Synergistic Nanoparticle Formulations against Multi-Drug Resistant Breast Cancers

Petro Pawlo Czupiel

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Chemical Engineering and Applied Chemistry
Institute of Biomaterials and Biomedical Engineering
University of Toronto

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Abstract

Current breast cancer treatments lack specificity, leading to dose-limiting cardiotoxicity, which is often coupled with multi-drug resistance (MDR), and thus severely impedes efficacious cancer treatment. Current efforts to utilize chemotherapeutics and MDR inhibitors have failed in the clinic due to low efficacy, off-target toxicities, and altered pharmacokinetics. Synergistic nanoparticles (NPs) offer spatiotemporal control of therapeutics from the injection site to the tumor site. Synergistic NPs co-encapsulate two or more drugs, whereby the co-encapsulated drugs exhibit anti-cancer activity greater than the sum of the anti-cancer activity exhibited by the single-drug loaded NPs. This thesis demonstrates that doubly-loaded NPs are more efficacious against multi-drug resistant (MDR) breast cancer cells in vitro, relative to each drug alone or combined, singly-loaded drug NPs. The NPs, comprising poly(D,L-lactide-co-2-methyl-2-carboxytrimethylene carbonate)-graft-poly(ethylene glycol)-azide (P(LA-co-TMCC)-g-PEG-N₃), were used to co-encapsulate two therapeutics and evaluate their synergistic cytotoxicity of MDR cancer cells: vitamin E succinate modified with octahistidine-octaarginine (VES-H₈R₈) and a pH-responsive prodrug of doxorubicin (pDox).

The mechanism of the anti-cancer activity of VES-H₈R₈ comprised MDR efflux inhibition, mitochondria depolarization with inhibited bioenergetics, and induction of reactive oxygen species, apoptosis and G1 cell cycle arrest. When VES-H₈R₈ was co-encapsulated with pDox in
NPs, synergistic anti-cancer activity against MDR breast cancer cells was observed, but only when co-encapsulated NPs were administered.

Work towards NP-mediated delivery of siRNA targeted against an essential protein, eukaryotic translation initiation factor 3 subunit B (eIF3B), is also presented; however, limitations associated with endo/lysosomal accumulation terminated the use of NPs comprising P(LA-co-TMCC)-g-PEG-N₃.

This thesis provides the groundwork to study synergistic anti-cancer activity against MDR breast cancer in vivo.
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1 Introduction

1.1 Rationale

Among Canadian women, breast cancer is a devastating disease representing 25.5% of new cancer cases and 13.1% of cancer deaths.\cite{1} Current breast cancer chemotherapeutics are poorly soluble and lack specificity, which leads to dose-limiting cardiotoxicity.\cite{2, 3} Moreover, the common occurrence of multi-drug resistance (MDR) severely impedes full cancer treatment. MDR arises from cancer cells expressing efflux pumps that actively pump out chemotherapeutics, thereby reducing the efficacy of the chemotherapeutic.\cite{2} Current MDR inhibitors failed in the clinic due to low efficacy, off-target toxicities, and altered pharmacokinetics with other chemotherapeutics.\cite{4} Traditional co-administration of chemotherapeutics and MDR inhibitors are also limited by the absence of control over spatiotemporal distribution whereby each drug exhibits different pharmacokinetics.\cite{5} As a result, delivery vehicles on the nanometer scale have been developed to solve the problems of toxicity, low efficacy and local synergy.

Nanoparticles (NPs) have been established as a delivery platform for poorly soluble drugs, with the capacity to encapsulate multiple drugs.\cite{6} Furthermore, NPs can passively target rapidly grown tumors in vivo by exploiting the enhanced permeability and retention (EPR) effect.\cite{7} The EPR effect helps to explain the greater accumulation of NPs in tumors relative to normal organs, whereby the tumors exhibit leaky vasculature with immature lymphatic drainage. This passive targeting reduces off-target toxicities by altering the biodistribution of the drugs. Importantly, co-encapsulating multiple drugs in NPs permits a synergistic drug ratio to be maintained from injection site to tumor tissue. The synergistic NP formulation of cytarabine and daunorubicin, Vyxeos, is a notable example of clinical success for the treatment of acute myeloid leukemia that paved the way towards other synergistic chemotherapeutic combinations.\cite{4}

In this work, I propose to engineer a NP formulation capable of delivering two drugs: (1) a peptide-modified vitamin E succinate, (2) a pH-sensitive doxorubicin prodrug. Work is also presented towards the nanoparticle-mediated delivery of a small interfering ribonucleic acid (siRNA) targeting eukaryotic transcription initiation factor 3 subunit B (si3B). The peptide-modified vitamin E succinate affects mitochondrial function and reduces drug efflux in MDR
cells, the pH-sensitive doxorubicin prodrug releases doxorubicin in lysosomes and inhibits DNA replication. The si3B targets an essential protein required for messenger ribonucleic acid (mRNA) transcription. The outlook of the synergistic NP formulation encompasses a lower total dose of drugs administered in vivo with a potential reduction in incidence of MDR.

1.1.1 Hypothesis and Objectives

The hypotheses governing the body of this work is:

*When compared to each drug alone, or single drug encapsulated nanoparticles (NPs), NPs co-encapsulating peptide-modified vitamin E succinate, and a pH-sensitive prodrug of doxorubicin will be more efficacious against multi-drug resistant (MDR) breast cancer cells in vitro.*

To test this hypothesis, this work was divided into two primary objectives:

1) Investigate the mechanism of cell death induced by vitamin E succinate modified H₈R₈ and similar lipid modified peptides.

In Chapter 2, I synthesized lipid modified and vitamin E succinate modified octaarginine-octahistidine (H₈R₈) and investigated the in vitro mechanism of anticancer activity against parental and MDR breast cancer cells. I established that a hydrophobic threshold existed for the lipid modified H₈R₈ in order to exert potent anticancer activity. Importantly, the vitamin E succinate modified H₈R₈ exhibited significant efflux inhibition, suitable for co-encapsulation in NPs with other chemotherapeutics.

2) Investigate the synergism between vitamin E succinate modified H₈R₈ (VES-H₈R₈) and a pH-sensitive prodrug of doxorubicin (pDox) on MDR breast cancer.

In Chapter 3, I investigated the synergism of co-encapsulated VES-H₈R₈ and pDox. Here, a stable co-encapsulated NPs was prepared and validated for pH-dependent release of doxorubicin. Importantly, only the co-encapsulated NPs exhibited synergistic anti-cancer activity against the MDR breast cancer cells in vitro, whereas the synergistic anti-cancer activity was lost when either free drugs or singly-loaded NPs were combined.

Additionally, work is presented in the investigation of the following hypothesis:
Targeted NP delivery of eIF3B siRNA (si3B) will effectively knockdown eIF3B mRNA and protein, and induce cell death in human epidermal growth factor receptor 2 (HER2) overexpressing breast cancer cells in vitro.

To test this hypothesis, the following objective was pursued:

3) Design an effective NP-mediated delivery system for si3B.

In Chapter 4, I investigated the in vitro NP delivery of siRNA targeting eukaryotic translation initiation factor 3 subunit B (eIF3B), an essential protein in breast cancers. A potent siRNA targeting eIF3B was established and knockdown was verified at the mRNA and protein levels of eIF3B. Efforts were made to observe knockdown mediated by NP delivery of the siRNA; however, our strategies were ultimately unsuccessful, thereby requiring further investigations in other siRNA delivery vehicles.

1.2 Breast Cancer Treatment, Multi-Drug Resistance and Cancer Mitochondria

1.2.1 Current Treatment Options

Breast cancer is a traumatic disease while being the most common cancer in women worldwide, claiming an estimated 627,000 women in 2018.[8, 9] Localized cancers are treated with surgery and/or radiation while systemic cancer treatment involves either a monotherapy or a combination treatment of chemotherapy, immunotherapy, hormonal, or radiopharmaceutical therapy. Chemotherapy encompasses small molecules that exhibit potent but typically, non-selective anti-cancer activity associated with adverse dose-limiting side effects. Immunotherapy utilizes full or partial antibodies, such as Herceptin (anti-human epidermal growth factor receptor 2 (HER2)), to specifically bind to overexpressed receptors, either inducing apoptosis or inhibiting an intracellular downstream cascade. Hormonal therapy seeks to either add or inhibit hormones, such as estrogen receptor blockers, or aromatase inhibitors. Radiopharmaceuticals encompass radioactive agents such as radium and iodine that are administered orally or intravenously, and kill cancer cells through the released radiation. However, there is a growing concern regarding the potential leukemogenic and carcinogenic effects of radiopharmaceuticals, in addition to the required extensive radiation protection, making chemotherapeutics a more favorable option.[10] While immunotherapy and hormonal therapy are applied to a subset of
breast cancer patients dependent on specific criteria, such as the overexpression of HER2, the majority of the patients are treated with chemotherapeutics. The treatment response is highly variable and dependent on health, age, and cancer stage of the patient.[11] Additionally, tumor heterogeneity plays a major role in treatment response and is a major contributor to resistant tumors that are capable of thriving under clinically used doses of the chemotherapeutic.[12] Current chemotherapeutics used in the clinic typically target rapidly dividing cells and are categorized as alkylating agents (cisplatin), antimetabolites (5-fluoro uracil), topoisomerase inhibitors (irinotecan, doxorubicin), and mitotic inhibitors (docetaxel, paclitaxel).[13] The majority of the chemotherapeutics are hydrophobic molecules requiring large volumes of surfactants for solubilization that leads to allergic reactions in patients.[14] The use of toxic surfactants, poorly selective chemotherapeutics, and prevalence of resistant tumors have resulted in the need for the development of cancer-specific intracellular targets, as well as safer delivery vehicles, such as NPs.

1.2.2 Multi-Drug Resistance

Despite advances in chemotherapeutics against cancers, the 5-year survival rates have not significantly increased, primarily due to multi-drug resistance (MDR).[15] MDR arises from cancer cells becoming resistant upon continuous exposure to a chemotherapeutic, permitting the cancer cells to proliferate under clinically used doses.[16] MDR cancer cells are resistant either through drug dependent, target dependent, or drug and target independent pathways. Drug dependent resistance arises mainly through overexpression of drug efflux pumps on the plasma membrane that effectively reduces the intracellular concentration of the drug, requiring higher doses to kill the cancer cell.[15] Unfortunately, the higher doses of the chemotherapeutic required to kill the now resistant cancer cells are complicated by off-target toxicities, such as hepatotoxicity. Target dependent resistance arises from the cancer cells adopting an alternative pathway for survival through deletion, mutation, translocation, or amplification of the target.[17] Target and drug independent resistance arises from cancer cells altering the cell signaling pathways genetically or epigenetically to become less sensitive to the drug.[18] The largest contributor to resistance observed in the clinic is drug dependent resistance that relies heavily on the overexpression of the adenosine triphosphate-binding cassette (ABC) superfamily of transporters.[17] The ABC transporters actively pump out a variety of hydrophobic and hydrophilic chemotherapeutics, consuming adenosine triphosphate (ATP) in the process (Figure
1.1. ABC transporters consist of 48 members broken down into seven subfamilies and are composed of two nucleotide-binding domains and two transmembrane domains. The largest contributors to MDR in the clinic are plasma membrane-bound transporters such as breast cancer resistance protein (BRCP/ABCG2), multidrug resistance protein (MRP2/ABCC2) and permeation-glycoprotein (Pgp/ABCB1).[19, 20]

First identified in 1975 by Ling et al. as a 170 kDa membrane glycoprotein, Pgp functions to protect against toxicants and xenobiotics and is widely expressed in the body such as in the liver, luminal blood-brain barrier, placenta, and kidney.[21] Of note, Pgp substrates often include molecules with a protonated nitrogen and aromatic rings, but other molecules missing these properties have been identified as substrates as well.[18] Efforts have been made to directly inhibit Pgp efflux in the clinic, pushing forward three generations of inhibitors. The Pgp inhibitors and the pitfalls will be further discussed in Chapter 1.3.1. To date, there are no Food and Drug administration (FDA) approved efflux inhibitors, prompting the search for efflux inhibition through the indirect method of mitochondrial targeting. The MDR cancer cells efflux a chemotherapeutic through ABC transporters in an ATP-dependent pathway, whereby ATP is generated solely in the mitochondria.[22] Therefore, targeting the mitochondria can sufficiently deplete the levels of ATP, and thus inhibit efflux pumps.

![Figure 1.1 Schematic representation of permeation glycoprotein (Pgp) effluxing a chemotherapeutic out of cell. Pgp is a 170 kDa protein comprised of transmembrane domains (blue) and nucleotide binding domains (grey) located in the cytosol (light green). A membrane bound chemotherapeutic or a chemotherapeutic in the cytosol binds onto Pgp, and adenosine triphosphate (ATP) binds onto the nucleotide binding domains. ATP is hydrolyzed to adenosine](image)

[5]
diphosphate (ADP) and phosphate (Pi) in order to pump out the chemotherapeutic extracellularly

1.2.3 Cancer Cell Mitochondria

The mitochondria of eukaryotic cells are remnants of an ancient endosymbiotic event preserved by evolution with a semi-autonomous status.[23] The mitochondria houses its own mitochondrial DNA coupled with transcription/translation machinery while providing eukaryotic cells with cellular energy through oxidative phosphorylation (OXPHOS) generating ATP, as well as regulating death pathways, such as apoptosis.[24] Secondary functions of the mitochondria include fatty acid oxidation, amino acid metabolism, harboring the urea and tricarboxylic acid cycles, regulating reactive oxygen species (ROS) and calcium homeostasis.[25] As the mitochondria houses many complicated pathways, the structure of the mitochondria is equally as complicated. For instance, the mitochondria consist of two membranes: the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM) (Figure 1.2).[25] The IMM differs from the plasma membrane of eukaryotic cells in that the IMM contains three times more protein than lipid relative to the plasma membrane, as well as containing more hydrophobic and saturated phospholipids, such as cardiolipids.[26, 27] Specifically, the cardiolipin composition in mitochondrial membranes are approximately 20% of the total lipid content, and harbor lower levels of sphingolipids and cholesterol relative to plasma membranes.[28-30] The high cardiolipin composition in the inner mitochondrial membrane supports a functional ETC for OXPHOS as cardiolipin has been demonstrated to be essential for optimal activity of complexes I, III, IV, and V.[30] ATP is generated through OXPHOS which consists of the electron transport chain (ETC) and ATP synthase located on the IMM.[23] Furthermore, the ETC consists of four complexes, known as complex I, II, III, IV, and primarily acts to accept and transfer electrons in a coordinated way to produce water and ATP.[31] The four complexes of the ETC are organized into supercomplexes for efficient electron transport while minimizing premature escape of electrons that ultimately result in harmful ROS.[32] Coupled with the ETC is the active transport of protons across the IMM, which generates an IMM membrane potential ($\Delta \Psi_m$) meaning that the mitochondria matrix within the IMM is negatively charged. ATP synthase (complex IV) exploits the proton gradient to balance the $\Delta \Psi_m$, for the generation of ATP.[31] Interestingly, the mitochondria of cancer cells has been documented to be more polarized relative to non-
cancerous cells, in order to keep up with the growing demands of the tumor and provide sufficient ATP, thereby providing an exploitable intracellular target in cancer therapy.

A century ago, the mitochondria of cancer cells were thought to be OXPHOS-defective, where cancer mitochondria relied heavily on glucose metabolism and lactate production.[33] This work stemmed from Otto Warburg and was related to inefficient aerobic glycolysis.[34] The ‘Warburg’ effect is the dysregulation of growth signaling due to the inactivation of tumor suppressors in cancer and activation of oncogenes.[35] As mitochondria act as central hubs for cell death induction, ROS generation, and cellular homeostasis, they represent a unique intracellular target in cancer therapy. Importantly, cancer mitochondria are hyperpolarized relative to non-cancer cells. For example, Neu4145 cancer cells exhibit a mitochondrial membrane potential of -210 mV, whereas healthy cells typically exhibit mitochondrial membrane potentials in the range of -108 to -159 mV.[36, 37] For reference, the plasma membrane potential of cancer cells has been measured to be -10 - -45 mV.[38] The hyperpolarization of cancer cell mitochondria provides an exploitable opportunity for intracellular targeting, through electrophoretic attraction to the negatively charged mitochondria membrane. Targeting the mitochondria has the potential to reverse MDR by inhibiting ATP, and thus reducing the capabilities of the efflux pumps while also simultaneously inducing apoptosis that commences within the mitochondria. Furthermore, tumor initiating cells, a subpopulation found within tumors that are responsible for the recurrence and metastatic propensity for cancer cells, have been shown to heavily depend on functional ETC and OXPHOS in several cancers.[39] By targeting the mitochondria, both tumor initiating cells and aggressively growing cancer cells can be eliminated resulting in easier to treat tumors with a suppression of tumor metastasis.[40] Small molecules, antibodies, and cationic peptides have been designed to target the mitochondria, with limited clinical success due to toxic side effects, such as the detrimental effects on the mitochondria of cardiomyocytes, as well as delivery challenges.
Figure 1.2 Schematic representation of eukaryotic mitochondria. The mitochondria are comprised of outer mitochondrial membranes and inner mitochondrial membranes (IMM) that houses the mitochondrial matrix. Adenosine triphosphate (ATP) is generated through oxidative phosphorylation which consists of the ATP synthase and the electron transport chain (ETC) located on the IMM. The ETC is comprised of complexes I, II, III, and IV where electrons are efficiently transferred, and using the proton gradient, ATP is produced. The ETC attempts to transfer electrons efficiently, however some premature release occurs where reactive oxygen species (ROS) are produced. The mitochondrial matrix also harbors the tricarboxylic acid cycle (TCA), of which pyruvate (Pyr) and acetyl coenzyme A are fed into the TCA.

1.3 Emerging Cancer Therapeutics

Although clinically administered chemotherapies have remained relatively unchanged for the past 20 years, novel and exciting chemotherapeutics are in the pipeline to clinical trials. For example, MDR inhibitors, mitochondrial targeted therapies, doxorubicin analogs and RNAi have gained traction in the past decade (Figure 1.3).
Figure 1.3 Overview of some of current and emerging cancer chemotherapeutics. A) Structure of the efflux and mitochondrial inhibitor, vitamin E succinate. B) Structure of a mitochondrial penetrating peptide with the following amino acid sequence: Fx-R-Fx-K, where Fx, R and K are single letter amino acid codes for cyclohexylalanine, arginine and lysine, respectively. C) Structure of the clinically administered anthracycline, doxorubicin. D) Representation of a single guanine-cytidine hybridization pair that would be found in small interfering ribonucleotides (siRNA) where siRNA typically comprise of 21-27 paired nucleotides.

1.3.1 Multi-Drug Resistance (MDR) Inhibitors

One of the obvious ways to circumvent MDR is to co-administer efflux inhibitors with chemotherapeutics to increase the intracellular concentration of the chemotherapeutic. However, there are no FDA approved efflux inhibitors, mainly due to ineffective tumor reduction or major toxicity concerns.[41] Progress to reduce MDR in cancer has pushed the development of three generations of efflux inhibitors. The first generation of efflux inhibitors included verapamil and cyclosporin A and were mainly non-specific Pgp inhibitors that exhibited similar toxicity in cancer and normal cells.[42] Second generation efflux inhibitors included PSC-833 and Valspodar (VX-710) with the added benefits of higher potency and selectivity on efflux inhibition, with less toxic side effects.[43, 44] While the administration of PSC-833 with some chemotherapeutics provided minimal advantage in the clinic, the combination altered the absorption, distribution, metabolism and excretion of both drugs that led to significant toxicity in
cancer patients.[45] Therefore, drug-drug interactions confined the progress of second generation inhibitors.[46] The third generation of efflux inhibitors include LY335979, XR9576, R101933, and GF120918, and were generated with enhanced selectivity to specific efflux pumps relative to second generation inhibitors, however the clinical benefits are dismal.[45] One potential reason is that third generation inhibitors are specific to one efflux pump, resulting in compensation through other efflux pumps, thereby cancelling out effective efflux inhibition. Another potential reason is that the efflux inhibitor and the chemotherapeutic may not be colocalizing at the tumor site due to differences in pharmacokinetics and physicochemical properties of both drugs.[15] Therefore, the efflux capability of the MDR cancer cell is ineffectively inhibited, and the cancer cell proliferates. Furthermore, ABC efflux transporters are ubiquitously expressed in normal human tissues, including the kidney and liver, where efflux inhibitors can ultimately amplify undesired toxicity by means of saturating drug-metabolizing enzymes.[46] Altogether, there is a need to deliver efflux inhibitors and chemotherapeutics together to ensure that both are exposed to the cancer cells for effective efflux inhibition while increasing the intracellular concentration of the chemotherapeutic.

1.3.1.1 MDR and the Mitochondria Connection

Drug efflux is an active transport requiring ATP that is exclusively produced in the mitochondria.[47] This process, coupled with the hyperpolarization of cancer mitochondria has driven mitochondrial-targeting drug development. Chemotherapeutics that target any of the functions of the mitochondria of cancer cells are termed “mitocans,” an acronym for mitochondria and cancer. Eight classes of mitocans exists based on the molecular target of the mitocan starting from interaction with proteins on the surface of the outer mitochondrial membrane, to affecting pathways within the mitochondrial matrix, such as mitochondria DNA or the tricarboxylic acid cycle.[48] Mitocans exhibit selective anti-cancer activity as cancer cells harbor more polarized mitochondria and show lower levels of antioxidant defenses with higher levels of oxidative stress, priming them to be more sensitive to induced ROS and apoptosis.[49] Using mitocans in the clinic is a challenge where toxic side effects impede the continued use of the mitocan.[23] Notably, vitamin E succinate (VES) exhibits potent and selective anti-cancer activity and is recognized as a class 5 mitocan, affecting the ETC by specifically inhibiting complex II (succinate dehydrogenase) (Figure 3A). [50] VES is able to suppress angiogenesis through an ROS-dependent disruption of the fibroblast growth factor 2 (FGF2) pathway.[51]
Moreover, mitochondrial targeted VES (MitoVES) efficiently eliminated tumor initiating breast cancer cells in a complex II-dependent manner while exhibiting enhanced apoptosis with superior anti-cancer activity in animal models.[52-54] VES or its analogs have not progressed to FDA approval either as a monotherapy or combination therapy due to insufficient benefits in the clinic. One reason may be the hydrolysable ester found within VES, that is rapidly degraded in the blood, calling for a delivery vehicle to be used to protect the ester.[55] Other mitocans have been designed through peptide chemistry, termed mitochondrial penetrating peptides (MPPs), with alternating amino acids bearing cationic and hydrophobic side groups. The MPPs exhibited selective uptake in mitochondria due to electrophoretic targeting of the negatively charged mitochondrial membrane (Figure 3B). These mitochondrial penetrating peptides have been linked with other drugs to focus the chemotherapeutic activity within the mitochondria.[56] Generally, MPPs have not been investigated in multi-drug resistant cancers, potentially missing an opportunity to reverse efflux inhibition. Furthermore, none of the developed MPPs have entered clinical trials, possibly due to delivery challenges and rapid in vivo degradation of the peptides. Specifically, VES may be coupled with peptides similar to the MPPs to potentially target and inhibit the mitochondria, ultimately reversing MDR.

