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Investigating Novel Block Copolypeptide Vesicles for the Enhanced Delivery of Therapeutics

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Author
Choe, Uh-Joo

Publication Date
2013

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Investigating Novel Block Copolypeptide Vesicles
for the Enhanced Delivery of Therapeutics

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Biomedical Engineering

by

Uh-Joo Choe

2013
ABSTRACT OF THE DISSERTATION

Investigating Novel Block Copolypeptide Vesicles for the Enhanced Delivery of Therapeutics

by

Uh-Joo Choe

Doctor of Philosophy in Biomedical Engineering

University of California, Los Angeles, 2013

Professor Daniel T. Kamei, Chair

Delivery of naked drugs in the body faces many challenges, such as poor solubility in blood and interstitial fluids, enzymatic and proteolytic degradation in the liver, clearance by the kidneys, recognition by the immune system, inability to cross the cell membrane, and nonspecific toxicity to normal cells. Over the years, drug encapsulation and delivery through nanocarriers have been sought as a potential approach to overcome many of these obstacles. Traditionally, research in this area has been dominated by vesicles assembled from lipids. These lipid based vesicles, also known as liposomes, are currently the most widely used vesicle for drug delivery with commercial formulations (DOXIL® and Myocet™) in the market. However, liposomes are intrinsically unstable in vivo unless they are coated with polymers such as
polyethylene glycol (PEG), which decreases their clearance from the body. Even with the addition of PEG, there are limitations as the added PEG chain increases the hydrophilic head group of the lipids, increasing their tendency to form micelles instead of bilayers. Due to these limitations, researchers have investigated alternative systems, such as polymer-based vesicles or polymersomes. The disproportionality that exists between the hydrophilic PEG head group and the hydrophobic tail of a lipid can be overcome by synthesizing synthetic PEG amphiphilic polymers with longer hydrophobic segments. In addition, by synthesizing building blocks that are larger than lipids, membrane stability can be improved along with slower leakage of drugs. However, some polymersomes suffer from lacking the ability to incorporate biofunctionality without impairing their ability to self-assemble into vesicles.

In order to contribute to this exciting drug delivery field and find a material that could potentially improve upon current lipids and synthetic polymers, our lab investigated the use of novel amino acid-based polymers as the building blocks for forming vesicles. The use of amino acids offers many advantages. They have an intrinsic biocompatibility, which implies great potential for low immunogenicity and toxicity. The variety of amino acids and their many possible sequence combinations can be used to custom design polypeptides with different chemical properties and biofunctionalities. Moreover, recent advances in polymerization techniques allow the synthesis of long chains of monodisperse polypeptides. Vesicles formed from polypeptides can offer increased stability over conventional liposomes by providing thicker membranes that contribute to increased attractive interactions between the building blocks. We have investigated vesicles comprised of polypeptides that have been synthesized by Dr. Timothy Deming’s group (UCLA), which employs a transition metal-mediated α-amino acid N-carboxyanhydride (NCA) polymerization technique to synthesize the amphiphilic block
copolypeptides. The vesicles formed from these polypeptides can be prepared in different sizes in bulk quantities.

This thesis focused on investigating the potential for using these polypeptide vesicles as drug delivery vehicles. Initially, vesicles comprised of positively charged polypeptides were studied, specifically the lysine-leucine (K₆₀L₂₀) and arginine-leucine (R₆₀L₂₀) block copolypeptides. Due to their positive charge, these vesicles have a disadvantage of being toxic to cells through interactions with the net-negatively charged plasma membranes. Therefore, in order to identify a design criterion for producing positively charged vesicles with low cytotoxicity, we focused on optimizing the hydrophilic/hydrophobic ratio of the polypeptide by varying the length of the hydrophobic block while maintaining a constant length for the hydrophilic block. The K₆₀L₂₀ polypeptides were used for this study due to their relative ease of preparation compared to the R₆₀L₂₀ polypeptides. It was found that varying the hydrophilic/hydrophobic ratio in the lysine-leucine block copolypeptide affects its ability to form vesicles, where polypeptides with long hydrophobic segments formed less toxic vesicles with appropriate sizes for drug delivery. Among the copolypeptides investigated, the K₆₀L₂₀ copolypeptide composition showed the most potential for drug delivery applications, as this copolypeptide was able to form monodisperse nanoscale vesicles with the least amount of micelles and small aggregates that were more toxic than the vesicles themselves.

The optimized block copolypeptide composition was then applied to the arginine-leucine block copolypeptide, and the ability of these arginine-based vesicles to transfect mammalian cells was systematically investigated. The arginine-leucine R₆₀L₂₀ block copolypeptide was studied, since the arginine residues had previously been shown to enhance the delivery of cargo into cells. Plasmid DNA was used as our model therapeutic, since only low concentrations of
plasmid DNA are required for an effect. This was an important point, since high concentrations of $R_{60}L_{20}$ vesicles will lead to significant cytotoxicity. Our transfection results with our $R_{60}L_{20}$ vesicles demonstrated that there is potential for using this novel material as a transfection agent, as they were able to achieve transfection with low cytotoxicity and immunogenicity.

In contrast to plasmid DNA, high concentrations of the vesicles are required when delivering small molecule chemotherapeutics due to their IC$_{50}$ values being in the micromolar range. Since the $R_{60}L_{20}$ vesicles themselves become cytotoxic at high concentrations, we also investigated the negatively charged vesicle formed from the glutamate-leucine ($E_{60}L_{20}$) block copolypeptide. A main advantage of these vesicles, over the positively charged vesicles, is that they are less toxic to cells. However, this is also a disadvantage, since the electrostatic repulsive interactions between the vesicles and the net-negatively charged cell membranes can inhibit the vesicles from entering cells. We were able to overcome this limitation by conjugating the vesicle surface with transferrin (Tf), a ligand that has been commonly used for targeting many types of cancer cells. We investigated the intracellular trafficking behavior of these Tf-conjugated EL vesicles, and they were shown to recycle back to the cell surface once they were internalized. This behavior was similar to what has been observed for other nanoparticle systems conjugated with Tf.

Even with this recycling behavior, other Tf-conjugated nanoparticle systems were found to exhibit improved drug delivery efficacy. Accordingly, we investigated the ability of our Tf-$E_{60}L_{20}$ vesicles to deliver the chemotherapeutic doxorubicin (DOX). Before performing the *in vitro* efficacy study, the vesicle surface was conjugated with poly(ethylene glycol) (PEG) to improve its stability for future *in vivo* applications. The targeting Tf ligand was then conjugated to the resulting vesicle, and DOX was then encapsulated in the vesicle interior. A previously
developed mathematical model was applied to our system to predict trends that helped guide experiments and allowed for reduced experimentation and faster optimization. Once the carrier was complete, *in vitro* cytotoxicity studies were performed to demonstrate proof-of-concept that the newly developed Tf-conjugated vesicles exhibit a significant improvement in drug delivery efficacy over the non-targeted version.
The dissertation of Uh-Joo Choe is approved.

Timothy Deming

Kathleen Kelly

Min Lee

Daniel Kamei, Committee Chair

University of California, Los Angeles

2013
This work is dedicated to my wife, Minnie, my heart and soul,

and to God, my heavenly father
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ACKNOWLEDGMENTS

I first and foremost thank God for the opportunity he has provided and the people with whom he has surrounded me. Never in my wildest dream did I envision myself completing a degree that is worthy of so much discipline, dedication, passion, and perseverance. I can happily say that my journey would nowhere be complete without the support and help from many people. My sincerest gratitude and thanks goes to my principle investigator, Dr. Daniel T. Kamei. Throughout my five-plus years in his lab, Dan has been more than just a mentor and an advisor to me. On any given day, Dan was a friend, big brother, father, counselor, someone whom I could trust, and someone to whom I could go to when I was down. It is not just the lessons that Dan preached everyday to become a good scientist and a good person that inspired me. It is not just his consistency to follow his own principles, even under difficult circumstances, that moved me. It is all of the above and his unconditional love to support his lab members without demanding anything in return that I find so rare in today’s society. Although Dan would be the first to acknowledge that he doesn’t follow the “Christian” way in his daily life, the irony is that I find him to be one of the most Jesus-like people I have ever seen.

This journey to obtaining a Ph.D. degree would not have been as rewarding and meaningful without the help and support from my friends in the laboratory. I have to first give thanks to Dr. Victor Sun, who was my mentor in my first year. For better or worse, my experimental techniques were shaped by Victor, and truthfully, I could not have asked for a better example than Victor, who was patient, care-free, and clumsy in real life, but extremely precise during an experiment and strict, as well as demanding, with me whenever necessary. Victor set the foundation for the vesicle project, which I inherited and pushed to where it is now. I have to also thank Kristine Mayle for her dedication to the project and her willingness to be a
team player and learn. Her passion towards research and kind heart towards people will serve her well as the successor of the vesicle project, and I am certain that the project is in good hands. I have also received help from several undergraduate students. Sergey Boyarskiy not only helped me with the initial vesicle processing and toxicity assays, but always welcomed me with his warm heart and wit. Howard Dai helped me with the transfection experiments, and I have always been thankful for his unending support for the lab. Kevin Tan was instrumental in developing the drug-loading protocol for our vesicles, and always a good person to talk to about shopping for designer clothes and accessories for my wife. Brian Lee helped me develop the targeted vesicles and establish the early protocols for the cytotoxicity assays; while Allison Yip, my last undergraduate student, helped me finalize the study. To work with all these people was my pleasure and honor. The rest of the Kamei group, Foad Mashayekhi, Dennis Yoon, Ricky Chiu, Tracy Ying, Garret Mosley, and David Pereira, and the rest of the Kamei undergrads have made every moment in graduate school much more gratifying. This “Kamei-zing” lab has truly been a family to my wife and me in the United States, and every single member, from past to present, has impacted my life and experiences in more ways than I could ever explain. I have experienced the most ups and downs in life during the last six years, and I am truly grateful to have shared every single moment with them, even the bad ones.

The vesicle project has been a collaborative effort with the Deming lab who synthesizes the polypeptides. I truly thank Dr. Timothy Deming for his guidance throughout all the meetings and his jokes that he would make, although at my expense. I would like to express my appreciation to April Rodriguez who grew and struggled with me from the beginning. I cannot thank her enough for cranking out the polymers, and I would like to say to her that we did it!
Finally, I would like to thank my family, whose love and prayers have always supported and lifted me. I thank my wife, Hyunsil Lee, also known as Minnie, for supporting me throughout the years, and for having to endure all the hardships associated with being a wife of a student. Not only does her confidence and belief in me give me the strength and courage to face this world, but her love and charm gives me the reason to live and hope for a bright future. My father, who is a professor at Seoul National University, not only inspired me with his work ethic, but his honest principles became the cornerstone of my values and shaped the way I view and love this world. My mom, a 9-year breast cancer survivor, is the most courageous woman I have ever met, whose faith in God and me has always kept me strong against fear.

Chapter 1 is a version of: U.-J. Choe, V. Z. Sun, J.-K. Y. Tan, and D. T. Kamei, Self-Assembled Copolypeptide and Polypeptide Hybrid Vesicles: From Synthesis to Application. Top Curr Chem, 2012, 310, 117-134, Springer Copyright © 2012. Reprinted with permission of Springer. D. T. Kamei was the director of research for this article. This work was supported by the Department of Defense Prostate Cancer Research Program under award number W81XWH-09-1-0584, the National Science Foundation DMR 0907453, and the UCLA Specialized Program of Research Excellence in Prostate Cancer P50 CA092131-08.

Chapter 2 is a version of: U.-J. Choe, A. R. Rodriguez, Z. Li, S. Boyarskiy, T. J. Deming, and D. T. Kamei, Characterization and Minimization of Block Copolypeptide Vesicle Cytotoxicity using Different Hydrophobic Chain Lengths, Macromol Chem Physic, 2012, 10.1002/macp.201200591, Wiley Periodicals, Inc. Copyright © 2012 Wiley Periodicals, Inc. Reprinted with permission of John Wiley & Sons, Inc. D. T. Kamei was the director of research for this article. This work was supported by the Department of Defense Prostate Cancer Research Program under award number W81XWH-09-1-0584, the National Science Foundation DMR 0907453,
and the UCLA Specialized Program of Research Excellence in Prostate Cancer P50 CA092131-08.

Chapter 3 is a version of: V. Z. Sun, U.-J. Choe, A. R. Rodriguez, H. Dai, T. J. Deming, and D. T. Kamei, *Transfection of Mammalian Cells using Block Copolyptide Vesicles*, Macromol Biosci, 2012, 10.1002/mabi.201200383, Wiley Periodicals, Inc. Copyright © 2012 Wiley Periodicals, Inc. Reprinted with permission of John Wiley & Sons, Inc. D. T. Kamei was the director of research for this article. This work was supported by the Department of Defense Prostate Cancer Research Program under award number W81XWH-09-1-0584 and the National Science Foundation DMR 0907453. We also acknowledge the use of the SPM facility at the Nano and Pico Characterization Lab at the California NanoSystems Institute.

Chapter 4 is a recently submitted version of: U.-J. Choe, A. R. Rodriguez, B. S. Lee, S. Knowles, A. M. Wu, T. J. Deming, and D. T. Kamei, *Endocytosis and Intracellular Trafficking Properties of Transferrin-Conjugated Block Copolyptide Vesicles*, Biomacromolecules, 2013. D. T. Kamei was the director of research for this article. This work was supported by the Department of Defense Prostate Cancer Research Program under award number W81XWH-09-1-0584 and the National Science Foundation DMR 0907453. We also acknowledge the use of the SPM facility at the Nano and Pico Characterization Lab at the California NanoSystems Institute.

Lastly, the work in Chapter 5 was funded by the Department of Defense Prostate Cancer Research Program under award number W81XWH-09-1-0584.
VITA

2004  B.S., Chemical Engineering
University of California, Los Angeles
Los Angeles, California

2012  M.S., Biomedical Engineering
University of California, Los Angeles
Los Angeles, California

2008-2013  Teaching Assistant
Department of Bioengineering
University of California
Los Angeles, California

PUBLICATIONS


1. Motivation and Background

1.1. Introduction

Vesicles composed of synthetic lipids (liposomes) and polymers (polymersomes) have been quite successful in delivering drugs both in vitro and in vivo. These nanoscale drug delivery vehicles have improved current therapeutics by providing benefits, such as protection from degradation inside the body, increased circulation time, targeted delivery to decrease toxic side effects, and controlled release of drugs to widen the therapeutic window. Liposomes have been extensively studied for their ability to deliver DNA into cells, while two formulations of doxorubicin (DOX) encapsulated in liposomes have been approved for clinical use. DOXIL®️, an FDA approved DOX formulation encapsulated in pegylated liposomes, is currently being used in the treatments of ovarian cancer and Kaposi’s sarcoma. Myocet™️, a non-pegylated liposomal DOX, is approved in Europe and Canada for the treatment of metastatic breast cancer, in combination with cyclophosphamide. Polymersomes, while still in the research phase, offer certain advantages over liposomes, such as greater chemical diversity with regard to composition and the potential to be more stable and highly functionalized. For example, Discher and coworkers were able to encapsulate both hydrophilic and hydrophobic drugs inside their polymersomes and show enhanced therapeutic efficacy in vivo over the drugs in free form. Although delivery of drugs with liposomes and polymersomes have met with some success, there is still room for improvement in the field of drug delivery, especially since many therapeutics, such as small interfering RNA (siRNA), still lack a suitable delivery option.
In regards to drug delivery, the investigation of self-assembled vesicles comprised of amino acids is relatively new, but has been gaining popularity. Since the amino acid building blocks are naturally occurring, these vesicles have the potential to be non-toxic and non-immunogenic. The availability of various natural and synthetic residues also suggests that these materials can be tailored to exhibit a wide variety of chemical properties. In addition, recent advances in polymerization techniques have led to precise control over the length of the polypeptides, leading to more customization and uniformity. Most importantly, polypeptides have an inherent ability to adopt stable secondary structures, which allows them to self-assemble into precisely defined structures, such as vesicles. We are using the term “polypeptide” in this review to refer to polymers consisting of 20 or more amino acids, which is consistent with definitions found in the literature.\textsuperscript{6-8} In addition, the term “polypeptide hybrid” is being used in this review to refer to a macromolecule comprised of a polypeptide and another type of polymer, where the polypeptide portion plays a role in the formation of the vesicular structures. Accordingly, liposomes or polymersomes conjugated to an oligopeptide (less than 20 amino acids) are not included in this review. Moreover, although the self-assembly of peptides into vesicles was first reported in 1996 with helical oligopeptides\textsuperscript{9} and much work has been performed with oligopeptide-based vesicles,\textsuperscript{9-16} this review will not include vesicles formed from oligopeptides.

Historically, after the development of oligopeptide-based vesicles, several groups developed and characterized vesicles using polypeptide hybrid systems consisting of polypeptide and synthetic polymer blocks.\textsuperscript{17-19} Soon thereafter, vesicles formed entirely from polypeptides, such as \textit{poly(\textit{l}-lysine)}-\textit{b}-\textit{poly(\textit{l}-leucine)} and \textit{poly(\textit{l}-lysine)}-\textit{b}-\textit{poly(\textit{l}-glutamate)}, were developed.\textsuperscript{20, 21} This review will focus on the recent developments in the formation of vesicles
composed of polypeptide hybrid or polypeptide systems, as well as the potential promise of these systems as effective drug delivery vehicles. A specific example of a polypeptide-based vesicle is shown in Figure 1.1, where the hydrophobic segment is α-helical while the hydrophilic segment is a random coil.

![Figure 1.1. Schematic of a vesicle construct formed from poly(L-lysine)-b-poly(L-leucine) polypeptides, where the poly(L-leucine) block corresponds to the α-helical hydrophobic segments and the poly(L-lysine) block corresponds to the random coil hydrophilic segments. Note that this is one specific example and not all vesicle constructs have α-helical and random coil blocks. Moreover, the amphiphilic copolymer can be comprised of either a pure block copolypeptide or a macromolecule consisting of a polypeptide and another type of polymer. Adapted with permission from 20. Copyright 2010 American Chemical Society](image)

**1.2. Synthesis of Polypeptide Materials**

The preparation of polypeptide and polypeptide hybrid vesicles with predictable properties begins with proper synthesis of a primary structure. This section focuses on three different classes of synthesis procedures that are used to prepare polypeptides. Although
conjugation between the polypeptide and non-polypeptide blocks to form polypeptide hybrids is discussed briefly in the third class of the synthesis procedures, more detailed information regarding the synthesis and generation of polypeptide hybrid macromolecules are reviewed elsewhere.22-26

1.2.1. Solid-Phase Peptide Synthesis
Solid-phase peptide synthesis (SPPS) has been a useful method for preparing oligopeptides and polypeptides up to approximately 50 amino acid residues in length.26-28 In this method, an amino acid is covalently linked to a resin (solid bead), and onto the amino acid, a peptide chain is assembled. Once the peptide is synthesized, the peptide chain is cleaved from the resin by a mixture of reagents, one reagent being trifluoroacetic acid (TFA). The main principle behind this process is repeated steps of coupling and deprotection for every amino acid addition. Briefly, a resin-anchored amino acid with a free amine group is coupled to a single amine-protected amino acid, producing a single dipeptide. The protected amine of the new dipeptide is then deprotected, revealing a new free amine, upon which a new amino acid can be added for coupling, and this process continues. Because each amino acid can be added one at a time, primary sequences of oligopeptides and polypeptides can be prepared with precise control. Even though a high yield (~99%) is associated with each amino acid addition step of SPPS, the final yield of the entire polypeptide can become quite low for large amino acid chain lengths (~60% for 50 amino acids compared to ~37% for 100 amino acids), since there will be a certain percentage of chains acquiring a defect at each step.26 Due to this exponential increase in defects with increasing chain length, the concentration of polypeptides containing defects will increase
as the number of amino acids increases.\textsuperscript{29, 30} Thus, the application of SPPS has been limited to synthesizing long chains of block copolypeptides of \textasciitilde 50 amino acids.\textsuperscript{31}

Over the past 15 years, selective chemical or enzymatic ligation methods have been developed to address this limitation and combine polypeptides formed by SPPS. One method is the native chemical ligation (NCL) method, where an unprotected synthetic polypeptide segment with a C-terminal \( \alpha \)-thioester is reacted in a chemoselective manner with another unprotected polypeptide segment containing an N-terminal cysteine residue.\textsuperscript{32} Because the ligation technique requires a cysteine group at the N-terminus, polypeptides lacking this amino acid at this position will first need to add a cysteine. This addition, however, may end up disrupting the polypeptide construct. Other methods, which improve upon the drawbacks of NCL, are enzymatic ligation techniques, one of them being the subtiligase catalyzed fragment condensation (SCFC) method. Unlike NCL, SCFC only requires a free N-terminal amino group, which removes the need for an N-terminal cysteine residue.\textsuperscript{33} In this case, the enzyme subtiligase is used to catalyze the condensation reaction selectively between an activated polypeptide ester segment and a second polypeptide segment with a free N-terminal amino group, forming the ligation. Thus, polypeptide blocks of approximately 50 amino acids, formed by SPPS, can be ligated by NCL or SCFC to generate even longer polypeptide chains with higher overall yields.\textsuperscript{31}

\textbf{1.2.2. Protein Engineering}

Recombinant DNA technology can also be used to design genes that encode for proteins with desired features.\textsuperscript{34} The gene can be incorporated into a plasmid, which then gets transformed into a bacterial host, such as Escherichia coli (\textit{E. coli}). Finally, the production of the
desired amino acid polymer is performed by the host with a precisely defined sequence and near absolute monodispersity.\textsuperscript{29, 35}

An early challenge with protein engineering was the limitation of the polypeptide to the 20 naturally occurring amino acids, making it difficult to incorporate synthetic functional groups, such as alkenes and alkynes.\textsuperscript{36, 37} However, new molecular biology techniques now allow the incorporation of non-natural amino acids. A thorough review on this topic of incorporating non-natural amino acids in protein engineering can be found in another review by Beatty and Tirrell.\textsuperscript{38} Although protein engineering has primarily been used to synthesize oligopeptides in the field of peptide-forming vesicles,\textsuperscript{12-14} this approach may be used in the future to synthesize polypeptides.

