity of the murine BBB model. In addition to using the BMEC BBB
model, optimizing the molar percentage of angiopep present in the shell of the LPN will need to be performed in order to more accurately exploit its transcytosis capacity. Cytotoxicity of plated breast cancer cells located across the transwell BBB was seen, with the Dox-PLGA LPNs showing higher cytotoxicity than the Dox LPNs. Optimization of this experimental design was discussed to increase the cytotoxic potential of the LPNs after transcytosis.

This overall hypothesis of this work was that a Dox-PLGA conjugate-based LPN is more effective at controlling drug release, maintaining cytotoxicity, and preventing BBB damage than a NP with passively loaded doxorubicin. It is the belief of this author that this hypothesis was supported by the data presented in this dissertation. However, there is much work yet to be done to fully understand, develop, and optimize this drug delivery system. As an extension of the work presented in aim 2, the drug release kinetics experiment should be repeated with the inclusion of serum proteins in the dialysis solution to more accurately simulate an in vivo environment. This change will allow for a more thorough understanding and better predictive model of the role and potential of a conjugate-loaded LPN in a clinical setting.

There are myriad future experiments to be done regarding the application of the Dox-PLGA LPNs for use in crossing the BBB and treating tumors post-transcytosis. As mentioned, optimization of the angiopep content will create a better platform for its incorporation. Differentiation of iPSCs to human BMEC cells should continue to be performed for BBB experiments, as they produce a much stronger BBB and more accurate model when compared to the animal equivalents. Experimental design will need
to be altered as discussed for the transwell experiments in order to gain a better understanding of the cytotoxic potential of the LPNs after transcytosis across the BBB.

On a more far-reaching scale of future applications for the work previously presented, options are extensive. While the work in this dissertation focused on a doxorubicin-PLGA conjugate, the intention of this work was to introduce a chemotherapy delivery system platform that can be altered to the specifications of each application. Many other drug-polymer conjugates can be used in lieu of doxorubicin-PLGA; indeed, multiple chemotherapeutics could be encapsulated in the same LPN either through use of a second drug-polymer conjugate or through intentional passive loading to induce fast release of the second component. Regarding the lipid shell, many targeting moieties can be incorporated depending on the tumor target and its receptor expression. pH-sensitive lipids incorporated into the LPN shell could further control drug release in an environment-dependent manner.

While the benefits of further developing the LPN drug delivery system are compelling, the limitations and requirements of this design need to be acknowledged. In order to create an effective drug-polymer conjugate, the drug’s chemical structure must allow room for polymer conjugation without interfering with the drug’s mechanism of action. Otherwise, the cytotoxicity is limited by the time it takes for the bond connecting the drug to the polymer to degrade, creating a decidedly suboptimal system. A second limitation of the LPN system is the need for use of a solvent that easily evaporates from aqueous solution. The drug-polymer conjugate must be soluble in an organic solvent that can be pulled out of an aqueous solution either through rotary evaporation or stirring at
room temperature. One example of an attempted system that was limited by this factor was the synthesis and subsequent struggle to encapsulate a cisplatin-polylactide conjugate in an LPN. While the conjugate was successfully synthesized, it was only soluble in dimethyl sulfoxide (DMSO). DMSO’s vapor pressure is such that it is incredibly difficult to remove from an aqueous solution, and is typically only done so by extended dialysis. Dialysis was attempted; however, dialysis either incompletely removed the DMSO from the suspension or somehow interfered with the self-assembly of the LPNs as the LPNs never fully formed. DLS of the resultant LPN suspension showed NPs that ranged from under 100nm to several microns in size, with polydispersity values just over 0.5. This experiment demonstrated the significance of the organic solvent evaporation step in the proper preparation of the LPNs as well as the need for future drug-polymer conjugates to be soluble in an organic solvent that can be easily removed from the aqueous solution.

As is evidenced, there are numerous variations on the LPN that could be used for different clinical applications. Those variations could eventually be exploited through customization of the LPNs in the field of personalized medicine. With a future library of chemotherapy-polymer conjugates to be incorporated in an LPN treatment, a patient’s cancer treatment can contain whichever combination of LPNs necessary to deliver the chemotherapy cocktail of choice. The ability to change the pharmacokinetics of the chemotherapy drugs to drive a better clinical outcome is the ultimate goal of LPN drug delivery. The LPN’s capacity for long-term drug release would be a tremendous asset in clinical chemotherapy administration. The use of a conjugate causes longer drug
retention within the LPN, making dosing less frequent, with higher doses able to be used to more effectively treat the tumor while minimizing off-target toxicities. From a design standpoint, it is the modularity of the LPN that makes it such an exciting platform for drug delivery. While not as well-studied as liposomes or polymeric nanoparticles, the lipid-polymer hybrid nanoparticle has remarkable potential for the future of drug delivery.
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