1.3.2 Doxorubicin

Doxorubicin (Dox) is part of the anthracycline family and is among the most active agents used against solid tumors, transplantable leukemias, lymphomas such as breast cancer, and hepatocellular carcinoma (Figure 3C).[57] Dox primarily kills cancer cells by inhibiting macromolecular biosynthesis by intercalating with DNA as well as inhibiting topoisomerase II, responsible for unwinding DNA before DNA transcription.[57, 58] A secondary mode of action for doxorubicin is the induction of ROS that damages DNA, proteins, and cellular membranes.[59] One of the limitations of doxorubicin is cardiotoxicity, primarily due to induction of ROS, resulting in extremely limited doses in the clinic. For example, Dox-induced cardiotoxicity can lead to heart failure with an estimated prevalence of 5%, 26%, and 48% in patients treated at doses of 400, 550, and 700 mg/m², respectively.[60] Unfortunately, the Dox-induced toxicity severely impedes repeated dosing that is required in the clinic for sufficient tumor reduction.[61] The side effects of Dox can be reduced by increasing the infusion time of the chemotherapeutic as well as co-administering chelators or antioxidants, such as VES, as a cardioprotective agent.[61] Alternative methods to mitigate the Dox-induced toxicity is to
encapsulate Dox in NPs, however other side effects may occur such as skin toxicity, mucositis, weight loss, depression, drowsiness, nephrotoxicity, and bone marrow depression.[57] NPs are controlled aggregates with diameters smaller than 200 nm and are used to encapsulated various hydrophobic and hydrophilic drugs, and will be further discussed in Section 0. Doxil, the first NP formulation of Dox approved by the FDA, was developed by Johnson and Johnson and is used for the treatment of breast and ovarian cancers. Compared to free Dox, the reduced toxicity profile and cardiotoxicity of Doxil makes it an attractive option for patients with high risk factors for cardiac diseases.[57] As a first-line therapy of metastatic breast cancer, Doxil treatment exhibits similar tumor response to free doxorubicin but with significantly reduced risk of adverse reactions such as alopecia, vomiting, cardiotoxicity, neutropenia and myelosuppression.[58, 62] Unfortunately, due to the prolonged circulation of Doxil relative to free Dox, new side effects arise such as palmar-plantar erythrodysesthesia (hand foot syndrome), impeding subsequent dosing.[57] Doxil exhibits similar tumor reduction to free Dox, prompting investigations to investigate ways to either improve the therapeutic effects of Doxil or related formulations, or co-administer with other therapeutics for enhanced, synergistic anti-cancer activity.

1.3.3 RNA Interference

Unlike the non-specific clinically used chemotherapeutics, RNA interference (RNAi) has the potential for selective gene silencing of targets that are overexpressed in cancer cells using small interfering RNA (siRNA). RNAi was first discovered in mammalian cells by Fire and Mello whereby exogeneous double stranded RNA were able to silence or downregulate complementary messenger RNA (mRNA) in the nematode worm, Caenorhabditis elegans.[63] This discovery along with major developments in the RNAi therapeutic field has pushed the advancement of RNAi to the point of confirming RNAi-mediated knockdown in humans in 2010.[64, 65] RNAi utilizes exogenous double stranded RNA, such as siRNA, to selectively target and degrade a specific mRNA, thereby inhibiting its translation to a protein. siRNAs are attractive not only for the potent and selective knockdown of a target protein, but are 21-27 nucleotides long, allowing for facile assembly in or on NPs (Figure 3D). siRNAs are composed of two RNA strands, one that is the passenger strand sharing an identical sequence as the target mRNA and a guide strand that is complementary to the target mRNA. Once siRNA enter the cytoplasm, they are loaded into an RNA-induced silencing complex (RISC), which contains the argonaute protein (Ago-2) that removes the passenger strand of the siRNA.[66] The RISC
complex loaded with the guide strand then finds complementary mRNA and induces cleavage and degradation of the mRNA, potently and effectively inhibiting the correct translation of the target mRNA into a functional protein.[67]

### 1.3.3.1 siRNA Delivery Challenges

While siRNAs are extremely attractive for the potent and selective inhibition of specific protein targets, major hurdles exist in vivo and in the clinic that impedes its translation. As naked siRNA are injected in the blood plasma, 3’ exonucleases as well as endonucleases rapidly degrade the siRNA with a half-life of less than 5 minutes.[68] Furthermore, the positively charged glomerular membrane of the kidney electrostatically interacts with the negatively charged siRNA, and shifts the biodistribution.[67] In addition to these factors, immune stimulation and the reticulo-endothelial system (RES), including tissue macrophages and circulating monocytes, recognize the siRNA as a foreign body, resulting in rapid degradation.[68] The largest concern for siRNA is the strongly hydrophilic and negatively charged phosphate backbone that impedes cell penetration into the cytoplasm. In order for the siRNA to function properly, the siRNA must be transported into the cytoplasm where the siRNA machinery is located. These factors are mostly circumvented with the use of chemically modified siRNA and carriers, such as NPs, that can facilitate the transfer into the cytosol.[69]

Furthermore, chemical modifications such as fluoro-modifications of the sugar backbone of siRNA were proven to reduce nuclease degradability as well as increase the potency of the siRNA in vitro.[70] siRNA are most typically packaged into cationic NPs in order to increase tumoral accumulation and endosomal escape into the cytosol.[65] Clinical targets for the use of siRNA encompass overexpressed proteins such as vascular endothelial growth factor (VEGF) $^{237}$, P53 $^{239}$, Ribonucleotide Reductase Regulatory Subunit M2 (RRM2) $^{238}$, polo-like kinase 1 (PLK1) $^{236}$ and Apolipoprotein B-100 (Apo B) $^{240}$. While in theory, any mRNA target can be selectively targeted by siRNA, there are only a few siRNA formulations that have made it through FDA approval. The first siRNA therapies in the 2000s focused on local delivery at the diseased site or tumor, while most formulations didn’t proceed past Phase II due to the inability to achieve primary objectives.[71] NP carriers were then used for systemic administration of siRNA, such as the CALAA-01, however dose-limiting toxicities, immune stimulation, and transient reductions in cholesterol levels were evident.[72, 73] While monotherapies of siRNA formulations failed in the clinic, the co-administration of siRNA with other chemotherapeutics
has the potential for enhanced anti-cancer activity, while reducing the dose-limited toxicities observed in the clinic.

1.4 Nanoparticle Drug Delivery Systems

NPs, specifically liposomes, were discovered in the 1960s with diameters in the range of 20-400 nm.[74, 75] Typical compositions of NPs include liposomes, polymeric micelles, inorganic nanoparticles, nanoconjugates, and drug colloids, of which liposomes make up nearly all of the FDA approved NPs.[76] NPs must be non-immunogenic, non-inflammatory, biodegradable, non-toxic, and avoid uptake through the RES.[77] NPs face challenging in vivo barriers such as shear forces, diffusion flow, protein adsorption, phagocytic sequestration, aggregation, and renal clearance.[78-81] Specifically, NPs have to be rationally designed to limit interactions with phagocytic cells in the liver and spleen, but also be able to assemble into particles with hydrodynamic diameters larger than 5.5 nm to bypass kidney filtration.[80, 82] NPs are a particularly exciting development in the chemotherapeutic field for their simplistic solubilization of hydrophobic and hydrophilic drugs as well as mounting clinical evidence for reduced adverse effects in the clinic.[83] For example, in replacement of toxic surfactants to solubilize taxanes, such as paclitaxel or docetaxel, taxanes can be encapsulated in NPs, such as Genexol-PM, to reduce adverse side effects.[84] Similarly, the toxic side effects of water-soluble Dox can be mitigated by encapsulation in NPs, such as Doxil.[85] Here, Doxil attenuates drug accumulation from the heart, reducing cardiomyopathy, while permitting better tolerability and prolonging drug dosing.[86] However, since the approval of Doxil, mostly all subsequent NPs formulations have shown no significant tumor reduction in the clinic.[64]

1.4.1 Passive Targeting

The initial excitement of NP formulations was that tumors could be targeted passively. Passive targeting is referring to the enhanced permeability and retention (EPR) effects of some tumors whereby tumor vasculature grows rapidly, producing fenestra large enough for NPs to enter from the bloodstream.[87, 88] The NPs are then retained at the tumor site as the rapid growth of the tumors cannot support a functioning lymphatic system, resulting in poor lymphatic drainage.[72] In vivo results of NPs encapsulating various chemotherapeutics on rapidly grown human tumors in mice were at first promising, however translation into the clinic has proven difficult. The primary reasons for the difficult clinical translation include irrelevant in vivo
models, heterogeneity of human tumors, reduced prevalence of EPR in humans, and unfavorable tumor microenvironments. Human tumors are rapidly grown in immuno-compromised mice, which have a reduced number of complement proteins. However, NPs have been shown to bind with plasma proteins, especially complement proteins, which alter the pharmacokinetic parameters of NPs in immunocompetent vs immunocompromised mice. [89] Moreover, patients in NP-related clinical trials are rarely screened for tumors sensitive to the EPR effect, thereby drastically reducing the response rates observed in patients. [90] Patient selection on the susceptibility of the tumor to the EPR effect can be potentially beneficial as pre-screened patients can respond better to NPs through passive targeting. Furthermore, as tumors typically take years to grow in humans, and thus grow mature vasculature with an effective lymphatic drainage system, NP treatment may be less effective relative to in vivo studies where human tumors grow in mice typically over 6 weeks. [91] The rapid tumor growth in mice provides a biased advantage for NPs in demonstrating superior efficacy in animal studies, but becomes less relevant in clinical trials using humans, especially if a pre-selection for EPR-sensitive tumors is absent. [61] Lastly, some tumor microenvironments exhibit increased interstitial fluid pressure along with highly organized and densely packed extracellular matrices, making it difficult for NPs to extravasate into tumors. [92, 93] Specifically, interstitial fluid pressure can be 10-40 fold higher relative to normal tissue, inhibiting NP extravasation into the tumor. Reducing interstitial fluid pressure, by use of anti-angiogenic antibodies, can normalize tumor vasculature while remaining permeable to NPs with diameters less than 60 nm. [94] Moreover, the collagen mesh size of 40-60 nm in diameter in the extracellular matrix presents a diffusion challenge for NPs, reducing NP uptake into cancer cells. [95] Investigations into the heterogeneity and exploitability of EPR in vivo and in the clinic, as well as the effects of immunocompetent mice on NPs, are ongoing.

1.4.2 Active Targeting

Another method of targeting the tumors included active targeting of nanoparticles, which drove the field into the development or various targeting agents. Active targeting of nanoparticles encompasses decorating the nanoparticles with targeting agents, such as antibodies, aptamers, or peptides, that bind to overexpressed receptors on the surface of cancer cells. [96] A subset of actively targeted NPs includes antibody-drug-candidates where chemotherapeutics are covalently attached to antibodies. The main goal was to exploit an overexpressed receptor in cancer cells with nanoparticles decorated with a relevant targeting agent to specifically deliver
anticancer agents to the tumor, while reducing off-target toxicities.[97] Although in vitro studies show very promising results of active vs passive targeting, with minimal serum protein exposure and homogeneous cell populations, the translation of active targeting in vivo and into the clinic has been dissatisfactory. Interestingly, a meta-analysis of nanoparticles investigated nanoparticle delivery in vivo over the past ten years, indicating that active targeting delivered more chemotherapeutic into the tumor vs. passive targeting (0.9% vs 0.6% of the injected dose), however there are no actively targeted NPs approved by the FDA.[98] In addition to the problems with passive targeting, active targeting also suffers from targeting ligand and receptor exposure, and heterogeneity of the tumor. Specifically, NPs are initially characterized in silico with the targeting agents on the surface of the NPs. However, upon in vivo administration, the NPs are covered in serum proteins on the surface, therefore hampering exposure of the targeting ligand to its receptor. Moreover, tumors are heterogeneous whereby only a subset of the cancer cells overexpress the receptor. For example, Herceptin (Anti-HER2) is prescribed to patients harboring tumors where only 10% of the cells overexpress HER2.[99] Therefore, if anti-HER2 decorated NPs or relevant antibody-drug-candidates were administered to these patients, then at most, only 10% of the cancer cells would be actively targeted. This problem extends to other receptors as only a subset of the tumor will overexpress the receptor, permitting other cancer cells to proliferate. Active targeting of NPs has had many complications, along with the added manufacturing complexity and costs, however it is still currently under investigation at the clinical stages.

1.4.3 Clinical Success and Failures

By 2015, 43 formulations containing NPs were approved by the FDA, of which 18 formulations encapsulated anticancer drugs.[100, 101] Although NPs used for chemotherapy have not shown increased tumor reduction or survival rates, the reduced toxic side effects warrant their use in the clinic. For example, Abraxane, a paclitaxel containing NP, and Doxil are estimated to bring in $1 billion US per year each in 2019 and 2022, respectively, simply because of reduced toxic side effects and increased dosing, rather than improved efficacy[102, 103] Relative to free paclitaxel, Abraxane is advantageous for increased drug dosing, shorter infusion times, and reduced usage of corticosteroids or antihistamines.[104] However, clinical failures come with major losses to both investors and the scientific community. For example, Merck purchased siRNA therapeutics for $1.1 billion in 2007, but later sold it to Alnylam for $175
million, losing $925 million. Furthermore, it has become difficult to learn from the failed or terminated clinical trials as the data are not publicly available. Even when the failed clinical data are publicly available, the interpretation of the results are difficult due to the small sample sizes. NPs loaded with one chemotherapeutic have yet to show significant tumor reduction relative to free drug formulations, prompting investigations into other methods of improving the therapeutic index, such as co-encapsulation of multiple chemotherapeutics.

1.5 Synergistic Nanoparticle Formulations

While cancer therapy was initially given as a monotherapy, combination treatments have gained traction in the clinical community due to reduction of tumor volumes and decreasing the prevalence of MDR. For example, Herceptin (Anti-HER2) combined with cyclophosphamide and free Dox were demonstrated to enhance the tumor response rate with prolonged progression-free survival, relative to administering single agents in Phase III clinical trials. However, clinical outcomes of combination treatments are still unsatisfactory. The initial goal of combination treatments was that drugs could be co-administered to elicit a synergistic tumor response. Synergy is commonly evaluated by the established median effect analysis, whereby a combination index (CI) is obtained. Therefore, combination treatments can be antagonistic (CI > 1), additive (CI = 0.9-1.1), or synergistic (CI < 1). Additive anticancer effects are observed when the effect of the drug combination is equal to the sum of the effects of each individual drug. Antagonistic and synergistic effects are observed when the anticancer effect of the drug combination is either smaller or greater than the sum of the effects of each drug, respectively. Ideally, “good” drug combinations are chosen to exhibit synergistic anticancer effects. Drug combinations are primarily chosen with the idea to offer anticancer synergy by using clinically proven drugs as single agents with different mechanisms of action while not exhibiting cross-drug resistance. However, free drug combinations are challenging in that they lack synergistic anti-cancer activity in vivo. Here, free drug combination treatments lacked the desired spatiotemporal distribution whereby the synergistic ratio of both drugs are not maintained at the tumor site. The combined drugs may display different clearance and metabolic rates, exhibit drug-to-drug interactions, and amplify off-target toxic effects. Moreover, some combination therapies are administered sequentially, and not simultaneously, to reduce prominent adverse effects, while missing an opportunity for anticancer synergy. One of the main concerns with simultaneous delivery is oversaturation of metabolizing enzymes, as
observed in the clinic with hepatotoxicity.[4] For example, combining Herceptin with doxorubicin is favorable as synergistic anti-cancer activity is demonstrated in vitro.[113] However, this leads to problems in vivo where cardiac dysfunction is a major concern. Here, Herceptin inhibits the dimerization of HER2 and human epidermal growth factor receptor 4 (HER4), leading to the downstream induction of ROS in cardiomyocytes. This effect, coupled with the Dox induced ROS, activates the apoptosis signal-regulating kinase 1 (ASK-1) pathway, resulting in apoptosis of cardiomyocytes, and ultimately leading to heart failure.[99] The lack of spatiotemporal distribution is attributed to differences in pharmacokinetic and physicochemical properties of free drugs; however, co-encapsulation in NPs has the potential to mediate this problem.[4]

1.5.1 Advantages of Co-encapsulation

NPs co-encapsulating multiple drugs have shown the potential to exhibit anticancer synergism in vivo and in the clinic. Co-encapsulating multiple drugs in NPs is advantageous for maintaining synergistic drug ratios in a temporal and spatial manner from injection site to tumor site, as well as being therapeutically superior relative to free drug combinations or co-administration of singly-loaded NPs.[114, 115] For example, a NP co-administering Dox and mitomycin C was demonstrated to maintain the molar ratio of encapsulated drugs from injection site to the tumor tissue for up to 24 h.[116] Maintaining the synergistic ratio of drugs in vivo was lost when co-administered as free drugs, or singly-loaded NPs, and both were therapeutically inferior to the co-encapsulated NPs. Specifically, the co-encapsulated NPs were effective at killing MDR breast tumors at 30-fold lower doses than the free drugs, highlighting the importance of co-encapsulation. Another notable example is Triolimus, a NP co-encapsulating 17-(AllylAmino)Geldanamycin (17-AAG), paclitaxel, and rapamycin at a synergistic molar ratio of 3:2:1.[117] Triolimus has demonstrated satisfactory in vitro and in vivo stability while synergistically enhancing the tumor reduction relative to singly- or doubly-loaded NPs. The synergistic mechanism of action of Triolimus utilizes paclitaxel as a mitotic inhibitor, binding with tubulin during mitosis, and its anti-cancer activity is enhanced with rapamycin by inhibiting the phosphorylation of p70s6K. Then, the mechanism of action of rapamycin activates the compensatory protein kinase B (Akt) pathway by phosphorylation of extracellular- signal-regulated kinase 1/2 (Erk1/2), which is then blocked with 17-AAG.[3] Co-encapsulation of multiple drugs also presents new challenges that are non-existent in singly-loaded NPs. For
example, loading multiple drugs in NPs may affect the in vitro and in vivo stability.[117] Importantly, co-encapsulated NPs face formulation challenges such as increased aggregation, drug leakage, and fusion, all of which must be accounted for before administration in vivo or in the clinic.[118] While co-encapsulated NPs have been demonstrated to be therapeutically superior relative to free drug combinations or the co-administration of singly-loaded NPs in clinical trials, none have passed FDA approval although some formulations are showing great potential.

1.5.2 Clinical Success and Future Directions

While singly-loaded NPs failed to reduce the tumor size and survival rate in the clinic, NPs co-encapsulating multiple drugs have been demonstrated to improve the survival rate of patients with acute myeloid leukemia (AML) for the first time in 40 years.[86] Vyxeos (formerly CPX-351), developed by Celator, is a NP formulation co-encapsulating cytarabine and daunorubicin at a 5:1 molar ratio, which has revealed a 31% reduction in risk of death in AML patients relative to the free drug combination. Furthermore, patients treated with Vyxeos lived 9.56 months compared to 5.95 months when treated with the free drug combination, prompting the FDA to grant Vyxeos a Fast Track designation which then attracted Jazz Pharmaceuticals to acquire Celator for US $1.5 billion.[7, 119] An alternative NP formulation developed by Celator is CPX-1, co-encapsulating irinotecan and floxuridine at a molar ratio of 1:1, and is currently in phase II clinical trials for the treatment of advanced colorectal cancer.[86] A previously mentioned, but notable example by Co-D Therapeutics includes Triolimus, encapsulating rapamycin, paclitaxel, and 17-AAG, which was granted orphan drug designation for the treatment of angiosarcoma, and is currently in late stage preclinical evaluation for the treatment of angiosarcoma, non-small-cell lung carcinoma, and breast cancer.[117] The results thus far are promising for the future of NPs for cancer treatments, but the issue of cancer resistance remains largely unanswered.

The majority of co-encapsulated NPs focus on combining drug agents that have been clinically proven as single agents, however there is a gap in treating multi-drug resistant cancers. The synergistic anti-cancer activity of co-encapsulated NPs in the clinic do not have an ability to reverse MDR. As in the case of Vyxeos, the cancer cells that are sensitive to both cytarabine and daunorubicin will be the initial cells that die off, leaving behind more resistant cancer cells. The
surviving cancer cells are then resistant in a primarily drug-dependent pathway as discussed in Chapter 1.2.2. Currently, Vyxeos does not contain any efflux inhibitors to affect the resistance, pushing for the development of a NP formulation that will affect these now drug-resistant cancer cells.

1.6 Types of Nanoparticles

The compositions of NPs varies greatly and includes nanoconjugates, lipids and polymers, of which lipids can be found in the majority of the NP formulations currently approved or being investigated for FDA approval.[76] Nanoconjugates typically include proteins or enzymes modified with a hydrophilic polymer, such as PEG, or with chemotherapeutics, such as those found in antibody-drug-conjugates. Nanoconjugates are restricted to specific cancer cells that are dependent on the protein or enzyme, or other cancer cells that overexpress receptors for antibody recognition.[120] Liposomes are spherical vesicles with a lipid bilayer that harbors a hydrophobic layer, with a hydrophilic core.[121] Lipids are used to make the bulk of the liposomes and can vary in lipid composition, size, and surface charge engineered for the specific application.[122] For example, liposomes that are engineered to deliver siRNA would typically include cationic lipids, such as dioleoyl phosphatidylethanolamine (DOPE), whereas liposomes delivering hydrophobic chemotherapeutics would include pegylated lipids such as N-(carbonylmethoxy(polyethyleneglycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanol-amine (DSPE-PEG).[123, 124] Liposomal formulations are engineered for stability in in vivo circulation coupled with limited aggregation with other liposomes.[121] The approval by the FDA in 1995 as well as the successful commercialization of Doxil, a liposomal formulation of doxorubicin, led the push for the majority of the FDA approved NP containing formulations, and investigational formulations to also include liposomes.[103] However, Doxil circulates in the body for extended periods of time, relative to free doxorubicin, eliciting new side effects, such as mucositis and palmar-plantar erythrodysesthesia.[57] These new side effects prompted the use of other materials for NPs, such as polymers.