\subsection*{1.2.3. NCA Polymerization}

The most general and frequently used method to synthesize long chains of block copolypeptides for vesicle assembly is successive ring opening polymerizations (ROPs) of \(\alpha\)-amino acid-\(N\)-carboxyanhydride (NCA) monomers.\textsuperscript{18, 20, 21, 39-51} NCA monomers are readily prepared from commercially available amino acids, most commonly through direct phosgenation.\textsuperscript{52}

The first step of NCA polymerization is usually accomplished by the use of nucleophilic initiators. These initiators can be alkoxides, alcohols, amines, transition metals, and even water.\textsuperscript{53, 54} In order to synthesize a copolymer diblock, the polymerization of the second block and its connection to the previously formed block are performed in a single process. This is achieved by initiating the polymerization of the second NCA monomer using the first
homopolypeptide as a macroinitiator. Precipitation and purification processes follow to isolate the block copolypeptides. To synthesize polypeptide hybrids where synthetic polymers are combined with homopolypeptide sequences, typically the synthetic polymer block is first synthesized using controlled polymerization methods, followed by NCA polymerization using the synthetic polymer block as a macroinitiator.18,55,56

Commonly, NCA monomers with ionic functional side chains are initially protected to avoid side reactions. Once polymerization is complete, a deprotection process is used to free these functional groups, such as amine groups from lysine and carboxylic groups from glutamate or aspartate. Other protecting agents exist, but amine groups are typically protected with benzyloxycarbonyl groups and later removed by 33% hydrobromic acid in acetic acid. In contrast, carboxylic groups are generally protected by benzyl groups and removed by potassium hydroxide.57

Primary amines, which are more nucleophilic than basic, are one of the most commonly used initiators for the polymerization of NCA monomers. The n-hexylamine molecule is a popular choice among several research groups to initiate polymerization of the first block of the block copolypeptides.21,41,43 Once the first block is synthesized, the primary amine in the N-terminus of this block is used to initiate the synthesis of a new block. Lecommandoux’s research group has used this initiator to synthesize block copolypeptides of poly(L-glutamic acid)-b-poly(L-lysine) that formed pH responsive vesicles. Jing and coworkers also used this approach to synthesize poly(L-lysine)-b-poly(L-phenylalanine) polypeptides which also formed vesicles. Kono and coworkers used the primary amine located at the focal point of a polyamidoamine (PAMAM) dendron to initiate the ring opening polymerization of ε-benzyloxycarbonyl-L-lysine NCA monomers.49 The resultant PAMAM dendron-poly(L-lysine) block copolymer formed
monodisperse vesicles. In addition, Forster’s group used amine functionalized polybutadiene to initiate the synthesis of the polypeptide portion of the polybutadiene-\textit{b}-poly(l-glutamate) polymer, which was also found to self-assemble into stable vesicles.\textsuperscript{18}

Deming and coworkers used transition metal complex initiators to synthesize a series of poly(l-lysine)-\textit{b}-poly(l-leucine) block copolypeptides, of which the poly(l-lysine)\textsubscript{60}-\textit{b}-poly(l-leucine)\textsubscript{20} polymer was found to form stable vesicles.\textsuperscript{20} Jan’s group also employed this method to synthesize the poly(l-lysine)\textsubscript{200}-\textit{b}-poly(l-glycine)\textsubscript{50} block copolypeptide, which also formed vesicles.\textsuperscript{48} The sizes of these poly(l-lysine)\textsubscript{200}-\textit{b}-poly(l-glycine)\textsubscript{50} vesicles, irrespective of the vesicle processing method, could be controlled by cycling the pH of the external solution between 7 and 11. Although transition metals can be toxic, cytotoxicity results have demonstrated that polypeptides synthesized with this approach can be purified to remove trace metal ions and are reasonably non-cytotoxic after extensive dialysis.\textsuperscript{44}

Recently, Schlaad and coworkers used amine hydrochloride salts as initiators for NCA polymerization.\textsuperscript{58} Huang and Chang also used this method to synthesize the polypeptide segment of the block copolymer, poly(N-isopropylacrylamide)-\textit{b}-polylysine that could self-assemble into vesicles.\textsuperscript{59} The Hadjichristidis group employed the use of a high vacuum technique (HVT) to allow the use of amine initiators for the living polymerization.\textsuperscript{34} Schue’s lab was also able to significantly reduce the side reactions of the NCA polymerization to only 1\% by lowering the reaction temperature to 0\textdegree C.\textsuperscript{60} Moreover, Lee and coworkers demonstrated that, by adding hydrochloric acid to a primary amine initiator, the resulting initiator was able to begin the living polymerization of poly(1-glutamic acid)-\textit{b}-poly(1-phenylalanine). This block copolypeptide was found to self-assemble into pH responsive vesicles.\textsuperscript{61} In addition, Cheng and Lu recently reported a controlled NCA polymerization using \textit{N}-trimethylsilyl (TMS) amines.\textsuperscript{62, 63}
The researchers demonstrated that this polymerization could be performed with a variety of N-TMS amines containing different functional groups, which could be used to further modify the polypeptide.

A unique copolypeptide was also synthesized by Kros and coworkers, where they combined a block of poly(γ-benzyl L-glutamate) (PBLG) with poly(ethylene glycol) (PEG) through a unique complexation. Specifically, the PBLG block, synthesized through NCA polymerization, was ligated with a coiled-coil peptide sequence (called peptide $E$), which itself was synthesized through SPPS. They then conjugated a hydrophilic peptide sequence (called peptide $K$) to PEG. Upon complexing peptide $E$ with the complementary sequence $K$, the PBLG-$E/K$-PEG suspension formed vesicles. These researchers also discovered that the PBLG-$E$ polypeptides could form vesicles on their own if the polypeptides were initially solubilized by detergent micelles in aqueous buffer, followed by dialysis to dilute the detergent concentration. This process transformed the mixed micelles into vesicles in an aqueous solution. A unique graft polypeptide was also synthesized by Tian’s group, where a PBLG block was grafted with PEG to created a PBLG-g-PEG polymer. By tuning the degree of grafting and the composition of the common solvent, these graft polymers were able to form different structures, such as vesicles, spindle-like micelles, and spherical micelles. This work corresponded to the first time a graft copolymer with the polypeptide as the backbone molecule was able to self-assemble into vesicles.

1.3. Vesicle Processing Techniques

Amphipilic polypeptides that are synthesized with appropriate ratios of hydrophilic to hydrophobic blocks can form ordered vesicular shapes. Although many polypeptides can self-
assemble into vesicles when simply dissolved into the correct solvent, others require more processing steps. This section provides an overview of the techniques that have been developed to process various polypeptide and polypeptide hybrid systems into vesicles.

1.3.1. Direct Dissolution

Many polypeptide based materials are able to self-assemble into vesicles when directly dissolved into the appropriate solvent. In fact, this method was used to form some of the earliest polypeptide vesicles in the literature. Lecommandoux and coworkers studied polypeptide and polypeptide hybrid systems that self-assembled into pH responsive vesicles. In their work, they synthesized a series of polypeptide hybrids, including polybutadiene-\(b\)-poly(\(l\)-glutamic acid) and polyisoprene-\(b\)-poly(\(l\)-lysine). When these hybrid macromolecules were directly dissolved in aqueous solution, vesicles were formed that could change size with changes in pH.\(^{45,67,68}\) They also synthesized vesicles composed of poly(\(l\)-glutamic acid)-\(b\)-poly(\(l\)-lysine) which also formed vesicles by direct dissolution and could respond to changes in pH.\(^{21,45,67}\)

Recently, Kataoka and coworkers developed a novel micron-sized vesicle with a membrane comprised of polyion complexes, also called a PICsome, using the direct dissolution method.\(^{56}\) The PICsomes could be formed in aqueous media through self-assembly of oppositely charged block copolymers, where they used PEG-\(b\)-poly(\(\alpha,\beta\)-aspartic acid) (PEG-poly(Asp)) as the anionomer and PEG-\(b\)-poly((5-aminopentyl)-\(\alpha,\beta\)-aspartamide) (PEG-poly(Asp-AP)) as the cationomer. Similar to Lecommandoux’s pH responsive vesicles, these PICsomes could also reversibly respond to changes in pH.\(^{69}\) While the PICsomes maintain their vesicular structures at pH 7.4, they dissociate into small particles at or below pH 5.7. Increasing the pH back to 7.4 or
above restores the vesicular structures, and this transition is reversible. Interestingly, by simply changing the partner of the PEG-poly(Asp) from PEG-poly(Asp-AP) to the homocatiomer poly(Asp-AP), they showed that this new combination allowed nanometer-sized vesicle formation with controllable sizes ranging from 100 to 400 nm (Figure 1.2). Other groups have also adopted direct dissolution to prepare vesicles from polypeptides (diblock and triblock) and polypeptide hybrids.  

**Figure 1.2.** PICsomes formed from oppositely charged building blocks. (a) Chemical structures of the hybrid polypeptides for PICsomes and a schematic representation of the PICsome preparation. (b) Cryo-transmission electron microscopy (TEM) image of 100 nm sized PICsomes (Scale bar = 50 nm). Arrows are placed to highlight vesicle walls. Adapted with permission from 70. Copyright 2010 American Chemical Society

The advantage of the direct dissolution method is its simplicity. Systems that form vesicles under this method are easily dissolved in aqueous solution, unlike many other systems that form irregular aggregates due to strong hydrophobic interactions. Also, since the aqueous environment is relatively mild, this method allows the encapsulation of biologically active materials, such as proteins and DNA, with a low risk of denaturation. However, this method does not work with all block copolypeptide systems. For instance, when block copolypeptides containing poly(l-leucine) blocks are dissolved in water, hydrogels or aggregates of no particular shape were observed. Solvation in water also usually produces smaller vesicles from 50 nm to
200 nm in diameter, although large micron-sized vesicles were reported for poly(l-lysine)-b-poly(l-phenylalanine). Although these vesicles were shown to be stable for up to 2 months, they were found to lose their vesicular structure when relatively low concentrations of sodium chloride (10 mM) were present, making them difficult to be used for in vivo applications.

1.3.2. Dual Solvent Method

Researchers studying polypeptide and polypeptide hybrid systems have also processed vesicles using two solvents. This method usually involves a common organic solvent that solubilizes both blocks and an aqueous solvent that solubilizes only the hydrophilic block. The two solvents can be mixed with the polypeptide or polypeptide hybrid system at the same time or added sequentially. The choice of organic solvent depends heavily upon the properties of the polypeptide material, and commonly used solvents include dimethylformamide (DMF), methanol (MeOH), dimethyl sulfoxide (DMSO), and tetrahydrofuran (THF). Vesicles are usually formed when the organic solvent is slowly replaced with an aqueous solution via dialysis or removed through evaporation; however, some vesicles have been reported to be present in the organic/aqueous mixture.

This dual solvent method is suitable for polypeptides with a block that does not dissolve easily in aqueous solution due to being or having a hydrophobic segment. For instance, poly(l-lysine)$_{60}$-b-poly(l-leucine)$_{20}$ (K$_{60}$L$_{20}$) and poly(l-arginine)$_{60}$-b-poly(l-leucine)$_{20}$ (R$_{60}$L$_{20}$) block copolypeptides synthesized using transition metal-mediated NCA polymerization were previously assembled into vesicular structures with this method. The poly(l-leucine) block in this material is highly difficult to dissolve due to the α-helical hydrophobic region, which only
swells in organic solvents, such as THF and DMF. The organic solvents used in this method, however, can be removed with careful and exhaustive dialysis to minimize and remove their cytotoxic effects. In a recent report, Kros and coworkers were able to dissolve poly(γ-benzyl L-glutamate)_{36}-b-E(EIAALEK)_{3}-NH_{2}, which is a rigid polypeptide, using the surfactant sodium cholate to form micelles. The surfactant was then slowly removed via dialysis to yield vesicles. Since some surfactants are significantly milder than organic solvents, this method represents an approach to directly encapsulate sensitive biomacromolecules.

1.4. Characterization of Vesicle Formation and Biocompatibility

Many techniques used in other fields have been adapted to visualize and characterize vesicles composed of polypeptide and polypeptide hybrid systems. This section will describe these different approaches.

1.4.1. Microscopy

The most direct way of visualizing vesicle formation is through microscopy. Vesicle sizes typically range from 10^2 to 10^4 nm, thus electron microscopy techniques provide the best method for visualization. Both scanning electronic microscopy (SEM) and cryo-transmission electron microscopy (TEM) techniques are frequently used to characterize vesicles with diameters as small as 20 nm. Unfortunately, these techniques do not always allow for the best three-dimensional (3D) characterization. For surface and 3D studies, atomic force microscopy (AFM) has been used. However, Jing and coworkers reported that their poly(l-lysine)_{53}-b-poly(l-phenylalanine)_{12} vesicles collapsed upon AFM sample preparation, citing the
possible escape of water from the vesicle core. Other groups did not report this problem, but in those instances, longer hydrophobic chains were used which may have prevented water from leaving.

Laser scanning confocal microscopy (LSCM) can also be a useful tool in characterizing the vesicle shape and interior, whether by labeling the polypeptides themselves via conjugation of a fluorescent dye or by encapsulating a fluorescent molecule inside of the vesicles. This method is particularly beneficial when a biologically relevant molecule is being delivered, since it can be visualized within live cells for a qualitative measure of uptake efficiency and endocytic trafficking. Although LSCM does not have as high of a resolution as SEM or TEM, the advantage of LSCM is its ability to filter out unfocused signal, thus allowing the distinction between cellular uptake and mere association with the cellular membrane. This is particularly important if the cargo delivered within the vesicles has no way of entering the cell on its own. Specifically, Deming and coworkers used LSCM to visualize the delivery of a hydrophilic cargo, Texas-Red-labeled dextran, by a polypeptide vesicle that has the ability to enter cells (Figure 1.3).
Figure 1.3. Polypeptide vesicle with endocytosis capability. (a) A schematic diagram of vesicles formed from poly(L-arginine)$_{60}$-b-poly(L-leucine)$_{20}$. The poly(L-arginine) block provides an added cell penetrating feature to the vesicles. LCSM images of internalized vesicles (green) containing Texas-Red-labeled dextran (red) in (b) epithelial and (c) endothelial cells. Colocalization of the vesicles and Texas-Red-labeled dextran appears as a yellow fluorescent signal. Adapted by permission from Macmillan Publishers Ltd: [Polyarginine segments in block copolypeptides drive both vesicular assembly and intracellular delivery] 44, Copyright (2007)

1.4.2. Light Scattering, Polarization, and Zeta Potential

Scattering experiments can be performed to help determine the size and shape of the vesicles without the need for extensive sample preparation required for electron microscopy and atomic force microscopy. Dynamic (DLS) and static light scattering (SLS) are widely used to determine the size and possible shape of vesicle systems. 40, 42, 48, 49, 51, 56, 61, 64, 72, 73, 75, 76 DLS and SLS experiments provide the hydrodynamic ($R_H$) and gyroscopic ($R_G$) radii of the vesicles, respectively. A linear angular dependence of the $R_H$ implies a spherical shape, and if the value for the $R_H$ is greater than a few times the predicted polypeptide length, a hollow spherical shape indicative of a vesicle can be assumed. Further confirmation can be obtained by noting the $R_G /
R_H ratio. Hollow spheres will have a ratio close to 1, whereas uniform spheres (micellar like structures) and random coil conformations have ratios close to 0.774 and 1.50, respectively.\textsuperscript{77} Small-angle neutron or X-ray scattering experiments can also be performed to further characterize the vesicles.\textsuperscript{67,72}

Circular dichroism (CD) experiments can provide valuable data pertaining to the conformation of the peptides within the vesicles.\textsuperscript{48, 49, 51, 64, 75, 78} It is common for hydrophobic or uncharged hydrophilic blocks to form \( \alpha \)-helical conformations in aqueous solution, while charged hydrophilic blocks tend to form random coils. CD is especially useful for the study of stimuli-responsive vesicles where a change in polypeptide conformation is caused by the stimulus. Lecommandoux and coworkers, for example, have demonstrated that both blocks of their poly(l-glutamic acid)-\textit{b}-poly(l-lysine) vesicles undergo a change from an \( \alpha \)-helical to a random coil conformation with a change in pH.\textsuperscript{45, 67} CD was also employed to observe pH triggered structural shifts in other diblock and triblock polypeptide and polypeptide hybrid systems.\textsuperscript{40, 42, 47}

Measuring the zeta potential of the polypeptide or polypeptide hybrid vesicle has recently been widely used to characterize the surface charge of the vesicle.\textsuperscript{64} The zeta potential describes the electrostatic potential near the surface of the particle. Higher absolute values of the zeta potential confer better particle stability as the particles will resist aggregation. Particles with zeta potentials ranging from +40 to +60 (or -40 to -60) mV are accepted as having good colloidal stability, whereas particles with zeta potentials ranging from +30 to +40 (or -30 to -40) mV are moderately stable. Lastly, particles with zeta potentials with absolute values lower than 30 mV will start showing instability, i.e., the particles will begin to coagulate. For example, Lee’s group synthesized a poly(l-glutamic acid)-\textit{b}-poly(l-phenylalanine) block copolypeptide that self-assembles into
vesicles. These vesicles were found to have negative zeta potentials below -30 mV at pH 7, which indicated moderate stability and ionization of the glutamate block. However, when the surrounding pH was dropped to pH 4, the zeta potential values ranged between -10 and -12 mV, indicating that the glutamate amino acids were being protonated. The resulting neutral amino acids therefore decreased the electrostatic repulsions between the vesicles, which were previously providing colloidal stability.

1.5. Cytotoxicity, Encapsulation, and Delivery

Although the field is still relatively new and only a handful of groups have started investigating polypeptide and polypeptide hybrid vesicles for drug delivery, there has been an increase in recent reports demonstrating that these vesicles are able to encapsulate biologically relevant molecules and deliver them to cells and animals, without causing toxicity towards cells and tissues. In this section, we will discuss the progress made in this area.

Kataoka’s group was able to encapsulate myoglobin inside the hydrophilic core of their PICsomes. In the same study, they showed that the 100 nm PICsomes have a long in vivo circulation time in the bloodstream of mice, comparable to typical long-lived liposomes and polymersomes. More recently, Lecommandoux’s group encapsulated DOX into their hyaluronan-b-poly(γ-benzyl glutamate) vesicle system to take advantage of hyaluronan’s ability to bind to CD44 for cancer targeting (Figure 1.4). When administered intravenously into a mouse model for cancer, the encapsulated DOX had better tumor shrinking properties due to the combined effects of controlled release, targeting ability of hyaluronan, and the EPR effect of the vesicles. They also showed that the empty vesicles were nontoxic using in vitro cytotoxicity

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assays, where 90% of the cells were viable at copolymer concentrations up to 650 μg/mL. In addition to DOX, Lecommandoux’s group was able to encapsulate docetaxel, a hydrophobic anticancer drug, into the hydrophobic membrane of the same vesicles, showing the versatility of these vesicles in loading different drugs.\textsuperscript{81} Compared to free docetaxel, these docetaxel-loaded vesicles showed high \textit{in vitro} cytotoxicity, especially towards cells that express high levels of CD44 receptors, demonstrating the vesicle’s ability to target the receptor.

\textbf{Figure 1.4.} Polypeptide hybrid vesicle that was used to load DOX. (a) A schematic representation of the hyaluronan-\textit{b}-poly(γ-benzyl glutamate) vesicle. Adapted with permission from \textsuperscript{50}. Copyright 2009 American Chemical Society. (b) Tumor regression data after administration of free DOX and DOX-loaded hyaluronan-\textit{b}-poly(γ-benzyl glutamate) vesicles (PolyDOX). Reprinted from Biomaterials, 31/10, Kamal K. Upadhyay, Anant N. Bhatt, Anil K. Mishra, Bilikere S. Dwarakanath, Sanyog Jain, Christophe Schatz, Jean-François Le Meins, Abdullah Farooque, Godugu Chandraiah, Amit K. Jain, Ambikanandan Misra, Sébastien Lecommandoux, The intracellular drug delivery and anti tumor activity of doxorubicin loaded poly(γ-benzyl l-glutamate)-\textit{b}-hyaluronan polymersomes, 11., Copyright (2010), with permission from Elsevier

Uchegbu and coworkers have studied the complexation and delivery of DNA using a unique poly(amino acid) based polymer vesicle. A polymer of either poly(L-lysine) (PLL) or poly(L-ornithine) (PO) was functionalized with methoxy-poly(ethylene glycol) (mPEG) and hydrophobic palmitic acid chains to synthesize an amphiphilic triblock of either mPEG-\textit{b}-PLL-\textit{b}-
palmitoyl or mPEG-\(b\)-PO-\(b\)-palmitoyl. Vesicles formed from these polymers were complexed with DNA and showed improved transfection \textit{in vitro} over poly(amino acid) complexed with DNA or DNA alone.\(^8^2\)

Pure polypeptide vesicles have also been used for encapsulation and delivery of biologically relevant material, but with limited success. Kimura’s group has reported the encapsulation of FITC-labeled dextran within their vesicles formed from a copolymer composed of poly(sarcosine) (PSar) and poly(\(\gamma\)-methyl-\(\epsilon\)-glutamate) (PMLG) (Figure 1.5).\(^4^1\) Sarcosine is an amino acid found in muscle, which can be degraded to glycine by sarcosine dehydrogenase. They also labeled their vesicles with the near-infrared fluorescence (NIRF) probe, indocyanine green (ICG),\(^4^1\) and showed that the labeled vesicles are retained within mouse tumors due to the enhanced permeability and retention (EPR) effect\(^8^3\) of tumor vasculature.

**Figure 1.5.** Polypeptide vesicles demonstrate the ability to utilize the EPR effect. (a) Chemical structure of the amphiphilic block polypeptide, PSar-\(b\)-PMLGs. (b) Fluorescence image using fluorescently labeled PEG. Fluorescence is not observed in the cancer site although accumulation is observed in the bladder. (c) Fluorescence image using ICG-labeled vesicles, showing evidence of vesicle accumulation due to the EPR effect. Adapted with permission from \(^4^1\). Copyright 2008 American Chemical Society.
The Jing group investigated their poly(1-lysine)-b-poly(1-phenylalanine) vesicles for the development of synthetic blood, since PEG-lipid vesicles were previously used to encapsulate hemoglobin to protect it from oxidation and to increase circulation time. They extended this concept and demonstrated that functional hemoglobin could be encapsulated into their vesicles. The same polypeptide material was also used to complex DNA, which caused the vesicles to lose their spherical shape and form irregular aggregates of less than 2 µm.\textsuperscript{43} Encapsulation of DNA inside polypeptide vesicles has also been reported by Hadjichristidis’s group.\textsuperscript{74} Moreover, Deming and coworkers were able to employ poly(1-arginine) as both a structural component of the poly(1-arginine)\textsubscript{60}-b-poly(1-leucine)\textsubscript{20} vesicles as well as a cell penetrating moiety to transport Texas-Red-labeled dextran into both endothelial and epithelial cell cultures (Figure 1.3).\textsuperscript{44} The vesicles were also shown to be relatively non-cytotoxic to T84 and HULEC-5A cells. The endocytosis mechanism and intracellular trafficking pathway of these vesicles were further investigated.\textsuperscript{84} Macropinocytosis was found to be the dominant mechanism for endocytosis. Immunostaining experiments demonstrated that the vesicles enter early endosomes but not lysosomes, suggesting that they recycle back to the cell surface. This last finding suggests that incorporating a mechanism for endosomal disruption into the polypeptide vesicles would improve their release into the cytoplasm.

1.6. Concluding Remarks and Proposal for Thesis

The field of polypeptide and polypeptide hybrid vesicles is steadily growing. Using polypeptides to form vesicles is very promising, since they are stable, can be tailored to incorporate a variety of functions, and have the potential to exhibit good biocompatibility. The
aqueous core of vesicles also allows for the isolation of the interior environment from the bulk solution, which if combined with proper release mechanisms, could greatly enhance the delivery of hydrophilic therapeutics. The delivery of therapeutics with these systems is just beginning to emerge, demonstrating the potential for this new class of materials as effective drug carriers.

In this thesis, we will investigate the potential of using vesicles comprised of positively charged polypeptides, as well as those composed of negatively charged polypeptides, to deliver therapeutics. The positively charged polypeptides that will be investigated include the K$_{60}$L$_{20}$ and R$_{60}$L$_{20}$ block copolypeptides described in this chapter. The vesicles formed from these polypeptides can encapsulate a therapeutic inside the aqueous core, and can also take advantage of their positive charges to complex negatively charged cargo, such as plasmid DNA or siRNA. Moreover, the R$_{60}$L$_{20}$ vesicles could cross the net-negatively charged plasma membrane, which is essential for delivering a drug that has an intracellular target. Chapter 2 summarizes our efforts to optimize the positively charged polypeptide vesicles by varying the length of the hydrophobic block while maintaining a constant length for the hydrophilic block. In Chapter 3, we discuss our investigation of using these positively charged vesicles for transfecting mammalian cells.

In addition to the positively charged vesicles, we also studied the properties of a negatively charged vesicle. One advantage of these vesicles is that they are less toxic to cells, which have net-negatively charged membranes. However, this can also be a disadvantage, since the electrostatic repulsive interactions between the vesicles and the cell membranes also inhibit the ability of the vesicles to enter cells. Thus, in Chapter 4, we overcome this challenge by conjugating to the vesicles a ligand that allows specific cancer cell targeting and internalization. Finally, in Chapter 5, we investigate the drug loading and delivery efficacy of the ligand-conjugated, negatively charged vesicles.
2. Characterization and Optimization of Block Copolypeptide Vesicles with Different Hydrophobic Chain Lengths

2.1. Overview

Polymeric drug delivery vehicles have the potential to improve the efficacy of current therapeutics. In our research, we have been investigating polymers comprised of amino acids, or polypeptides, that self assemble into vesicles. We previously synthesized the amphiphilic block copolypeptide, poly(L-lysine)$_{60}$-b-poly(L-leucine)$_{20}$ (K$_{60}$L$_{20}$), that was able to form vesicles that could be manipulated to different sizes and be prepared in large quantities. This chapter further expands upon that work by varying the length of the hydrophobic segment to optimize the vesicles so that they are monodisperse and low in toxicity. Polypeptides with longer oligoleucine segments were found to exhibit lower toxicity than vesicles formed from polypeptides with shorter hydrophobic domains due to having fewer toxic micelles, small aggregates, and unstable vesicles. Too long of an oligoleucine segment, however, resulted in a very rigid hydrophobic domain that prevented the processed vesicle sample to be extruded into a monodisperse population of vesicles.

2.2. Motivation and Background

Block copolypeptides represent an emerging class of materials that has recently gained interest in the drug delivery field. Polypeptides carry the promise of large chemical diversity, ability to incorporate secondary structures, and possible improved biocompatibility due to being comprised of natural amino acids. We previously developed the block copolypeptide poly(L-
lysine)_{60}-b$-\text{poly}(L\text{-leucine})_{20} (K_{60}L_{20})$ that self-assembles into versatile vesicles that could be extruded into different sizes and encapsulate hydrophilic cargo.$^{20}$

This communication summarizes our efforts to further understand and optimize these vesicles as potential drug delivery vehicles. To identify guidelines for producing a monodisperse population of vesicles with minimal cytotoxicity, the effect of varying the hydrophilic/hydrophobic ratio of the copolypeptide on these properties was investigated by fine-tuning the length of the hydrophobic segment while keeping the hydrophilic segment constant. Changing this ratio alters the dimensions of the polymer in an aqueous solution, which affects the supermolecular structures formed during self-assembly.$^{86}$ As the hydrophobic oligoleucine segment becomes shorter, the $\alpha$-helix of the hydrophobic moiety becomes less stable, resulting in a disordered hydrophobic segment.$^{20}$ Block copolypeptides with such shortened and disordered oligoleucine segments are expected to assume a conical shape that favors the self-assembly of the copolypeptides into spherical micelles and non-vesicular aggregates,$^{86}$ as shown in Figure 2.1. In contrast, copolypeptides with longer leucine segments are predicted to adopt a truncated-cone shape that aids self-assembly into a vesicle morphology.

Thus, by tuning the length of the hydrophobic segment, we expect to alter the polypeptide's ability to form vesicles in an aqueous solution. Different supermolecular structures, such as micelles and vesicles, in the population could potentially give rise to different levels of toxicity. In addition, the hydrophobic length may also affect the ability of the vesicles to be extruded into a monodisperse population, as different lengths may affect the stability and rigidity of the $\alpha$-helical hydrophobic segment, which in turn affects their packing in the self-assembled structures. In this study, we explored how the length of the hydrophobic domain
affects the formation, cytotoxicity, extrudability, and stability of the vesicles by preparing block copolypeptides of varying hydrophobic chain lengths.

Figure 2.1. Effect of the hydrophobic chain length on the self-assembly of block copolypeptides. Block copolypeptides with shorter oligoleucine segments are expected to give rise to disordered segments that could favor the formation of micelles or non-vesicular aggregates.

2.3. Materials and Methods

2.3.1. Materials

HeLa cell lines were obtained from the American Type Culture Collection (Manassas, Virginia). Minimum essential medium (MEM) with Earl’s balanced salt solution, penicillin-streptomycin, sodium pyruvate, phosphate-buffered saline (PBS), and 0.25% trypsin with
ethylenediaminetetraacetic acid (EDTA) were purchased from Invitrogen (Carlsbad, California). Fetal bovine serum (FBS) was obtained from Hyclone (Waltham, Massachusetts). The MTS cell proliferation assay kit was purchased from Promega (Madison, Wisconsin). The Bradford reagent was obtained from Bio-Rad (Hercules, California). Dialysis membranes were purchased from Spectrum Laboratories, Inc (Rancho Dominguez, California). All other tissue culture reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri).

2.3.2. Preparation of Block Copolypeptides

The block copolypeptides were synthesized using the transition metal-initiated living α-amino acid-N-carboxyanhydride polymerization technique as previously described.20 $K_{60}L_y$ block copolypeptides were synthesized maintaining the lysine domain at 60 residues, while varying the size of the leucine domain from 10 to 25 residues in increments of 5. The characteristics of the synthesized blocks are shown in Table A1 (Appendix).

2.3.3. Vesicle Formation

The copolypeptides were processed into vesicles by first dissolving 10 mg of the copolypeptides in 1 mL of a 1:1 tetrahydrofuran (THF):sterile Milli-Q water mixture by volume. Subsequently, 250 μL of THF was added four times to yield a polypeptide concentration of 0.5\% w/v and a 3:1 THF:sterile Milli-Q water ratio by volume. The mixture was placed in a dialysis bag (MWCO = 1,000 Da), and dialyzed against sterile Milli-Q water overnight to remove the THF, where the water was changed every hr for the first 4 hrs. The next day, the suspension was
collected and imaged using the Zeiss Axiovert 200 DIC/Fluorescence Inverted Optical Microscope (Carl Zeiss Inc., Thornwood, New York) to confirm the formation of the vesicles.

2.3.4. Transmission Electron Microscope (TEM)

\( K_{60}L_{10} \) and \( K_{60}L_{20} \) copolypeptide suspensions (0.1% w/v) were processed into vesicles as described above. One drop of each respective sample was placed on a sheet of parafilm, and a carbon coated copper grid was placed on the droplet and allowed to sit for 90 seconds. Filter paper was then used to remove the residual sample and liquid. One drop of 2% w/v uranyl acetate (negative stain) was then placed on parafilm, and the grid was placed on the droplet and allowed to stand for 30 sec. Excess liquid was removed by wicking away with filter paper. The resulting samples were imaged using a JEM1200-EX transmission electron microscope (JOEL, Tokyo) at 80 keV and ambient temperature.

2.3.5. Cell Culture

The HeLa cell line is a human cervical cancer cell line widely used in scientific research. These cells were maintained in MEM supplemented with 26.2 mM sodium bicarbonate, 10% v/v FBS, 100 units mL\(^{-1}\) penicillin, 100 μg mL\(^{-1}\) streptomycin, and 1 mM sodium pyruvate at a pH of 7.4 in a 37°C humidified atmosphere with 5% CO\(_2\).
2.3.6. Toxicity Assay

The MTS cell proliferation assay was performed according to the manufacture supplied instructions. Briefly, HeLa cells were seeded onto a 48-well tissue culture plate at 40,000 cells/cm² and incubated overnight in a 37°C humidified atmosphere with 5% CO₂. The next day, the medium was aspirated from each well, and the cells were incubated with different concentrations of vesicles for 5 hrs. Subsequently, the medium containing polypeptide vesicles was aspirated. Fresh medium containing 20% MTS was then added to the cells. The cells were placed back into a CO₂ incubator for 1 hr, and the absorbance at 490 and 700 nm was measured with an Infinite F200 plate reader (Tecan Systems Inc., San Jose, California).

2.3.7. Vesicle Purification and Quantification

Vesicle suspensions of either K₆₀L₁₀ or K₆₀L₂₀ were dialyzed against sterile Milli-Q water using a 1,000,000 MWCO membrane (estimated pore diameter = 80 nm) in order to purify the vesicles from the micelles and small aggregates (Figure 2.2). The dialysis was conducted overnight with four water changes using sterile Milli-Q water, and the contents inside the dialysis bag were collected the next day. After dialysis, the concentration of polypeptide in each dialysis bag was quantified using the Bradford protein assay, according to the manufacture supplied instructions, using the predialyzed vesicles as the standard.
2.3.8. Vesicle Stability Assay

The stability of the $K_{60}L_{10}$ and $K_{60}L_{20}$ vesicles was assessed in the presence of ethanol. Vesicle suspensions (0.2% w/v) were subjected to an equal volume of ethanol, and the resulting samples were allowed to stand for 30 min. The mixtures were then examined using differential interference contrast (DIC) optical microscopy.

2.4. Results and Discussions

2.4.1. Vesicle Formation

In order to investigate the effect of varying the hydrophobic chain length on the assembly properties of the copolypeptides, four diblock copolypeptides with different leucine lengths were
synthesized ($K_{60}L_{10}$, $K_{60}L_{15}$, $K_{60}L_{20}$, and $K_{60}L_{25}$) and processed into vesicles. DIC microscopy images of the self-assembled vesicles from these block copolypeptides are shown in Figure 2.3. Within the range of the hydrophobic segments investigated, all of the block copolypeptides formed some micron-scale vesicles, and the TEM images confirmed the presence of vesicles for the $K_{60}L_{10}$ and $K_{60}L_{20}$ block copolypeptides (Figures 2.3e and g). Although we had previously found that the $K_{60}L_{10}$ copolypeptide predominantly forms micelles, we have found that our improved processing method of using a THF:water ratio of 3:1 by volume leads to the formation of some vesicles along with micelles and irregular small aggregates. As indicated by the higher magnification images (Figures 2.3f and h), these micelles and small aggregates were present at a much higher concentration in the $K_{60}L_{10}$ sample than in the $K_{60}L_{20}$ sample. Overall, these results demonstrate that copolypeptides with longer hydrophobic blocks lead to self-assembled supermolecular structures that are predominantly comprised of vesicles.

![Figure 2.3. DIC images of vesicles prepared from (a) $K_{60}L_{10}$, (b) $K_{60}L_{15}$, (c) $K_{60}L_{20}$, and (d) $K_{60}L_{25}$ (Scale bar = 10 μm). TEM images of the processed vesicle solutions for $K_{60}L_{10}$ ((e) and (f)) and $K_{60}L_{20}$ ((g) and (h)) at different magnifications.](image-url)
2.4.2. Toxicity of Vesicles from Different Hydrophobic Chain Lengths

The samples with different hydrophobic segments were then investigated with respect to toxicity. Figure 2.4 shows that the increase in cell viability plateaus at $K_{60L_{20}}$ for the processed suspensions, which is consistent with our previous report that the $\alpha$-helix of the oligoleucine domain becomes stable at approximately 20 residues.\textsuperscript{20} The $K_{60L_{20}}$ and $K_{60L_{25}}$ vesicles were subsequently serially passed through 1.0, 0.4, and 0.2 μm polycarbonate (PC) membranes to compare their sizes after extrusion (Figure A2, Appendix). The $K_{60L_{20}}$ samples were found to yield a nanoscale vesicle population that was more monodisperse and closer in size to the final pore size, suggesting that the polyleucine segment of the $K_{60L_{25}}$ may be too long and rigid to form nanosized vesicles.

![Graph showing relative survival with different concentrations and vesicle lengths.]

**Figure 2.4.** 5 hr cytotoxicity results of processed vesicle solutions where the length of the hydrophobic block was varied. Error bars represent the standard deviation from an average of three measurements.
2.4.3. Vesicle Purification, Quantification, and Subsequent Cytotoxicity

Figure 2.4 also shows that the hydrophobic chain length does have an impact on toxicity, where suspensions of samples with shorter hydrophobic blocks were more toxic. To determine if the micelles and small aggregates present at higher concentrations in the processed vesicle suspensions of the shorter hydrophobic block copolypeptides were contributing to the additional cytotoxicity, dialysis was performed to separate micelles and small aggregates from the much larger vesicles. The cytotoxicity of the samples retained within the membranes was then examined. Specifically, a 1,000 kDa MWCO dialysis membrane (estimated pore size = 80 nm) was used, where unextruded vesicles (diameter > 100 nm) were retained, while micelles and small aggregates were dialyzed away from the sample. For this experiment, we investigated K₆₀L₁₀, the most toxic copolypeptide, and K₆₀L₂₀, the least toxic copolypeptide that also yielded a monodisperse vesicle population. The amount of copolypeptide before and after the dialysis treatment is shown in Figure 2.5a. Both K₆₀L₁₀ and K₆₀L₂₀ show a decrease in the overall amount of copolypeptide, verifying the existence of micelles and small aggregates in both samples prior to dialysis. Furthermore, less of the K₆₀L₁₀ copolypeptide was retained after dialysis, which confirmed our previous observation from the TEM images that the K₆₀L₁₀ copolypeptide has more micelles and small aggregates within the population. The toxicities of the dialyzed samples were then investigated to see if the toxicity decreases after removing the micelles and smaller aggregates. As shown in Figure 2.5b, the K₆₀L₂₀ vesicle suspension, which had the micelles and aggregates dialyzed away, demonstrated a lower cytotoxicity than the K₆₀L₂₀ vesicle suspension that did not undergo dialysis. This implies that the vesicles are less toxic than the micelles and smaller aggregates, which are likely the main source of toxicity within a population of processed copolypeptides. In addition, we investigated the cytotoxicity of
micelles formed from \( \text{K}_{60}(\text{rac-L})_{20} \) polypeptides (Figure A3), which, in terms of chemical composition, are identical to the \( \text{K}_{60}\text{L}_{20} \) polypeptides. The \( \text{K}_{60}(\text{rac-L})_{20} \) polypeptides form spherical micelles instead of vesicles, due to the racemization of the leucine segments, which destabilizes the alpha-helices into random coils. \( \text{K}_{60}(\text{rac-L})_{20} \) micelles were found to be more cytotoxic than both the predialyzed and dialyzed \( \text{K}_{60}\text{L}_{20} \) vesicles, where the relative survival of the micelles at each concentration point was significantly lower than the relative survival of both predialyzed and dialyzed vesicles. It is also possible that the polypeptide unimers, which are always present in a dynamic macromolecular population, could contribute to cytotoxicity. However, these block copolypeptides are reported to have very slow dynamic and low critical aggregation concentrations (CAC),\(^{20}\) which substantiates the dominant presence of these assemblies, even under high dilution.

For the case of \( \text{K}_{60}\text{L}_{10} \), the effect of the dialysis treatment was not apparent, as indicated by the similar levels of toxicity between the dialyzed and predialyzed \( \text{K}_{60}\text{L}_{10} \) samples. Moreover, the dialyzed \( \text{K}_{60}\text{L}_{10} \) sample was still more toxic than the dialyzed \( \text{K}_{60}\text{L}_{20} \) sample even though both of these samples should be mostly populated with vesicles. These results together suggest that the copolypeptides with the shorter leucine domain not only form fewer vesicles, but may also form less stable vesicles that have a greater tendency to destabilize into micelles and small aggregates that are toxic.
2.4.4. Vesicle Stability Assay

To compare the stability of the K$_{60}$L$_{10}$ and K$_{60}$L$_{20}$ vesicles, both vesicle suspensions were subjected to an equal volume of ethanol, such that the final ethanol concentration was 50% v/v. Treatment with ethanol has been previously used by other research groups to assess the stability of vesicles formed from positively-charged surfactants.$^{88,89}$ The presence of ethanol was found to disrupt the K$_{60}$L$_{10}$ vesicular structures (Figure 2.6b), while the K$_{60}$L$_{20}$ vesicles were stable to the ethanol treatment (Figure 2.6d). The lack of vesicles or any other detectable structures in Figure 2.6b suggests that the vesicles were destabilized into the smaller toxic micelles and aggregates. This result demonstrates that K$_{60}$L$_{20}$, or copolypeptides with longer hydrophobic segments, form more stable vesicles, contributing to a lower cytotoxicity. The stability of the
hydrophobic α-helical segments in these materials therefore not only influences vesicle assembly, but also the stability of the formed vesicles.

**Figure 2.6.** DIC images of vesicles in different solutions. (a) K\textsubscript{60}L\textsubscript{10} in sterile Milli-Q water, (b) K\textsubscript{60}L\textsubscript{10} in a 50% v/v ethanol/sterile Milli-Q water mixture, (c) K\textsubscript{60}L\textsubscript{20} in sterile Milli-Q water, and (d) K\textsubscript{60}L\textsubscript{20} in a 50% v/v ethanol/sterile Milli-Q water mixture (Scale bar = 10 μm).

**2.5. Conclusions**

Varying the hydrophilic/hydrophobic ratio in lysine-leucine block copolypeptides affects their ability to form less toxic vesicles with controllable sizes. We have shown that samples with longer leucine segments form vesicles that are less toxic than vesicles formed from copolypeptides with shorter hydrophobic domains. The shorter hydrophobic domains are
associated with unstable α-helical structures that lead to the presence of more toxic micelles and small aggregates during the vesicle formation process. In fact, our previous circular dichroism studies also show that α-helical content within the oligoleucines decrease with shorter leucine domains. Moreover, it was observed that vesicles formed from these copolypeptides with shorter hydrophobic domains were also less stable, potentially contributing to additional micelles and small aggregates in the suspension during the cytotoxicity experiment. Among the copolypeptides investigated, the K_{60}L_{20} copolypeptide composition showed the most potential for drug delivery applications, as this copolypeptide was able to form vesicles with the least amount of micelles and small aggregates, and was most easily extruded into monodisperse nanoscale vesicles.