Polymers are attractive for the NP-mediated delivery of chemotherapeutics mainly due to facile synthesis, engineered biodegradation of the polymer, and facilitating drug release for extended periods of time.[64] The facile synthesis allows for bulk production of the polymer, while the biodegradability of the polymer ensures that the injected polymer can be fully
metabolized and cleared from the body.[125] Polymers used for NPs are typically amphiphilic, where separate portions of the polymer are either hydrophilic or hydrophobic, allowing for instantaneous self-assembly in aqueous media forming NPs with hydrophobic cores. The hydrophilic portions are mainly composed of PEG while other alternatives include zwitterionic polymers, such as poly(methacryloyloxyethyl phosphorylcholine), or poly(sulfobetaine methacrylate).[126] The hydrophobic portions typically include poly(lactic-co-glycolic acid), poly(lactide), or poly(caprolactone).[127] Many polymeric NPs are approved for cancer treatment, with more in the pipeline. For instance, Genexol-PM is a polymeric NP consisting of PEG and poly(lactide) encapsulating paclitaxel, and has been approved in South Korea for the treatment of lung and metastatic breast cancers.[128] Using a similar polymer to Genexol-PM, Triolimus co-encapsulates rapamycin, 17-AAG, and paclitaxel, and demonstrates synergistic anti-tumor activity in breast tumor-bearing mice.[3] Interestingly, the developers of Triolimus, Co-D Therapeutics, received an orphan drug designation by the FDA for market exclusivity to treat angiosarcoma.[129] Polymeric NPs have a proven track record as carriers for chemotherapeutics in the clinic.

1.7 P(LA-co-TMCC)-g-PEG-N₃ Graft Copolymer for Nanoparticle-Mediated Drug Delivery

The NPs used in this thesis comprises a self-assembling, graft copolymer, poly(\(D,L\)-lactide-co-2-methyl-2-carboxytrimethylenecarbonate)-graft-poly(ethylene glycol)-azide (P(LA-co-TMCC)-g-PEG-N₃) (Figure 1.4). The P(LA-co-TMCC) component is biodegradable and synthesized through a ring opening polymerization catalyzed by a thiourea organocatalyst of benzyl protected 2-methyl-2-carboxytrimethylenecarbonate (TMCC) and \(D,L\)-lactide (LA).[130] The thiourea organocatalyst used is advantageous as this helps synthesize polymers with narrow polymer molar mass distributions, while avoiding the use of toxic lewis acid catalysts, such as tin.[130] Notably, other aliphatic polycarbonates and polyesters have been employed for the synthesis of hydrophobic polymers, such as poly(caprolactone) (PCL), poly(trimethylene carbonate) (PTMC), and poly(lactic-co-glycolic acid) (PLGA).[131-133] However, using the TMCC comonomer is beneficial as this introduces functional acids into the copolymer that can then react with hydrophilic polymers or other functional agents.[134] Additionally, the P(LA-co-TMCC) is a random copolymer likely resulting in enriched LA and TMCC repeats due to differences in the monomer reactivity ratios.[130] Multiple PEG polymers are grafted onto
P(LA-co-TMCC) through carbodiimide chemistry. A majority of the TMCC comonomers would remain as free acids as it is unlikely that all of the TMCC would be modified with PEG primarily due to steric hinderance, where the accessibility of the acids within P(LA-co-TMCC) would be limited. The grafted PEG polymers are end terminated with an azide, making it ideal for conjugation of either targeting agents or siRNA. For instance, NPs comprising of P(LA-co-TMCC)-g-PEG-N₃ were surface modified with alkyne modified siRNA through a copper catalyzed azide-alkyne cycloaddition (CuAAC). As the copper catalyst is cytotoxic to cells and CuAAC is relatively slower, compared to other click chemistry routes, a copper free alternative reaction would employ a strained alkyne, such as a dibenzylcyclooctyne (DBCO), to react with an azide in a commonly used click reaction termed strain promoted azide-alkyne cycloaddition (SPAAC). Importantly, P(LA-co-TMCC)-g-PEG exhibits sub-micromolar critical micelle concentrations, suggesting good thermodynamic stability suitable for in vivo studies. Furthermore, P(LA-co-TMCC)-g-PEG has also been used to deliver docetaxel and doxorubicin in breast cancer cells in vitro. For instance, P(LA-co-TMCC)-g-PEG has been safely used in vivo to deliver docetaxel while allowing for a 1.6-fold higher maximum tolerated dose that resulted in an enhanced therapeutic index, relative to the conventional docetaxel formulation. While the in vivo results were promising, the prevalence and effects of MDR in the NP treated tumors were not explored. Therefore, P(LA-co-TMCC)-g-PEG-N₃ is a suitable copolymer for the potential encapsulation of chemotherapeutics and surface modification of siRNA.
Figure 1.4 Structure of the copolymer used in the nanoparticles (NPs) and schematic of the click chemistry utilized in this thesis. A) Structure of the copolymer poly(D,L-lactide-co-2-methyl-2-carboxytrimethylene carbonate)-graft-poly(ethylene glycol)-azide (P(LA-co-TMCC)-g-PEG-N3) where the hydrophobic segment of the copolymer is in black, while the hydrophilic segment of the copolymer is in orange. P(LA-co-TMCC)-g-PEG-N3 is shown here with 2 PEGs/ backbone. An azide (N₃) is shown at the terminal end of the PEG. Upon self-assembly in water, P(LA-co-TMCC)-g-PEG-N3 NPs would have azides exposed on the surface of the NP. B) Reaction mechanism of a strain promoted azide-alkyne cycloaddition (SPAAC) between the azide on the NP with a dibenzylcyclooctyne modified targeting agent or drug (denoted as X).
2 Cationic block amphiphiles show anti-mitochondrial activity in multi drug resistant breast cancer cells

2.1 Abstract

Currently, there are limited treatment options for multi-drug resistant breast cancer. Lipid modified, cationic peptides have the potential to reach the mitochondria, which are attractive targets for the treatment of multi-drug resistant (MDR) breast cancer; yet, little is known about their mitochondrial targeting and anti-cancer activity. Interestingly, lipid modified cationic peptides, typically used as gene transfection agents, exhibit similar structural features to mitochondrial targeted peptides. Using octahistidine-octaarginine (H₈R₈) as a model cationic peptide for cell penetration and endosomal escape, we explored the anti-cancer potential of lipid-modified cationic peptides as a function of amphiphilicity, biodegradability and lipid structure. We found that cationic peptides modified with a lipid that is at least 12 carbons in length exhibit potent anti-cancer activity in the low micromolar range in both EMT6/P and EMT6/AR-1 breast cancer cells. Comparing degradable and non-degradable linkers, as well as L- and D-amino acid sequences, we found that the anti-cancer activity is mostly independent of the biodegradation of the lipid modified cationic peptides. Two candidates, stearyl-H₈R₈ (Str-H₈R₈) and vitamin E succinate-H₈R₈ (VES-H₈R₈) were cytotoxic to cancer cells by mitochondria depolarization. We observed increased reactive oxygen species (ROS) production, reduced cell bioenergetics and drug efflux, triggering apoptosis and G1 cell cycle arrest. Compared to Str-H₈R₈, VES-H₈R₈ showed enhanced cancer cell selectivity and drug efflux inhibition, thereby serving as a potential novel therapeutic agent. This study deepens our understanding of lipid modified cationic peptides and uncovers their potential in multi-drug resistant breast cancer.
Figure 2.1 Graphical abstract outlining the mechanism of action of vitamin E succinate modified octahistidine-octaarginine (VES-H₈R₈). VES-H₈R₈ penetrates into multi-drug resistant breast cancer cells and targets and depolarizes the mitochondria, inducing apoptosis and G1 cell cycle arrest and inhibits permeation glycoprotein efflux.

2.2 Introduction

Mitochondria are the powerhouse of the cell and serve as an attractive target for cancer treatment. In cellular processes, these organelles provide adenosine triphosphate (ATP) through oxidative phosphorylation, constitute the main source of reactive oxygen species (ROS), and participate in oxidative signaling, thereby playing a critical role in the rapid proliferation of cancer cells.[2, 27, 142] Oncogenic activation leads to increased mitochondrial metabolism and higher mitochondrial membrane potential compared to that of non-cancer cells.[2, 143] Moreover, multi-drug resistant (MDR) cancer cells exhibit increased mitochondrial mass with more polarized mitochondria relative to non-MDR cells.[144] As MDR arises from the overexpression of drug efflux pumps, which requires ATP from mitochondria, mitochondrial targeting is a particularly sensible option for the treatment of drug-resistant cancer cells.[2, 145, 146] Hence, the more polarized mitochondria membranes in cancer cells together with the ATP-dependent drug efflux introduces an important target in MDR cancer cells.

Targeting mitochondria requires penetration through cellular and mitochondrial membranes.[56] Cationic amphiphilic structures are excellent candidates for mitochondrial targeting as they can interact with negatively charged cellular and mitochondrial membranes. [56] [36, 147] Mitochondrial-penetrating peptides (MPPs) have been designed with alternating amino acids containing delocalized lipophilic cationic side groups, such as arginine, and hydrophobic amino acids, such as cyclohexylalanine.[56] Primarily used at low concentrations for mitochondria...
imaging, these peptides have been shown to potentiate the activity of chemotherapeutics or reduce the side effects of antimicrobials.[56, 148] At a threshold concentration, they inhibit oxidative phosphorylation and electron transport chain, collapse mitochondria and induce apoptosis.[56] Similarly, cationic amphiphilic polymers, such as poly(ethyleneimine) (PEI), form pores in mitochondria membranes, causing proton leakage and depolarization, and thereby induce apoptosis.[149] Interestingly, lipid-modified cationic peptides, composed of a cationic cell penetrating peptide and a fatty acid chain, closely resemble MPPs in structural properties; yet, their anti-mitochondrial activity remain understudied and poorly understood. This knowledge gap prompted us to investigate the activity of lipid-modified cationic peptides as mitochondria-targeting drugs for cancer treatment. We hypothesized that such lipid-modified cationic peptides could selectively target and depolarize the mitochondria of MDR breast cancer cells (Figure 2.2).

Using the octahistidine-octaarginine (H₈R₈) peptide as a common cationic cell penetrating peptide with endosomal escape capabilities, we investigated, for the first time, the intracellular fate and effects of lipid modified cationic peptides in breast cancer cells.[150, 151] First, we studied the anticancer activity of H₈R₈-based amphiphiles in relation to hydrophobicity and degradability. Comparing a biologically inert hydrophobic stearyl chain-modified H₈R₈ to the bioactive MDR sensitizer, vitamin E succinate (VES)-modified peptide, we investigated cancer cell toxicity and cancer selectivity relative to healthy cells.[152, 153] We explored the intracellular effects of H₈R₈-based cationic lipids and evaluated mitochondrial inhibition, induction of ROS accumulation, apoptosis, necrosis, and cell cycle arrest. VES-H₈R₈ was also investigated for inhibition of permeation glycoprotein (Pgp) efflux in MDR breast cancer cells.
Herein, we highlight H$_8$R$_8$-based amphiphiles, and specifically vitamin E-based prodrugs for MDR breast cancer cell targeting.

![Diagram](image)

**Figure 2.2** Schematic of the mechanism of cancer cell death following treatment with vitamin E succinate modified octaarginine-octahistidine (VES-H$_8$R$_8$). VES-H$_8$R$_8$ is taken up through electrostatic interaction with the plasma membrane and depolarizes the mitochondria through a mitochondrial permeability transition pore (mPTP)-dependent pathway (dotted red line). Consequently, bioenergetics of the mitochondria are inhibited, ROS are elevated, both apoptosis and necrosis are induced, and cells are arrested at the G1 phase. VES-H$_8$R$_8$ was shown to decrease the efflux capability of permeation glycoprotein (Pgp). VES represents vitamin E succinate moiety and Str represents a stearyl moiety.

### 2.3 Materials and Methods

All solvents and reagents were purchased from Sigma-Aldrich and used as received unless otherwise noted. Peptide synthesis reagents were purchased from AnaSpec (Fremont, CA).

#### 2.3.1 Peptide synthesis

H$_8$R$_8$ was synthesized by conventional solid-phase microwave assisted peptide synthesis techniques (CEM Liberty 1) where double histidine and arginine couplings were employed. To make amide peptides, rink amide ProTide Resins were used (CEM Corp, NC, USA). Standard
microwave-assisted Fmoc deprotection was used.[154] Peptides were prepared at 0.25 mmol scale using HOBt/DIC/Oxyma for activation. Fmoc-Arg(Pbf)-OH was purchased from Ark Pharm (Illinois, USA) and Fmoc-His(Trt)-OH were from EMD Millipore (Massachusetts, USA).

2.3.2 Synthesis of Vitamin E-oxy-butyric acid

Vitamin E was modified according the schematic in Figure S 2.1A and as follows: in a 250-mL round-bottom flask, 417.69 mg (1.46 eq) of NaH were dissolved in 50 mL of THF and stirred on ice under argon for 10 min. 5.12 g (1 eq) of Vitamin E (α-tocopherol) were dissolved in 50 mL of THF and added to the NaH solution, before stirring on ice for 15 min under argon. 2.492 mL (1.46 eq) of ethyl 4-bromobutyrate were added to the solution before stirring under argon first on ice for 20 min and then at room temperature overnight. The solution was transferred to a large beaker and diluted with 300 mL of CH₂Cl₂. Liquid-liquid extraction with excess DI water was used to purify the Vitamin E-oxy-ethylbutyrate intermediate (ESI-MS⁺: C₃₅H₆₀O₄ expected m/z 544.9, found m/z 545.5 corresponding to M+H). Vitamin E-oxy-ethylbutyrate was hydrolyzed in a mix of 10% w/v KOH (5 mL) and THF (10 mL) overnight. The solution was then quenched with 10 mL DI water, adjusted to pH 3 with concentrated HCl (5-10 drops), and extracted 3 times with CH₂Cl₂. The organic phase was washed 2 times with saturated NaCl solution, dried on MgSO₄ and evaporated under vacuum to give the final product. 1.0 gram of the crude was then purified on a silica column, using chloroform as eluting solvent. Impurities were washed off in chloroform, and the final product was eluted in chloroform:methanol (9:1 v/v). The organic solvent was removed by rotary evaporation and dried in an oven. (360 mg, 36% yield, ESI-MS: C₃₃H₅₆O₄ expected m/z 516.8, found 515.4 corresponding to M-H, Figure S 2.1B) ¹H NMR (500 MHz, CDCl₃, δ in ppm): 3.69 (t, CH₂), 2.67 (t, CH₂), 2.56 (t, CH₂), 2.15 (s, aromatic), 2.11 (s, aromatic), 2.07 (s, aromatic), 1.22 (s, CH₃), 0.87-0.83 (s, CH₃) ppm (Figure S 2.1C).

2.3.3 N-terminal peptide acylation

To modify the N-terminus of the peptide (0.125 mmol), 4.9 eq. of 2-(6-Chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) were used to activate 5.0 eq. of acid (stearic acid or vitamin e succinate) in DMF for 15 min at room temperature. The activated acid was then added to the peptides on resin, while adding 1 eq. of N,N-diisopropylethylamine (DIPEA). The reaction was allowed to proceed for 24 h before the resin was washed 2 times with
DMF, then 2 times with DCM. Conjugation was evaluated with the 2,4,6-Trinitrobenzenesulfonic acid (TNBS) test.[155]

2.3.4 Fluorescein labeling of peptide

To synthesize the fluorescein-modified peptide, Fmoc-Lys(alloc)-OH (EMD Millipore, Massachusetts, USA) was used and inserted between H₈ and R₈ as NH₂-H₈K(Alloc)-R₈-Resin. VES and stearic acid were separately conjugated to the N-terminus using HCTU as above. Then the alloc protecting group was deprotected 3-times, 20 min each, using 0.1 eq. of tetrakis(triphenylphosphine) palladium(0) (Pd(PPh₃)), and 10 eq. of borane dimethylamine complex (Me₂NH·BH₃). Finally, 5(6)-Carboxyfluorescein was activated using HCTU and conjugated to the peptide as stated above. Fmoc-NH₂-H₈K(Alloc)-R₈-Resin was used to prepare the fluorescein modified H₈R₈ where alloc was deprotected as above, and fluorescein was added to the free amine on lysine as above. Fmoc was then deprotected using 20% piperidine in DMF.

2.3.5 Cleavage and purification

Final deprotection and cleavage off of the resin was completed using a cleavage cocktail composed of trifluoroacetic acid:water:triisopropylsilane (TFA:H₂O:TIS, 95:2.5:2.5 v/v/v) for 5 h. The peptide was then precipitated in cold ether, centrifuged and washed with cold ether and allowed to dry overnight. The precipitate was then dissolved in TFA, diluted in acetonitrile:water (ACN:H₂O:TFA, 90:9.9:0.1 v/v/v), and purified through a C18 reverse phase column (Silicycle, Quebec, Canada) using a gradient of acetonitrile from 10 to 100 %. Peptide mass was verified through electrospray ionization (EI) or matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry using the Agilent 6538 Q-TOF mass spectrometer. Peptide characterization results are shown in Table S 2.1. Stock solutions of peptides were prepared in DMSO.

2.3.6 Nanoparticle characterization and Critical micelle concentration measurements

Str-H₈R₈ and VES-H₈R₈ were dissolved in DMSO at a concentration of 250 mg/mL, and diluted in PBS (pH 7.4), 1.5 μM citric acid in PBS (pH 6.3), or 3 μM citric acid in PBS (pH 5.3). The peptides were passed through a 0.2 μm polyethylysulfone (PES) filter and nanoparticle diameters were measured using a Malvern Zetasizer Nano ZS (4 mW, 633 nm laser) at a concentration of
2.5 mg/mL. Critical micelle concentration (CMC) was calculated based on the scattering intensity measured by dynamic light scattering as previously described.[156] The scattering intensity was measured using a DynaPro Plate Reader II (Wyatt Technologies) configured with a 60 mW, 830 nm laser and a detector angle of 158°. Str-H₈R₈ and VES-H₈R₈ were added to clear bottom 96-well plates at a peptide concentration of 16.6 mg/mL in PBS (pH 7.4), and serially diluted with measurements of three acquisitions per sample. Curve fitting algorithms were used to determine the CMC of the nanoparticles.

2.3.7 Cell culture

Both parental (EMT6/P) and doxorubicin resistant (EMT6/AR1) EMT6 cells were generously provided by Dr. X.Y. Wu (University of Toronto), originally from Dr. Ian F. Tannock at the Ontario Cancer Institute, (Toronto, ON, Canada) and maintained in our laboratory. Non-cancerous fibroblast cells (NIH/3T3) were grown in DMEM media supplemented with 10% fetal calf serum and 1% penicillin/streptomycin and grown as above. EMT6/P cells were grown in α-MEM medium supplemented with 10% FBS, 1% penicillin/streptomycin at 37 °C in a humidified incubator with 5% CO₂ atmosphere. EMT6/AR-1 cells were grown as above, with the addition of doxorubicin at 1 µg/mL to maintain doxorubicin resistance and permeation glycoprotein (Pgp) overexpression.

2.3.8 Cell culture cytotoxicity assay

Cells were seeded into 96-well flat-bottomed tissue culture plates at a density of 3,000 cells per well and allowed to adhere for 24 h. H₈R₈ peptides dissolved in DMSO were serially diluted in PBS and then into full medium, and incubated with cells for 72 h. The doses chosen were based on the observed inhibitor concentration to kill 50% of the cells (IC₅₀). Similar DMSO concentration was used as a control. DMSO was used at concentration < 0.5 % v/v. After the addition of peptides, cells were allowed to grow for 72 h at 37 °C in a 5% CO₂ and 95% air humidified incubator. Presto Blue (Life Technology) was added to fresh medium as per the manufacturer’s protocol and incubated with cells for 1.5 h. Viable cells are able to reduce the resazurin dye in Presto Blue to a highly red fluorescent resorufin (ex/em 540/590 nm) which can be read by a microplate fluorescent reader (Infinit m200 Pro, Tecan Group Ltd, Switzerland). Each measurement is an average of 3 separate passages of cells. Dose response curves and inhibitor concentration to kill 50% of the cells (IC₅₀) were obtained from Graph Pad Prism version 6.00 for
Windows (Graph Pad Software, CA, USA, www.graphpad.com). Relative viability was calculated as the fluorescence intensity of the treated group divided by the fluorescence intensity of a control group.

2.3.9 Laser scanning confocal microscopy

EMT6/P cells were seeded at a density of 20,000 cells per well in a 8-well Nunc Lab-Tek II chambered cover glass (Thermo Fisher Scientific, MA, USA) and allowed to adhere for 24 h at 37 °C in a 5% CO₂ and 95% air humidified incubator. 0.8 µM of fluorescein-modified peptide was added to the cells and incubated for 3 h. The peptide containing media was aspirated off, and 200 nM of MitoTracker Deep Red FM (Thermo Fisher, MA, USA) in full media was added to the cells. After 15 min incubation, the cells were washed 3-times with PBS, and then Hoescht 33342 nuclei acid dye (Molecular Probes, Inc., Eugene, OR, USA) in PBS was added to the cells. Live cell imaging was done using an Olympus FV1000 confocal microscope equipped with an oil immersion 60x lens. Excitation and emission wavelengths are as follows: Hoescht 33342 (ex/em: 405/460 nm) fluorescein (ex/em 488/520 nm), MitoTracker Deep Red FM, (ex/em: 640/670 nm). Unlabeled control cells were used to set the laser power to avoid fluorescent bleed over between channels.

2.3.10 Fluorescein-modified peptide uptake in intact cells and isolated mitochondria

The uptake of the VES-H₈R₈, Str-H₈R₈ in the mitochondria of intact cells was quantified as previously reported.[157] 5x10⁶ cells were seeded in T-25 flasks and allowed to adhere for 24 h at 37 °C in a 5% CO₂ and 95% air humidified incubator. Non-toxic concentrations of peptides were used (<0.8 µM). The EMT6/AR-1 cells were treated the next day with the fluorescein-modified peptides of VES-H₈R₈, Str-H₈R₈, or H₈R₈ (0.8 µM), or free fluorescein as a dye control (0.8 µM). The cells were incubated with peptides or dye control for 3 h in full medium, and then the cells were harvested with trypsin. After centrifugation, mitochondria from the pelleted cells were extracted according to the instructions of the Mitochondria Isolation Kit for cultured cells (Thermo Fisher Scientific, MA, USA). cOmplete Mini, EDTA free protease inhibitor cocktail was added to reagents A and C of the mitochondrial isolation kit with 1 tablet / 10 mL of extraction buffer. A more purified fraction of mitochondria was obtained by centrifuging the post-nuclear supernatant at 3,000 x g. The mitochondria pellet was then suspended in PBS, transferred into a 96-well clear
bottom black plate and fluorescein was quantified (ex/em 490/520 nm) against a standard curve in PBS using a Tecan Plate Reader. The average of three biological repeats was used to measure the amount of fluorescein uptake.