A few research groups have studied the effects of altering hydrophilic/hydrophobic ratios in polypeptide containing block copolymers to design nanostructures for the purpose of drug delivery. However, most of these studies involved exploring a broad range of ratios, resulting in different self-assembled structures from spherical micelles to vesicles to nanotubes to sheets. In this study, we have focused on a narrow range, and have shown that fine-tuning this ratio within a range that yields primarily vesicles is one method for designing less toxic nanoscale carriers that are suitable for drug delivery applications.
3. Transfection of Mammalian Cells using $R_{60}L_{20}$ Vesicles

3.1. Overview

Gene replacement therapy has great potential for treating many diseases that currently lack an effective treatment option. However, a safe and effective means of delivering genetic material remains to be the major challenge in the field. Therefore, materials that can deliver DNA with low cytotoxicity and immunogenicity are highly desirable. Block copolypeptides have the potential to fulfill this role, because they are comprised of naturally occurring building blocks and can be tuned to have a wide variety of chemical properties. Chapter 2 summarized our work in identifying an optimal block copolypeptide composition comprised of 60 positively charged amino acids followed by 20 leucine residues, and this chapter investigates arginine as the positively charged amino acid. The poly(arginine)$_{60}$-block-poly(leucine)$_{20}$ polypeptide ($R_{60}L_{20}$) was previously found by our research group to be capable of forming vesicles with controllable sizes, able to transport hydrophilic cargo across the cell membrane, and exhibit relatively low cytotoxicity. In this study, we demonstrated that the $R_{60}L_{20}$ vesicles also possess the ability to deliver DNA into mammalian cells for transfection. Although the transfection efficiency was lower than that of the commercially-available transfection agent Lipofectamine™ 2000, the $R_{60}L_{20}$ vesicles were able to achieve transfection with significantly lower cytotoxicity and immunogenicity. We hypothesize that this behavior is potentially due to its stronger interaction with DNA which subsequently provides better protection against anionic heparin.
3.2. Motivation and Background

The ability to manipulate gene expression in vitro and in vivo has helped investigators gain a better understanding of many diseases, such as atherosclerosis, severe combined immune-deficiency disorder, and multiple types of cancer. Moreover, delivering genes also has therapeutic potential for treatment of diseases, such as Parkinson’s, Alzheimer’s, and Huntington’s diseases. However, the method of delivery for these genetic materials represents the major challenge in this field as DNA molecules cannot cross the cell membrane on their own. To address this issue, much effort has been spent on evaluating the efficacy of different materials as transfection agents. Transfection agents currently available for research can be broadly categorized into viral vectors and non-viral vectors. Viral vectors hijack the ability of viruses and are efficient vehicles for delivering genetic materials into target cells. Among the different viral vectors under investigation, retroviruses, lentiviruses, and adenoviruses have been greatly investigated and are continuously under research and development. Although viral vectors can achieve high levels of transfection, concerns regarding immunogenicity have hindered their development for commercial use in human gene therapy. Non-viral systems, on the other hand, depend on the ability of the transfection agent to complex or entrap the genetic material and deliver it across the cell membrane. Since DNA is negatively charged, many cationic systems have been studied as potential gene carriers. Among these, effective in vitro transfection agents have been developed from lipid-based materials and polyethyleneimine (PEI). Although these synthetic systems can be tailored to be less immunogenic than viruses, their transfection efficiency is generally lower than viral systems. This is due to the fact that several biological barriers exist before the genetic materials reach the target cells.
material can reach the nucleus, such as crossing the cell membrane, escaping the endosome, and crossing the nuclear membrane.\textsuperscript{110}

Block copolypeptides are a new class of materials that have the potential to become efficient gene delivery vehicles.\textsuperscript{85,111,112} These polypeptides are comprised of amino acids that are naturally occurring so they may be nontoxic and less immunogenic. They can also be tailored to include a wide variety of chemical properties due to the availability of many natural and synthetic residues. We previously described the poly(L-lysine)\textsubscript{60}-block-poly(L-leucine)\textsubscript{20} block copolypeptide (K\textsubscript{60}L\textsubscript{20}) synthesized using a transition metal mediated living N-carboxyanhydride polymerization reaction.\textsuperscript{113} This approach was used to synthesize block copolypeptides with a narrow molecular weight distribution, and the resulting K\textsubscript{60}L\textsubscript{20} polypeptides possessed the ability to self-assemble into vesicles when processed in two steps with the organic solvent tetrahydrofuran (THF) and water. These vesicles had controllable sizes, could encapsulate hydrophilic cargo, and were relatively stable in both water and phosphate-buffered saline (PBS).\textsuperscript{20} Because of the presence of lysine residues on the outer surface, we reasoned that the K\textsubscript{60}L\textsubscript{20} vesicles could be used as gene delivery vehicles, since polylysines and polyarginines have been investigated as potential transfection agents due to their cationic nature.\textsuperscript{114,115} However, initial uptake studies showed that the K\textsubscript{60}L\textsubscript{20} vesicles were not able to cross the cell membrane.\textsuperscript{20} In order to engineer an alternative vesicle construct possessing the ability to cross the cell membrane, we studied peptides such as the HIV TAT sequence (Tat\textsubscript{48-59}: GRKKRRQRRRAP) and oligoarginine.\textsuperscript{116} These arginine-rich peptides have been previously shown to be able to cross the cell membrane barrier due to a bidentate binding conformation which allows for two hydrogen bonds between the guanidinium groups and the anions on the plasma membrane, such as phosphates and sulfates.\textsuperscript{117,118} We reasoned that, since
arginine and lysine have similar charge properties, the lysine residues in K_{60}L_{20} could be replaced with arginine residues without disrupting the polypeptide’s ability to form vesicles while also imparting to the vesicles the ability to cross the cell membrane. To test this idea, poly(L-arginine)$_{60}$-block-poly(L-leucine)$_{20}$ block copolypeptides (R$_{60}$L$_{20}$) were synthesized and characterized. As predicted, the R$_{60}$L$_{20}$ copolypeptides were able to form vesicles similar to their K$_{60}$L$_{20}$ predecessor, and the R$_{60}$L$_{20}$ vesicles possessed the added ability to enter cells.\textsuperscript{44}

In this report, we explored the potential of using the R$_{60}$L$_{20}$ vesicle system as a transfection agent. There are other studies that report the use of poly(L-arginine) to deliver plasmid DNA into cells.\textsuperscript{119,120} In these studies, the use of arginine was limited to its polymer chain form. In contrast, we have synthesized a block copolypeptide comprised of a poly(L-arginine) block and a poly(L-leucine) block that self-assembles into vesicles. These poly(L-arginine)$_{60}$-block-poly(L-leucine)$_{20}$ vesicles have the potential to be more effective than just the poly(L-arginine) homopolymer, since transfection using self-assembled cationic vesicles has been shown to yield higher transfection efficiencies than when using the polymer building blocks separately as homopolymers.\textsuperscript{119} To our knowledge, this is the first time the transfection capability of a self-assembled vesicle formed from a block copolypeptide has been reported. Our results demonstrate that the R$_{60}$L$_{20}$ vesicles possess the ability to deliver a reporter gene into HeLa cells. We also performed experiments to optimize the transfection efficiency of the R$_{60}$L$_{20}$ vesicles and compared them with the commercially-available Lipofectamine\textsuperscript{TM} 2000 in terms of transfection efficiency and cytotoxicity. Immunogenicity of the R$_{60}$L$_{20}$ vesicles complexed to the plasmid DNA was also compared to the Lipofectamine\textsuperscript{TM} 2000/DNA complex.
3.3. Materials and Methods

3.3.1. Materials

HeLa and RAW264.7 cell lines were obtained from the American Type Culture Collection (Manassas, Virginia). Minimum essential medium (MEM) with Earl’s balanced salt solution, Dulbecco's Modified Eagle Medium (DMEM) with Earl’s balanced salt solution, penicillin-streptomycin (P/S), sodium pyruvate (NaPyr), PBS, 0.25% trypsin with EDTA, and the Lipofectamine™ 2000 reagent were purchased from Invitrogen (Carlsbad, California). Fetal bovine serum (FBS) was obtained from Hyclone (Waltham, Massachusetts). Eight-well chambered coverglasses were obtained from Lab-Tek (Rochester, New York). The DNA vector pDsRed Express-2 was a product of Clontech (Mountain View, California). The MTS cell proliferation assay kit was purchased from Promega (Madison, Wisconsin). Flowtubes from Fisher Scientific (Pittsburgh, Pennsylvania) were used for fluorescence-activated cell sorting (FACS). The Bradford reagent was obtained from Bio-Rad (Hercules, California). The mouse interleukin-6 (IL-6) ELISA kit was purchased from BD Biosciences (San Jose, California). DNAse I was obtained from New England Biolabs (Ipswich, Massachusetts). All other reagents, such as heparin (>500,000 MW), were purchased from Sigma-Aldrich (St. Louis, Missouri).

3.3.2. Synthesis and processing of polypeptide vesicles

The K₆₀L₂₀ block copolypeptide was synthesized using the transition metal initiated living N-carboxyanhydride polymerization technique as previously described.¹¹³ Fluorescein isothiocyanate (FITC) conjugation was performed by mixing 1% w/v polypeptide in sodium...
borate buffer at a pH of 8.0. A 6:1 molar ratio between $K_{60}L_{20}$ and FITC dissolved in DMSO was used, and the mixture was allowed to incubate at room temperature for at least one hr. Following the conjugation, free primary amines on lysines unconjugated to FITC were converted to guanidinium groups using 3,5-dimethyl-1-pyrazolylformaminidium nitrate. The resulting copolypeptide was freeze-dried and referred to as poly($L$-homoarginine)$_{60}$-block-poly($L$-leucine)$_{20}$, which has properties indistinguishable from poly($L$-arginine)$_{60}$-block-poly($L$-leucine)$_{20}$, and hence designated as $R_{60}L_{20}$.[31] The $R_{60}L_{20}$ polypeptide was processed into vesicles using a modification of a method previously reported.$^{20, 44}$ Specifically, the vesicles were formed by first dissolving 10 mg of polypeptide in 1 mL of a 1:1 mixture between THF and sterile Milli-Q water. Subsequently, 250 μL of THF was added four times to yield a final polypeptide concentration of 0.5 % w/v. The mixture was placed in a dialysis bag (MWCO = 1,000 Da), and dialyzed against sterile Milli-Q water overnight to remove the THF, where the sterile Milli-Q water was changed every hr for the first 4 hrs. The resulting vesicles were extruded through a polycarbonate membrane with 100 nm pores to obtain uniformly sized vesicles. The sizes of the vesicles and their distribution were analyzed with dynamic light scattering (DLS), and the Bradford assay was performed to quantify the final concentration of the polypeptide vesicles according to the manufacture supplied instructions, using the predialyzed samples as the standard.

### 3.3.3. Dynamic light scattering (DLS) and zeta potential

DLS and zeta potential measurements were performed on $R_{60}L_{20}$ vesicles complexed with the DsRed plasmid at various charge ratios. The DsRed plasmid and 100 nm extruded $R_{60}L_{20}$
vesicles were first diluted separately in blank media (MEM containing 26.2 mM sodium bicarbonate) and left undisturbed for 5 min. These two solutions were then mixed and incubated for 20 min to allow the formation of complexes. The resulting mixtures were analyzed with the Malvern Zetasizer Nano ZS model Zen 3600 (Malvern Instruments Inc, Westborough, Massachusetts).

3.3.4. Cell culture

The HeLa cell line was grown in MEM supplemented with 26.2 mM sodium bicarbonate, 10% FBS, 1 mM sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin at a pH of 7.4. The RAW264.7 cell line was grown in DMEM supplemented with 26.2 mM sodium bicarbonate, 10% FBS, 1 mM sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin at a pH of 7.4. Both cell lines were maintained in a 37°C humidified atmosphere with 5% CO₂ and handled with standard sterile tissue culture protocols.

3.3.5. Transfection

HeLa cells were seeded at a density of 4x10⁴ cells/cm² the night before transfection. These cells were seeded onto 8-well chambered coverglasses for confocal microscopy experiments and 24-well tissue culture plates for fluorescence-activated cell sorting (FACS). The DsRed plasmid, 100 nm extruded FITC-labeled R₆₀L₂₀ vesicles, and the Lipofectamine™ 2000 reagent were separately diluted in blank media and left undisturbed for 5 min. Following the dilutions, the concentration of Lipofectamine™ 2000 was 2.5 μg/mL as suggested by the
manufacturer, and the concentration of polypeptide vesicles was kept at 15 μg/mL. After the 5 min incubation, the DsRed plasmid solution was mixed with either the R₆₀L₂₀ vesicles or the Lipofectamine™ 2000 reagent and incubated for 20 min to allow complexes to form. The complexes were then diluted further with blank media and incubated with HeLa cells for up to 4 hrs to allow the complexes to be internalized into the cells. Subsequently, the medium containing the complexes was aspirated to remove the complexes, and was replaced with complete medium containing serum. Cells were then given 48 hrs to express the protein and subjected to either confocal microscopy or FACS to determine the transfection efficiency.

3.3.6. Laser scanning confocal microscopy (LSCM)

LSCM images were taken on a Leica Inverted TCS-SP MP Spectral Confocal and Multiphoton Microscope (Heidelberg, Germany) equipped with an argon laser (488 nm blue excitation: JDS Uniphase), a diode laser (DPSS; 561 nm yellow-green excitation: Melles Griot), a helium-neon laser (633 nm red excitation), and a two photon laser setup consisting of a Spectra-Physics Millenia X 532 nm green diode pump laser and a Tsunami Ti-Sapphire picosecond pulsed infrared laser tuned at 768 nm for UV excitation.

3.3.7. MTS cell proliferation assay

The MTS cell proliferation assay (CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay) was performed to assess the cytotoxicity level of the transfection process using Lipofectamine™ 2000 and polypeptide vesicles with HeLa cells. Transfection
experiments were performed with HeLa cells seeded on 48-well plates with quadruplicates of each transfection condition. At the end of the 48-hr incubation period allotted for protein synthesis, the medium was aspirated and fresh medium containing 20% MTS reagent was added to the cells. The cells were placed back into a humidified CO₂ incubator at 37°C for 2 hrs, and the absorbance at 490 nm was measured.

3.3.8. Fluorescence-activated cell sorting (FACS)

FACS analyses of transfected HeLa cells were performed on a BD FACScan™ (BD Bioscience, San Jose, CA) system equipped with an argon laser (488 nm blue excitation) and two filters: a green filter (530 ± 30 nm) and an orange filter (585 ± 42 nm).

3.3.9. IL-6 ELISA assay

The amount of IL-6 stimulated from either Lipofectamine™ 2000 or polypeptide vesicles was quantified using the mouse IL-6 ELISA kit. RAW264.7 cells were seeded at a density of 1x10⁵ cells/cm² in a 48-well plate and incubated overnight. The DsRed plasmids were complexed to Lipofectamine™ 2000 or polypeptide vesicles in the same manner as the transfection experiments. The complexes were then incubated with RAW264.7 cells for 24 hrs, at which time the culture media from the wells were collected. The collected samples were then centrifuged at 2,000 g for 10 min, and the resulting supernatant was collected for each sample. The level of IL-6 in each supernatant was then determined by the ELISA kit following the manufacturer-supplied protocol.
3.3.10. Stability against anionic polymers

The stability of the DsRed complexes against heparin, an anionic polymer, was investigated. An equal mass of plasmid was added to either Lipofectamine™ 2000 or the polypeptide vesicles, and each mixture was diluted in PBS and incubated at room temperature for 20 min to allow complexes to form. Different concentrations of heparin were added to the complexes at varying molar ratios of heparin to DsRed plasmid from 0 to 800, and the mixtures were incubated at room temperature for 1 hr. The mixtures were then combined with a DNA loading buffer and run on a 1% w/v agarose gel with a 100 V potential applied for 45 min. Plasmid alone was also loaded as a control. The plasmids were detected by staining the gel with ethidium bromide (EtBr) and visualized under UV light.

3.3.11. Stability against DNase I

The R_{60L20} vesicles and Lipofectamine™ 2000 complexed to the same mass of DNA were exposed to 1 unit of DNase I for 10 min at 37°C. The samples were then treated with 0.25% trypsin with EDTA® for 30 min at 37°C. EDTA stops the DNase I activity, while trypsin cleaves the polypeptides of the vesicles to help release the DNA. This was followed by a 30-min incubation with heparin to separate the plasmid from the delivery vehicles. This mixture was then analyzed on an agarose gel to examine the integrity of the DNA.
3.4. Results and Discussions

3.4.1. Characterization of R₆₀L₂₀ vesicle/plasmid DNA complexes

The polypeptide vesicles were expected to associate with DNA due to attractive electrostatic interactions. To further characterize this phenomenon quantitatively, R₆₀L₂₀ vesicles were complexed to plasmid DNA at various +/- charge ratios, and their sizes and stabilities were analyzed with DLS (Figure 3.1) and zeta potential (Figure 3.2) measurements, respectively. The +/- charge ratio has been shown to affect the stability of many cationic lipid vesicle/plasmid complex systems, which ultimately affected the ability of the complex to transfect cells.¹²¹-¹²³ The DLS measurements showed that the vesicles extruded through a 100 nm PC membrane possessed a mean diameter of approximately 160 nm and exhibited excellent colloidal stability with a zeta potential exceeding +60 mV.

![Figure 3.1. DLS size measurements of R₆₀L₂₀ vesicles and R₆₀L₂₀ vesicle/DNA complexes at various charge ratios. Error bars represent the standard deviation from an average of three measurements.](image-url)
As plasmid DNA was added, the size of the complexes initially decreased. The vesicles still exhibited good stability at the +/- charge ratio of 10:1 with a zeta potential greater than +30 mV. As the charge ratio decreased to 5:1, the mean diameter of the vesicle complexes increased. This was expected since the vesicle complexes were becoming less stable as shown by zeta potential measurements and were probably starting to aggregate. The aggregation of the vesicle complexes was more apparent at the charge ratio of 2.5:1 when visible precipitates were observed (data not shown).

![Figure 3.2.](image)

**Figure 3.2.** Zeta potential measurements of R₆₀L₂₀ vesicles and R₆₀L₂₀ vesicle/DNA complexes at various charge ratios. Error bars represent the standard deviation from an average of three measurements.

### 3.4.2. Transfection using R₆₀L₂₀ vesicles

In order to investigate the potential of the R₆₀L₂₀ vesicles to transfet mammalian cells, we attempted to deliver the DsRed reporter plasmid into HeLa cells. Successful transfection of this plasmid into the cells would result in the production of a red fluorescent protein.
Transfection with the commercially-available Lipofectamine™ 2000 was also performed in parallel as a positive control and a means for comparison. The results of the transfection experiment are shown in Figure 3.3. For wells incubated with polypeptide vesicles, the FITC channel images were also included to show the uptake of vesicles into the cells. In addition to the positive control, negative controls were performed with the plasmid alone, the vesicles alone, and Lipofectamine™ 2000 alone. The control cells incubated with only DsRed or only Lipofectamine™ 2000 showed no fluorescence as expected. Cells incubated with only R₆₀L₂₀ vesicles showed only green fluorescence due to the FITC label on the polypeptide, indicating that the vesicles were indeed taken up by the cells. The cells incubated with the complex of Lipofectamine™ 2000 and the DsRed plasmid showed that the reporter gene is functional. In fact, the red channel showed that many cells were successfully transfected, as signified by the production of red fluorescent proteins (Figures 3.3e and h). Finally, the cells incubated with the complex of R₆₀L₂₀ vesicles and DsRed plasmid demonstrated that the R₆₀L₂₀ vesicles were able to transflect the cells (Figures 3.3f and i). We also examined the poly(L-lysine)₆₀-block-poly(L-leucine)₂₀ block copolypeptide (K₆₀L₂₀) vesicles, and found they were not able transfecct cells (data not shown), which was not surprising since they were previously shown to be unable to cross the cell membrane.²⁰
Figure 3.3. Confocal images of the HeLa cell transfection experiment. The controls in the experiment were: (a) cells incubated with only DsRed plasmid, (b) cells incubated with only Lipofectamine™ 2000, and (c) cells incubated with only R_{60}L_{20} vesicles and (d) the corresponding green channel image. The experimental conditions were as follows: (e) cells incubated with complexes of Lipofectamine™ 2000 and DsRed taken at 10x magnification, (f) cells incubated with complexes of R_{60}L_{20} vesicles and DsRed at 10x magnification, (g) the corresponding green channel image, (h) cells incubated with complexes of Lipofectamine™ 2000 and DsRed at 100x magnification, (i) cells incubated with complexes of R_{60}L_{20} vesicles and DsRed at 100x magnification, and (j) the corresponding green channel image. The concentration of the R_{60}L_{20} vesicles was maintained at 15 μg/mL, using a DNA complexation +/- charge ratio of 10:1 (final DNA concentration = 2.25 μg/mL), while the concentration of Lipofectamine™ 2000 was maintained at 2.5 μg/mL, using a +/- charge ratio of 2:1 (final DNA concentration = 0.75 μg/mL).
Although the R<sub>60</sub>L<sub>20</sub> vesicles were able to transflect many cells, fewer cells were transfected with the polypeptide vesicles when compared to Lipofectamine™ 2000. In addition, the higher (100x) magnification images in Figures 3h and i show that the cells transfected with R<sub>60</sub>L<sub>20</sub> vesicles do not appear as bright as the cells transfected with Lipofectamine™ 2000, which may indicate that more plasmids were introduced by Lipofectamine™ 2000 into each cell leading to a greater production of red fluorescent proteins.

3.4.3. Factors influencing the transfection efficiency of the R<sub>60</sub>L<sub>20</sub> vesicles

In order to improve the transfection efficiency of the R<sub>60</sub>L<sub>20</sub> vesicles, different parameters in the transfection protocol were investigated. These parameters included the +/- charge ratio, amount of time allowed for the vesicles to complex with DNA, concentration of vesicles used, amount of time allowed for the vesicle/DNA complex to be taken up by the cells, and amount of time allowed for protein synthesis. Transfection experiments were performed as these parameters were varied, and a representative example of comparing the relative transfection efficiencies is shown in Figure 3.4.
Figure 3.4. Influence of various factors on the transfection efficiency of the R_{60}L_{20} vesicles: (a) +/- charge ratio, (b) time allowed for vesicles to complex to DNA, (c) concentration of vesicles, (d) time allowed for vesicle/DNA complexes to be taken up by the cells, and (e) time allowed for protein synthesis. Relative intensities were obtained from LSCM data using image analysis software. For the experiments in (a), the concentration of the vesicles was maintained at 15 μg/mL, while varying the DNA complexation ratio. For the experiments in (b), (d), and (e), the concentration of the vesicles was maintained at 15 μg/mL, using a DNA complexation +/- charge ratio of 10:1 (final DNA concentration = 2.25 μg/mL). For the experiments in (c), the concentration of the vesicles was varied, while keeping the DNA complexation +/- charge ratio of 10:1.

Varying the charge ratio showed that a 10:1 +/- ratio had the highest transfection efficiency (Figure 3.4a). The decrease in efficiency for lower charge ratios can be explained by the DLS and zeta potential measurements presented in Figures 1 and 2, since the vesicles began to aggregate at lower charge ratios. The decrease in transfection efficiency at higher charge
ratios may be a result of less DNA being introduced into the cells. The charge ratio of 10:1 represents the greatest amount of DNA that can be added to the vesicles before the complexes become unstable.

Varying the time allowed for the vesicles to complex to DNA showed that longer incubation times yield higher transfection, and this trend plateaus at the 20-min incubation period. This result implies that the 20-min time period is sufficient to allow the formation of complexes, and longer incubation times do not significantly improve the transfection efficiency (Figure 3.4b).

The amount of vesicles used for transfection also influences the transfection efficiency (Figure 3.4c), as our results revealed an increasing trend in transfection efficiency with increasing vesicle concentrations up to 15 μg/mL, most likely due to more DNA being brought into the cells. However, concentrations higher than 15 μg/mL did not exhibit any improvements in transfection efficiency. This may be due to the higher vesicle concentrations harming the cells, making it difficult for them to express the protein of interest.

Varying the amount of time allowed for the vesicle/DNA complex to be taken up by the cells showed a peak at 4 hrs (Figure 3.4d). Incubation times below 4 hrs exhibited lower transfection efficiencies, which may be due to fewer complexes being internalized into the cell. For incubation times greater than 4 hrs, the efficiency most likely decreased because the cells were in an unfavorable environment due to the lack of serum in the medium during this time period.

Lastly, 48 hrs was found to be the optimal time to allow protein synthesis to occur (Figure 3.4e). This time corresponds to approximately twice the doubling time of HeLa cells. The decrease in transfection efficiency at shorter times probably corresponds to not allowing
enough time for protein synthesis, while the decrease in transfection efficiency for longer times may be due to the amount of plasmids inside the cells being diluted as the cells continued to divide.

3.4.4. Cytotoxicity of the R_{60}L_{20} vesicle/DNA complexes

In addition to optimizing the protocol for our system, we wanted to evaluate the toxic levels of the transfection conditions for both delivery vehicles to ensure that the cells were being transfected under minimally toxic conditions. Previous reports have shown that materials exhibiting higher cytotoxicity often have better transfection efficiencies.\textsuperscript{124,125} However, low cytotoxicity is still desirable for transfection agents. For example, although certain PEI molecules do not transfect as efficiently as current commercially available agents, they maintain their usefulness by exhibiting lower cytotoxicity.\textsuperscript{126} The cytotoxicities of Lipofectamine\textsuperscript{TM} 2000 and R_{60}L_{20} vesicles were compared using the MTS cell proliferation assay and HeLa cells (Figure 3.5). The results show that, at the conditions used for transfection in Figure 3.3, the DsRed plasmid, R_{60}L_{20} vesicles, and Lipofectamine\textsuperscript{TM} 2000 at the manufacturer-recommended concentration of 2.5 μg/mL did not cause any significant cell death. However, when complexed to DNA, Lipofectamine\textsuperscript{TM} 2000/DNA complex had significant cytotoxicity, while the R_{60}L_{20} vesicle/DNA complexes did not cause any significant cell death. These results are in agreement with other reports that have documented that liposome-based transfection agents, such as Lipofectamine\textsuperscript{TM} 2000, can exhibit high levels of cytotoxicity.\textsuperscript{126}
Due to the large disparity in toxicity levels between Lipofectamine™ 2000 and $R_{60}L_{20}$ vesicles complexed to DNA, we wanted to compare the transfection efficiencies between these two materials at the same toxicity level. In order to determine the concentration of the Lipofectamine™ 2000/DNA complexes that exhibited comparable toxicity to the $R_{60}L_{20}$ vesicle/DNA complexes, we held the w/w ratio between Lipofectamine™ 2000 and DNA at 2.5:1 (the recommended ratio) and tested the cytotoxicity of the complex at varying concentrations to construct a dose-response curve (Figure 3.6). Using this curve, we found that

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**Figure 3.5.** Cytotoxicity measurements for different conditions. Error bars represent the standard deviation from an average of three measurements. The concentration of the $R_{60}L_{20}$ vesicles was maintained at 15 μg/mL, using a DNA complexation +/- charge ratio of 10:1 (final DNA concentration = 2.25 μg/mL), while the concentration of Lipofectamine™ 2000 was maintained at 2.5 μg/mL, using a +/- charge ratio of 2:1 (final DNA concentration = 0.75 μg/mL). Error bars represent the standard deviation from an average of three measurements.
0.9 μg/mL of Lipofectamine™ 2000 matched the cytotoxicity of the 15 μg/mL R_{60}L_{20} vesicle solution previously used in the transfection experiments.

![Graph showing dose-response curve for Lipofectamine™ 2000/DNA complexes.](image)

**Figure 3.6.** Dose-response curve for the Lipofectamine™ 2000/DNA complexes. The concentration of Lipofectamine™ 2000 was varied, while fixing the complexation ratio with DNA at a 2:1 +/- charge ratio. Error bars represent the standard deviation from an average of three measurements.

### 3.4.5. Quantitative comparison of transfection efficiency with normalized cytotoxicity

After identifying the Lipofectamine™ 2000/DNA condition that gave rise to the same cytotoxicity as that observed for the R_{60}L_{20} vesicle/DNA complexes, FACS analysis was performed on cells transfected with Lipofectamine™ 2000 at a concentration of 0.9 μg/mL, and quantitatively compared to cells transfected with R_{60}L_{20} vesicles using optimized parameters from Figure 3.4. As shown in Figure 3.7, the results indicated that Lipofectamine™ 2000 transfected 28% of the cells, while the R_{60}L_{20} vesicles transfected 18% of the cells.
Figure 3.7. FACS analysis of cells transfected with Lipofectamine™ 2000 and R60L20 vesicles at concentrations identified as exhibiting similar cytotoxicity. The concentration of the R60L20 vesicles was maintained at 15 μg/mL, using a DNA complexation +/- charge ratio of 10:1 (final DNA concentration = 2.25 μg/mL), while the concentration of Lipofectamine™ 2000 was maintained at 0.9 μg/mL, using a +/- charge ratio of 2:1 (final DNA concentration = 0.21 μg/mL). Error bars represent the standard deviation from an average of three measurements.

3.4.6. IL-6 ELISA assay

Although transfection using cationic lipid vesicles has yielded good results in vitro, their use in vivo and in clinical settings have been limited.127,128 One major concern is the immune response that is induced by DNA delivered through these vehicles, such as Lipofectamine™ 2000. In this study, we wanted to assess the immune response of the R60L20 vesicles by quantifying the amount of IL-6 produced in RAW264.7 cells, which is a widely used in vitro immunogenicity assay.129,130 As shown in Figure 3.8, both Lipofectamine™ 2000 and R60L20 vesicles did not induce IL-6 production when they were administered alone without DNA. However, upon complexation with the DsRed plasmid, Lipofectamine™ 2000 induced much higher levels of IL-6. Although the R60L20 vesicles show lower transfection efficiency compared to Lipofectamine™ 2000, the fact that they can deliver DNA with such a low immune response
is an advantage for gene delivery and RNA interference applications. Moreover, the data showed that both delivery vehicles were did not induce IL-6 secretion without the plasmid, implying that the IL-6 induction came from the plasmid and not the delivery vehicles themselves.

**Figure 3.8.** IL-6 expression from RAW264.7 cells after a 24 hr incubation with either uncomplexed or complexed R\textsubscript{60}L\textsubscript{20} vesicles and Lipofectamine\textsuperscript{TM} 2000. The concentration of the R\textsubscript{60}L\textsubscript{20} vesicles was maintained at 15 μg/mL, using a DNA complexation +/- charge ratio of 10:1 (final DNA concentration = 2.25 μg/mL), while the concentration of Lipofectamine\textsuperscript{TM} 2000 was maintained at 0.9 μg/mL, using a +/- charge ratio of 2:1 (final DNA concentration = 0.21 μg/mL). R\textsubscript{60}L\textsubscript{20} vesicles and Lipofectamine\textsuperscript{TM} 2000 alone were also investigated using the same concentrations as above. These concentrations were found to be non-toxic to cells. After a 24 hr incubation, the intracellular media was collected and analyzed for the presence of IL-6 using ELISA. Error bars represent the standard deviation from an average of three measurements.

A major cause for IL-6 secretion, which can indicate a likely immune response *in vivo*, is the CpG motif in the plasmid DNA. Reports in the literature have suggested that cationic
liposome/plasmid complexes first enter the endosome. Once inside the endosome, liposomes release the DNA cargo near the endosomal membrane due to the destabilization of the liposomal structure by anionic lipids comprising the endosomal membrane. At the same time, the endosomal membrane becomes destabilized so that free DNA can enter the cytosol. During this process, the CpG motif is recognized by the TLR-9 on the endosomal membrane. This recognition induces the production of proinflammatory cytokines such as IL-6 and tumor necrosis factor-alpha (TNF-α). Other mechanisms that are independent of the TLR-9 pathway have also been reported. Recently, the Z-DNA binding protein-1 (ZBP1) has been shown to act as a cytosolic double-stranded DNA receptor, leading to the induction of type I interferon (IFN) signals and proinflammatory immune responses, especially in RAW264.7 cells.

Similar to cationic liposomes, we have found that the R₆₀L₂₀ vesicles also traffic to the endosome after endocytosis. Because our vesicles also have a cationic surface charge, the complexed R₆₀L₂₀ vesicles may also go through a similar process of DNA release after internalization; however, the DNA/R₆₀L₂₀ vesicle complex may be more resistant against disruption by the anionic lipids near the endosomal membrane. Thus, less DNA is potentially released from the R₆₀L₂₀ vesicles, leading to less recognition by these immune response-initiating receptors.

### 3.4.7. Stability against anionic polymers

To compare the strength of the two delivery vehicles for binding DNA, both plasmid complexes of Lipofectamine™ 2000 and the R₆₀L₂₀ vesicles were exposed to increasing amounts of heparin (Figure 3.9a). Anionic polymers, such as heparin, have previously been shown to compete and displace DNA in a DNA/carrier complex. The first lane from the left, containing
the free plasmid, shows two distinct bands: a bottom band representing plasmid in the supercoiled state and top band representing the linear form. The next lane to the right, where heparin is added to free plasmid, shows that the heparin treatment does not affect the band mobility. The third lane, which contains the polypeptide vesicle/plasmid complex without any heparin treatment has no band appearing at all. This is most likely due to the plasmid being condensed or buried within the vesicle, which prevents EtBr from intercalating with the plasmid DNA. The following lane to the right, which corresponds to the polypeptide vesicle/plasmid complexes treated with heparin at a molar ratio of 5:1 heparin:plasmid, shows bands only near the well of the gel. This result suggests that the heparin treatment at this ratio was able to expose the plasmid to the EtBr, but was not able to fully release the plasmid. The following lanes with heparin treatment at higher molar ratios indicate that some of the plasmid dissociates from the vesicles, even though significant amount of the plasmid still remains bound to the vesicles. In the lanes that contain Lipofectamine™ 2000, a similar trend is observed; however, even at the starting molar ratio of 5:1, the plasmid DNA starts to dissociate from Lipofectamine™ 2000. In addition, significantly more DNA is released than in the case of the R_{60}L_{20} vesicles. These results show that the polypeptide vesicle/plasmid complexes are much more stable against anionic polymers than Lipofectamine™ 2000/plasmid complexes, suggesting that less DNA is likely to be released into the endosome and cytosol in the case of the polypeptide vesicles. However, we currently do not know if the increased stability affects the rate of intracellular DNA release and/or the absolute amount of DNA release from the vesicle. This investigation is of interest and will be pursued in the near future. Nonetheless, this discrepancy between the two carriers could potentially explain the lower transfection efficiency associated with the R_{60}L_{20} vesicles. However, note that heparin is present at a relatively high concentration in blood, and
therefore, a complex being stable in its presence can be beneficial in applications of intravenous drug administration.

**Figure 3.9.** Comparison of the stability of the DsRed plasmid complex formed from the R<sub>60</sub>L<sub>20</sub> vesicles and Lipofectamine™ 2000. (a) Plasmid complexed to either delivery vehicle was treated with increasing molar ratios of heparin (heparin: DsRed from 0 to 800) to estimate the stability of the complex in an anionic endosomal membrane environment. P indicates plasmid only. V+P and L+P indicates R<sub>60</sub>L<sub>20</sub> vesicle complexed with plasmid and Lipofectamine™ 2000 complexed with plasmid, respectively. (P = DsRed plasmid, V = 100 nm R<sub>60</sub>L<sub>20</sub> vesicles, L = Lipofectamine™ 2000). (b) Plasmid complexes were treated with 1 unit of DNase I to assess the stability of the complex against enzymatic digestion. Complexes with or without the DNase
treatment were then treated with trypsin and heparin to separate the plasmid from the delivery vehicle. The trypsin, prior to the heparin treatment, was added to breakdown the polypeptide vesicles into polypeptide monomers. In the “Control” lane, the free plasmid was sequentially treated twice with PBS, instead of trypsin and heparin. P and P+D indicate plasmid only and plasmid treated with DNase, respectively. V+P and L+P indicates R_{60}L_{20} vesicle complexed with plasmid and Lipofectamine™ 2000 complexed with plasmid, respectively. (V+P)+D and (L+P)+D indicate the R_{60}L_{20} vesicle complex treated with DNase and Lipofectamine™ 2000 complex treated with DNase, respectively. (P = DsRed plasmid, V = 100 nm R_{60}L_{20} vesicles, L = Lipofectamine™ 2000, D = DNase I).

### 3.4.8. Stability against DNase I

Lastly, the ability of the R_{60}L_{20} vesicles and Lipofectamine™ 2000 to protect complexed DNA against enzymatic degradation was investigated. Sensitivity of DNA to DNase I digestion while complexed to a vehicle is a method widely used to test the stability of complexes, and cationic liposomes have been shown to provide such protection. Free plasmid, plasmid complexed to R_{60}L_{20} vesicles, and plasmid complexed to Lipofectamine™ 2000 were each exposed to 1 unit of DNase I, followed by a sequential treatment of trypsin/EDTA and heparin. The trypsin cleaves the polypeptide vesicles into monomer blocks, which aids in the separation of the plasmid from the polypeptide during the heparin treatment. The EDTA in trypsin also stops the nuclease activity of DNase I, which prevents it from digesting the plasmids once they are released by the trypsin/heparin treatment.

The first and second lanes from the left of Figure 9b show bands from free plasmid and free plasmid treated with trypsin and heparin; both lanes do not incorporate DNase treatment. The identical bands from both lanes indicate that the treatment of trypsin and heparin does not affect the EtBr staining and mobility of the band. The third lane shows that the naked DsRed plasmid is completely digested by the treatment of 1 unit of DNase I. The fourth lane, which corresponds to the plasmid/R_{60}L_{20} vesicle complex treated with trypsin and heparin but no
DNase, contains mostly bands identical to those from free plasmid without DNase treatment, indicating that the heparin/trypsin treatment is able to almost entirely release the plasmids from the vesicles. The fifth lane contains the R₆₀L₂₀ vesicle/plasmid complex that was treated with DNase I, followed by the trypsin/heparin treatment. As with the previous lane, this lane shows bands that are the same as those observed in the case of the free plasmids without DNase treatment, demonstrating that the plasmid DNA complexed to the vesicles was not digested by DNase. The same trend was observed for the sixth and seven lanes containing plasmids complexed to Lipofectamine™ 2000. These results indicate that the two delivery vehicles are both effective in protecting DNA against enzymatic digestion.

3.5. Conclusions

In this study, we demonstrated the ability of vesicles composed of polyarginine-polyleucine block copolypeptides to transfec plasmid DNA into mammalian cells. Factors that could influence the transfection efficiency of R₆₀L₂₀ vesicles were explored and optimized. Although the in vitro transfection efficiency of the R₆₀L₂₀ vesicles was lower than the current commercially-available Lipofectamine™ 2000 under the optimized conditions, they were able to achieve successful transfection of HeLa cells with minimal cytotoxicity and potentially lower immunogenicity, which are attractive features. Even though other nanostructures, such as micelles and solid nanoparticles with cationic surfaces, can also complex DNA on their surfaces, we could potentially investigate the encapsulation and delivery of genetic material using the aqueous core of the vesicles, a feature that is unavailable for micelles and solid nanoparticles. Since the block copolypeptides that comprise these vesicles can be custom tailored with different
natural and synthetic amino acid residues, we can engineer additional features into future generations of these polypeptides to enhance their transfection efficiency.
4. Endocytosis and Intracellular Trafficking Properties of Transferrin-Conjugated Block Copolypeptide Vesicles

4.1 Overview

In Chapters 2 and 3, we explored the potential of using positively charged polypeptide vesicles as drug carriers through optimization of their building blocks and subsequent application in gene delivery. However, these positively charged vesicles begin to exhibit significant toxicity at higher concentrations, which prevents them from being used to deliver drugs that require high doses to achieve their therapeutic effects. Accordingly, this chapter focuses on our investigation of vesicles comprised of negatively charged block copolypeptides. We previously developed a negatively charged block copolypeptide, poly(L-glutamate)$_{60}$-block-poly(L-leucine)$_{20}$ (E$_{60}$L$_{20}$), which forms spherical vesicles in aqueous solution. The advantage of these vesicles is that they are negatively charged, which could pose minimal toxicity toward cells. However, this also inhibits these vesicles from effectively being internalized by cells, which can be disadvantageous as many therapeutics have intracellular targets. This limitation of the E$_{60}$L$_{20}$ vesicles was overcome by conjugating transferrin (Tf) onto the vesicle surface, since the receptor for Tf is overexpressed on cancer cells. The enhanced uptake of the Tf-conjugated vesicle was verified through confocal microscopy. Furthermore, endocytosis and immunostaining experiments confirmed that Tf conjugated on the vesicle surface plays a critical role in the internalization and subsequent intracellular trafficking of the vesicles.
4.2. Motivation and Background

Block copolypeptide vesicles are a new class of polymeric vesicles that have the properties to become effective carriers for therapeutics.\(^{85,111}\) The amino acid building blocks are naturally occurring, so there is potential for these materials to exert minimal toxic and immunogenic effects toward the host. The existence of many natural and synthetic residues of amino acids can also provide a wide range of chemical properties and biofunctionality. Recently, we developed a block copolypeptide, poly(L-arginine)\(_{60}\)-b-poly(L-leucine)\(_{20}\) (R\(_{60}\)L\(_{20}\)), that forms spherical vesicles in aqueous solution.\(^{44}\) The presence of arginine on the surface of these vesicles enhanced their ability to be internalized by cells, as arginine-rich peptides are known to penetrate across the cell membrane.\(^{142}\) However, one disadvantage of these vesicles is their concentration dependent toxicity, which may limit their application as drug carriers since the vesicles alone at higher concentrations can be quite toxic. One potential application for the R\(_{60}\)L\(_{20}\) vesicles would be to use them as carriers for DNA or siRNA, since the concentration of vesicles required for the delivery of these therapeutics is generally low, where studies have shown that gene expression can be manipulated with picomolar concentrations of genetic material.\(^{143-145}\) In order to be effective as a vehicle for other therapeutics such as doxorubicin (DOX) that require higher doses (micromolar in vitro), higher concentrations of the vesicles need to be administered as well.\(^{4,80}\) Unfortunately, at these higher concentrations, the toxicity of the R\(_{60}\)L\(_{20}\) vesicles alone can be quite significant, indicating that these vesicles would not be suitable for delivering these types of drugs.\(^{146}\)

In addition to the R\(_{60}\)L\(_{20}\) polypeptide, we previously reported an alternative polypeptide vesicle construct, poly(L-glutamate)\(_{60}\)-b-poly(L-leucine)\(_{20}\) (E\(_{60}\)L\(_{20}\)) (Figure 4.1).\(^{20}\) This polypeptide can also form spherical vesicles with sizes that can be controlled and can
encapsulate hydrophilic molecules. Most importantly, these vesicles are less cytotoxic than the R_{60}L_{20} vesicles due to their negative charge, as these vesicles do not have the detrimental effects associated with a positively charged drug carrier interacting with a net negatively charged cell membrane.\textsuperscript{146}

**Figure 4.1.** Schematic of the E_{60}L_{20} block copolypeptide and vesicle assembly.

However, one disadvantage of the E_{60}L_{20} (EL) vesicles, compared to the R_{60}L_{20} vesicles, is their inability to enter cells on their own. This disadvantage poses a limitation, since many therapeutics have intracellular targets. One approach to overcome this limitation is to conjugate a ligand that binds to a specific receptor overexpressed on the cell surface. In fact, transferrin (Tf) is a well-known ligand that binds to its receptor that is overexpressed on the surfaces of many cancer cells.\textsuperscript{147} Tf is an iron-binding glycoprotein (~78,000 Da) that transports iron, an essential nutrient for cellular growth and proliferation, throughout the body. Iron is delivered via
the transferrin receptor (TfR) through a receptor-mediated endocytosis pathway, specifically, a clathrin-mediated endocytosis pathway.\textsuperscript{148-150} Since cancer cells require more iron to sustain their rapid proliferation, TfR is overexpressed on the surfaces of many types of cancer cells.\textsuperscript{149,150} Accordingly, numerous investigations have been performed using Tf as a cancer-targeting agent.\textsuperscript{147,151}

In this study, Tf was conjugated onto the surface of the EL vesicles in order to give the vesicles the ability to target cancer cells and be internalized by them. The intracellular trafficking properties of the Tf-conjugated EL (Tf-EL) vesicles were evaluated using laser scanning confocal microscopy (LSCM) and immunostaining experiments.

4.3. Materials and Methods

4.3.1. Materials

The PSCA-transfected 22Rv1 cell line was generated by retroviral gene transfer, as previously described.\textsuperscript{152,153} The LAPC-4 cell line was kindly provided by Dr. Lily Wu (University of California, Los Angeles). Roswell Park Memorial Institute (RPMI) 1640 medium, Iscove's Modified Dulbecco's (IMDM), penicillin-streptomycin (P/S), sodium pyruvate (NaPyr), phosphate-buffered saline (PBS), and 0.25% trypsin with ethylenediaminetetraacetic acid (EDTA) were purchased from Invitrogen (Carlsbad, California). Fetal bovine serum (FBS) was obtained from HyClone (Waltham, Massachusetts). Dialysis bags (MWCO = 1,000 Da) were purchased from Spectrum Laboratories (Rancho Dominguez, California). The formalin solution used to fix cells was obtained from Thermo Fisher Scientific (Waltham, Massachusetts), and the antibodies for the immunofluorescence experiments were products of Abcam.
Eight-well chambered coverglass units were obtained from Lab-Tek (Rochester, New York). The MTS cell proliferation assay kit was purchased from Promega (Madison, Wisconsin). The Bradford reagent was obtained from Bio-Rad (Hercules, California). Zeba desalt spin columns, N-hydroxysuccinimide (NHS), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Pierce (Rockford, Illinois). Spin concentrators (MWCO = 100,000 Da) were purchased from Millipore (Billerica, California). All other reagents, such as apo-transferrin (apo-Tf), were purchased from Sigma-Aldrich (St. Louis, Missouri) unless otherwise noted.

4.3.2. Cell Culture

The PSCA-transfected 22Rv1 cell line was grown in RPMI 1640 supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at a pH of 7.4. The LAPC-4 cell line was grown in IMDM supplemented with 10% FBS, 1 mM sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin at a pH of 7.4. All cell lines were maintained in a 37°C humidified atmosphere with 5% CO₂ and handled with standard sterile tissue culture protocols.

4.3.3. Synthesis and Processing of EL Vesicles

The EL block copolypeptide was synthesized using the transition metal-mediated α-amino acid N-carboxyanhydride (NCA) polymerization technique, as described previously. The synthesized polymer was deprotected and dialyzed (MWCO = 1,000 Da) exhaustively against sterile Milli-Q water to remove any contaminants under pyrogen-free
conditions. Fluorescein isothiocyanate (FITC) and ethylene diamine (ED) were conjugated together to form fluorescein-ethylene diamine (FITC-ED), which was later used to label the polypeptide for visualization and quantification purposes. Briefly, 2.6 mmol of ED was cooled to 0°C in 2 mL of DMSO. Subsequently, 0.026 mmol of FITC was dissolved in 2 mL of DMSO and added drop-wise to the ED solution while stirring vigorously. The solution was sealed and covered with foil and allowed to react for 4 hrs at 0°C. To this solution, 2 mL of toluene were added, and the volatile compounds were removed by high vacuum. The addition of Toluene was repeated multiple times to ensure removal of all volatile compounds. The FITC-ED was subsequently conjugated to the EL polypeptide using EDC/NHS chemistry. Briefly, 1% w/v EL polypeptide in 2-(N-morpholino)ethanesulfonic acid (MES) buffer at a pH of 6.0 was prepared. EDC, NHS, and FITC-ED were added subsequently, such that the EL polypeptide:EDC:NHS:FITC-ED molar ratios were 4:10:1:1. The mixture was incubated overnight at room temperature. Afterwards, the solution containing the conjugated samples was purified through dialysis (MWCO = 1,000 Da) for 2 days, and the resulting samples were freeze-dried.