The uptake of the fluorescein-modified peptides of VES-H₈Rs₈, Str-H₈Rs₈, H₈Rs₈, or free fluorescein was also investigated with isolated mitochondria as reported previously.[157] Mitochondria from 1.1x10⁸ cells of untreated EMT6/AR-cells were extracted according to the previous section. The mitochondria of approximately 6x10⁶ cells were then suspended in mitochondria isolation buffer (10 mM Tris hydrochloride, 0.15 mM MgCl₂, 0.25 mM sucrose, 1 mM DTT, pH 6.7, and 1 tablet/10 mL of cOmplete, Mini, EDTA free protease inhibitor cocktail) and fluorescein-modified peptides of VES-H₈Rs₈, Str-H₈Rs₈, H₈Rs₈, or free fluorescein were added at a final concentration of 0.8 µM. The isolated mitochondria were incubated with the peptides or free fluorescein for 1 h at 37 °C, and then washed with PBS three times by centrifuging at 12,000xg for 5 minutes. The isolated mitochondria were then suspended in PBS, transferred into a 96-well clear bottom black plate and fluorescein was quantified (ex/em 490/520 nm) against a standard curve in PBS using a Tecan Plate Reader. The average of three biological repeats was used to measure the amount of fluorescein uptake.

2.3.11 Fluorescein-modified peptide uptake and retention studies

20,000 cells were seeded into 48-well plates and allowed to adhere for 24 h at 37 °C in a 5% CO₂ and 95% air humidified incubator. Non-toxic concentrations of peptides were used (<0.8 µM). The EMT6/AR-1 cells were treated with the fluorescein-modified peptides (0.8 µM) for 2, 5, or 24 h in full media. The fluorescein retention study was completed by incubating with either peptides alone or co-incubation of Str-H₈Rs₈ (0.8 µM) with free VES (20 µM) for 24 h, followed by 24 h in fresh media. A similar DMSO concentration was used as a control. After treatment, cells were washed 3 times with PBS, and harvested with trypsin. Cell fluorescence was analyzed using a BD Accuri C6 flow cytometer with excitation wavelength of 488 nm and emission filters of 533/30 nm (fluorescein, FL-2 channel). Cell debris and doublets were gated out using FSC-A vs FSC-H, and at least 10,000 events were collected. The mean fluorescence intensity in the FL-1 channel (ex/em 488/533(30) nm) for three biological repeats was used to measure the amount of fluorescein uptake.
2.3.12 Mitochondrial membrane polarization assay

Mitochondrial membrane potential was assayed using the JC-1 probe (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) (ex/em 488 nm/533-585 nm) (Biotium Inc., CA, USA).[158] 20,000 cells were seeded into 48-well plates and allowed to adhere for 24 h at 37 °C in a 5% CO₂ and 95% air humidified incubator. The cells were treated the next day for 2 or 5 h in full media. Control treatments and peptides were incubated at 8 μM, in the range of the IC₅₀ of both Str-H₈R₈ and VES-H₈R₈. A similar DMSO concentration was used as a control. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) at 50 μM was used as a positive control for mitochondrial depolarization. Following treatment, the cells were washed with PBS 3 times and then incubated at 37 °C with 10 μM of JC-1 in full media for 30 min. The EMT6/P cells were incubated with 2 μM of JC-1 in full media to avoid fluorescent saturation in the flow cytometer. The cells were then washed 3 times in PBS, trypsinized, and placed on ice before measuring fluorescence in a flow cytometer within 1 h. Cell debris and doublets were gated out using FSC-A vs FSC-H, and at least 10,000 events were collected. A gate was set according to DMSO and CCCP treated cells in the FL-2 channel (ex/em 488 nm/585(40) nm) to measure the proportion of JC-1 aggregate fluorescence versus JC-1 monomer fluorescence in the FL-1 channel (ex/em 488 nm/533(30) nm). To assess the mitochondrial permeability transition pore formation, cyclosporine A was used as previously described.[158] Averages were obtained from three biological repeats. Changes in the mitochondria membrane potential (ΔΨm) were expressed using the following equation:

\[
\text{Relative Mitochondrial Membrane Potential (ΔΨm)} = \frac{JC_{1\text{aggregate}}}{JC_{1\text{monomer control}}} \times 100\%
\]

2.3.13 Real-time investigation of Oxygen Consumption Rate (OCR) and Extracellular Acidification rate (ECAR)

Analyses of bioenergetics processes were performed in intact EMT6/P and EMT6/Ar-1 cells using the Seahorse XF Analyzer (Agilent, CA, USA). An optimized cell density of 10,000 cells / well were seeded in a XF96 V4 cell culture microplate and allowed to adhere for 24 h at 37 °C in a 5% CO₂ and 95% air humidified incubator. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured as cells were incubated with 30 μM of VES-H₈R₈ or Str-
H₈R₈ for 2 h before inhibitors were added. An optimized concentration of 1 µM of carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was used. Basal respiration, proton leak, ATP production and maximum-respiratory rate were calculated as reported before and averaged from three biological repeats.[159]

2.3.14 ROS production assay

Reactive oxygen species (ROS) in cells were detected using 5-(and-6)-carboxy-2,7-dichlorodihydrofluorescein diacetate (CDCFDA) (AAT Bioquest, CA, USA) as previously described.[160] 20,000 cells were seeded into 48-well plates and allowed to adhere for 24 h at 37 °C in a 5% CO₂ and 95% air humidified incubator. The cells were treated the next day for either 2 or 5 h in full media. Control treatments and peptides were incubated at 8 µM, in the range of the IC₅₀ of both Str-H₈R₈ and VES-H₈R₈. A similar DMSO concentration was used as a control. Hydrogen peroxide (H₂O₂) at 1 mM was used as a positive control. Following treatment, cells were washed with PBS 3 times, and then incubated at 37 °C in full media containing 2 µM CDCFDA for 30 min. The cells were washed 3 times with PBS, harvested, and placed on ice before measuring fluorescence using a flow cytometer within 1 h. Cell debris and doublets were gated out using FSC-A vs FSC-H, and 10,000 events were collected. The mean fluorescence intensity was collected in the FL-1 channel (ex/em 488/533(30) nm) and averaged from three biological replicates. The results were expressed as fold increase in mean fluorescence intensity of treated group relative to DMSO control. To assess the radical scavenger capability of free vitamin E, 100 µM of vitamin E was incubated simultaneously with peptide treatments (8 µM) for 5 h and ROS was measured as stated above.

2.3.15 Apoptosis induction assay

Apoptosis was measured using Annexin V-Cy5 (Biovision Inc, CA, USA) and 7-aminoactinomycin D (7-AAD) (AAT Bioquest, CA USA).[161, 162] 20,000 cells were seeded into 48-well plates and allowed to adhere for 24 h at 37 °C in a 5% CO₂ and 95% air humidified incubator. The cells were treated the next day with peptides at 5, 10, and 20 µM for 2 h in full media. A similar DMSO concentration was used as a control. Following treatment, floating cells were collected, adhered cells were harvested, washed with PBS, and then incubated with Annexin V-Cy5 (1 µL/mL) and 7-AAD (15 µg/mL) for 20 min at 25 °C as per the manufacturer’s protocol. Cells were then placed on ice before measuring fluorescence in a flow cytometer within an hour.
Cell debris and doublets were gated out using FSC-A vs FSC-H, and at least 10,000 events were collected. Using the untreated control group, gates were set in the FL-3 (ex/em 488/>670 nm) and FL-4 channel (ex/em 640/675(25) nm) for 7-AAD and Annexin-V-Cy5 respectively, and identical gates were used for the other treatment groups. Proportion of apoptotic or necrotic cells were averaged from 3 biological repeats.

2.3.16 Cell cycle analysis

Cell cycle analysis was performed using 7-AAD (7-aminoactinomycin D) (AAT Bioquest, CA USA). 20,000 cells were seeded into 48-well plates and allowed to adhere for 24 h at 37 °C in a 5% CO₂ and 95% air humidified incubator. The cells were treated the next day for 24 h in full media. Control treatments and peptides were incubated at 15 µM. A similar DMSO concentration was used as a negative control. Following treatment, the cells were harvested, washed with PBS, then incubated with 25 µg/mL of 7-AAD in PBS containing 1% bovine serum albumin (BSA) and 0.15% Triton X for 25 minutes. Cells were placed on ice before measuring fluorescence in a flow cytometer. Cell debris and doublets were gated out using the FSC-A vs FSC-H, and 10,000 events were collected. The FL-3 channel (ex/em 640/>670 nm) was set in a linear range, and cell cycle analysis was completed using the FlowJo software (Flowjo, OR, USA) using the Watson Pragmatic Mod Fit algorithms. The results were expressed as proportion of cells in the G1, S, and G2 phases relative to DMSO control, and averaged from three biological repeats.

2.3.17 Statistical analysis

All statistical analyses were performed using Graph Pad Prism version 6.00 for Windows (Graph Pad Software, San Diego, California, www.graphpad.com). Differences among groups were assessed by one-way ANOVA with Tukey’s multiple comparison test. Alpha levels were set at 0.05 and a p-value of <0.05 was set as the criteria for statistical significance. Graphs are annotated with p-values as *p < 0.05, **p < 0.01, or ***p < 0.001. All data are presented as mean ± standard deviation.
2.4 Results and Discussion

2.4.1 H₈R₈-based cationic lipids have selective anti-cancer activity

To investigate the effect of the lipid modification of H₈R₈ on its anti-cancer properties, the half maximum inhibitory concentration (IC₅₀) was evaluated with two breast cancer cells, parental breast cancer cells, EMT6/P, and the permeation glycoprotein (Pgp) overexpressing, MDR variant, EMT6/AR-1. We used the resazurin-based Presto Blue metabolic assay as a proxy to evaluate cell survival. Both Str-H₈R₈ and VES-H₈R₈ exhibited an IC₅₀ in the low micromolar range while the unmodified peptide control, H₈R₈, exhibited an IC₅₀ above 300 µM in both cancer cell lines (Figure 2.3A, Figure S 2.3A). Similarly to unmodified H₈R₈, poly(ethylene glycol) (PEG)-modified H₈R₈ showed no cytotoxicity, confirming the necessity of providing a lipophilic character to H₈R₈ cationic peptides for anti-cancer activity (Figure S 2.3A). While not attributed to the cationic amphiphilic structure by the authors, a similar strategy was employed with the cationic Tat peptide, which, when conjugated to paclitaxel, resulted in enhanced anti-cancer activity with increased paclitaxel uptake in MDR cancer cells relative to paclitaxel alone.[163] Here, the unmodified Tat peptide was non-toxic to the cancer cells while the Tat modified paclitaxel exhibited potent anti-cancer activity in both parental and MDR cancer cells. In our study, both Str- and VES-H₈R₈ had similar activities on each cell line, with an IC₅₀ on EMT6/P of 4.2 ± 0.1 µM and 4.4 ± 0.1 µM, respectively; and an IC₅₀ on EMT6/AR-1 of 6.8 ± 0.3 µM and 7.3 ± 0.3 µM, respectively. While the pKₐ of histidine is 6.0 and intratumoral pH can range between 6.5-6.9, we do not anticipate that the more acidic tumor environment would enhance the anti-cancer activity of both Str-H₈R₈ and VES-H₈R₈ as the histidine would remain mostly deprotonated.[164-166] Interestingly, due to the amphiphilic nature of the modified peptides, Str-H₈R₈ and VES-H₈R₈ formed nanoparticles in PBS exhibiting diameters of 11.1 nm ± 0.3 nm and 10.9 nm ± 0.2 nm, respectively, and nanoparticle diameters did not change in acidic buffer with pH 5.3 (Figure S 2.1A,B and Table S 2.2). Importantly, both Str-H₈R₈ and VES-H₈R₈ nanoparticles exhibited high critical micelle concentrations greater than 257 µM, indicating that both peptides exist as non-aggregated unimers in the low 0-20 µM range used (Figure S 2.1C,D and Table S 2.2). For both lipid-modified cationic peptides, there was a significant increase in IC₅₀ (p < 0.001) on MDR cancer cells compared to the parental cell line, suggesting that the activity of the H₈R₈-based amphiphiles was partially dependent on Pgp efflux activity. Pgp is able to efflux both hydrophobic and hydrophilic drugs, thereby reducing the effective intracellular drug concentration. For example, Pgp has been shown
to efflux docetaxel and doxorubicin, requiring 100-fold more drug to kill MDR cancer cells than non-resistant cancer cells, whereas lipid-modified cationic peptides bypass the efflux capabilities.[167, 168] Consistent with the literature, VES alone exhibited some anti-cancer activity, with IC$_{50}$ values of 23.0 ± 2.0 µM and 36.0 ± 4.6 µM on EMT6/P and EMT6/AR-1, respectively.[169, 170] Covalent modification of VES to H$_8$R$_8$ led to a 5-fold decrease in IC$_{50}$ on both EMT6/P and EMT6/AR-1 relative to VES alone.

We compared the anti-cancer activity of protease resistant D-amino acid-based lipid modified cationic peptides to that of their faster degrading L-amino acid counterparts.[171] Surprisingly, only a modest decrease in the IC$_{50}$ was observed using the D-amino acid containing H$_8$R$_8$-based amphiphiles (Table S 2.1[Error! Reference source not found.]). The enhanced activity may be attributed to the lower binding affinity to membrane-associated heparin sulfates and/or slower rates of internalization, as previously reported for similar cell penetrating peptides.[172] Thus, we used L-amino acid-based amphiphiles going forward as the L-amino acid peptides are naturally present in the body and the reduced degradability of D-amino acid containing peptides may be toxic to non-cancer cells.[171]

To confirm the anticancer selectivity of H$_8$R$_8$-based amphiphiles, the IC$_{50}$ of each amphiphile against MDR cancer cells was compared to that of non-cancer cells. NIH/3T3 cells were selected as the non-cancer cell line as fibroblasts are major stromal cells and they are typically in close proximity to cancer cells.[173] Cancer selectivity is typically deduced from the difference in IC$_{50}$ between cancer and endothelial or fibroblast cells.[174] The IC$_{50}$ of VES-H$_8$R$_8$ and Str-H$_8$R$_8$ on healthy cells was up to 8.4-fold and 4.3-fold higher, respectively, than that of breast cancer cells (p > 0.001, Figure 2.3A), demonstrating greater selective toxicity to cancer vs. healthy cells. Interestingly, the selectivity that we observed is consistent in order of magnitude with that observed of the clinical chemotherapeutic, docetaxel, where the IC$_{50}$ on NIH/3T3 cells is 6.4 fold higher than that on breast cancer EMT6/P cells (50 nM vs 7.8 nM).[175, 176] In both breast cancer cell lines investigated, VES-H$_8$R$_8$ was twice as selective compared to Str-H$_8$R$_8$, which may be due to the cancer selective activity reported for VES alone.[174, 177, 178] The anti-cancer selective activity of both VES-H$_8$R$_8$ and Str-H$_8$R$_8$ may arise from differences in cell membrane composition (e.g., o-glycosylated mucin concentration), mitochondrial polarization and greater cell proliferation of cancerous vs healthy cells.[179-181] The difference in membrane potential is attributed to healthy cells exposing more zwitterionic phospholipids vs cancer cells exposing more
anionic phospholipids and negatively charged glycoproteins.[181, 182] The mitochondrial membrane potential is typically more negative in cancerous than healthy cells. For example, Neu4145 cancer cells exhibit a mitochondria membrane potential of -210 mV whereas healthy cells typically exhibit a mitochondrial membrane potential in the range of -108 to -159 mV, which makes cancer cells more sensitive to mitochondria selective treatment.[36, 152] As drug sensitive and MDR cancer cells exhibit more polarized mitochondria relative to non-cancerous cells, we anticipate that VES-H₈R₈ and Str-H₈R₈ will be potent against other drug sensitive and MDR cancer cells.

To gain greater insight into the role of the hydrophobic tail to anti-cancer activity, we measured the IC₅₀ of a series of H₈R₈-based amphiphiles as a function of the lipid length of the amphiphilic peptides on EMT6/AR-1 (Figure 2.3B) and EMT6/P cells (Figure S 2.3B). Both unmodified H₈R₈ and butyl-modified H₈R₈ showed limited anti-cancer activity, with IC₅₀ above 200 µM. However, octyl-modified H₈R₈ exhibited an IC₅₀ of 25.6 ± 1.9 µM, and longer hydrophobic segments (C₁₂ and longer) exhibited IC₅₀ in the low µM range (<7 µM). These results suggest that H₈R₈-based amphiphiles modified with a lipid greater than or equal to 12 carbons is the threshold for anti-cancer activity. The enhanced anti-cancer activity of longer hydrophobic tails is likely due to enhanced membrane association and cell penetration, as was observed for C₁₀-C₁₆ modified heptaarginine (R₇).[183] Interestingly, mitochondrial-penetrating peptides with a logP in the range of -1.0 to -1.4 exhibited superior mitochondrial targeting relative to less hydrophobic peptides, highlighting a potential relationship between mitochondrial colocalization and anti-cancer activity.[56] We chose to further investigate Str-H₈R₈ and VES-H₈R₈ for cytotoxicity and targeting as the stearyl and VES moieties share similar logP values. While VES is known to be bioactive and Str-H₈R₈ has been used in gene delivery and nanoparticle formulations, there may be important and untapped synergistic effects encapsulating VES-H₈R₈ or Str-H₈R₈.[184, 185] Moreover, stearyl-modified cationic peptides have been used for nanoparticle-based drug delivery systems in vivo, where we anticipate that safe doses may be used with VES-H₈R₈ or Str-H₈R₈, prompting further in vivo investigations.[186, 187]
Figure 2.3 H₈R₈-based amphiphiles exhibit potent and selective anti-cancer activity. (A) Comparison of the IC₅₀ of Str-H₈R₈ and VES-H₈R₈ on the parental breast cancer cell line, EMT6/P, the multi-drug resistant breast cancer cell line, EMT6/AR-1, and the healthy fibroblast cell line, NIH/3T3. (B) Relationship between the anti-cancer activity on EMT6/AR-1 cells and the lipid length of the various H₈R₈-based amphiphiles. Data are presented as a mean ± SD (n=3) and statistical analyses was performed using one-way ANOVA and Tukey’s multiple comparison test (***p < 0.001).

2.4.2 Bioactive VES-modified cationic peptides enhances their retention in breast cancer cells

To investigate mitochondrial targeting of the conjugated peptides, EMT6/AR-1 cells were incubated with fluorescein-labeled Str-H₈R₈ and VES-H₈R₈. Within 3 h of incubation in full medium, membrane association and cell penetration were evident. The fluorescence from both Str-H₈R₈ and VES-H₈R₈ indicate colocalization with the Mitotracker dye, which stains the mitochondria (Figure S 2.4A, B).[188] Similarly to lipid modified octaarginines, the cell uptake of both Str-H₈R₈ and VES-H₈R₈ are expected through the endo-lysosomal pathway.[189] The resultant bright and punctate signals, suggestive of endo-lysosomes, make it difficult to visualize peptide uptake into the mitochondria. Therefore, we quantified the peptide uptake in isolated mitochondria from peptide treated EMT6/AR-1 cells. Intact cells were incubated with either fluorescein-labeled Str-H₈R₈, VES-H₈R₈, H₈R₈, or free fluorescein for 3 h, and mitochondria were isolated through differential centrifugation.[190] The concentration of fluorescein in the isolated mitochondria was quantified and both Str-H₈R₈ and VES-H₈R₈ treated cells exhibited significantly higher uptake relative to H₈R₈ and free fluorescein (p < 0.001, Figure 2.4A). Relative to Str-H₈R₈, VES-H₈R₈ treated cells exhibited significantly higher peptide uptake in the mitochondrial, possibly due to efflux inhibition on the plasma membrane and enhanced mitochondrial targeting (p < 0.05).[153] Importantly, both Str-H₈R₈ and VES-H₈R₈ treated cells exhibited significantly higher...
peptide uptake in the mitochondria relative to H₈R₈, suggesting that the lipid modification of H₈R₈ is crucial for mitochondrial targeting (p < 0.001). Lipid modified cationic peptides exhibit increased cell uptake due to enhanced membrane association and translocation, and hence increased mitochondrial uptake.[183] Therefore, we investigated the uptake of fluorescein-labeled Str-H₈R₈, VES-H₈R₈, H₈R₈, and free fluorescein in isolated mitochondria from untreated EMT6/AR-1 cells for 1 h. Isolated mitochondria treated with either Str-H₈R₈ or VES-H₈R₈ exhibited significantly higher peptide uptake relative to H₈R₈, confirming that lipid modification is required for mitochondrial uptake (p < 0.001, Figure 2.4B). Interestingly, isolated mitochondria treated with VES-H₈R₈ exhibited significantly higher peptide uptake relative to Str-H₈R₈ (p < 0.001). VES is more hydrophobic than stearyl, which may be beneficial in penetrating the hydrophobic inner mitochondrial membrane.[56] Doxorubicin-resistant breast cancer cells have been shown to express efflux pumps on the mitochondrial membranes, such as breast cancer related protein (BRCP) and multi-drug resistance protein (MRP1).[47] Relative to Str-H₈R₈, VES-H₈R₈ may also inhibit efflux pumps found on the mitochondrial membranes. The localization of VES-H₈R₈ and Str-H₈R₈ within the mitochondria is unclear, prompting further investigations to verify if both peptides penetrate into the mitochondria matrix.