In order to form the FITC-labeled EL vesicles, equal volumes of 1% w/v FITC-labeled polypeptide and 1% w/v unlabeled polypeptide were separately prepared in tetrahydrofuran (THF) and then combined together to form a mixture with a 1:1 molar ratio of FITC-labeled to unlabeled polypeptide. Subsequently, an equal volume of sterile Milli-Q water was slowly added to yield a final polypeptide concentration of 0.5 % w/v. The mixture was dialyzed (MWCO = 1,000 Da) against sterile Milli-Q water overnight to remove the THF, where the water was changed every hr for the first 4 hrs. The large polydisperse vesicles were then extruded serially through 1000, 400, and 200 nm polycarbonate membranes to form vesicles with
diameters of approximately 100 nm. The sizes of the vesicles and their distribution were analyzed with dynamic light scattering (DLS) measurements using the Malvern Zetasizer Nano ZS model Zen 3600 (Malvern Instruments Inc., Westborough, Massachusetts). The Bradford assay was performed to quantify the final concentration of polypeptide vesicles according to the manufacture-supplied instructions using the predialyzed samples as the standard.

4.3.4. Iron loading of Tf

Prior to all experiments, apo-Tf was iron loaded to make holo-Tf. Briefly, 20 µL of the iron chelating agent nitrilotriacetate (NTA) was mixed with 10 µL of 250 mM iron (III) chloride. An appropriate amount of this mixture was subsequently added to apo-Tf, dissolved in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer containing 20 mM sodium bicarbonate in a 10:1 iron:Tf molar ratio. The mixture containing iron and Tf was incubated at room temperature for 2 hrs to ensure all the Tf molecules were iron loaded. Excess free iron from the iron-loaded samples was removed with the Zeba desalt spin columns.

4.3.5. Conjugation of Tf ligands to EL Vesicles

In order to conjugate holo-Tf onto these vesicles, the carboxylate groups on the vesicle surfaces were activated using NHS and EDC. 10 mL of vesicles in sterile Milli-Q water (1 mg/mL) were mixed with NHS and EDC, using molar ratios of 5000:1 NHS:vesicle and 5000:1 EDC:vesicle. In order to achieve these ratios, the molecular weight of a single vesicle was estimated (Supporting Information) and used to quantify the number of vesicles in a given
sample. The reaction proceeded for 25 min and then was quenched by adding 10x phosphate buffer (0.5 M) to raise the pH to 7.0. To conjugate Tf to the vesicle surface, holo-Tf (5000:1 Tf:vesicle molar ratio) was added to the suspension of activated vesicles. The solution was then stirred for 2 hrs at room temperature to form the Tf-EL vesicles. Afterwards, the conjugated vesicles were purified using a spin concentrator (MWCO = 100,000 Da) to remove the free Tf and EDC/NHS and to have them in a final buffer of 50 mM HEPES supplemented with 20 mM sodium bicarbonate.

4.3.6. Characterization of Tf-EL Vesicles

The sizes of the Tf-EL vesicles were determined with DLS measurements. The concentrations of the vesicles were obtained by measuring the fluorescence of the FITC conjugated to the vesicle and using a standard curve that was previously constructed with known concentrations of vesicles. The concentration of Tf was quantified with the bicinchoninic acid (BCA) protein assay, using a standard curve that was constructed with known concentrations of Tf in the presence of fixed amounts of vesicles.

4.3.7. Cytotoxicity Study with Tf-EL Vesicles

The CellTiter 96® AQueous Non-radioactive Cell Proliferation Assay (MTS assay) was used to determine cell viability. The cytotoxicity of the Tf-EL vesicles was evaluated using LAPC-4 and PSCA-transfected 22Rv1 cells. All cells were seeded onto each well of a 96-well tissue culture plate with a density of 8x10^4 cells/cm². EL and Tf-EL vesicles were prepared in
growth medium, and their concentrations were varied from 20 to 100 µg/mL. After aspirating the original medium from each well, 100 µL of the prepared medium containing the vesicles was added to each well and incubated for 5 hrs in a humidified environment (37°C, 5.0% CO₂). Subsequently, 20 µL of the MTS reagent was added to each well, and the plate was incubated for an additional 1 hr. Cell viability relative to control wells (cells incubated in media without vesicles) was quantified by reading the visible light absorbance values at 490 nm and 700 nm with an Infinite F200 plate reader (Tecan System Inc., San Jose, California).

4.3.8. Laser Scanning Confocal Microscopy (LSCM)

LSCM images were taken on a Leica Inverted TCS-SP MP Spectral Confocal and Multiphoton Microscope (Heidelberg, Germany) equipped with an argon laser (488 nm blue excitation: JDS Uniphase), a diode laser (DPSS; 561 nm yellow-green excitation: Melles Griot), a helium-neon laser (633 nm red excitation), and a two photon laser setup consisting of a Spectra-Physics Millenia X 532 nm green diode pump laser and a Tsunami Ti-Sapphire picosecond pulsed infrared laser tuned at 768 nm for UV excitation.

4.3.9. Uptake of Tf-EL Vesicles

LAPC-4 and PSCA-transfected 22Rv1 cells were seeded onto eight-well chambered coverglass units using a density of 8x10⁴ cells/cm². FITC-labeled Tf-EL and EL vesicles were diluted in blank media to a concentration of 100 µg/mL. The cells were then incubated with the vesicles in serum-free media for 5 hrs in a 37°C humidified atmosphere with 5% CO₂.
Following this incubation, the media was aspirated, and the cells were washed with PBS to remove any free-floating polypeptide vesicles that were not internalized before the confocal images were taken.

### 4.3.10. Investigating the Endocytosis Mechanism of Tf-EL Vesicles

To elucidate which endocytosis pathways play a role in the uptake of the Tf-EL vesicles, various drugs were used to block specific pathways, and the effect of each drug on the internalization of vesicles was observed. LAPC-4 and PSCA-transfected 22Rv1 cells were seeded onto eight-well chambered coverglass units at a density of 8x10⁴ cells/cm². At the beginning of the experiment, the cell culture media was aspirated, and the cells were pretreated with various endocytosis inhibitors diluted in serum-free media at different concentrations for 30 min. Afterwards, the media containing the inhibitors was aspirated, and the cells were washed with PBS to remove any residual amount of inhibitors on the cell surface. The cells were then incubated in media containing 100 μg/mL of Tf-EL vesicles for 5 hrs. Afterwards, the cells were washed and visualized with the confocal microscope. In addition, the internalization behavior of the Tf-EL vesicle was investigated in the presence of excess Tf. In this case, LAPC-4 and PSCA-transfected 22Rv1 cells were incubated in medium containing both Tf-EL vesicles (100 μg/mL) and an excess of free Tf molecules (100 times the concentration of conjugated-Tf in media).
4.3.11. Intracellular Trafficking of Tf-EL Vesicles

Immunostaining of cells was performed to identify whether the Tf-EL vesicles were localized with the early endosome or lysosome after internalization. LAPC-4 and PSCA-transfected 22Rv1 cells were seeded onto eight-well chambered coverglass units using a density of 8x10^4 cells/cm^2. In the experiments which involved the staining of the early endosomes, cells were incubated in serum-free media containing Tf-EL vesicles (100 μg/mL) for 5 hrs at 16°C instead of 37°C. The purpose of lowering the incubation temperature from 37°C to 16°C was to aid visualization by decreasing the rate at which the Tf-EL vesicles were recycled, since this decrease in temperature has been used when studying the Tf ligand itself. At the end of this incubation period, the vesicles were removed, and the cells were briefly washed with PBS to remove any vesicles on the cell surface. The cells were subsequently fixed with a 10% formalin solution for 10 min at room temperature and washed three times with PBS, where each wash lasted 5 min. After fixation, the cells were permeabilized with a 0.25% Triton X-100 solution for 10 min, followed by three 5 min PBS washes. Nonspecific antibody binding sites on and in the cells were then blocked by incubating the fixed and permeabilized cells with a blocking solution containing 5% nonfat milk in PBS for 30 min. Rabbit polyclonal primary antibodies specific for human early endosome antigen-1 (EEA-1) were then added at a 1:500 dilution in blocking solution and incubated overnight at 4°C. Cells were then washed three times in PBS, where each wash lasted 10 min. These washes were followed by incubation with goat polyclonal antibodies against rabbit IgG conjugated with Cy5. The secondary antibodies were added at a 1:500 dilution in the blocking solution and incubated for 1 hr at room temperature, followed by three more 10 min PBS washes. At the last step, the cells were incubated for 5 min with a 300 nM DAPI solution, followed by three 5 min PBS washes before the LSCM images were taken. Each
image consisted of three channels: green for Tf-EL vesicles, blue for cell nucleus, and red for the labeled intracellular compartments (either the early endosomes or lysosomes). In order to perform the experiments that involved the staining of lysosomes, the above procedure was repeated except for the use of primary antibodies specific for the lysosomal-associated membrane protein-1 (LAMP-1) to stain the lysosomes.

4.4. Results and Discussions

4.4.1. Characterization of Tf-EL Vesicles

After conjugating Tf to the EL vesicles with an incubation ratio of 5000 Tf per vesicle, the size and polydispersity index (PDI) of the conjugated vesicles were measured using DLS. The average diameter of the vesicles was 121 ± 6 nm with an average PDI of 0.243 ± 0.056, which is within the size range that could take advantage of the enhanced permeability and retention (EPR) effect. Fluorescence measurements of the FITC-labeled vesicles and the BCA protein assay were used to quantify the vesicle and Tf concentrations, respectively. These values were subsequently used to quantify the number of Tf conjugated per vesicle, which was approximately 1930 ± 190, representing an average conjugation yield of 38%.

4.4.2. Cytotoxicity Study with Tf-EL Vesicles

The *in vitro* cytotoxicity of the Tf-EL vesicles was assessed using the prostate cancer cell lines and the MTS assay. Figure 4.2 shows that the Tf-conjugated EL vesicles had no toxic effect on the LAPC-4 and PSCA-transfected 22Rv1 cells at concentrations ranging from 20 to
100 μg/mL, as the relative survival of the cells incubated with the vesicles was approximately 100%. This was not surprising since the EL vesicles and Tf molecules are individually nontoxic, and therefore, the conjugate formed from the two should also be nontoxic.

**Figure 4.2.** The results of 5 hr cytotoxicity experiments for the Tf-EL vesicles incubated with LAPC-4 (red) and PSCA-transfected 22Rv1 (blue) cells. Error bars represent the standard deviation from an average of three measurements.

### 4.4.3. Uptake Study with Tf-EL Vesicles

In order to investigate whether the addition of Tf would increase the cellular uptake of the E<sub>60</sub>L<sub>20</sub> vesicles, LAPC-4 and PSCA-transfected 22Rv1 cells were incubated with the FITC-labeled Tf-EL and EL vesicles, and the level of fluorescence inside the cells was visualized using confocal microscopy. For both cell lines, a low level of fluorescence inside the cell was observed when the cells were incubated with the non-targeted EL vesicles, indicating minimal vesicular uptake (Figures 4.3a and c). However, when Tf was conjugated to the FITC-labeled
vesicles, the level of fluorescence was significantly enhanced, implying greater vesicle uptake and improved targeting towards cancer cells (Figures 4.3b and d).

![Figure 4.3. LSCM images of LAPC-4 cells incubated with 100 μg/mL of either (a) non-targeted FITC-labeled EL vesicles or (b) FITC-labeled Tf-EL vesicles for 5 hrs in serum-free media. PSCA-transfected 22Rv1 cells were also incubated with either (c) non-targeted FITC-labeled EL vesicles or (d) FITC-labeled Tf-EL vesicles. Scale bar = 30 μm.](image)

### 4.4.4. Investigating the Endocytosis Mechanism of Tf-EL Vesicles

Once we confirmed the increased uptake of vesicles due to Tf, we sought to determine the mechanism of internalization for these vesicles. Identifying this property can help us determine where the vesicles traffic after internalization, as different mechanisms of endocytosis affect intracellular fates differently. There are three well-studied mechanisms by which molecules are endocytosed by cells: macropinocytosis, clathrin-mediated endocytosis, and caveolae-mediated endocytosis. Macropinocytosis is a nonspecific mechanism for internalizing molecules in the extracellular environment via the formation of membrane ruffles, where the internalized macropinosomes can have sizes up to 5 μm in diameter.\(^\text{155}\) Clathrin-mediated endocytosis is a specific type of receptor-mediated endocytosis, where molecules, such as Tf, are taken up by clathrin-coated vesicles, which have diameters that range from 150 to 200 nm.\(^\text{156, 157}\) Caveolae-mediated endocytosis involves the formation of vesicles in the lipid raft region of the
plasma membrane in a caveolae-dependent manner. The internalized vesicle from the caveolae is the smallest among the three with a diameter between 50 and 80 nm.  

To determine the mechanism of cellular uptake for the Tf-EL vesicles, we used drugs that could inhibit each specific pathway, and observed their effects on the endocytosis of the vesicles. Specifically, LAPC-4 or PSCA-transfected 22Rv1 cells were separately treated with cytochalasin D and amiloride to observe the effect of inhibiting macropinocytosis. In addition, chlorpromazine and dynasore were used to inhibit clathrin-mediated endocytosis, and nystatin and filipin III were used to inhibit caveolae-mediated endocytosis. Initially, the toxic effects of the drugs were investigated with both cell lines, since cytotoxic effects are known to increase membrane permeability, which could confound our endocytosis results. For both cell lines, a range of concentrations for each drug was investigated, and the maximum concentration that exhibited at least 80% survival was chosen for the endocytosis inhibition experiments (Figures A5-A10 in the Appendix).

After determining drug concentrations that were minimally toxic to the cells, the effect of each drug on the uptake of the vesicles was investigated. Incubating LAPC-4 cells with the Tf-EL vesicles without the presence of an inhibitor leads to a high level of vesicle uptake as shown in Figure 4.4a. Pretreating the cells with inhibitors of clathrin-mediated endocytosis, namely, chlorpromazine and dynasore, had the most dramatic effect on inhibiting the vesicle’s uptake (Figures 4.4b and c). In addition, when cells were incubated with an excess amount of free Tf, which enters cells through clathrin-mediated endocytosis, the Tf-EL vesicle’s ability to be endocytosed also decreased, indicating that the excess Tf was able to outcompete the Tf on the vesicles for receptor binding and effectively inhibited the endocytosis of the vesicles (Figure 4.4d). The effects of inhibiting the other pathways, such as macropinocytosis and caveolae-
mediated endocytosis, were also investigated. Treatment with macropinocytosis inhibitors, such as cytochalasin D and amiloride, led to some level of inhibition, but not as significant as the inhibition observed with inhibitors of clathrin-mediated endocytosis (Figures 4.4e and f). Finally, inhibitors of caveolae-mediated endocytosis, namely, nystatin and filipin III, were also tested. In this case, there was minimal reduction in uptake due to these caveolae-mediated endocytosis inhibitors (Figures 4.4g and h). Based on these results, we concluded that clathrin-mediated endocytosis dominates the endocytosis pathway of the Tf-EL vesicles, which implies that the binding of Tf on the vesicles to Tf receptors on the cell surface is responsible for the internalization, since the Tf ligand itself enters cells via this mechanism. It is plausible that macropinocytosis can contribute to the vesicle internalization as well, where membrane ruffles can nonspecifically internalize the Tf-vesicles that are bound to the cell surface through Tf/TfR interactions. To test whether this phenomenon is cell-line dependent, the uptake inhibition experiments were repeated on the PSCA-transfected 22Rv1 cell line (Figures 4.4i-p). The inhibition results were consistent with those from the LAPC-4 cells, suggesting that the endocytosis behavior of the Tf-EL vesicle is not dependent on the cell-line.
Figure 4.4. Inhibition of cellular uptake of the Tf-EL polypeptide vesicles (green) using inhibitors prior to incubation with the vesicles. (a) LAPC-4 control cells with no inhibitor pretreatment. For the inhibition of clathrin-mediated endocytosis, LAPC-4 cells were pretreated with either (b) chlorpromazine or (c) dynasore, or simultaneously treated with (d) excess free Tf (100 times the concentration of conjugated-Tf in media) along with the vesicles. For the inhibition of macropinocytosis, LAPC-4 cells were pretreated with either (e) cytochalasin D or (f) amiloride. For the inhibition of caveolae-mediated endocytosis, LAPC-4 cells were treated with either (g) nystatin or (h) filipin III. The inhibition experiments were repeated with the PSCA-transfected 22Rv1 cells, where the cells were pretreated or simultaneously treated either with (i) media only, (j) chlorpromazine, (k) dynasore, (l) excess free Tf, (m) cytochalasin D, (n) amiloride, (o) nystatin, or (p) filipin III. Scale bar = 30 μm.
4.4.5. Intracellular Trafficking of Tf-EL Vesicles

Molecules and nanoparticles internalized via clathrin-mediated endocytosis and macropinocytosis are generally first trafficked to early endosomes, where they are typically either sorted to lysosomes for degradation or are recycled back to the cell surface.\textsuperscript{160} Moreover, the Tf ligand is well known for being trafficked to early endosomes and then recycled out of the cell.\textsuperscript{161-163} Accordingly, we expected a similar intracellular fate for our Tf-EL vesicles, and immunostaining experiments were performed with cells after they internalized the Tf-EL vesicles to investigate the vesicle trafficking behavior. Initially, LAPC-4 and PSCA-transfected 22Rv1 cells were incubated with the Tf-EL vesicles for 5 hrs at 37\textdegree C, followed by immunostaining and confocal microscopy to visualize the early endosomes, which were stained using EEA-1 (Figures 4.5a and e). Results from this initial study indicated that the vesicles did not colocalize with the early endosomes, which was unexpected as molecules endocytosed by clathrin-mediated endocytosis and macropinocytosis are generally routed to early endosomes. However, reports from other research groups have shown that the Tf ligand recycles very quickly (~5 min), making it difficult to maintain and observe Tf in the early endosomes during the washing steps preceding fixing of the cells for imaging.\textsuperscript{154,164} These same research groups also reported that, by lowering the incubation temperature and the temperature of the wash steps to 16\textdegree C, the recycling of the Tf ligand out of the early endosomes was decreased, allowing significantly more Tf to remain in the early endosomes immediately prior to fixing the cells. After we confirmed that we could observe the Tf ligand itself colocalized with early endosomes at this lower temperature (Figure A11 in the Appendix), we incubated FITC-labeled Tf-EL vesicles with LAPC-4 and PSCA-transfected 22Rv1 cells at 16\textdegree C, and the results of the early endosome immunostaining using EEA-1 are shown in Figures 4.5c and g. Colocalization
between the vesicles and the EEA-1 antibody are represented by yellow or orange fluorescence, since the fluorescent tag on the vesicle is FITC (green), and the fluorescent label on the secondary antibody for the primary antibody for EEA-1 is Cy5 (red). The figures show that there is significant colocalization of Tf-EL vesicles with the early endosomes. These results indicate that the vesicles are routed to the early endosomes, which is consistent with the behavior of other molecules and nanoparticles that are internalized via clathrin-mediated endocytosis and macropinocytosis.

Figure 4.5. Colocalization of Tf-EL vesicles with intracellular compartments. LAPC-4 cells were incubated with Tf-EL vesicles (green) at 37°C, and stained either with (a) EEA-1 (red) or (b) LAMP-1 (red). The same cells were also incubated with the vesicles at 16°C, and stained with either (c) EEA-1 or (d) LAMP-1. PSCA-transfected 22Rv1 cells were incubated with Tf-EL vesicles at 37°C, and stained either with (e) EEA-1 or (f) LAMP-1. The same cells were also incubated with the vesicles at 16°C, and stained with either (g) EEA-1 or (h) LAMP-1. The nucleus for each cell was stained with DAPI (blue). Scale bar = 20 μm.

To determine if the vesicles are routed from the endosomes to the lysosomes for degradation, LAPC-4 and PSCA-transfected 22Rv1 cells were again incubated with FITC-labeled Tf-EL vesicles at temperatures of 37°C and 16°C, followed by immunostaining for LAMP-1 (Figures 4.5b, d, f, and h). There was no colocalization observed between the green
fluorescence signal from the vesicles and the red fluorescence signal from the LAMP-1 immunostaining in either cell line for both temperatures. These results suggest that the Tf-EL vesicles do not traffic to the lysosomes, but probably recycle back to the cell surface. This same recycling fate was expected since de Witte and coworkers used fluorescence uptake experiments to demonstrate that their Tf-conjugated polyethylene glycol (PEG)-liposomes exhibited similar localization and trafficking patterns as the Tf ligand.\textsuperscript{165} Based on our findings, we expect drugs encapsulated in the Tf-EL vesicles to be routed to the early endosomes, and then recycled back to the cell surface. Although one could argue that the recycling of Tf could decrease intracellular release of drugs, and thus decrease their potency, many groups have rather reported increased efficacy from their drug-loaded nanocarriers by conjugating Tf on their surfaces.\textsuperscript{165-167}

4.5. Conclusions

Stable and permanent conjugation between Tf and the E\textsubscript{60}L\textsubscript{20} vesicles allows the formation of nontoxic Tf-conjugated EL vesicles that enhance the cellular uptake of these negatively charged nanocarriers. Subsequent endocytosis and immunostaining studies have shown that the presence of transferrin on the vesicles affects their internalization and intracellular trafficking properties. Clathrin-mediated endocytosis, the pathway by which the Tf ligand is internalized by cells, was identified as the dominant mechanism by which these vesicles enter cells with a smaller contribution from the macropinocytosis pathway. The Tf-EL vesicles were also found to traffic to the early endosomes but not the lysosomes, suggesting that the vesicles are recycled back to the cell surface. The next step is to see whether the increased
internalization of the vesicles could lead to an enhanced therapeutic efficacy of a drug, and this will be discussed in the upcoming Chapter 5.
5. Drug Encapsulation and Drug Carrier Efficacy of Transferrin-Conjugated Block Copolypeptide Vesicle

5.1. Overview

In Chapter 4, we investigated the conjugation of Tf to the EL vesicles by conjugating them with Tf. The Tf-EL vesicles showed enhanced cellular uptake, compared to the EL vesicles, which could potentially enhance the potency of therapeutics that have intracellular targets. In order to test this possibility, we first loaded a common cancer therapeutic, doxorubicin (DOX), inside our vesicles. The drug loading procedure was investigated and optimized using both mathematical modeling and experimentation. In addition, poly(ethylene glycol) (PEG) was conjugated to the vesicle surface to enhance the circulation and stability of the vesicles for future in vivo studies. The PEGylated DOX-loaded vesicles were then conjugated with Tf to form our next generation of vesicle, and the efficacy of the new drug-loaded vesicle was investigated through in vitro cytotoxicity assays.

5.2. Motivation and Background

Spherical vesicles formed from negatively charged E₆₀L₂₀ (EL) polypeptides have certain advantages as delivery vehicles, such as the ability to be prepared with nanoscale diameters, to encapsulate hydrophilic cargo, and exhibit very low cytotoxicity. One of their main disadvantage is that they cannot enter cells efficiently. In Chapter 4, we overcame this challenge by conjugating the vesicle surface with transferrin (Tf) and observed an improved ability of the EL vesicles to be internalized by cells. In addition, results from the immunostaining experiments
showed that these vesicles are likely to recycle back to the cell surface once they are internalized, which is similar to other nanoparticle systems conjugated with Tf. Nonetheless, many systems have still reported an enhanced delivery and efficacy of therapeutics due to the presence of Tf on their nanoparticle surface, leading to an enhanced targeted uptake.

In this study, we wanted to investigate whether the enhanced internalization of our targeted vesicles could also lead to an enhanced therapeutic efficacy of drugs encapsulated in the vesicles. To do this, we first examined the loading of a common cancer therapeutic, doxorubicin (DOX), inside our vesicles. DOX must be inside the cell to exert its chemotherapeutic effect by intercalating DNA and disrupting the cell cycle, making it an ideal candidate to test the effect of our Tf-vesicle, which has the potential to increase the intracellular delivery of DOX to cells.

In addition, in order to make our vehicles more suitable for in vivo applications, we decided to decorate the vesicle surface with PEG. Since Tf and the EL vesicle are both negatively charged, opsonization and eventual clearance by the liver in the in vivo environment is highly likely, as other negatively charged nanoparticles or vesicles have been subject to this outcome in the past. Conjugating PEG onto the EL vesicle surface can create a layer that reduces protein binding and adsorption, and ultimately improves the vesicle’s circulation and biodistribution characteristics. The two features were incorporated to transform the Tf-EL vesicle to a Tf-conjugated, DOX-loaded, and PEGylated EL (Tf-DPEL) vesicle.

DOX was loaded into the EL vesicles using a pH ion-gradient loading method, which is one of the most widely used methods to load this drug into spherical vesicles (Figure 5.1). The driving forces for drug encapsulation in this method are the gradients of pH and ammonium sulfate established across the hydrophobic bilayer of the vesicles. The ammonium sulfate gradient is created by maintaining a high concentration of ammonium sulfate within the aqueous
core of the vesicle, while the outer environment contains a relatively low amount of this salt. The ammonium sulfate in the aqueous core will dissociate into its respective ions, ammonium and sulfate, where the ammonium will dissociate further into ammonia and hydrogen ions. The neutral, hydrophobic ammonia molecule will pass through the vesicle bilayer to the outer environment, while the charged, hydrophilic sulfate molecule will remain trapped inside, leading to a high concentration of sulfate ions within the vesicle. The pH gradient is established by encapsulating an acidic solution in the interior of the vesicles, which is further aided by the generation of hydrogen ions as described above, and buffer exchanging the outside solution to a basic solution, such as tris(hydroxymethyl)aminomethane (Tris) buffer at pH 9.0.

Figure 5.1. Schematic representation of the pH ion-gradient DOX loading method. EL vesicles will be prepared with ammonium sulfate within the aqueous interior. At the basic pH of the outer environment, DOX becomes neutral and hydrophobic, and is able to diffuse into the vesicle interior through the hydrophobic bilayer. Once inside, DOX becomes protonated in the acidic environment and can no longer escape through the vesicle bilayer. DOX then reacts with the...
sulfate anion in the interior to form an insoluble DOX-sulfate complex, effectively trapping it in the vesicle interior.

When DOX is introduced in the outer environment, the outer basic pH favors the equilibrium towards the deprotonated form of DOX, which is hydrophobic and can cross the bilayer. Once inside the acidic inner environment, DOX becomes protonated. A concentration gradient therefore develops for neutral DOX between the interior and exterior environment of the vesicle. The concentration gradient drives the deprotonated DOX into the inner aqueous solution of the vesicle. The positively charged DOX then interacts with the negatively charged sulfate ions to form a neutral DOX-sulfate complex that gets trapped inside the vesicle interior.

There are many different factors that can affect the efficiency of drug loading using the pH ion-gradient loading method: the amount of DOX introduced in the outer environment of the vesicle, the concentration of vesicles used during the procedure, and the amount of ammonium sulfate encapsulated. Even though the above parameters have been optimized for DOX loading in other vesicle systems, such as liposomes, applying the loading procedure to our vesicles required reexamining these parameters so that the loading efficiency could be maximized for our system. One method to achieve this is to experimentally investigate the effect of each parameter. However, due to the number of variables listed above, this method could be time consuming and inefficient. Another method that is more efficient involves using a mathematical model that describes the mechanism of the pH ion-gradient loading to help guide our experiments. We employed a model, developed by Lasic and Ceh, which simulates this loading mechanism into negatively-charged liposomes.170, 171 The model was applied to our EL system to predict trends in DOX loading that improved our understanding of the process and minimized experimentation.
5.3. Proposed Materials and Methods

5.3.1. Materials

The PC3 cell line was obtained from the American Type Culture Collection (Manassas, Virginia). Roswell Park Memorial Institute (RPMI) 1640 medium, penicillin-streptomycin (P/S), sodium pyruvate (NaPyr), phosphate-buffered saline (PBS), and 0.25% trypsin with ethylenediaminetetraacetic acid (EDTA) were purchased from Invitrogen (Carlsbad, California). Fetal bovine serum (FBS) was be obtained from Hyclone (Waltham, Massachusetts). Dialysis bags (MWCO = 1,000 Da) were be purchased from Spectrum Laboratories (Rancho Dominguez, California). The poly(ethylene glycol)$_{5000}$ (PEG$_{5000}$) to conjugate onto the vesicles were be purchased from Nanocs (New York, New York). The MTS cell proliferation assay kit was be purchased from Promega (Madison, Wisconsin). The Bradford reagent was be obtained from Bio-Rad (Hercules, California). Zeba desalt spin columns, N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and 2-iminothiolane (IT) were be purchased from Pierce (Rockford, Illinois). Spin concentrators were purchased from Millipore (Billerica, California). All other reagents, such as apo-transferrin (apo-Tf), were purchased from Sigma-Aldrich (St. Louis, Missouri) unless otherwise noted.

5.3.2. Model of pH Ion-Gradient Loading of DOX into EL Vesicles

Before investigating the DOX loading experimentally, we employed a mathematical in order to predict the effects of varying certain parameters on drug loading. A mathematical model for DOX loading in negatively charged liposomes was developed by Lasic and Ceh.$^{171}$ This
model takes into account the acid-base equilibria of all the species in both interacting compartments (aqueous solutions inside and outside the vesicle), as well as possible binding of the protonated drug onto the inner/outer bilayer surface and formation of a precipitate DOX-sulfate complex inside the vesicle. This model incorporated three assumptions. First, a concentration gradient (or to be rigorous, a chemical potential gradient) drives permeable species toward achieving equilibrium. Second, the permeation of charged species is negligible. Third, all activity coefficients are equal to 1, since all species in the vesicle interior and exterior were assumed to be dilute. Three governing equations were derived, and in a subsequent paper, the authors specified these three equations as necessary for predicting the loading of DOX.\textsuperscript{170} The finalized equations rely on three nonlinear variables: the H\textsuperscript{+} concentration inside the liposome, the H\textsuperscript{+} concentration outside the liposome, and the ratio of the concentration of immobilized drug to free drug inside the liposome. These variables were represented by x, y, and R\textsuperscript{p}, respectively. The electroneutrality of the equilibrated state in the outer vesicle solution is described by the first governing equation:

\[
\begin{align*}
    c_1 \frac{1 - K_{11}K_{12}}{K_p^{-1}(\frac{x}{y})(1+R_p)\left(1+\frac{K_{11}}{x+\frac{K_{11}K_{12}}{x^2}}\right) + \left(1+\frac{K_{11}}{x}+\frac{K_{11}K_{12}}{y^2}\right)} \\
    + 2c_2 \frac{\frac{x}{y}(1+\frac{K_{31}}{x})+K_p\left(1+\frac{K_{31}}{y}\right)}{1+(\frac{K_{31}}{x})+K_p\left(1+\frac{K_{31}}{y}\right)} + \left(y - \frac{K_{\omega}}{y}\right) - c_1 = 0
\end{align*}
\]

The electroneutrality of the equilibrated state of the inner vesicle solution is described as follows:
Finally, the governing equation represents the solubility product (DOX-sulfate complex for our case), $L_p$, is described as follows:

$$
L_p = \left[ \frac{c_1}{K_p^{-1}} \left( \frac{1-K_{11}K_{12}}{x^2} \right) \left( \frac{y}{x} \right) \right]^2
$$

$$
\times \left[ \left[ c_2 - \left( \frac{1}{2} \right) R_p \left( \frac{1+K_{11}K_{12}}{x^2} \right) \left( \frac{y}{x} \right) \right] \left( \frac{K_{42}}{K_{42}+x} \right) \right]
$$

where $K_w$ is the dissociation constant of water. The other parameters are summarized in the following table:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$x$</td>
<td>Equilibrium $[H^+]$ inside vesicles (M)</td>
<td>Unknown</td>
</tr>
<tr>
<td>$y$</td>
<td>Equilibrium $[H^+]$ outside vesicles (M)</td>
<td>Unknown</td>
</tr>
<tr>
<td>$R_p$</td>
<td>Ratio of immobilized</td>
<td>Unknown</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
<td>Value</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>-------</td>
</tr>
<tr>
<td>$K_v$</td>
<td>Ratio of outer volume to inner volume</td>
<td>Depends on Experiment</td>
</tr>
<tr>
<td>$c_1$</td>
<td>Administered concentration of DOX (M)</td>
<td>Depends on Experiment</td>
</tr>
<tr>
<td>$c_2$</td>
<td>Concentration of ammonium sulfate inside vesicles (M)</td>
<td>Depends on Experiment</td>
</tr>
<tr>
<td>$K_{11}$</td>
<td>1st acid dissociation constant for DOX</td>
<td>pKa = 8.2</td>
</tr>
<tr>
<td>$K_{12}$</td>
<td>2nd acid dissociation constant for DOX</td>
<td>pKa = 10.2</td>
</tr>
<tr>
<td>$K_{31}$</td>
<td>Acid dissociation constant for ammonia</td>
<td>pKa = 9.26</td>
</tr>
<tr>
<td>$K_{42}$</td>
<td>Acid dissociation constant for sulfuric acid</td>
<td>pKa = 1.92</td>
</tr>
<tr>
<td>$K_w$</td>
<td>Acid dissociation constant for water</td>
<td>pKa = 13.9</td>
</tr>
<tr>
<td>$L_p$</td>
<td>Solubility product of immobilized drug</td>
<td>$1.1 \times 10^{-7}$</td>
</tr>
</tbody>
</table>

Most values in Table 5.1 were taken from those provided by Lasic and coworkers\textsuperscript{170}. $K_v$ is the volume ratio between the inner aqueous space of the vesicle and the exterior aqueous environment, which is assumed to be dictated by the vesicle concentration in this model. For example, Lasic and coworkers assumed a vesicle concentration of 0.1 v/v % to have a $K_v$ value of $99.9/0.1 \sim 1000$ as described below.

\[
0.1 \text{ v/v } \% = \frac{0.1 \text{ mL vesicle}}{100 \text{ mL total}} \approx \frac{0.1 \text{ mL inner aqueous core}}{100 \text{ mL total}} \quad (4)
\]

\[
K_v = \frac{100 \text{ mL total} - 0.1 \text{ mL inner aqueous core}}{0.1 \text{ mL inner aqueous core}} = 999 \approx 1000 \quad (5)
\]
Parameter $c_1$ is the administered concentration of DOX, and $c_2$ is the initial concentration of the ammonium sulfate in the vesicles during DOX loading. All three of these variables can be determined experimentally. These equations are then solved for $x$, $y$, and $R^p$, which used to calculate the drug loading efficiency, $\alpha$, as described by the following equation:

$$\alpha = \frac{\left(1 + \frac{K_{11}}{x} + \frac{K_{11}K_{12}}{x^2}\right)(1+R^p)}{\left(1 + \frac{K_{11}}{x} + \frac{K_{11}K_{12}}{x^2}\right)(1+R^p) + K_p(\frac{y}{x})\left(1 + \frac{K_{11}}{y} + \frac{K_{11}K_{12}}{y^2}\right)} \tag{6}$$

The above equations were used to predict trends in DOX-loading for our EL vesicles.

### 5.3.3. Cell Culture

PC3 cell lines were grown in RPMI 1640 supplemented with 10% FBS, 100 units/mL penicillin, and 100 $\mu$g/mL streptomycin at a pH of 7.4. The cells were maintained in a 37°C humidified atmosphere with 5% CO$_2$ and handled with standard sterile tissue culture protocols.

### 5.3.4. Synthesis and Processing of EL Vesicles

The EL block copolypeptide was synthesized using the transition metal-mediated NCA polymerization technique as previously described.$^{113}$ Polydisperse vesicles, prior to extrusion, were prepared using the EL polypeptides, as previously described in Chapter 4.
5.3.5. Preparation of Tf-Conjugated, DOX-Loaded, and PEGylated EL (Tf-DPEL) Vesicles

In order to form Tf-DPEL vesicles, the EL vesicle surface was first coated with PEG, followed by the loading of DOX using the pH ion-gradient method. Tf was subsequently conjugated onto the PEG surface of these vesicles to complete the formation of the Tf-DPEL vesicles. Briefly, polydisperse vesicles in sterile Milli-Q water were mixed with an equal volume of 100 mM ammonium sulfate (pH 5.0). The mixture was extruded serially through 1000, 400, and 200 nm polycarbonate membranes in order to encapsulate ammonium sulfate inside the aqueous cores of the vesicles. The sizes of the vesicles and their distributions were analyzed with dynamic light scattering (DLS) measurements using the Malvern Zetasizer Nano ZS model Zen 3600 (Malvern Instruments Inc., Westborough, Massachusetts). The Bradford assay was performed to quantify the final concentration of the polypeptide vesicles according to the manufacture supplied instructions, using the predialyzed samples as the standard.

To conjugate PEG onto the vesicle surface, EDC/NHS chemistry was first performed to activate the carboxylate groups on the EL vesicle surface. Twenty-five thousand-fold excess of EDC and 25,000-fold excess of NHS per vesicle were incubated with the vesicles for 25 min at room temperature. The reaction was then quenched by raising the pH to 7.0 by adding 10x phosphate buffer. Afterwards, a solution containing methoxy-poly(ethylene glycol)\textsubscript{5000}-amine (mPEG) and orthopyridyl disulfide-poly(ethylene glycol)\textsubscript{5000}-amine (biPEG) was incubated with the vesicles for 2 hrs at room temperature, where fold ratios of 12,500 and 12,500 excess per vesicle were used for mPEG and biPEG, respectively. The amine group on both PEG chains allowed them to be conjugated onto the vesicle surface, while the orthopyridyl disulfide (OPSS) group on the biPEG was used later to conjugate Tf.
After the 2 hr incubation period, the reacted mixture was purified using a spin concentrator (MWCO = 10,000 Da) to remove the free mPEG, biPEG, and EDC/NHS. During this process, the exterior buffer of the vesicles was exchanged to 50 mM Tris (pH 9.0) to create a pH gradient between the interior and exterior of the vesicles. DOX (5 mg/mL) was then added to the vesicle solution, and the mixture was incubated at 65°C for 1 hr with intermittent mixing every 10 min. The spin concentrator (MWCO = 10,000 Da) was used again to remove free DOX.

In order to conjugate Tf onto the PEG surface, Tf was initially thiolated with IT. As described in Chapter 4, iron-loaded Tf was prepared in 100 mM borate buffer (pH 8.0), and the iron-loaded Tf was reacted with IT using a molar ratio of 10:1 IT:Tf for 1 hr at room temperature. Afterwards, free IT was removed by centrifugation through Zeba desalt spin columns (Pierce) in 50 mM HEPES buffer (pH 7.0), supplemented with 20 mM sodium bicarbonate. Subsequently, the thiolated Tf was mixed with the PEGylated vesicles in a 10,000:1 Tf:vesicle molar ratio, and incubated for 24 hrs at room temperature. The free thiolated Tf was removed using a sepharose CL-4B column and eluted with 50 mM HEPES (pH 7.0) supplemented with 20 mM sodium bicarbonate. Note that DPEL vesicles were also prepared using the same procedure as above, except that mPEG was exclusively used to PEGylate the vesicles.

5.3.6. Characterization of Tf-DPEL Vesicles

The sizes of the DPEL and Tf-DPEL vesicles were quantified by DLS measurements. The concentration of DOX in the vesicle solution was determined by measuring its UV absorbance at 490 nm. The measured absorbance was correlated to the concentration of DOX using a standard curve that was previously constructed with known DOX concentrations. The concentration of
vesicles was determined by the Bradford assay, as explained in Chapter 4. The DOX encapsulation efficiency was determined by the following equation:

$$\text{Encapsulation Efficiency (\%) = \frac{\text{mass of encapsulated DOX}}{\text{mass of initial DOX}} \times 100} \quad (7)$$

In the addition, the DOX loading ratio was also determined as follows:

$$\text{Loading ratio (\%) = \frac{\text{mass of DOX in vesicle}}{\text{mass of vesicle}} \times 100} \quad (8)$$

### 5.3.7. In Vitro Cytotoxicity Studies with Tf-DPEL Vesicles

PC3 cells ($7.5 \times 10^3$ cells/cm$^2$) were seeded into each well of a 96-well tissue culture plate. After incubating the cells overnight, the growth medium was aspirated and incubated for 96 hrs with 100 μL of fresh growth medium containing varying concentrations of vesicles (0.01 to 10 μM) to allow the vesicles to be internalized by the cells. Subsequently, 20 μL of the MTS reagent was added to each well, and the plate was incubated for an additional 1 hr. Cell viability relative to control wells (cells incubated in media without DOX-loaded vesicles) was quantified by reading the visible light absorbance values at 490 and 700 nm. Growth inhibition of cells at each drug concentration was then defined as 1 minus the relative survival.
5.4. Results and Discussions

5.4.1. Model of pH Ion-Gradient Loading of DOX into EL Vesicles

There are many factors that can affect the loading efficiency of DOX using the pH ion-gradient loading method, such as the concentration of the vesicles (since $K_v$ is directly related to the vesicle concentration as mentioned earlier), concentration of DOX administered, and concentration of ammonium sulfate in the vesicle interior. In order to optimize the encapsulation efficiency of DOX using the ammonium sulfate gradient method, the effects of varying the different parameters need to be evaluated. One way to achieve this is to investigate the effect of each parameter experimentally, which can be time consuming and inefficient. Another way to accomplish this is to use a mathematical model to predict trends that can help guide experiments and allow for reduced experimentation and faster optimization.

In order to model DOX-loading into our E$_{60}$L$_{20}$ vesicles, we adapted a mathematical model developed by Leh and Casic, which predicts the loading of DOX into negatively charged vesicles.$^{170, 171}$ To implement the model, parameters from Table 5.1 were used, where unknown experimental parameters, such as the administered DOX concentration ($c_1$), ratio of outer to inner vesicle volume ($K_v$), and encapsulated inner ammonium sulfate concentration ($c_2$), were varied. Specifically, the DOX encapsulation efficiency as a function of $c_1$ was investigated for different values of $c_2$ (Figure 5.2a) and $K_v$ (Figure 5.2b). Figure 5.2 shows that increasing the DOX concentration decreases its encapsulation efficiency. This is intuitive as the amount of DOX that can be encapsulated is limited by the volume associated with the aqueous cores, while the amount of DOX administered can increase up to its solubility limit. In addition to verifying this expected trend, the model was also beneficial as it predicted that the maximum DOX
encapsulation efficiency could be achieved for a range of DOX concentrations (see plateau regions in the curves of Figure 5.2). This predicted result was not expected \textit{a priori}, and it informed us that we have some flexibility in the concentration of DOX that can be administered to achieve a target DOX encapsulation efficiency. As mentioned in the experimental results section below, this trend was qualitatively observed in our experiments as a range of DOX concentrations yielded similar DOX encapsulation efficiencies. Moreover, the predictions also indicate that the width of the plateau region and the rate at which the encapsulation efficiency decreases with increasing DOX concentration depends on ammonium sulfate and vesicle concentration.

Focusing on Figure 5.2a, the plots represent the predictions from varying the DOX concentration for various values of the ammonium sulfate concentration inside the aqueous core for a fixed $K_v$ value of 100. As indicated in the figure, the model predicts that a higher concentration of ammonium sulfate inside the vesicles allows the vesicles to maintain high encapsulation efficiencies even at high DOX concentrations. This makes sense because higher concentrations of ammonium sulfate correspond to higher concentrations of sulfate ions that can form salt complexes with the positively charged protonated DOX molecules inside the vesicles. This not only traps DOX inside the vesicles, but also helps maintain a high concentration gradient for the deprotonated, hydrophobic form of DOX between the inside and outside of the vesicles, which ultimately drives its mass transfer from the outer to inner environment of the vesicles. Based on these predictions, we experimentally maximized the concentration of ammonium sulfate that could be loaded into our EL vesicles. Specifically, we found that we could load up to 0.05 M ammonium sulfate into our EL vesicles via extrusion without affecting the vesicle stability.
Focusing on Figure 5.2b, the curves represent the predictions from varying the DOX concentration for various values of the vesicle concentration (which is directly related to $K_v$) for a fixed ammonium sulfate concentration in the vesicle of 0.05 M. Higher concentrations of vesicles were found to maintain high encapsulation efficiencies even at higher administered DOX concentrations. This was most likely due to the fact that having higher concentrations of vesicles corresponds to increasing the overall inner space volume that could be used to encapsulate the drug even as the DOX concentration increases. Accordingly, we experimentally maximized the concentration of vesicles during DOX loading. We found that we could perform this loading experiment with a vesicle concentration as high as 0.2 v/v % ($K_v \sim 500$). Any higher concentration led to problems as the higher concentrations led to noticeable clogging of the extrusion filters that we use to process the vesicles into a smaller, monodisperse population.
Figure 5.2. Percent of loaded DOX as a function of administered DOX concentration for varying (a) ammonium sulfate concentrations, $c_2$, and (b) ratios of outer to inner vesicle volume values, $K_V$, which is directly related to vesicle concentration. For (a), the value of $K_V$ was fixed at 100, and the different values of ammonium sulfate concentrations are represented above the curves in units of molarity. For (b), the value of $c_2$ was fixed at 0.05 M and the different values of $K_V$ are represented above the curves. Note that $K_V$ is dimensionless.
Note that the mathematical model developed by Ceh and Lasic is a thermodynamic equilibrium model, representing an upper limit to loading. In addition, if the experimental loading procedure does not achieve equilibrium, other parameters can play a role in the kinetics of drug loading. In particular, the initial pH gradient between the inner and outer aqueous environments can affect the rate of the loading process, where a larger pH gradient is expected to improve the rate of mass transfer. Accordingly, we used an initial inner pH value of 5 and an initial outer pH value of 9 as suggested in the literature.\textsuperscript{173}

### 5.4.2. Characterization of Tf-DPEL Vesicles

PEGylation and DOX loading of the EL vesicles to form the DPEL vesicles was investigated. PEGylated EL (PEL) vesicles were initially formed by conjugating 25,000 mPEG onto one vesicle surface. DOX loading was subsequently investigated, using the understanding gained from the predictions of the model. The drug to polypeptide feed ratio was varied from 2:10 to 4:10 g DOX/g polypeptide. The drug loading ratio, which is the mass of DOX loaded per mass of polypeptide, is a commonly used metric that quantifies the amount of DOX loading within a vesicle system. Table 5.2 shows that the loading ratio increases as the feed ratio (which is directly related to the administered DOX parameter in the model) increases. At the highest feed ratio of 4:10, a drug loading ratio of 0.150 (0.150 g DOX/ g polypeptide) and an encapsulation efficiency of 37.4\% were achieved, while maintaining a stable vesicle size of approximately 94.3 nm in diameter. These diameters are in the appropriate size range that allows the vesicles to elucidate the EPR effect (diameter range = 60 to 400 nm), and
internalization into cells by the clathrin-mediated endocytosis (upper limit of diameter = 150 to 200 nm)\textsuperscript{156,157} and macropinocytosis (upper limit of diameter = upto 5 μm).\textsuperscript{155}

Table 5.2. Drug loading ratio and encapsulation efficiency of DOX in DPEL, Tf-DPEL, Tf-DPEL vesicles.