To investigate the role of VES-H₈R₈ on efflux pump inhibition,[191] we compared the uptake and retention of fluorescein-labeled VES-H₈R₈ to that of Str-H₈R₈ with MDR breast cancer EMT6/AR-1 cells by flow cytometry. Interestingly, cell uptake was similar after 2 h of incubation; however, cells treated with VES-H₈R₈ showed significantly more fluorescein uptake at 5 and 24 h compared to those treated with Str-H₈R₈ (p < 0.001, Figure 2.4C). To evaluate long-term retention of the peptides in the EMT6/AR-1 cells, the peptide-containing medium was removed after 24 h, and cells were left incubating for an additional 24 h in fresh medium. In these conditions, VES-H₈R₈ led to a ~8-fold higher retention in cells relative to that of Str-H₈R₈. Given that both amphiphiles should exhibit similar proteolytic stability, the increased uptake and retention observed for VES-H₈R₈ likely results from specific Pgp efflux inhibition. This observation is consistent with other studies where both vitamin E and VES have been shown to inhibit Pgp efflux in cancer cells.[191] Furthermore, PEGylated-VEs is an established Pgp efflux inhibitor that has higher anti-cancer activity and greater efflux inhibition than VES alone.[153] Co-incubating VES and fluorescein-Str-H₈R₈ with EMT6/AR-1 cells led to a 12-fold increase in retention at 48 h vs. fluorescein-Str-H₈R₈ alone, further confirming the role of VES in Pgp efflux inhibition (Figure S 2.5 Retention
of fluorescein-labeled Str-H8R8 with or without vitamin E succinate (VES) in EMT6/AR-1 after 48 h. Peptides were removed after 24 h, and the cells were incubated with fresh media for another 24 h. Data are presented as a mean ± SD (n=3) and statistical analysis performed using one-way ANOVA and Tukey’s multiple comparison test (***(p < 0.001)). Thus, VES-H₈R₈ is an attractive amphiphile towards MDR treatment and would be more efficacious with the fluorescein replaced with a chemotherapeutic, such as doxorubicin, which would otherwise be effluxed by Pgp.[192]

Figure 2.4 Mitochondrial uptake and time-dependent uptake and retention of the H₈R₈-based amphiphiles. (A) Concentration of the dye retained in mitochondria isolated from EMT6/AR-1 cells upon a 3 h treatment of intact cells with either free dye, fluorescein, or fluorescein labelled H₈R₈-based peptides. (B) Concentration of the dye retained in mitochondria isolated from EMT6/AR-1 cells upon a 1 h treatment of isolated mitochondria with either free dye or fluorescein labelled H₈R₈-based peptides. Free dye was used as a negative control, whereas
fluorescein modified H₈R₈ was used as a peptide control. (C) Time-dependent uptake and retention of fluorescein-labeled H₈R₈-based amphiphiles in EMT6/AR-1 cells during 2, 5 and 24 h incubation. Peptides were removed after 24 h, and the cells were incubated with fresh media for another 24 h. Data are presented as a mean ± SD (n=3) and statistical analysis performed using one-way ANOVA and Tukey’s multiple comparison test (*p < 0.05, ***p < 0.001).

2.4.3 Lipid-modified cationic peptides affect mitochondria polarization and bioenergetics

We compared the effect of H₈R₈-based amphiphiles on mitochondria depolarization, using the JC-1 probe, to a series of controls, including the positive control of carbonyl cyanide m-chlorophenyl hydrazone (CCCP), which showed the lowest mitochondrial membrane potential (Figure 2.5A). EMT6/AR-1 cells treated with H₈R₈-based amphiphiles showed significantly reduced mitochondrial membrane potential relative to all the controls (i.e., VES, stearic acid, unmodified peptide (H₈R₈), PEG-H₈R₈ and DMSO). Similar results were obtained with the parental cell line (Figure S 2.6A). These results suggest that mitochondria depolarization is involved in the cytotoxic mechanism of action of lipid-modified cationic peptides.

To better understand mitochondrial depolarization, we investigated VES-H₈R₈ and Str-H₈R₈ on the induction of mitochondrial permeability transition pore (mPTP). mPTP consists of adenine nucleotide translocase (ANT), cyclophilin D (CypD), and a voltage-dependent anion channel (VDAC), which together form a pore through the outer and inner mitochondrial membrane allowing for solutes < 1500 Da to leak out into the cytosol.[193] The presence of mitochondrial proteins in the cytoplasm induces intrinsic apoptosis.[194] We investigated the involvement of mPTP by inhibiting CypD binding with cyclosporine A (CsA), as previously reported;[149] however, since CsA is also a potent inhibitor of the Pgp efflux pump in the EMT6/AR-1 cells (and would result in JC-1 accumulation), we used EMT6/P cells to investigate the role of mPTP (Figure 2.5B).[195] While both Str-H₈R₈ and VES-H₈R₈ depolarized the mitochondria, in the presence of CsA, mitochondria polarization was maintained at levels similar to those of DMSO and CsA controls (p > 0.05), demonstrating the involvement of mPTP induction in the mechanism of action of H₈R₈-based amphiphiles. The CsA-sensitive mitochondrial depolarization that we observed is consistent with that of guanidine-containing streptomycin and other cationic amphiphilic peptides, such as melittin and mastoparan.[196, 197] Interestingly, decreased mitochondria membrane fluidity can induce mPTP, as may be the case with VES-H₈R₈ and Str-H₈R₈ treatment. This mechanism was observed with mastoparan, which interacts with the lipid phase of the
mitochondrial membrane.[198, 199] VES alone can also induce mPTP, further supporting this mechanism for the VES-H₈R₈ treatment. Interestingly, with cationic polymers of higher molecular weight, such as PEI (> 25 kDa), CsA treatment did not maintain mitochondria polarization, possibly because PEI itself may form pores in the mitochondria membranes, allowing solutes to leak out.[149, 200]

Mitochondria bioenergetics were studied by measuring mitochondrial respiratory states in intact EMT6/AR-1 cells treated with H₈R₈-based amphiphiles using real-time measurements of oxygen consumption rates (OCR), as previously reported (Figure 2.5C and Figure S 2.6B).[201] Oligomycin was used to inhibit the mitochondrial F₀/F₁-ATP synthase, representing mitochondrial proton leak across the mitochondria inner membrane as a proxy for ATP production. Carbonylcyanide-p-trifluoromethoxyphenylhydrazone was used to measure the highest capacity of the electron transport chain, providing the maximum respiratory rate. All mitochondrial bioenergetics states of EMT6/AR-1 cells were significantly inhibited when treated with H₈R₈-based amphiphiles (Figure 2.5C). Following a 2 h treatment, the basal respiration of EMT6/AR-1 cells were significantly inhibited compared to DMSO controls (p < 0.001). Furthermore, both cationic amphiphiles significantly inhibited proton leak, ATP production, and maximum respiratory rate relative to DMSO controls, which was expected as the proton current generated by basal respiration supports proton leak and ATP production (p < 0.001).[201] Similar results were obtained in the parental cell line, EMT6/P (Figure S 2.6C). A decrease in ATP production directly affects Pgp-mediated drug efflux. For example, curcumin inhibited ATP production in doxorubicin resistant MCF7 cells, which led to efflux inhibition, and increased accumulation and retention of doxorubicin.[167] The changes in mitochondrial respiration states are consistent with other studies using cationic polymers where, for example, H1299 and C2C12 cells treated with linear or branched PEI had decreased basal respiration, proton leak, maximum respiration rate, and ATP production.[159]
Figure 2.5 H₈R₈-based amphiphiles depolarize mitochondria via a mitochondrial permeability transition pore (mPTP) dependent pathway and alter mitochondria bioenergetics. (A) The mitochondria membrane polarization of EMT6/AR-1 cells was measured after a 5 h treatment with Str-H₈R₈, VES-H₈R₈, or their controls using the JC-1 probe and flow cytometry. (B) Using EMT6/P cells, mPTP-dependent depolarization was validated by co-incubating H₈R₈-based amphiphiles with cyclosporine A (CsA), an mPTP inhibitor, for 2 h. (C) Mitochondrial bioenergetic states of EMT6/AR-1 cells measured after incubation with H₈R₈-based amphiphiles for 2 h. Mitochondrial membrane potential and oxygen consumption rates are normalized to DMSO treated cells. Data are presented as mean ± SD (n=3) and statistical analyses were performed using one-way ANOVA and Tukey’s multiple comparison test (N.S. > 0.05, *p < 0.05, **p < 0.01, ***p < 0.001).

2.4.4 Lipid-modified cationic peptides induce ROS

mPTP induction and mitochondrial depolarization typically involve increased cellular levels of ROS, triggering complex signaling cascades that lead to cell death.[2] To test whether this was
happening during the treatment of EMT6/AR-1 cells with H₈R₈-based amphiphiles, ROS levels were evaluated after treatment with Str-H₈R₈ and VES-H₈R₈, and compared to controls using 5-carboxy-2,7-dichlorodihydrofluorescein diacetate (CDCFDA) (Figure 2.6). After 5 h of incubation with Str-H₈R₈ or VES-H₈R₈, ROS levels significantly increased to 200.9 ± 43.5% and 179.2 ± 26.3%, respectively (p<0.01), which is similar to ROS levels with exposure to the positive control, hydrogen peroxide, of 266.7 ± 28.6%. Controls including VES, stearic acid, PEG-H₈R₈ and H₈R₈, did not increase ROS levels relative to DMSO controls (p > 0.05). Similar results were observed with the EMT6/P cells (Figure S 2.7A). Str-H₈R₈ significantly induced ROS after 2 h of incubation relative to controls whereas, surprisingly, VES-H₈R₈ did not (p < 0.001, Figure S 2.7B). Vitamin E has well known antioxidant properties whereby the phenolic alcohol acts as a radical scavenger.[202, 203] We therefore hypothesized that the delayed induction of ROS by VES-H₈R₈ was attributed to the cleavable ester present within vitamin E succinate, and subsequent radical scavenging. To test this hypothesis, we synthesized a vitamin E-modified H₈R₈ with a more stable butyl ether linker (VEB- H₈R₈) (Figure S 2.7C). Unexpectedly, however, treating EMT6/AR-1 cells for 2 h with the ether-linked VEB-H₈R₈ induced ROS levels comparable to those of the ester-linked VES-H₈R₈ (Figure S 2.7B). The ether linkage may be oxidized by intracellular reactive oxygen intermediates, cleaving vitamin E and thereby enabling ROS scavenging.[204, 205]

**Figure 2.6** Reactive oxygen species (ROS) levels are significantly increased upon treatment with H₈R₈-based amphiphiles. EMT6/AR-1 cells were incubated with treatment groups for 5 h and

[45]
the CDFDA probe. Flow cytometry was used to determine relative ROS levels to DMSO controls. Data are presented as a mean ± SD (n=3) and statistical analyses were performed using one-way ANOVA and Tukey’s multiple comparison test (N.S. p > 0.05, **p < 0.01).

2.4.5 Lipid-modified cationic peptides induce apoptosis, necrosis, and cell cycle arrest

Mitochondria depolarization and increased ROS levels often lead to apoptosis;[201] however, synthetic cationic polymers, such as PEI, have been reported to damage cell membranes and cause necrotic cell death.[149, 159] To investigate the mechanism of cell death upon treatment with lipid-modified cationic peptides, annexin-V-Cy5 and 7-AAD were used to monitor the levels of apoptosis and necrosis, respectively.[206] Both Str-H₈R₈ and VES-H₈R₈ induced death of EMT6/AR-1 cells by apoptosis and necrosis significantly more than DMSO controls (Figure 2.7A-C) and in a concentration-dependent manner (Figure 2.7D-E). Consistent with their IC₅₀ values, 5 µM of each of the lipid-modified cationic peptides had minimal effect compared to the DMSO control in terms of necrosis and apoptosis (p > 0.05). Interestingly, at 20 µM, VES-H₈R₈ treatment led predominately to apoptosis (46.9 ± 2.8%) which is similar to that observed with VES alone[207] whereas Str-H₈R₈ resulted in greater necrosis (44.2 ± 2.0%) which may be due to plasma membrane damage, as was observed for other amphiphilic peptides.[208, 209]

H₈R₈-based amphiphile treatments were expected to arrest cells in a specific cell cycle as mitochondria depolarization and increased ROS levels should inhibit progression past cell cycle checkpoints.[210] We studied cell cycle distributions of EMT6/AR-1 cells treated with the H₈R₈-based amphiphiles by flow cytometry, using 7-AAD as a nucleic dye. Cells treated with Str-H₈R₈ and VES-H₈R₈ were arrested in the G1 cell cycle phase relative to DMSO controls (Figure 2.7F and Figure S 2.8A-C, p < 0.01), which is consistent with the reported mechanisms of VES, pegylated-VEs and melittin.[207, 211-213] Consequently, there were fewer cells in the S (proliferation) and G2 cell cycle phases when treated with Str-H₈R₈ and VES-H₈R₈ vs. DMSO (p<0.001) Similar results were observed in the EMT6/P cells (Figure S 2.8D). None of the controls, including VES, stearic acid, PEG-H₈R₈ and H₈R₈, significantly arrested the EMT6/P and EMT6/AR-1 cells in any phase (Figure S 2.8E, F). While it is not clear whether H₈R₈-based amphiphiles interact with any of the cell cycle regulatory or checkpoint proteins, sonic hedgehog signaling may be implicated based on its involvement with other cationic peptides.[213]
Figure 2.7 Apoptosis, necrosis, and G₁ cell cycle arrest are induced upon treatment of EMT6/AR-1 with H₈R₈-based amphiphiles. Representative histograms of (A) VES-H₈R₈, (B) Str-H₈R₈, and (C) DMSO treated cells showing annexin-V-Cy5 (apoptosis) and 7-aminoactinomycin D (7-AAD) (necrosis) staining. Identical gates were set up to demonstrate the proportion of live, apoptotic (apop) and necrotic (necro) populations. (D) The proportion of cells in the apoptotic state upon treatment with either H₈R₈-based amphiphiles at 5, 10, and 20 uM or DMSO controls for 2 h. (E) The proportion of cells in a necrotic state upon treatment with either H₈R₈-based amphiphiles at 5, 10, and 20 uM or DMSO controls for 2 h. (F) The proportion of cells in G₁, S or G₂ cell cycle when treated with the H₈R₈-based amphiphiles relative to that when treated with DMSO. Data are presented as a mean ± SD (n=3) and statistical analyses were performed using one-way ANOVA and Tukey’s multiple comparison test (N.S. p > 0.05, **p < 0.01, ***p < 0.001).
2.5 Conclusion

Two lipid-modified cationic peptides, Str-H$_8$R$_8$ and VES-H$_8$R$_8$, were selectively cytotoxic to multi-drug resistant breast cancer cells in the low micromolar range, with VES-H$_8$R$_8$ showing greater selectivity and drug efflux inhibition. Similar to other cationic amphiphilic structures, lipid-modified cationic peptides target, disrupt and depolarize mitochondria, inhibit mitochondrial bioenergetics, decrease Pgp efflux, and induce ROS production. These effects led to apoptosis and/or necrosis, and G1 cell cycle arrest. These properties of VES-H$_8$R$_8$ suggest its utility for chemotherapeutic delivery to MDR cancer cells in terms of enhanced uptake, retention, and anti-cancer activity.

2.6 Acknowledgements

We are grateful to the Shoichet Lab for thoughtful review and to the following funding agencies NSERC (Discovery to MSS, CREATE M3 to PCC) and CIHR (Foundation to MSS).
## 2.7 Supplemental Information

### Table S 2.1 Peptide characterization and anti-cancer activity in EMT6/P and EMT6/AR-1

<table>
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<tr>
<th>Peptide</th>
<th>MS Expected (g/mol)</th>
<th>MS Obtained (g/mol)</th>
<th>IC_{50} (µM)</th>
<th>IC_{50} (µM)</th>
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<tr>
<td>H_{8}R_{8}</td>
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<td>2363.40</td>
<td>301.3 ± 43.0</td>
<td>330.0 ± 7.2</td>
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<td>Butyl-H_{8}R_{8} (C4-H_{8}R_{8})</td>
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<td>2545.50</td>
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<td>Pentadecyl-H_{8}R_{8} (C15-H_{8}R_{8})</td>
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<td>2587.39</td>
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**Figure S 2.1** Characterization of the nanoparticles formed from VES-H$_8$R$_8$ and Str-H$_8$R$_8$. Dynamic light scattering measurements of the nanoparticles formed from (A) VES-H$_8$R$_8$ or (B) Str-H$_8$R$_8$, in PBS with pH 7.4, 6.3 or 5.3. Critical micelle concentration measurements of the nanoparticles formed by (C) VES-H$_8$R$_8$ or (D) Str-H$_8$R$_8$ as determined by dynamic light scattering in PBS (pH 7.4). (n=3, mean ± SD)

**Table S 2.2** Characterization of the nanoparticles formed by VES-H8R8 and Str-H8R8 at various pH, and critical micelle concentrations as determined by dynamic light scattering.

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<th>Name</th>
<th>Diameter @ pH 7.4 (Polydispersity Index)</th>
<th>Diameter @ pH 6.3 (Polydispersity Index)</th>
<th>Diameter @ pH 5.3 (Polydispersity Index)</th>
<th>Critical Micelle Concentration (CMC) [µM]</th>
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<td>VES-H$_8$R$_8$</td>
<td>10.9 nm ± 0.2 nm (0.04 ± 0.01)</td>
<td>11.3 nm ± 0.1 nm (0.13 ± 0.02)</td>
<td>9.8 nm ± 0.2 nm (0.05 ± 0.02)</td>
<td>257.0 ± 61.7</td>
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<tr>
<td>Str-H$_8$R$_8$</td>
<td>11.1 nm ± 0.3 nm (0.10 ± 0.05)</td>
<td>11.2 nm ± 0.2 nm (0.12 ± 0.01)</td>
<td>10.7 nm ± 0.1 nm (0.12 ± 0.03)</td>
<td>350.6 ± 62.6</td>
</tr>
</tbody>
</table>
Figure S 2.2 Synthesis and characterization of Vitamin E-oxy-butyric acid (A) Schematic representing the synthesis of Vitamin E-oxy-butyric acid. (B) Mass Spectrometry of the Vitamin E-oxy-butyric acid. (ESI-MS: C_{33}H_{56}O_{4} expected m/z 516.8, found 515.4 corresponding to M-H). (C) $^1$H NMR spectra of Vitamin E-oxy-butyric acid where the letters on the molecule correspond to the assigned $^1$H NMR peaks observed.

Figure S 2.3 Anti-cancer activity of H$_8$R$_8$-based amphiphiles and controls. (A) IC$_{50}$ of the H$_8$R$_8$-based amphiphiles and controls in EMT6/p and EMT6/AR-1 cells. ^ denotes toxicity was not observed at the highest tested concentration, 125 µM. (B) The IC$_{50}$ of various H$_8$R$_8$-based amphiphiles in the EMT6/P cells. Data are presented as a mean ± SD (n=3) and statistical
analysis was performed using one-way ANOVA and Tukey’s multiple comparison test (**p<0.001).

**Figure S 2.4** Intracellular localization of the H₈R₈-based amphiphiles in EMT6/AR-1 cells. Confocal image cross-sections of the intracellular localization of (A) fluorescein-labeled VES-H₈R₈ and (B) Str-H₈R₈ in EMT6/AR-1 cells after a 3 h incubation period. Mitochondria were stained with MitoTracker Deep Red (red), nuclei were stained with Hoesht 3342 (blue), and peptides were modified with fluorescein (green). Co-localization between peptides and mitochondria are shown as yellow and indicated by white arrows. (scalebar = 10 µm)

**Figure S 2.5** Retention of fluorescein-labeled Str-H₈R₈ with or without vitamin E succinate (VES) in EMT6/AR-1 after 48 h. Peptides were removed after 24 h, and the cells were incubated with fresh media for another 24 h. Data are presented as a mean ± SD (n=3) and statistical
Figure S 2.6 H₈R₈-based amphiphiles depolarize the mitochondria in EMT/P and alter the bioenergetics of the mitochondria. (A) Mitochondria membrane polarization was measured in EMT6/P after treatment with H₈R₈-based amphiphiles or controls. (B) Real-time oxygen consumption rates (OCR) in EMT6/Ar-1 when pre-treated with H₈R₈-based amphiphiles and addition of inhibitors. (C) Mitochondrial bioenergetics measured in EMT6/P cells where the bioenergetic states were normalized relative to DMSO. Data are presented as a mean ± SD (n=3) and statistical analysis performed using one-way ANOVA and Tukey’s multiple comparison test (N.S. p> 0.05, *p< 0.05, **p< 0.01, ***p< 0.001).
**Figure S 2.7** H₈R₈-based amphiphiles induce ROS production in EMT6/P and only Str-H₈R₈ induces ROS in EMT6/AR-1 within 2 h. (A) ROS are significantly increased upon a 5 h treatment with H₈R₈-based amphiphiles in EMT6/P. (B) Str-H₈R₈ significantly induces ROS within 2 h in EMT6/AR1. (C) Chemical structure of Vitamin E succinate-H₈R₈-NH₂ (VES-H₈R₈) and Vitamin E-butyl-H₈R₈-NH₂ (VEB-H₈R₈) Data are presented as a mean ± SD (n=3) and statistical analysis performed using one-way ANOVA and Tukey’s multiple comparison test (N.S. p>0.05, **p < 0.01, ***p < 0.001)
**Figure S 2.8** H₈R₈-based amphiphiles induce a G1 cell cycle arrest. Representative histograms of DNA content of (A) VES- H₈R₈, (B) Str- H₈R₈, and (C) DMSO treated EMT6/AR-1 cells. Control treatments did not significantly increase the proportion of cells in a cell cycle relative to DMSO in (D) EMT6/Ar-1 and (E) EMT6/P cells. (F) Treatment of H₈R₈-based amphiphiles in EMT6/P cells led to increased proportions of cells in the G1 cell cycle relative to DMSO controls. Data are presented as a mean ± SD (n=3) and statistical analysis performed using one-way ANOVA and Tukey’s multiple comparison test (N.S. p> 0.05, *p < 0.05, ***p < 0.001)
Mitochondrial targeting VES-H8R8 and pH-sensitive palmityl-doxorubicin, co-encapsulated in polymeric nanoparticles, act synergistically against multi-drug resistant breast cancer cells.

3.1 Abstract

Multi-drug resistance remains a major obstacle in cancer treatment due to active drug efflux driven by mitochondria. By targeting mitochondria with cationic lipids, such as vitamin E succinate modified with octahistidine-octaarginine (VES-H₈R₈), mitochondria are depolarized and indirectly inhibit drug efflux in multi-drug resistant (MDR) breast cancer cells. We hypothesized that the co-delivery of a chemotherapeutic, such as doxorubicin, with VES-H₈R₈ would achieve greater cytotoxicity than either alone. To investigate the synergism of this system, doxorubicin was first modified with palmityl via an acid-labile hydrazone bond, resulting in a pH-sensitive prodrug of doxorubicin (pDox) which resulted in greater encapsulation efficiency. VES-H₈R₈ and pDox were co-encapsulated in polymeric nanoparticles and shown to be more cytotoxic to MDR breast cancer cells than either alone and at lower concentrations. The mechanism of synergism was demonstrated with pDox uptake and retention, Pgp efflux inhibition, mitochondrial depolarization, enhanced induction of ROS and apoptosis. Thus, doubly-loaded nanoparticles of pDox and VES-H₈R₈ more effectively kill MDR breast cancer cells than singly-loaded nanoparticles of each alone.

3.2 Introduction

Multi-drug resistance remains a major obstacle in cancer treatment because certain cancer cells are able to survive and proliferate under clinical doses of chemotherapeutic agents.[143] Nonspecific acquired resistance arises when cancer cells overexpress drug efflux pumps, such as breast cancer resistance protein, multidrug resistance-associated protein, and permeation glycoprotein (Pgp).[145, 214, 215] For example, Pgp expression in cancer tissue increases from 11% in untreated patients to 30% in those who have undergone chemotherapy, resulting in an increased prevalence of MDR tumors.[216] Drug efflux is an active process that requires adenosine triphosphate (ATP) to pump various chemotherapeutics out of the cell and lower the effective intracellular drug concentration.[142] While multi-drug resistant (MDR) inhibitors that target drug efflux pumps have been investigated in clinical trials, none are currently used in the clinic due to
unacceptable toxicity and undesired drug-drug interactions.[42, 46] Both intrinsic and acquired MDR depend on the mitochondria, making these organelles attractive intracellular targets.

The mitochondria of cancer cells are hyperpolarized and remain so after chemotherapy, opening new opportunities for tumor targeting.[146] The difference in mitochondrial membrane potential between normal epithelial cells and cancer cells is at least 60 mV as demonstrated in 200 cell lines derived from tumors of breast, prostate, liver and others.[217-221] For example, Neu4145 cancer cells have a mitochondrial membrane potential of -210 mV whereas non-cancer cells have a potential of -138 mV.[36, 152, 222] Mitochondrial targeting of MDR cancer cells with chemotherapeutics can be achieved with delocalized lipophilic cations, such as triphenylphosphine- and guanidine-containing compounds (e.g., arginine), and mitochondrial-penetrating peptides, composed of alternating hydrophobic and cationic amino acids.[56, 144] The vitamin E succinate modified octahisitidine-octaarginine (VES-H$_8$R$_8$) conjugate targets cancer mitochondria and selectively kill MDR cancer cells by mitochondria depolarization, inducing reactive oxygen species (ROS) production, apoptosis, and G1 cell cycle arrest.[223] Herein, we take advantage of these properties of VES-H$_8$R$_8$ and investigate its co-delivery with a prodrug of the potent chemotherapeutic, doxorubicin, from polymeric nanoparticles.

Polymeric nanoparticles (NPs) solubilize hydrophobic drugs and enable encapsulation of multiple therapeutics for delivery to the tumor.[96, 224, 225] Relative to free drug combinations, co-encapsulation strategies offer enhanced therapeutic advantages, such as spatiotemporal release in vivo, wherein the initial drug ratio is maintained from the injection site to the tumor.[4] For example, relative to the free drug combination, the co-encapsulation of doxorubicin and mitomycin-C in NPs resulted in prolonged systemic circulation, enhanced tumor accumulation, and maintenance of synergistic drug ratios over 24 h.[115] The synergistic co-encapsulation of cytarabine and daunorubicin also demonstrated clinical success against high-risk acute myeloid leukemia (AML), improving the survival of patients with AML.[7, 119] Thus, NPs are suitable carriers for multiple drugs and support synergistic anti-cancer activity against tumors.

We explored, for the first time, the potential synergism of co-encapsulated VES-H$_8$R$_8$ and a prodrug of doxorubicin with MDR breast cancer cells. As doxorubicin is water soluble (> 50 mg/mL) and rapidly leaks out of NPs, we synthesized palmityl-modified doxorubicin (pDox) using a pH-sensitive hydrazone linkage, resulting in a hydrophobic prodrug of doxorubicin that is suitable for
encapsulation.[226, 227] We anticipated that pDox would be water-insoluble at neutral pH and release doxorubicin from NPs in the acidic endolysosomal environment after endocytosis.[228, 229] pDox was successfully synthesized and the pH-dependent release of pDox from NPs was characterized. We hypothesized that delivering co-encapsulated VES-H₈R₈ and pDox in NPs would be more cytotoxic to MDR cancer cells than each drug alone because VES-H₈R₈ not only impacts viability through a mitochondria-mediated mechanism, but it also enhances pDox uptake and retention and therefore greater nuclear cytotoxicity (Figure 3.1). We tested this hypothesis by investigating the co-delivery of VES-H₈R₈ and pDox in NPs against both parental and MDR breast cancer cells in terms of NP uptake and retention, Pgp efflux inhibition, mitochondria depolarization, induction of ROS, and cell death. We demonstrate effective killing of MDR breast cancer cells with VES-H₈R₈ and pDox co-released from the same NPs.
Figure 3.1 Schematic representation of the proposed mechanism against MDR breast cancer cells of polymeric nanoparticles co-encapsulated with vitamin E succinate modified octahistidine-octaarginine (VES-H₈R₈) and palmityl-doxorubicin (pDox). Polymeric nanoparticles are composed of: (A) poly(D,L-lactide-co-2-methyl-2-carboxytrimethylene carbonate)₁₂K-g-poly(ethylene glycol)₁₀K (P(LA-co-TMCC)-g-PEG) (black wavy line represents hydrophobic backbone and orange wavy line represents grafted PEG); (B) Vitamin E succinate (red rectangle) modified octahistidine-octaarginine (blue wavy line) (VES-H₈R₈); and (C) a pH-responsive hydrazone bond links palmityl (green wavy line) to doxorubicin (pink square) (pDox). (D) Drugs and polymers are combined in an aqueous solution to form self-assembled nanoparticles. (E) VES-H₈R₈ and pDox loaded NPs (VDNPs) are endocytosed through the endolysosomal pathway where VES-H₈R₈ and hydrolyzed Dox (pink square) escape into the cytosol. VES-H₈R₈ decreases the mitochondrial membrane potential and indirectly inhibits the permeation glycoprotein (Pgp) efflux.
of Dox, allowing its cytosolic accumulation. Accumulated Dox poisons topoisomerase II and, together with VES-H₈R₈, effectively kills multi-drug resistant breast cancer cells.

### 3.3 Materials and Methods

All solvents and reagents were purchased from Sigma-Aldrich and used as received unless otherwise noted. Peptide synthesis reagents were purchased from AnaSpec (Fremont, CA).

#### 3.3.1 Vitamin E Succinate modified Octahistidine-Octaarginine (VES-H₈R₈) Synthesis

VES-H₈R₈ was synthesized as previously reported.[223] Briefly, H₈R₈ was synthesized by conventional solid-phase microwave assisted peptide synthesis techniques (CEM Liberty 1) on rink amide ProTide Resins (CEM Corp, NC, USA). The N-terminus of H₈R₈ was modified using 5.0 eq of vitamin E succinate activated with 4.9 eq of 2-(6-Chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethyleniminium hexafluorophosphate (HCTU) for 24 h. After washing the resin, the peptide was cleaved using trifluoroacetic:water:triisopropylsilane (TFA:H₂O:TIS, 95:2.5:2.5) for 5 h and purified through a C18 reverse phase column (Silicycle, Quebec, Canada). Peptide mass was verified through electrospray ionization or matrix assisted laser desorption ionization-time of flight mass spectrometry using the Agilent 6538 Q-TOF mass spectrometer.

#### 3.3.2 Palmityl-Doxorubicin Synthesis

Palmitic acid hydrazide was reacted with the ketone of doxorubicin, to produce the pH-sensitive hydrazone bond of palmityl-doxorubicin (pDox), as previously described (Figure S 3.3A). [226, 230] Briefly, 150 mg of doxorubicin free base (MedKoo Biosciences, Inc, NC, USA) and 82 mg of palmitic acid hydrazide (1.1 eq) were added to 300 mL of MeOH:DCM solution (1:1, v/v) containing 15 µL of trifluoroacetic acid. The reaction proceeded for 24 h during which the reaction progress was monitored by thin layer chromatography (TLC) using MeOH:DCM (1:3, v/v). After the reaction was complete, the solvent was removed by rotary evaporation, and the crude was dissolved in a 2 mL volume of MeOH:DCM (1:20, v/v). The dissolved crude was purified by silica column chromatography using DCM, and eluted with increasing concentrations of MeOH until the product, pDox, was eluted. Fractions containing pDox were collected, solvent removed by rotary evaporation, and dried in a vacuum oven to yield a red oil (142 mg, yield = 65%). ^1H NMR (500 MHz, DMSO-d₆, δ in ppm): 7.65-7.92 (m, aromatics), 5.44 (t, CH₂), 5.28 (t, CH₂), 4.40 (CH₂),
3.98 (s, CH₃), 3.54 (m, CH), 2.34 (s, CH₃), 1.27-1.10 (m, CH₂), 1.20 (s, CH₃) ppm. (Figure S 3.3B)

m/z calculated for protonated pDOX ([M + H]⁺): 795.97 found: 796.63. (Figure S 3.3C)

3.3.3 Polymer Synthesis

Poly(D,L-lactide-co-2-methyl-2-carboxytrimethylene carbonate)₁₂K-graft poly(ethylene glycol)₁₀k-azide (P(LA-co-TMCC)-g-PEG-N₃) was synthesized following previously established protocols.[130, 137, 141] Benzyl ester protected P(LA-co-TMCC) (P(LA-co-TMCC-Bn)) was synthesized by ring opening polymerization of D,L-lactide (1.50 g, 10.40 mmol) and benzyl ester protected 2-methyl-2-carboxy-trimethylenecarbonate (TMCC-Bn) (0.59 g, 2.34 mmol) catalyzed by 1-[3,5-bis-(trifluoromethyl)phenyl]–3-[(1R,2R)-(−)-2(dimethylamino)cyclohexyl] thiourea (0.14 g, 0.34 mmol) and initiated by a pyrenebutanol (23.50 mg, 85.50 µmol) in 10 mL of distilled dichloromethane (DCM) under argon for 7 days. The copolymer was precipitated twice in hexanes and vacuum dried. The crude copolymer was then purified on a silica column using a solvent mixture of DCM:methanol:ammonium hydroxide (97.5:2.0:0.5). Solvent was removed from the purified copolymer by rotary evaporation, dissolved in DCM, and precipitated in hexanes twice before placing in a dry oven overnight. (1.23 g, 58.5% yield) ¹H NMR(CDCl₃): δ8.25–7.23 (s, CH from pyrene initiator), 7.32 (m, aromatic from TMCC-Bn), 5.15 (m, CH from LA), 4.32 (m, CH₂ from TMCC), 1.57 (m, CH₃ from LA) ppm (Figure S 3.1A). According to the end group analysis of the pyrene initiator calculated by ¹H NMR, there are on average 123 lactide units (LA), and 12 2-methyl-2-carboxytrimethylene carbonate (TMCC) units per copolymer, with a number average molecular weight of 10,776 g/mol.

The benzyl ester group in P(LA-co-TMCC-Bn) was deprotected by using activated palladium on carbon (20 wt%) in a 1:1 mixture of tetrahydrofuran:ethylacetate solution under hydrogen gas for 7 days. The palladium was filtered off, solvent was removed by rotary evaporation, and P(LA-co-TMCC) was placed in a dry oven overnight.(0.95 g, 77.2% yield) Successful benzyl deprotection was verified by ¹H NMR by the disappearance of the benzene peaks at 7.33 ppm.

P(LA-co-TMCC)-g-PEG-N₃ was obtained by activating the acids in P(LA-co-TMCC) (200 mg, 17.4 µmol) using N,N’-diisopropyl-carbodiimide (DIC, 32.9 mg, 261 µmol) and hydroxybenzotriazole (HOBt, 37.6 mg, 278 µmol) in dimethylformamide (DMF, 6 mL) for 30 minutes. Amino-poly(ethylene glycol)₁₀K-azide (696 mg, 69.6 µmol) was dissolved in DMF (6 mL) and added to the activated P(LA-co-TMCC) and allowed to react for 24 h. Borate buffer (0.5
mL, pH 9, 500 mM) was then added, and the solution was dialyzed against distilled water for 24 h (2 kg/mol MWCO). Unreacted PEG was removed using a Sepharose CL-4B column equilibrated with distilled water. Fractions containing the copolymer were verified using Bradford reagent, and these fractions were collected and freeze-dried to afford a fluffy, white solid. (573 mg, 64% yield). ^1H NMR (CDCl3): δ5.16 (m, CH from LA), 4.33 (m, CH₂ from TMCC), 3.64 (bs, CH₂ from PEG) 1.57 (m, CH₃ from LA) ppm. PEG conjugation was calculated by group analysis comparing area under the curve for PEG (3.64 ppm) vs. LA (5.16 ppm) as previously reported (Figure S 3.1B). [130] There are approximately 2.7 PEGs per copolymer chain.

### 3.3.4 Nanoparticle Preparation

1.2 mg of VES-H₈R₈ and 1.5 mg of pDox were added to 6 mg of P(LA-co-TMCC)-g-PEG-N₃ in 100 µL of methanol:acetonitrile solution (1:1, v/v), and then diluted with 2 mL of distilled water. Singly loaded NPs were prepared using an identical NP protocol, except only one drug was added at the same respective wt%. The remaining methanol and acetonitrile was removed by rotary evaporation at 45 °C. NPs were filtered through a 0.45 µm PES filter, and aliquots were frozen at -80 °C until used. The diameters and zeta potential of NPs were measured using a Malvern Zetasizer Nano ZS (4 mW, 633 nm laser) at a concentration of 1 mg/mL, and NPs were diluted in a solution of sodium chloride to a final concentration of 10 mM for the zeta potential measurements.

### 3.3.5 Critical micelle concentration (CMC) measurements

CMC was calculated based on the scattering intensity measured by dynamic light scattering as previously described[156, 231] The scattering intensity was measured using a DynaPro Plate Reader II (Wyatt Technologies) configured with a 60 mW, 830 nm laser and a detector angle of 158°. Blank NPs, doxorubicin-NPs (DNPs), VES-H₈R₈-NPs (VNPs) or doubly-loaded NPs (VDNPs) were added to clear bottom 96-well plates at a NP concentration of 2.5 mg/mL in PBS (pH 7.4), and serially diluted with measurements of three acquisitions per sample. Curve fitting algorithms were used to determine the CMC of the NPs.

### 3.3.6 Drug Loading Characterization

NP formulations containing pDox were quantified by fluorescence using the Tecan Infinite M200 Pro fluorescent plate-reader. NP formulations were diluted in DMSO:PBS (200:25, v/v), and pDox
encapsulation was quantified by fluorescence measurement (ex/em 480/580 nm) against a standard curve. VES-H₈R₈ encapsulation was quantified using standard amino acid analysis protocols. Briefly, samples submitted for amino acid analysis were subjected to acidic hydrolysis for 24 h, labelled with a fluorophore and quantified by high-performance liquid chromatography coupled to a fluorometer. Amino acid analysis was completed at the SicKids Proteomics, Analytics, Robotics and Chemical Biology Centre (SPARC) Biocenter. Peptide concentration in the samples were calculated by dividing the quantified histidine and arginine amino acids by 8, as per the peptide sequence, and averaging the values from both histidine and arginine. Average drug loading was obtained from three separate NP batches, calculated according to the following equation:

\[
\text{Drug Loading (\%)} = \frac{\text{Mass}_{pDox} + \text{Mass}_{VHR}}{\text{Mass}_{pDox} + \text{Mass}_{VHR} + \text{Mass}_{\text{Polymer}}} \times 100
\]

3.3.7 pDox release study

Doxorubicin control, doxorubicin-NPs (DNPs), or doubly-loaded NPs (VDNPs), were diluted in either PBS (pH 7.4) or PBS/citric acid (pH 5.0) at a NP concentration of 100 µg/mL and sealed in dialysis tubing (Spectra/Por 6, 3.5 kDa, Spectrum Labs, CA, USA). The formulations were then allowed to dialyze against 40 mL of either PBS (pH 7.4) or PBS/citric acid (pH 5.0), under shaking at 37 °C. At indicated times, 25 µL of the dialysate was sampled, diluted with 200 µL of DMSO and measured by fluorescence (ex/em: 480/580 nm) as a proxy for Dox release. After 48 h, the remaining encapsulated pDox was quantified as above. Three batches of each formulation were tested. Released Dox was calculated by the amount detected according to the following equation:

\[
\text{Released Dox (\%)} = \frac{\text{Fluorescence Intensity of Dox}_{\text{Diasylate at Time } X}}{\text{Fluorescence Intensity of pDox}_{\text{Encapsulated at 48 h}}} \times 100
\]

3.3.8 Cell Culture

Both WT (EMT6/P) and doxorubicin resistant (EMT6/AR1) EMT6 cells were generously provided by Dr. X.Y. Wu (University of Toronto), originally from Dr. Ian F. Tannock (Ontario Cancer Institute, Toronto, ON, Canada), and maintained in our laboratory. Non-cancerous fibroblast cells (NIH/3T3) were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified incubator with 5% CO₂ atmosphere. EMT6/P cells were grown in α-MEM supplemented with 10% fetal bovine serum and 1%
penicillin/streptomycin at 37 °C in a humidified incubator with 5% CO₂ atmosphere. EMT6/AR-1 cells were grown as EMT6/P, with the addition of doxorubicin at 1 µg/mL to maintain doxorubicin resistance and Pgp overexpression.

3.3.9 NP potency assay

Cells were seeded into 96-well, flat-bottomed tissue culture plates at a density of 3,000 cells per well, and allowed to adhere for 24 h. Treatments were incubated with cells for 24 h in full medium, then replenished with fresh media and incubated for an additional 48 h. Singly-loaded NPs (DNPs and VNPs) were dose-matched to the respective drug concentration of doubly-loaded NPs (VDNPs). After the addition of NP formulations, cells were allowed to grow for 24 h at 37 °C in a 5% CO₂ and 95% air humidified incubator. The Presto Blue (Life Technology) assay was used as a proxy for cell relative viability, calculated according to the following equation:

\[
\text{Relative Viability (\%)} = \frac{\text{Fluorescence Intensity}_{\text{Treated}}}{\text{Fluorescence Intensity}_{\text{Untreated}}} \times 100\%
\]

3.3.10 Synergism Analysis

Combination indices (CI) were calculated using the median-effect analysis taking advantage of the Compusyn software (ComboSyn Inc. NY, USA) [108, 109] The median-effect analysis considers both the shape and potency of the dose response curves on cell viability. The dose response curves of individual and combination treatments were linearized into a median effect plot of \(\log((f_a)^{-1} - 1)^{-1}\) vs \(\log[D]\), where \(f_a\) is the fraction of dead cells, and \(D\) is the drug concentration. CI values > 1, 1, < 1 are indicative of antagonistic, additive, or synergistic effects, respectively.

3.3.11 Confocal Microscopy

EMT6/AR-1 cells were seeded at a density of 2 x 10⁴ cells per well in a 8-well Nunc Lab-Tek II chambered cover glass (Thermo Fisher Scientific, MA, USA) and allowed to adhere for 24 h at 37 °C in a 5% CO₂ and 95% air humidified incubator. The concentration of doxorubicin or pDox (Ex/Em: 488/550 nm) was maintained at 5 µM for doxorubicin controls, DNPs and VDNPs. After incubation times of 2, 5 and 24 h in full medium, cells were washed 3x with PBS, and then Hoescht 33342 nuclei acid dye (Molecular Probes, Inc., Eugene, OR, USA; ex = 405, em = 460 nm) in PBS was added. Live cell imaging was performed using an Olympus FV1000 confocal microscope equipped with an oil immersion 60x lens.
3.3.12  Doxorubicin and pDox Uptake and Retention Studies

2 x 10^4 cells were seeded into 48-well plates and allowed to adhere for 24 h at 37 °C in a 5% CO_2 and 95% air humidified incubator. The concentration of doxorubicin or pDox was maintained at 5 µM for doxorubicin controls, DNPs and VDNPs. DNPs were either incubated alone, or with VNP s dose-matched at the concentration of VES-H_8R_8 in VDNPs. To test Pgp inhibition, DNPs were also incubated with 20 µM of vitamin E succinate. Untreated cells were used as negative control to ensure minimal fluorescent noise during flow cytometry analysis. After 24 h of treatment in full medium, cells were washed 3-times with PBS, and harvested with trypsin. Cell fluorescence was analyzed using a BD Accuri C6 flow cytometer. Cell debris was gated out using FSC-A vs SSC-A and doublets were gated out using FSC-A vs FSC-H, and at least 1 x 10^4 events were collected. The mean fluorescence intensity was in the FL1 channel (ex = 488 nm, em = 533 nm / 30 nm) measured on three biological repeats to evaluate the doxorubicin or pDox uptake and retention.

3.3.13  Mitochondrial Membrane Polarization Assay

Mitochondrial membrane potential was assayed using the JC-1 probe (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolycarbocyanine iodide) (Biotium Inc., CA, USA). Both DNP- and VDNP-treated cells exhibited fluorescence in the FL-2 channel due to the fluorescence of pDox. As such, VNPs were used to explore the effects of mitochondrial membrane polarization. VNPs was incubated at its IC_50 (14 µM) as well as the IC_50 of the concentration of VES-H_8R_8 in VDNPs (4 µM). 2 x 10^4 cells were seeded into 48-well plates and allowed to adhere for 24 h at 37 °C in a 5% CO_2 and 95% air humidified incubator. The cells were treated the next day for 5 h in full media. Carbonyl cyanide m-chlorophenyl hydrzone (CCCP) is a rapid mitochondrial depolarizer and was used as a positive control (50 µM). Following treatment, the cells were washed with PBS 3-times, and then incubated at 37 °C with 10 µM of JC-1 in full medium for 30 min. The cells were then washed three times in PBS, harvested, and placed on ice before measuring fluorescence in a flow cytometer within an hour. Cell debris was gated out using FSC-A vs SSC-A and doublets were gated out using FSC-A vs FSC-H, and at least 10,000 events were collected. A gate was set according to DMSO and CCCP treated cells in the FL-2 channel (ex = 488 nm, em = 585 nm / 40 nm) to measure the proportion of JC-1 aggregate fluorescence versus JC-1 monomer fluorescence in the FL-1 channel (ex = 488 nm, em = 533 nm / 30 nm). Averages were obtained from three
biological repeats. Changes in the mitochondrial membrane potential ($\Delta \Psi_m$) were calculated according to equation:

\[
Relative \text{ Mitochondrial Membrane Potential} = \frac{\frac{JC - 1_{\text{aggregate}}}{JC - 1_{\text{monomer \ Treatment}}}}{\frac{JC - 1_{\text{aggregate}}}{JC - 1_{\text{monomer \ Control}}}} \times 100
\]

3.3.14 ROS Production Assay

Reactive oxygen species (ROS) in cells were detected using the cellular reactive oxygen species detection assay kit (ROS Deep Red) (Abcam, ON, Canada) as per manufacturer’s protocols. 2 x $10^4$ cells were seeded into 48-well plates and allowed to adhere for 24 h at 37 °C in a 5% CO$_2$ and 95% air humidified incubator. The cells were treated the next day for 2, 5, or 24 h in full medium at the IC$_{50}$ of VDNPs, where DNPs, VNPs, and DNPs+VNPs were dose-matched to the respective concentration of pDox or VES-H$_8$R$_8$ in VDNPs. Untreated cells were used as a control for background fluorescence in the FL-4 channel. Hydrogen peroxide (H$_2$O$_2$) at 1 mM was used as a positive control. Following treatment, the cells were washed with 3x PBS, and then the cells were incubated at 37 °C in full medium with ROS deep red for 30 min. The cells were washed with 3x PBS, harvested, and placed on ice before measuring fluorescence in a flow cytometer within an hour. Cell debris were gated out using FSC-A vs SSC-A and doublets were gated out using FSC-A vs FSC-H, and 10,000 events were collected. The mean fluorescence intensity was collected in the FL-4 channel (ex = 640 nm, em = 675 nm / 25 nm) and averaged from three biological replicates. The results were expressed as fold increase in mean fluorescence intensity of treated cells relative to the untreated control group.