<table>
<thead>
<tr>
<th>Vesicle Type</th>
<th>DOX/Polypeptide Feed Ratio (g DOX/g Polypeptide)</th>
<th>Loading Ratio (g DOX/g Polypeptide)</th>
<th>Encapsulation Efficiency (g Encapsulated DOX/g Initial DOX %)</th>
<th>Vesicle Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPEL</td>
<td>0.2</td>
<td>0.081</td>
<td>40.5</td>
<td>91.7</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>0.108</td>
<td>35.9</td>
<td>92.9</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.150</td>
<td>37.4</td>
<td>94.3</td>
</tr>
<tr>
<td>Tf-DPEL</td>
<td>0.4</td>
<td>0.108</td>
<td>27.0</td>
<td>126</td>
</tr>
</tbody>
</table>

In order to form the Tf-DPEL vesicles, the conditions that yielded the highest loading ratio and a similar encapsulation efficiency for the DPEL vesicles (25,000 PEG/vesicle molar feed ratio, 4:10 DOX/polypeptide weight feed ratio) were used to first form DPEL vesicles which were then conjugated to Tf. However, with these DPEL vesicles, instead of using only mPEG, a 1:1 mixture of mPEG and biPEG (12,500 mPEG and 12,500 biPEG per vesicle molar) was used for the PEGylation process. Due to also using biPEG, these DPEL vesicles have OPSS groups exposed from the surface that would allow the formation of a disulfide bond with thiolated Tf. The Tf-DPEL vesicles were found to have a lower drug loading ratio (0.108) and encapsulation efficiency (27.0 %) than the DPEL vesicles at the same feed ratio. One possible reason for this observation is that DOX could have leaked from the vesicles during the overnight Tf conjugation and purification procedures. Nevertheless, the loading ratios reported in this
study are comparable to results reported for other vesicle systems.\textsuperscript{174,175} Moreover, the diameter of 126 nm is still within the size range that enables the vesicles to exploit passive tumor targeting via the EPR effect and TfR-mediated endocytosis via clathrin-coated pits.

\textbf{5.4.3. In Vitro Cytotoxicity Studies with Tf-DPEL Vesicles}

The \textit{in vitro} drug delivery efficacies of the DOX-loaded vesicles were then compared to each other. Specifically, DPEL and Tf-DPEL vesicles were administered to PC3 prostate cancer cells over a range of concentrations for 96 hrs, as shown in Figure 5.3. The Tf-DPEL vesicles were more potent than the DPEL vesicles, since, for every percent inhibition of cellular growth value, a lower concentration of drug was required for the Tf-DPEL vesicles. Considering the \textit{IC}_{50} value, or the DOX concentration at which 50\% inhibition of cellular growth occurs, as a metric to quantify the potency of the different vesicle types, the \textit{IC}_{50} values were found to be 0.197 and 0.120 $\mu$M, for DPEL and Tf-DPEL vesicles, respectively.

\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{in_vitro_cytotoxicity_results}
\caption{In vitro cytotoxicity results for the DPEL and Tf-DPEL vesicles in PC3 cells.}
\end{figure}
Although this 1.64-fold difference is modest and currently less than the 2 to 4-fold differences in IC\textsubscript{50} differences reported in literature\textsuperscript{80, 176}, it demonstrates the potential for using these targeted polypeptide vesicles, and suggest that the increased internalization of the vesicles due to Tf enhances the drug carrier efficacy of our DOX-loaded vesicles. Moreover, this investigation represented a proof-of-concept study, and further improvements can definitely be made to the Tf-DPEL vesicle to enhance its capability. For example, the number of Tf ligands on the surface of the vesicle can be optimized. By performing cytotoxicity studies for varying ligand densities, we expect to identify the optimal Tf number for our vesicle system, and take full advantage of the Tf targeting. In addition, we can incorporate a variant form of Tf, previously developed by others in our lab\textsuperscript{177} that undergoes multiple rounds of recycling to potentially increase the conjugated vesicles’ time inside cells and improve drug delivery efficacy.

5.5. Conclusions

In Chapter 5, we were able to demonstrate proof-of-concept that we can develop targeted Tf-conjugated EL vesicles that can encapsulate and deliver DOX to prostate cancer cells. In this development, we employed a mathematical model of the pH-ion gradient loading method to predict trends in DOX loading to guide our experiments and better understand the drug loading process. At this present time, our current loading procedure yields high concentrations of DOX (loading ratios well over 0.10). In addition, the newly designed Tf-DPEL vesicles exhibited a greater potency in inhibiting the growth of prostate cancer cells than the non-targeted DPEL vesicles, demonstrating an improved \textit{in vitro} efficacy via Tf targeting. Through more optimizations of the amount of Tf on the surface and the ligand design itself, we believe that we
can further improve our current vesicle design to improve these systems as carriers for chemoth.
6. Appendix

6.1. Preparation of Block Copolypeptides

K\textsubscript{60}L\textsubscript{y} block copolypeptides were synthesized using the transition metal-mediated \(\alpha\)-amino acid \(\text{N-carboxy-anhydride (NCA)}\) polymerization technique as previously described.\textsuperscript{20} The actual compositions of the synthesized blocks are shown in Table A1.

Table A1. Properties of the K\textsubscript{60}L\textsubscript{y} block copolypeptides

<table>
<thead>
<tr>
<th>Copolypeptide</th>
<th>Actual Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>K\textsubscript{60}L\textsubscript{10}</td>
<td>K\textsubscript{60}L\textsubscript{9}</td>
</tr>
<tr>
<td>K\textsubscript{60}L\textsubscript{15}</td>
<td>K\textsubscript{60}L\textsubscript{13}</td>
</tr>
<tr>
<td>K\textsubscript{60}L\textsubscript{20}</td>
<td>K\textsubscript{60}L\textsubscript{18}</td>
</tr>
<tr>
<td>K\textsubscript{60}L\textsubscript{25}</td>
<td>K\textsubscript{60}L\textsubscript{25}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Determined by integration of proton peaks using \(^1\text{H} \text{NMR.} \)

Copolymer compositions were determined by removing an aliquot (50 \(\mu\text{L}\)) of poly(\(\text{N}_\epsilon\text{-Z-L-lysine})\) from the polymerization mixture after completion of the first segment, which was analyzed by FTIR to confirm that all of the \(\text{N}_\epsilon\text{-Z-Lysine NCA}\) had been consumed. The remainder of the aliquot was diluted to a concentration of 5 mg/mL in DMF containing 0.1 M LiBr for GPC/LS analysis (\(M_n = 15,710; M_w/M_n = 1.18\)). The remaining bulk of the living poly(\(\text{N}_\epsilon\text{-Z-L-lysine})\) reaction mixture was divided into four equivalent aliquots, and the required amounts of L-Leucine NCA were added to give the desired diblock copolypeptide compositions of K\textsubscript{60}L\textsubscript{10}, K\textsubscript{60}L\textsubscript{15}, K\textsubscript{60}L\textsubscript{20}, and K\textsubscript{60}L\textsubscript{25}. The number of leucine residues in each final sample was...
determined by comparing the integrated intensities of leucine and lysine resonances in the $^1$H NMR spectra. These relative compositions were used to calculate absolute compositions based on the known length of the poly(L-lysine) segment. A sample $^1$H NMR spectrum of K$_{60}$L$_{20}$ is shown in Figure A1.

![Figure A1](image.png)

**Figure A1.** $^1$H NMR spectrum of K$_{60}$L$_{20}$ dissolved in deuterated trifluoroacetic acid (d-TFA). (a) lysine methylene resonance, and (b) leucine methyl resonances.

### 6.2. Extrusion of Polypeptide Vesicles

Aqueous vesicle suspensions of the K$_{60}$L$_{20}$ and K$_{60}$L$_{25}$ samples were extruded using an Avanti Mini-Extruder (Avanti Polar Lipids Inc., Alabaster, Alabama). The vesicles were serially extruded through polycarbonate (PC) membranes with 1.0, 0.4, and 0.2 μm pore sizes. For each pore size, the vesicle suspensions were passed three times through the membrane using the mini-extruder. Subsequently, the size distribution of the vesicles was obtained with the Malvern Zetasizer Nano ZS model Zen 3600 (Malvern Instruments Inc., Westborough, Massachusetts). The resulting size distributions for the two samples are shown in Figure A2.
Figure A2. Size distributions of the $K_{60}L_{20}$ and $K_{60}L_{25}$ vesicle samples after serial extrusion through PC membranes with 1.0, 0.4, and 0.2 μm pores. Error bars represent the standard deviation from an average of three measurements.

6.3. Cytotoxicity of $K_{60}(\text{rac-L})_{20}$ Micelles

The $K_{60}(\text{rac-L})_{20}$ micelles were processed using previously reported methods. The MTS cell proliferation assay was subsequently performed to assess the cytotoxicity of the micelle suspension, according to the manufacturer’s supplied instructions. Briefly, HeLa cells were seeded onto a 48-well tissue culture plate at 40,000 cells/cm$^2$ and incubated overnight in a 37°C humidified atmosphere with 5% CO$_2$. The next day, the medium was aspirated from each well, and the cells were incubated with different concentrations of vesicles for 5 hrs. Subsequently, the medium containing polypeptide vesicles was aspirated. Fresh medium containing 20% MTS was then added to the cells. The cells were placed back into a CO$_2$ incubator for 1 hr, and the absorbance at 490 and 700 nm was measured with an Infinite F200 plate reader (Tecan Systems Inc., San Jose, California).
6.4. Individual Vesicle Molecular Weight

An estimate for the molecular weight of each vesicle can be calculated from the following equation:

\[
MW_{\text{ves}} = MW_{\text{pol}} \times n_{\text{pol}}
\]  

where \( MW_{\text{ves}} \) is the molecular weight of a single vesicle, \( MW_{\text{pol}} \) is the molecular weight of a single chain of polypeptide, and \( n_{\text{pol}} \) is the number of polypeptide chains comprising a single vesicle. \( MW_{\text{pol}} \) can be measured from GPC measurements. In order to estimate \( n_{\text{pol}} \), we modeled our polypeptide vesicle as shown in Figure A4.
Figure A4. Schematic representation of the E_{60}L_{20} vesicle and block copolypeptide. Note that the leucine segments of the block copolypeptide is represented as a cylinder with radius R_{L20} and height L_{L20}. The polypeptides are assumed to be organized in a hexagonal close packed conformation. As indicated in the figure, the length of each side of the hexagon is equal to 2R_{L20}. R_{ves} represents the radius of the vesicle, and L_{E60} represents the length of the hydrophilic segment of the E_{60}L_{20} polypeptide.

When the polypeptides self-assemble into spherical vesicles, the hydrophobic bilayer will assume a thickness equal to the length of the hydrophobic alpha-helix of the leucine segment, L_{L20}. Considering a hexagonal close packed configuration, six polypeptides immediately surround a central polypeptide as depicted in Figure A4. Considering only the alpha-helical tail segments, and assuming the alpha-helical tails have a cylindrical shape with a radius R_{L20}, the hexagon can be split into 6 equilateral triangles whose edge lengths are equal to 2R_{L20}. Since the triangles are equilateral, the area of the hexagon (A_{hex}) consists of the central polypeptide and 1/3 of the area of each surrounding polypeptide, totaling 3 polypeptide units. Assuming the surface of the spherical bilayer can be represented as many hexagons, we can calculate the total number of hexagons (n_{hex}) by dividing the surface area of the bilayer (SA_{bilayer}) by the area of one...
hexagon. $S_A_{\text{bilayer}}$ can be found if the values for $R_{\text{ves}}$, $L_{E60}$, and $L_{L20}$ are known, where $R_{\text{ves}}$ is the radius of the vesicle, $L_{E60}$ is the length of the hydrophilic segment, and $L_{L20}$ is the length of the hydrophobic segment of the $E_{60}L_{20}$ polypeptide. For this report, $R_{\text{ves}}$ was measured using the Malvern Zetasizer Nano ZS model Zen 3600 (Malvern Instruments Inc., Westborough, Massachusetts). In order to find $L_{L20}$ we used the fact that alpha helices typically have one turn per 3.6 residues, and the pitch (distance between each turn) is 5.4 Å. Thus, for a leucine segment with 20 residues, $L_{L20}$ would equal 20 residues multiplied by 5.4 Å per 3.6 residues. $R_{L20}$ is 5.5 Å as reported in another study. The average distance of 3.4 Å per residue was used to calculate $L_{E60}$, which is approximated as a random coil.

Once we evaluated $S_A_{\text{bilayer}}$, we estimated $n_{\text{pol}}$ with Eqs. (2) and (3) below:

\[
\frac{S_A_{\text{bilayer}}}{A_{\text{hex}}} = n_{\text{pol}} \quad (2)
\]

\[
n_{\text{pol}} = n_{\text{hex}} \times \frac{2 \text{ Polypeptides}}{\text{Hexagon}} \quad (3)
\]

Subsequently, $n_{\text{pol}}$ was substituted into Eq. (1) to estimate $MW_{\text{ves}}$.

### 6.5. Cytotoxicity of Endocytosis Inhibitors

Investigating the *in vitro* cytotoxicity of endocytosis inhibitors is important, since toxicity to cells is known to increase membrane permeability, which can confound the results of endocytosis experiments. LAPC-4 and PSCA-transfected 22Rv1 cells were seeded on 96-well plates, using a seeding density of $8 \times 10^4$ cells/cm$^2$ for both cell lines. After an overnight
incubation, both cell lines were subsequently exposed to the following inhibitors for 30 min: chlorpromazine, dynasore, cytochalasin D, amiloride, nystatin, and filipin III. For each cell line, a range of concentrations based on literature values was investigated for each drug.\textsuperscript{84} Afterwards, the MTS assay was used to determine cell viability. 20 µL of the MTS reagent was added to each well, and the plate was incubated for an additional 1 hr. Cell viability relative to control wells (cells incubated in media without vesicles) was quantified by reading the visible light absorbance values at 490 nm and 700 nm with an Infinite F200 plate reader (Tecan System Inc., San Jose, California).

The results of the inhibitor cytotoxicity study are shown below (Figures A5-A10). For each drug investigated for a particular cell line, the maximum concentration that exhibited at least 80% survival was used for the endocytosis inhibition experiments. Note that the LAPC-4 cells were much more sensitive to the filipin III treatment than PSCA-transfected 22Rv1 cells, suggesting that toxicity due to an inhibitor can be cell line dependent, consistent with other reports.\textsuperscript{159}

\textbf{Figure A5.} The results of the 30 min cytotoxicity assay for chlorpromazine with (a) LAPC-4 and (b) PSCA-transfected 22Rv1 cells. Error bars represent the standard deviation from an average of three measurements.
Figure A6. The results of the 30 min cytotoxicity assay for dynasore with (a) LAPC-4 and (b) PSCA-transfected 22Rv1 cells. Error bars represent the standard deviation from an average of three measurements.

Figure A7. The results of the 30 min cytotoxicity assay for cytochalasin D with (a) LAPC-4 and (b) PSCA-transfected 22Rv1 cells. Error bars represent the standard deviation from an average of three measurements.
Figure A8. The results of the 30 min cytotoxicity assay for amiloride with (a) LAPC-4 and (b) PSCA-transfected 22Rv1 cells. Error bars represent the standard deviation from an average of three measurements.

Figure A9. The results of the 30 min cytotoxicity assay for nystatin with (a) LAPC-4 and (b) PSCA-transfected 22Rv1 cells. Error bars represent the standard deviation from an average of three measurements.

Figure A10. The results of the 30 min cytotoxicity assay for filipin III with (a) LAPC-4 and (b) PSCA-transfected 22Rv1 cells. Error bars represent the standard deviation from an average of three measurements.

6.6. Intracellular Trafficking of Transferrin

Immunostaining of LAPC-4 cells was performed to confirm the localization of the fluorescein-labeled transferrin (FITC-Tf) proteins within the early endosome after internalization.
FITC conjugation was performed by mixing FITC and Tf in sodium bicarbonate buffer (pH = 8.0), using a molar ratio of 10:1 for FITC:Tf. LAPC-4 cells were seeded on eight-well chambered coverglass units using a seeding density of $8 \times 10^4$ cells/cm$^2$. After an overnight incubation, the cells were incubated with 25 μg/mL FITC-Tf molecules for 5 hrs at 16°C. The purpose of incubating Tf at this temperature was to decrease the rate at which the Tf molecules recycle out of the early endosome.$^{154}$ At the end of this incubation period, the FITC-Tf molecules were removed, and the cells were briefly washed with PBS. The cells were subsequently fixed with a 10% formalin solution for 10 min at room temperature and washed three times with PBS, where each wash lasted 5 min. After fixation, the cells were permeabilized with a 0.25% Triton X-100 solution for 10 min, followed by three 5 min PBS washes. Nonspecific antibody binding sites on the surface and inside the cells were then blocked by incubating the fixed and permeabilized cells with a blocking solution containing 5% nonfat milk in PBS for 30 min. Rabbit polyclonal primary antibodies specific for human early endosome antigen-1 (EEA-1) were then added at a 1:500 dilution in blocking solution and incubated overnight at 4°C. Cells were then washed three times in PBS, where each wash lasted 10 min. These washes were followed by incubation with goat polyclonal antibodies against rabbit IgG conjugated with Cy5. The secondary antibodies were added at a 1:500 dilution in the blocking solution and incubated for 1 hr at room temperature, followed by three more of the 10 min PBS washes. For the last step, the cells were incubated for 5 min with a 300 nM DAPI solution, followed by three 5 min PBS washes before images were taken with the laser scanning confocal microscope. Each image consisted of three channels: green for Tf, blue for cell nucleus, and red for the early endosomes. The result from Figure A11 shows that there is definitely colocalization between the green florescence signal from the Tf and the red florescence signal from the early
endosome, indicating the presence of Tf in the early endosome. This result is consistent with reports in the literature, where the transferrin protein has been shown to traffic to the early endosome upon endocytosis.\textsuperscript{154}

![Image](image.png)

**Figure A11.** Colocalization of FITC-Tf with the early endosome. LAPC-4 cells were incubated with FITC-labeled Tf (green) at 16°C and stained with EEA-1 (red). The nucleus for each cell was stained with DAPI (blue).

6.7. Matlab Code of Model of pH Ion Gradient Loading of DOX into EL Vesicles

The following Matlab code contains equations and constants that were used to model the DOX-loading efficiency within our EL polypeptide vesicle. Three governing equations, derived by Ceh and Lasic,\textsuperscript{170} were adapted to our EL vesicle system. The following Matlab code, SOLVEEQS.M, calls the EQNS.M function to calculate the DOX encapsulation efficiency while
varying the following parameters: the administered DOX concentration ($c_1$), the interior ammonium sulfate concentration ($c_2$), and the ratio of outer to inner vesicle volume ($K_v$).

```matlab
% SOLVEEQS.M

close all; clear all;
hold on;

% Administered DOX concentration (x-axis)
min=0;
step=.0001;
max=.01;

% Data Container Initialization
final=zeros(((max/step)+1), 5);

% Ratio of volume in the outer environment over volume in the inner aqueous core of the vesicle
Kv=[30 100 1000 10000];

% Starting interior ammonium sulfate concentration
c2=[.01 .05 .1 .2];

i=1; % Input increment
for j=1:4; % Loop over Kv
    for k=1:4; % Loop over c2
        for cl=min:step:max; % Initial DOX concentration
            guess=[1E-5, 1E-7, 450];
            [result,fval,exitflag,output]=fsolve(@(x) eqns(x,Kv(j),cl,c2(k)),guess);
            answer=(1+result(3))/((1+result(3))+Kv(j)*(result(2)/result(1)));
            final(i,:)=[(cl, answer, result(1), result(2), result(3));
            i=i+1;
        end
    end
    plot(final(:,1),final(:,2));
end

axis([0 .01 0 .1]);
ylabel('DOX Encapsulation Efficiency (%)');
xlabel('DOX Administered Concentration (M)');

%EQNS.M

function fcns=eqns(x,Kv,cl,c2)
```

117
%Constants
pK42=1.92; K42=10^-(pK42); %sulfuric acid
Lp=1.1E-7; %DOX-sulfate solubility product

%Governing Equations
fcns(1)= c1/(Kv^1*(x(1)/x(2))+(1+x(3))^1) + 2*c2/((x(1)/x(2))+Kv) + x(2) - c1;

fcns(2)= (c1/(Kv^1*(1+x(3))+(x(2)/x(1))))*(1-(1/2)*x(3)*(-1-(2*K42/x(1)))/(1+(K42/x(1)))) + c2*(-1-(2*K42/x(1)))/(1+(K42/x(1))) + 2*c2/(1+Kv*(x(2)/x(1))) + x(1);

fcns(3)= (c1/(Kv^1*(1+x(3))+(x(2)/x(1))))^2 * (c2-((1/2)*x(3)*c1/(Kv^1*(1+x(3))+(x(2)/x(1)))))*K42/(x(1)+K42)) - Lp;
7. References