3.3.15 Apoptosis and Necrosis Assays

Apoptosis was measured using Annexin V-Cy5 (Biovision Inc, CA, USA) and necrosis using the Live or Dead Fixable Dead Cell Staining Kit [Deep Red Fluorescence] (Catalog # 22604, AAT Bioquest, CA USA). Apoptotic cells have exposed phosphatidylserine on the external cell membrane that binds to Annexin V-Cy5, while necrotic cells exhibit membrane damage that allows Live or Dead Fixable Dead Cell Staining dye to bind to DNA. Briefly, 20,000 EMT6/AR-1 cells were seeded into 48-well plates and allowed to adhere for 24 h at 37 °C in a 5% CO$_2$ and 95% air humidified incubator. EMT6/AR-1 cells were analyzed for induction of apoptosis the next
day after treatment for 2, 5, or 24 h in full medium at the IC$_{50}$ of VDNPs, where DNPs, VNPs, and DNPs+VNPs were dose-matched to the respective concentration of pDox and VES-H$_8$R$_8$ in VDNPs. Identical treatment groups were used with a 24 h treatment period to analyze necrosis. Untreated control cells were used to measure background fluorescence in the FL-4 channel. Following treatment, floating cells were collected, adhered cells were harvested, washed with PBS, and then incubated with Annexin V-Cy5 (0.1 % v/v) or with Live or Dead Fixable Dead Cell Staining Kit for 20 min at 25 °C as per manufacturer’s protocol. Cells were analyzed through a flow cytometer as described in the previous section. Gates were set in the FL-4 channel (ex = 640 nm, em = 675 nm / 25 nm) for Annexin-V-Cy5 or Live or Dead Fixable Dead Cell Staining relative to unstained controls. The proportion of apoptotic or necrotic cells were averaged from 3 biological replicates.

### 3.3.16 Statistical Analysis

All statistical analyses were performed using Graph Pad Prism version 6.00 for Windows (Graph Pad Software, San Diego, California, www.graphpad.com). Differences among groups were assessed by one-way ANOVA with Bonferroni post hoc tests. Graphs are annotated where p-values are represented as *p < 0.05, **p < 0.01, or ***p < 0.001. All data are presented as mean ± standard deviation.

### 3.4 Results and Discussion

#### 3.4.1 NP Characterization and pDox Release

All of the nanoparticles were formulated by nanoprecipitation in water: blank NPs, singly-loaded NPs - VES-H$_8$R$_8$-NPs (VNPs) and pDox-NPs (DNPs) - and doubly-loaded VES-H$_8$R$_8$+pDOX NPs (VDNPs). In all of the conditions tested, the average hydrodynamic size of the NPs was ~60 nm, indicating that the nanoparticle size is independent of drug encapsulation (Table 3.1). Most of the NPs also exhibited polydispersity indices below 0.20, indicative of relatively narrow size distribution.[231] Blank NPs had a zeta potential of -5.9 ± 0.5 mV, DNPs had a zeta potential of +1.5 ± 0.2 mV, VNPs of +8.2 ± 0.3 mV and VDNPs of +17.1 ± 1.2 mV. While all the values fall within the typical neutral zeta potential range (i.e., -20 to +20 mV), the change in surface charge reflects the successful encapsulation of cationic VES-H$_8$R$_8$ and pDox. For DNPs, the protonated amine of pDox neutralizes the free acids of P(LA-"co-TMCC)-g-PEG, while for VDNPs the greater positive charge is attributed to both pDox and VES-H$_8$R$_8$. The critical micelle concentrations
(CMCs) of the blank and drug loaded NPs were in the low micromolar range, similar to previously reported CMCs (Figure S 3.2).[231] DNP achieved a pDox loading of 13.4 ± 1.6% while VNP achieved a VES-H₈R₈ loading of 15.1 ± 0.9%. Interestingly, while pDox alone precipitates, NPs solubilize pDox, indicating successful drug encapsulation (Figure S 3.4). VDNPs achieved drug loadings similar to each of the singly-loaded NPs, with 10.9 ± 1.3% and 11.7 ± 0.7% for pDox and VES-H₈R₈, respectively, yielding an average total drug loading of 22.6%.

**Table 3.1.** Physicochemical characteristics of nanoparticles: blank (NP), pDox-NPs (DNP), VES-H₈R₈-NPs (VNP) and doubly-loaded VES-H₈R₈+pDOX NPs (VDNP).

<table>
<thead>
<tr>
<th>Name</th>
<th>Diameter [nm]ᵃ</th>
<th>PDIᵇ</th>
<th>Zeta Potential (mV)ᶜ</th>
<th>Critical Micelle Concentration (µM)ᵃ</th>
<th>pDox Loading (%)ᵈ</th>
<th>VES-H₈R₈ Loading (%)ᵉ</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>64 ± 9</td>
<td>0.19 ± 0.04</td>
<td>-5.9 ± 0.5</td>
<td>8.2 ± 1.6</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>DNP</td>
<td>59 ± 3</td>
<td>0.20 ± 0.02</td>
<td>+1.5 ± 0.2</td>
<td>7.8 ± 0.9</td>
<td>13.4 ± 1.6</td>
<td>n/a</td>
</tr>
<tr>
<td>VNP</td>
<td>57 ± 3</td>
<td>0.21 ± 0.02</td>
<td>+8.2 ± 0.3</td>
<td>9.9 ± 3.4</td>
<td>n/a</td>
<td>15.1 ± 0.9</td>
</tr>
<tr>
<td>VDNP</td>
<td>68 ± 6</td>
<td>0.19 ± 0.01</td>
<td>+17.1 ± 1.2</td>
<td>12.5 ± 1.0</td>
<td>10.9 ± 1.3</td>
<td>11.7 ± 0.7</td>
</tr>
</tbody>
</table>

ᵃmeasured by dynamic light scattering (DLS)
bpolydispersity index (PDI) determined by DLS
czeta potential measurement calculated with NPs in 10 mM NaCl at 1 mg/mL
determined by doxorubicin fluorescence in 10:1, v/v DMSO:PBS against a standard curve,
eetermined by amino acid analysis

NP stability was monitored by hydrodynamic size and polydispersity index (PDI) as a function of time (Figure 3.2A, B). Interestingly, while NPs with high drug loadings typically exhibited limited stability, all formulations maintained size and PDI over at least 96 h at 37 °C.[232] These P(LA-co-TMCC)-g-PEG NPs showed similar stability to those previously tested in serum.[233]

The pH-dependent release of doxorubicin (Dox) from the pDox prodrug was validated by mass spectrometry, where all of the pDox was hydrolyzed within 24 h at pH 5.0 (Figure S 3.5). Importantly, Dox was modified with palmityl hydrazide, instead of modifying P(LA-co-TMCC)-g-PEG with Dox, as the trifluoroacetic acid would degrade the P(LA-co-TMCC) backbone.[234] Dox release was studied at both physiological and acidic pH, using a previously reported dialysis test.[235, 236] As free pDox is insoluble in water and precipitates, we used free Dox as the control in the dialysis test. (Figure S 3.4B). Free Dox controls showed the expected rapid release from the dialysis bag, with > 85% Dox released within 4 h at both pH 5.0 and 7.4 (Figure 3.2C). Both DNPs and VDNPs showed limited Dox release (< 20%) over 48 h at pH 7.4; however, the same
formulations released up to 81% of Dox under acidic conditions (pH 5.0). Interestingly for both DNPs and VDNPs at pH 5.0 within 4 h, a burst release of Dox was observed where changes in NP swelling in acidic media may expose more pDox for hydrolysis. Together, these results confirm the pH-sensitive release of Dox from the NPs. DNPs and VDNPs showed similar profiles for pDox hydrolysis and Dox release, indicating that VES-H₈R₈ co-encapsulation did not impact this process. The release study at pH 5.0 represents the acidity of the late lysosome, reflecting the trafficking for endolysosomal escape of Dox following cell uptake.[236]

Figure 3.2 Nanoparticle (NP) stability studies and pH-responsive release of palmityl-doxorubicin (pDox). (A) NP diameters were measured after incubation in PBS at 37 °C as a function of time for: singly-loaded NPs - pDox-NPs (DNPs) and vitamin E succinate modified octahistidineoctaarginine-NPs (VNPs) - and doubly-loaded vitamin E succinate modified octahistidineoctaarginine+pDox NPs (VDNPs). (B) Polydispersity indices of DNPs, VNPs and VDNPs, were consistent over 96 h. (C) pH-responsive release of Dox from DNPs or VDNPs dispersed in dialysis tubing at pH 7.4 vs pH 5.0 at 37 °C. To control for any barrier posed by the
dialysis membrane, the release of free doxorubicin (Dox) from the dialysis tubing was followed (n = 3, mean ± SD).

### 3.4.2 VDNP Synergistically Increases Cytotoxicity in a MDR Breast Cancer Model

We investigated the benefit of co-encapsulating VES-H₈R₈ and pDox in NPs (VDNPs) for MDR breast cancer cytotoxicity using the Presto Blue assay as a proxy for cell viability (Table 3.2 and Table S 3.1). The IC₅₀ of VDNPs in EMT6/AR-1 cells was 16.1 ± 0.7 µM (concentrations at the IC₅₀: [pDox] = 11.7 µM, [VES-H₈R₈] = 4.3 µM), whereas that of DNPs was 39.4 ± 1.0 µM. VNPs was 14.0 ± 0.8 µM and simply mixing DNPs and VNP resulted in an IC₅₀ of 26.3 ± 2.3 µM (concentrations at the IC₅₀: [pDox] = 19.2 µM, [VES-H₈R₈] = 7.1 µM). The IC₅₀ of VDNPs is lower than that of DNPs mixed with VNP, indicating that co-encapsulation results in more potent anti-cancer activity. Interestingly, the anti-cancer activity of Dox was enhanced when pDox was encapsulated in DNPs, with an IC₅₀ 2.1-fold lower than free Dox alone. This observation is consistent with the literature where drug-loaded NPs facilitate higher intracellular concentrations of the drug in MDR cancer cells.[192, 237, 238]

To assess the synergistic advantage of co-encapsulating VES-H₈R₈ and pDox in NPs, a combination index (CI) was calculated based on the median effect analysis.[109] A median effect plot was produced by converting the dose response curves (Figure S 3.6C, D) into log[(fa)⁻¹ − 1)]⁻¹ vs log(D), where fa is the fraction of non-viable cells and D is the drug concentration (Figure S 3.7). R² > 0.90 indicates statistical validity of the analysis and conforms to mass action law. Nonparallel lines in the median effect plots infer non-exclusivity in the mechanism of action of VES-H₈R₈ and pDox, and satisfies a condition required for the median effect analysis.[108] The median effect plot was analyzed to obtain a CI: CI > 1 reflects antagonistic effects, CI = 1 reflects additive effects, and CI < 1 shows synergistic effects.[109] The CI of VDNPs was 0.61 ± 0.04, suggesting synergism of the co-encapsulated drugs against the MDR EMT6/AR-1 cells whereas that of the mixture of DNPs and VNPs was 0.99 ± 0.07, indicating an additive anti-cancer activity. Interestingly, a similar CI of 0.5-0.9 was reported for NP formulations combining cytarabine and daunorubicin, which improved patient survival.[239, 240] The synergism of VDNPs may be attributed to the pH-dependent release of Dox from NPs, which poisons the topoisomerase II enzyme, coupled with the toxicity of VES-H₈R₈, which targets the mitochondria through depolarization.[241, 242] While the CI of VDNPs is similar to that of
pDox and VES-H₈R₈, the IC₅₀ of VDNPs is significantly lower than that of the mixture of pDox and VES-H₈R₈. VDNPs required approximately 3.4-fold less pDox than DNPs to achieve the IC₅₀ (11.7 µM in VDNPs vs. 39.4 µM in DNPs). Similarly, VDNPs required approximately 3.3-fold less VES-H₈R₈ than VNPs to achieve the IC₅₀ (4.4 µM in VDNPs vs. 14.00 µM in VNPs). The dose reduction indices for both pDox and VES-H₈R₈ are greater than 1, indicative of favorable dose-reduction.[109]

**Table 3.2** IC₅₀ and combination indices of free drugs and drug-loaded NPs on the multi-drug resistant breast cancer cell line, EMT6/AR-1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC₅₀ (µM, Total drug)</th>
<th>Combination Index</th>
<th>[(p)Dox] : [VES-H₈R₈] at IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDNPs</td>
<td>16.1 ± 0.7</td>
<td>0.61 ± 0.04</td>
<td>[11.7] : [4.4]</td>
</tr>
<tr>
<td>DNPs + VNPs</td>
<td>26.3 ± 2.3</td>
<td>0.99 ± 0.07</td>
<td>[19.2] : [7.1]</td>
</tr>
<tr>
<td>DNPs</td>
<td>39.4 ± 1.0</td>
<td>n/a</td>
<td>[39.4] : [0.0]</td>
</tr>
<tr>
<td>VNPs</td>
<td>14.0 ± 0.8</td>
<td>n/a</td>
<td>[0.0] : [14.0]</td>
</tr>
<tr>
<td>Dox</td>
<td>82.2 ± 7.4</td>
<td>n/a</td>
<td>[82.2] : [0.0]</td>
</tr>
<tr>
<td>VES-H₈R₈</td>
<td>12.0 ± 1.9</td>
<td>n/a</td>
<td>[0.0] : [12.0]</td>
</tr>
<tr>
<td>Dox + VES-H₈R₈</td>
<td>28.8 ± 2.9</td>
<td>0.60 ± 0.17</td>
<td>[21.0] : [7.8]</td>
</tr>
</tbody>
</table>

*a* obtained from Presto Blue analyses of drug treated cells  
*b* calculated using CompuSyn.  
*c* molar ratio in DNPs+VNPs and Dox + VES-H₈R₈ were dose matched to respective drugs in VDNPs

Having evaluated VDNP synergism in drug-resistant cells, we next investigated its synergism in the parental, drug-sensitive cells (EMT6/P, Table S 3.1). The IC₅₀ of VDNPs in EMT6 cells was 0.97 ± 0.14 µM (concentrations at the IC₅₀: [pDox] = 0.71 µM; [Ves-H₈R₈] = 0.26 µM) whereas that of DNPs was 0.68 ± 0.12 µM, VNPs was 8.34 ± 1.45 µM, and mixing DNPs and VNPs resulted in an IC₅₀ of 0.99 ± 2.30 µM (concentrations at the IC₅₀: [pDox] = 0.72 µM; [Ves-H₈R₈] = 0.26 µM). The combination index of 0.96 for VDNP indicates that the co-encapsulated NPs only exhibited additive anti-cancer effects against the parental, drug sensitive cancer cells. As expected, doxorubicin was cytotoxic to drug-sensitive cells whether delivered in VDNPs or DNPs; however, since VDNPs were more cytotoxic to MDR cells, in those cases where drug-sensitive cancer cells die and drug-resistant cancer cells persist, the synergistic activity of Ves-H₈R₈ and pDox will be required to achieve improved results.
### 3.4.3 VDNP Enhances Uptake, and Retention of pDox

To elucidate the synergistic mechanism of VDNPs, we investigated their time-dependent uptake relative to those of free Dox and DNPs in MDR EMT6/AR-1 cells using confocal microscopy (Figure 3.3A). As expected, the free Dox control showed minimal Dox fluorescence at all time points, confirming active drug efflux in MDR cancer cells. Both DNPs and VDNPs showed time-dependent Dox accumulation over the 24 h period. DNP- and VDNP-treated cells exhibited both punctate structures, suggesting endolysosomal entrapment, and diffuse cytoplasmic fluorescence, indicative of endosomal escape.[243] For both DNPs and VDNPs, Dox release from the NPs was evidenced by its localization in cell nuclei, the intracellular target of Dox.

We quantified the difference in Dox fluorescence between VDNPs, DNPs+VNPs, DNPs, and free Dox using flow cytometry (Figure 3.3B). Here, we incubated treatment groups for 24 h to measure time-dependent uptake followed by a subsequent 24 h in fresh media to measure retention. At all time points, VDNPs had significantly more Dox in the MDR EMT6/AR-1 cells relative to DNPs and free Dox controls (**p < 0.001): after the 24 h incubation, VDNP-treated cells exhibited a mean fluorescence intensity (MFI) of 122,000 ± 3,200, which was 2.4-times greater than that of DNP-treated cells (51,000 ± 3,000) and 16.5-times greater than free-Dox treated cells (7,407 ± 144). VDNPs are advantageous against the MDR EMT6/AR-1 cells due to the synergism of pDox and VES-H8R8 co-delivery, resulting in greater Dox efficacy.

To better understand the importance of co-encapsulation of VES-H8R8 and pDox in NPs, we compared the uptake and retention of VDNPs to that of a mixture of DNPs and VNPs having the identical composition of pDox and VES-H8R8. VDNP-treated cells at 24 h exhibited 1.6-fold greater pDox retention than the mixture of DNPs and VNPs. This difference may be attributed to both the more positive surface charge of VDNPs (+17.1 ± 1.2 mV) vs. DNPs (+1.5 ± 0.2 mV), which would improve cell uptake, and the enhanced endosomal escape afforded by VES-H8R8, thereby delivering more Dox into the cytosol.[244] This observation is consistent with the literature where cationic peptides enhanced cellular uptake of NPs due to electrostatic interactions with the negatively charged plasma membrane.[245]

We explored the retention of pDox in MDR cells as a function of delivery strategy by comparing Dox fluorescence at 48 h. VDNP-treated cells retained significantly more pDox compared to all other groups (**p < 0.001). Specifically, VDNP-treated cells exhibited approximately 2.1-fold
greater pDox retention relative to the mixture of DNPs and VNPs, or DNPs alone and supports the synergistic anti-cancer activity of VDNPs. The increased mean fluorescence intensity of VDNPs can be attributed to both increased cell penetration and reduced Pgp efflux afforded by VES-H₈R₈ in MDR EMT6/AR-1 cells.[191] This is consistent with the literature where VES-H₈R₈ alone reduced the Pgp efflux capability of MDR breast cancer cells.[223] The enhanced uptake and retention of pDox delivered from VDNPs is superior to that from DNPs alone or VNPs and DNPs in MDR breast cancer cells.
Figure 3.3 Multi-drug resistant (MDR) breast cancer cells treated with doubly-loaded nanoparticles (NPs) show a significant increase in doxorubicin (Dox) accumulation and retention compared to controls. (A) Free Dox, singly-loaded NPs - palmityl-doxorubicin-NPs (DNPs) - and doubly-loaded NPs - vitamin E succinate-modified octahistidinoctaarginine/palmityl-doxorubicin-NPs (VDNPs) - were incubated with MDR breast cancer cells for 2, 5 and 24 h. Images were obtained by confocal microscopy, showing nuclei stained with Hoechst 3342 (blue) and pDox or Dox (red). (B) Time-dependent quantification of Dox uptake in MDR breast cancer cells with either VDNPs, DNPs alone, DNPs mixed with vitamin E succinate modified octahistidinoctaarginine-NPs (VNPs), or free doxorubicin (Dox) control. Cells were incubated with each treatment for up to 24 h, followed by 24 h in fresh medium prior to pDox/Dox fluorescence quantification. Data are presented as a mean ± SD (n=3), and statistical analysis performed using one-way ANOVA and Tukey’s multiple comparison test showing statistical significance of VDNP treatment vs all of the other groups, except for the 2 h time point where the difference is to DNPs and Dox controls only (***p < 0.001).

3.4.4 Doubly loaded NPs Induce Mitochondrial Depolarization and ROS

To deepen our understanding of the role of VES-H₈R₈ in VDNPs, we monitored mitochondrial membrane potential with JC-1 – a mitochondria-specific polarization probe.[246] To avoid Dox fluorescence interference with the JC-1 assay, we used VES-H₈R₈ only NPs (VNPs). At the IC₅₀ concentration (14 µM), VNPs significantly depolarized the mitochondrial membrane relative to that of blank NPs and no treatment controls, confirming that VES-H₈R₈ is capable of mitochondrial depolarization when encapsulated in NPs (Figure 3.4A, ***p < 0.001). Moreover, VNPs significantly depolarized the mitochondria at 4 µM, the equivalent dose of VES-H₈R₈ at the IC₅₀ of VDNPs, relative to the no treatment control (*p < 0.05). This suggests that depolarization is involved in the synergistic anti-cancer activity of VDNPs. These results are consistent with mitochondria depolarization observed with free VES-H₈R₈.[247] Dox has also been shown to indirectly depolarize cancer cell mitochondria through Dox-induced oxidative stress, and thus the
combined effects of co-encapsulated VES-H₈R₈ and pDox on mitochondrial depolarization may contribute to the synergistic cytotoxicity observed.[248-250]

As both Dox and VES-H₈R₈ induce ROS, we investigated the effect of VDNPs at their IC₅₀ on ROS levels relative to those of the singly-loaded NPs (i.e., DNPs and VNPs) and the mixture of DNPs+VNPs. VDNPs significantly increased ROS levels relative to all other groups, underlining the importance of co-encapsulating VES-H₈R₈ and pDox to maximize synergism (Figure 3.4B, ***p< 0.001 at 5 and 24 h). The increase in ROS upon VDNP treatment is consistent with the oxidative stress observed with treatments of either Dox or VES-H₈R₈ alone.[250] Thus, the synergistic cytotoxicity of VDNPs is likely due to mitochondrial membrane depolarization and subsequent ROS induction.

![Figure 3.4](image-url)

**Figure 3.4** NPs containing vitamin E succinate modified octahistidine-octaarginine (VES-H₈R₈) (VNPs) depolarize the mitochondrial membrane, and vitamin E succinate modified octahistidineoctaarginine/palmityl-doxorubicin NPs (VDNPs) induce ROS in a time-dependent manner in multi-drug resistant (MDR) breast cancer cells. (A) Mitochondrial membrane potential of EMT6/AR-1 cancer cells treated with VNPs at a VES-H₈R₈ concentration of 14 µM or 4 µM relative to no treatment controls. A VES-H₈R₈ concentration of 14 µM represents the IC₅₀ of VNPs while 4 µM represents the concentration of VES-H₈R₈ at the IC₅₀ of VDNPs. Negative controls include no treatment (Cntl) and blank nanoparticles (NPs) (light orange bars). The positive control, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), decreased the mitochondria polarization. (B) VDNPs treatment induces more ROS relative to singly-loaded NPs, palmityl-doxorubicin (pDox) NPs (DNPs) and VNPs, or a mixture of singly-loaded NPs, VNPs+DNPs, in a time-dependent manner. ROS levels were normalized to no treatment control cells for each time point. VDNPs were incubated at their IC₅₀, while the other NP formulations were dose-matched to the respective drug concentration for either pDox or VES-H₈R₈. Data are presented as the mean (n=3
biological replicates) ± SD, and statistical analyses were performed using one-way ANOVA and Tukey’s multiple comparison test (*p < 0.05, ***p < 0.001).

3.4.5 Doubly loaded NPs Induce Apoptosis

Mitochondrial membrane depolarization and induction of ROS typically result in apoptosis.[149] To investigate the mechanism of cell death when MDR breast cancer cells were treated with VDNPs, treated cells were stained with both Annexin V-Cy5 and 7-AAD for apoptosis and necrosis, respectively. VDNPs incubated at their IC_{50} significantly increased the proportion of apoptotic cells at 2, 5 and 24 h of incubation, compared to singly-loaded NPs (i.e., DNPs and VNPs), and a mixture of DNPs+VNPs (Figure 3.5A, **p < 0.01). VDNPs also significantly increased the proportion of necrotic cells relative to DNPs, VNPs and no treatment controls (***p < 0.001) (Figure 3.5B). Since a greater percentage of cells expressed the Annexin V vs. 7-AAD marker, it is likely that VDNPs induce cell death primarily via apoptosis.

![Figure 3.5 A](image1)
![Figure 3.5 B](image2)

**Figure 3.5** Cell death by apoptosis or necrosis is more prevalent for MDR cells treated with VDNPs than controls. (A) Co-encapsulated Vitamin E succinate modified octahistidine-octaarginine (VES-H₈R₈) and palmityl-doxorubicin (pDox) NPs (VDNPs) induce apoptosis in a time-dependent manner whereas singly-loaded NPs, palmityl-doxorubicin NPs (DNPs) and VES-H₈R₈ NPs (VNPs), or their mixture (i.e., VNPs + DNPs) did not significantly induce apoptosis compared to untreated controls at 2, 5 and 24 h of incubation. (B) VDNPs increased the proportion of cells that were necrotic after a 24 h incubation, relative to singly-loaded NPs, DNPs and VNPs, or their mixture (VNPs+DNPs). VDNPs were incubated at their IC_{50}, while the other NP formulations were dose-matched to the respective drug concentration for either pDox or VES-H₈R₈. Data are presented as the mean (n=3 biological replicates) ± SD, and statistical
analyses were performed using one-way ANOVA and Tukey’s multiple comparison test (**p < 0.01, ***p < 0.001).

3.4.6 Conclusion

We demonstrate, for the first time, the synergistic effect of co-encapsulated VES-HsR₈ and pDox in polymeric nanoparticles against MDR EMT6/AR-1 breast cancer cells through mitochondrial depolarization and Pgp inhibition. The co-encapsulation strategy with VES-HsR₈ may be applicable to other chemotherapeutics prone to Pgp efflux. We demonstrate that incorporating VES-HsR₈ in NP formulations sensitizes drug-resistant breast cancer cells. We highlight the significance of co-encapsulating a novel mitochondria depolarizer and Pgp efflux inhibitor, VES-HsR₈, with a pH-sensitive prodrug of Dox in NPs to synergistically kill MDR breast cancer cells in vitro.

3.4.7 Competing interests

The author(s) declare no competing interests.

3.4.8 Acknowledgments

We thank the members of the Shoichet lab for thoughtful review of this manuscript. We are grateful to the Natural Sciences and Engineering Research Council of Canada (Discovery grant to MSS and NSERC CREATE in M3 scholarship to PC) and the Canadian Institutes for Health Research (CIHR Foundation Grant to MSS) for financial support.
Figure S 3.1 $^1$H NMR of A) P(LA-co-TMCC-Bn) and B) P(LA-co-TMCC)-g-PEG-N$_3$. (A) P(LA-co-TMCC-Bn): $\delta$ 8.25-7.23 (s, CH from pyrene), 7.32 (m, aromatic from TMCC-Bn), 5.15 (m, CH from LA), 4.32 (m, CH$_2$ from TMCC), 1.57 (m, CH$_3$ from LA) ppm. (B) P(LA-co-TMCC)-g-PEG-N$_3$: $\delta$ 5.16 (m, CH from LA), 4.33 (m, CH$_2$ from TMCC), 3.64 (bs, CH$_2$ from PEG) 1.57 (m, CH$_3$ from LA) ppm.
Figure S 3.2 The critical micelle concentration of blank and drug loaded NPs as determined by dynamic light scattering in PBS (pH 7.4). The scattering intensity of serially diluted blank NPs (A), palmityl-doxorubicin NPs (B), vitamin E succinate modified octahistidine-octaarginine (VES-H8R8) NPs (C), and doubly-loaded NPs, (D). (n=3, mean ± SD)
Figure S 3.3 Synthesis and characterization of pH-responsive palmityl-doxorubicin (pDox). (A) Schematic representing the synthesis of pDox using trifluoroacetic acid (TFA) as a catalyst in a
dichloromethane/methanol solution. (B) $^1$H NMR spectra of palmityl hydrazide, palmityl-
doxorubicin (pDox), and doxorubicin (top to bottom). The red boxes represent the $^1$H peaks
attributed to palmitic hydrazide while the blue box represents those of the hydrazone bond
formed in pDox. (C) Mass spectrometry results of purified (pDox) confirm successful synthesis
(expected: 796.63 g/mol; obtained: 796.44 g/mol).

Figure S 3.4 Palmityl-doxorubicin (pDox) alone precipitates in water. (A) pDox mixed with
poly(D,L-lactide-co-2-methyl-2-carboxytrimethylenecarbonate)$_{12k}$-grafted-poly(ethylene
glycol)$_{10k}$-azide [P(LA-co-TMCC)-g-PEG-N$_3$] spontaneously form nanoparticles (NPs) that can
be sterile-filtered. (B) pDox alone immediately precipitates and is completely removed by
filtration.

Figure S 3.5 Characterization of the pH-responsive release of doxorubicin from palmityl-
doxorubicin (pDox) by mass spectrometry (expected: 544.17 g/mol; obtained: 544.18 g/mol).
Free palmitic hydrazide is observed as a degradation product (expected: 270.46 g/mol; observed:
271.27 g/mol), while an expected molecular ion adduct is observed at 305.26 g/mol
(M+CH$_3$OH+H).
Figure S 3.6 Dose-response curves of free drugs in (A) EMT6/P and (B) EMT6/AR-1, and those of doubly-loaded nanoparticles (VDNPs) in (C) EMT6/P and (D) EMT6/AR-1. Dose response curves were obtained after a 24 h incubation, followed by 48 h in fresh medium to allow cells to grow. Singly-loaded NPs, palmityl-doxorubicin NPs (DNPs) and vitamin E succinate modified octahistidine-octaarginine (VES-H₈R₈) NPs (VNPs), doubly-loaded NPs, (VDNPs), and VES-H₈R₈ were tested for anti-cancer activity. Presto blue analysis was used to evaluate cell proliferation.

Table S 3.1 IC₅₀ and combination indices of free drugs and drug-loaded nanoparticles (NPs), in the parental breast cancer cell line, EMT6/P.

<table>
<thead>
<tr>
<th>Name</th>
<th>IC₅₀ (µM, drug)⁴</th>
<th>Total Combination Index⁵</th>
<th>[(p)Dox] : [VES-H₈R₈] at IC₅₀⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDNPs</td>
<td>0.97 ± 0.14</td>
<td>1.07 ± 0.10</td>
<td>[0.71] : [0.26]</td>
</tr>
<tr>
<td>DNPs + VNPs</td>
<td>0.99 ± 0.10</td>
<td>1.09 ± 0.08</td>
<td>[0.72] : [0.27]</td>
</tr>
<tr>
<td>DNPs</td>
<td>0.68 ± 0.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VNPs</td>
<td>8.34 ± 1.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dox + VES-H₈R₈</td>
<td>0.30 ± 0.03</td>
<td>1.51 ± 0.08</td>
<td>[0.22] : [0.08]</td>
</tr>
<tr>
<td>Dox</td>
<td>0.16 ± 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VES-H₈R₈</td>
<td>11.12 ± 2.56</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

⁴Obtained from Presto Blue analysis of drug treated cells.
⁵Calculated using CompuSyn
Molar ratio co-encapsulated in VDNPs, and in DNPs+VNP s and Dox + VES-H$_8$Rs were dose matched to respective drugs in VDNPs.

**Figure S 3.7** Linearized dose-response curves of palmityl-doxorubicin NPs (DNPs), vitamin E succinate modified octahistidine-octaarginine NPs (VNP s), and co-encapsulated vitamin E succinate modified octahistidine-octaarginine and palmityl-doxorubicin (VDNPs) in (A) EMT6-AR-1 cells and (B) EMT6/P cells. $fa$ represents the fraction of dead cells and $D$ is the total drug concentration in each formulation. Non-parallel lines suggest non-exclusivity in the mechanism of action of VNP s and DNPs. $R^2$ values greater than 0.9 indicate statistical validity of the analysis and conforms to mass action law.
4 Towards Nanoparticle delivery of siRNA targeting an Essential Protein in Breast Cancer

4.1 Abstract

Ribonucleic acid interference (RNAi) therapy uses small interfering ribonucleic acids (siRNA) and is a promising strategy for cancer treatment. However, siRNA is rapidly degraded in serum and efficient delivery remains a challenge. Chemical modifications of siRNA enhance serum stability, increase potency, and reduce immunostimulation. To improve delivery, immuno-nanoparticles (NPs) comprised of poly(ᴅ,ʟ-lactide-co-2-methyl-2-carboxytrimethylene carbonate)-g-poly(ethylene glycol) (poly(LA-co-TMCC)-g-PEG) targeting cells overexpressing human epidermal growth factor receptor 2 (HER2) have proven to be an effective vehicle for siRNA. A particularly attractive gene target is eukaryotic translation initiation factor 3 subunit B (eIF3B) which is an essential gene required for cancer cell proliferation. When compared to standard lipid transfections, it is hypothesized that the targeted delivery of eIF3B siRNA (si3B) by immuno-NPs will enhance eIF3B mRNA and protein knockdown, and decrease cell proliferation in HER2 overexpressing cancer cells. A si3B sequence was validated for the potent knockdown of eIF3B at the mRNA and protein levels, while exhibiting durable anti-cancer effects in breast cancer cells for up to 10 d. Dibenzylcyclocctyne modified si3B and Trastuzumab were successfully modified on the NPs, while exhibiting superior nuclease stability suitable for in vitro studies. si3B immuno-NPs failed to transfect breast cancer cells, prompting the use of various amphiphilic, endosomal escape peptides. Incorporating the peptides in si3B modified immuno-NPs significantly enhanced si3B conjugation onto the NPs while increasing cancer cell uptake relative to standard NPs. The si3B modified immuno-NPs encapsulating these peptides failed to transfect breast cancer cells in vitro, pushing forward investigations into alternative, established siRNA delivery vehicles, such as cationic liposomes.

4.2 Literature Review

*This sub-chapter was published in the Journal of Controlled Release.

4.2.1 Abstract

Ribonucleic acid interference therapy is a promising cancer treatment, which uses small interfering RNAs (siRNAs) to target and degrade messenger RNAs. Due to endogenous nuclease activity, siRNA is degraded rapidly, resulting in poor cell uptake and hence specificity. Moreover, it will not readily cross the cell membrane by passive diffusion. In order to take advantage of the therapeutic power of siRNA for the treatment of cancer, specialized delivery vehicles have been designed. In this review, we highlight advances in optimizing nanoparticle functionalization for guided siRNA delivery at the cellular level – that is, promoting cell uptake, escaping the endosome, and releasing siRNA from the delivery vehicle.

4.2.2 Introduction

Ribonucleic acid interference (RNAi) is a powerful tool for the regulation of gene expression, making it an ideal therapeutic for diseases caused by genetic mutations, such as cancer. Often, the cancer cells overexpress oncogenic genes, providing potential targets for gene knockdown [251, 252]. Small interfering ribonucleic acids (siRNAs) are short strands of ribonucleic acid typically composed of 21-30 base pairs with overhanging 3’ ends that can induce sequence-specific gene silencing at low (picomolar) concentrations when transfected into cells [253]. Although other silencing technologies are available, including the CRISPR/Cas9 system and antisense oligonucleotides, among others, siRNAs are advantageous due to their high potency and small size. Additionally, delivery of CRISPR/Cas9 components in vivo is still a major challenge, whereas there are a plethora of siRNA delivery strategies, as described herein. Naturally occurring small interfering ribonucleic acids (siRNAs) were first reported in 1999 in plants [254], and synthetic siRNAs were used to effect gene knockdown in mammalian cells two years later [255]. “Naked” siRNA therapeutics have been successful in clinical trials for ocular diseases when locally delivered at high concentrations, despite limitations including inflammation and increased ocular pressure [256]. The systemic delivery of these therapeutics presents additional challenges following intravenous injection in order to reach cancerous tissue.
When injected, siRNA formulations must (1) evade the immune system, (2) avoid interactions with non-target cells, (3) avoid premature renal clearance, and (4) reach target tissues. These requirements have been reviewed extensively [257-259], thus here we will focus on overcoming further roadblocks once siRNA formulations reach their target tissues, including degradation by extracellular nucleases, poor cell uptake, and trafficking into the lysosomal compartments where the RNA strands are quickly degraded [258, 260]. These challenges often require that siRNA therapeutics are combined with specialized delivery materials in order to be effective.

Nanotechnologies, encompassing a wide variety of formulations including metallic nanoparticles, micelles, liposomes, nanocrystals, nanogels/capsules, among others, are important delivery vehicles for a wide range of therapeutics including small molecule drugs, proteins, and siRNAs [261-263]. Advantages of nanoformulations include improved biodistribution and pharmacokinetics, stabilization of therapeutics, solubilization of hydrophobic drugs, and attenuating toxicity to off-target tissues [264-266]. The size, surface charge, and morphology of the delivery vehicle must be considered as they have a significant impact on pharmacokinetics and biodistribution [267-271]. The morphology of the vehicle can also have a significant impact on cellular internalization rates [272]. Moreover, in order for the nanoparticles to respond to both stimuli on the surface of the cell and within intracellular trafficking pathways, they must be flexible in terms of structure, functionalization, and resultant properties [273-275]. Although lipid-based nanoparticles have played an important role in the development of siRNA delivery strategies, lipid-based formulations are limited to a smaller number of well-established lipid components [276], whereas there are numerous monomers for polymer synthesis [277-280]. Therefore, while lipid-based strategies have been extensively studied and reviewed [281-283], this review will focus on fundamental and novel research in polymeric micelles, nanoparticles, and polyplexes for siRNA therapeutic delivery.

We begin with a brief discussion of stability of siRNA in the extracellular environment, and then examine some of the key challenges of siRNA delivery and trafficking in the target tissues using polymeric delivery vehicles, including: enabling cellular uptake, avoiding degradation within the cell, and successfully releasing the therapeutic payload. While there are many parameters that influence the success of an siRNA nanoparticle delivery system, including uptake specificity, rate of clearance and degradation, the key parameter is efficiency of knockdown and it is this
parameter on which we have based our review.

### 4.2.3 Stability of siRNA in the Extracellular Environment

In order to increase the delivery efficiency of siRNA payloads, siRNAs are often conjugated or complexed to nanoparticles that protect them from nucleases and rapid clearance (Figure 4.1). Within 15 minutes of injection in mice, more than 90% of standard 21-mer siRNAs are degraded by serum nucleases or lost via renal or lymphatic clearance [284], underlining the importance of the delivery vehicle. Polymeric nanoparticles can increase the stability of siRNAs against degradation: Raja et al. demonstrated that crosslinked chitosan nanoparticles increased the stability of siRNAs against serum during a 15 day storage at 4°C [285] while Zhu et al. increased the half-life of siRNA in the blood to approximately 8 hours by encapsulating it within a PLGA-based delivery vehicle, resulting in better tumor accumulation [286]. It is hypothesized that the nuclease resistance conferred by nanoparticle formulations is due to the steric bulk of the polymeric corona, preventing nucleases from reaching the siRNA. Therefore, with increasing density of the polymeric corona, the stability of siRNA in biologically relevant conditions is increased [287, 288].
**Figure 4.1** siRNA nanocarriers protect it from nuclease degradation. (A) Free siRNA (blue double helix) is rapidly degraded by nucleases (orange semi-circle) and (B) cleared by lymphatic drainage (pale blue ovals). (C) Nanoparticles may protect siRNA from nucleases and (D) reduce clearance.

siRNAs can also be chemically modified in order to increase their stability against nucleases. These modifications include any change to the native siRNA structure, typically employed on the phosphodiester bond or sugar ring (Figure 4.2). These modifications enhance siRNA stability and potency, provide longer knockdown duration, reduced off-target effects, and lower immunostimulatory effects [70, 289-291]. Modified siRNAs are now commonly used in research [292-294]. As shown in Figure 4.2, some of the most common modifications of oligonucleotides include modifications to the backbone or nucleosides. For example, backbone modifications include phosphorothioate [295] and boranophosphonate [296] linkages, which increase nuclease resistance, while nucleoside modifications include 2’-O-methyl [297, 298], 2’-deoxy-2’-fluoro [299], and locked nucleic acids [300], which increase stability and target binding affinity. Chemical modification of oligonucleotides and the effect on potency have been extensively reviewed by Deleavey *et al.* [70].
Common modifications to siRNA include modifications to both: (A) the phosphodiester linkage and (B) the 2’ sugar.

4.2.4 Cellular Internalization

Most clinically relevant hydrophobic small molecule drugs can passively diffuse through the cell membrane. SiRNAs are large, hydrophilic, and negatively charged, so their passage across the cell membrane in the absence of a specialized carrier is hindered or blocked entirely. In a nanoparticle formulation, several different internalization pathways are possible - clathrin/caveolar-mediated endocytosis, phagocytosis, macropinocytosis, and pinocytosis - but each ultimately leads to the endolysosomal pathway [301]. Although not directly addressed in the examples that we highlight herein, it has been shown that subsequent endocytic recycling pathways [302] or autophagy [303] may also play a role in limiting the dose of oligonucleotides delivered and should be more extensively studied for polymeric siRNA delivery systems. There are several different interactions that a polymeric vehicle can use to trigger one of these internalization pathways and carry siRNA across the cell membrane, including cationic charge, cell penetrating peptides (CPPs), antibodies, and aptamers (Figure 4.3). Non-specific uptake, via cationic charge or CPPs, is often efficient in vitro, but a more selective strategy requires ligands (antibodies or aptamers) to be taken up by specific cells. Selective strategies may be more
relevant in an *in vivo* setting in order to reduce off-target effects. Here, we present a variety of strategies used to cross the cell membrane from low to high specificity.

![Diagram of nanoparticle formulations crossing the cell membrane](image.png)

**Figure 4.3** Nanoparticle formulations can cross the cell membrane by multiple pathways: (A) deshielding of cationic charges triggered by cues in the tumor microenvironment, yields (B) positively charged nanoparticles that interact with the anionic cell membrane; (C) deprotection of cell penetrating peptides (CPPs) yields nanoparticles that cross cell membranes non-specifically; (D) Antibody-modified nanoparticles target specific receptors on the cell surface for internalization; (E) Nanoparticles modified with Aptamers, selected from phage-display libraries that bind specific cell membrane targets, trigger internalization.

### 4.2.5 Cationic polymers enhance penetration of the cell membrane

Cationic polymers are often used to facilitate siRNA penetration of the cell [304] because they interact with the anionic proteoglycans of the cell membrane, facilitating endocytosis [305, 306]. One classically used cationic polymer for siRNA delivery is polyethyleneimine (PEI) [307]. Highly branched and high molecular weight PEI (>20 kDa) is toxic, so low molecular weight PEI (<2 kDa) is often used [308]. In one example, Lee *et al.* used low molecular weight PEI for the delivery of ‘polymerized’ siRNA - that is, chains of repeating siRNA segments.
connected by disulphide bonds. By using this PEI delivery system, they were able to achieve 70% knockdown in vitro of red fluorescent protein (RFP) in RFP+ melanoma cells [309]. Despite efficient transfection, any cell will non-specifically take up PEI and other positively charged polymers. Since most nano-scale formulations naturally accumulate in the liver [310, 311], many strategies deliver therapeutics against diseases of the liver [311, 312]. In order to target other tissues, the positively charged polymer must be shielded until it reaches the tumor site. To temporarily shield their positive surface charge, cationic nanoparticles are modified with sheddable poly(ethylene glycol) (PEG) coronas using various stimulus-responsive coupling strategies. For example, Li et al. developed a polymeric nanoparticle that is responsive to matrix metalloproteinase 7 (MMP-7), an enzyme that is overexpressed by breast cancer cells and found at high concentrations in the tumor microenvironment [313]. The nanoparticle corona is composed of a PEG block linked by an MMP-7 cleavable peptide to a cationic block. When the nanoparticle reaches the tumor microenvironment, extracellular MMPs cleave the peptide, shedding the PEG layer and exposing the cationic layer, raising the zeta-potential of the nanoparticle from +5.8 to +14.4 mV and increasing cellular internalization 2.5-fold. Nanoparticles pre-treated with MMP-7 resulted in knockdown efficiency of approximately 50% in vitro; however, this system was not studied in vivo, so it is still unclear whether this strategy will result in improved biodistribution [313]. Despite the shielded cationic charge, significant toxicity was observed at high nanoparticle:siRNA ratios, underlining the importance of nanoparticle safety to their utility. Perche et al. took advantage of the hypoxic tumor microenvironment to develop a nanoparticle shielded by PEG conjugated through an azobenzene moiety that undergoes reduction-mediated cleavage under hypoxic conditions [314]. Once the PEG layer has been shed, an siRNA-PEI complex, conjugated to a hydrophobic anchor, is revealed that readily enters the surrounding cells. Using green fluorescent protein (GFP) as a model target, knockdown of ~30% was achieved in vitro. In an in vivo mouse model, the nanoparticle formulation significantly reduced tumor GFP expression compared to a scrambled control [314]. In this case, no significant cytotoxicity was observed.

4.2.5.1 Cell penetrating peptides for cellular uptake

CPPs have been exploited to bring “cargo” into cells. The CPPs are typically < 40 amino acids, cationic, and viral-derived [315]. There have been many reviews focused on the
characteristics and mechanisms of CPPs [315-317] and while there are numerous CPP sequences (Table 4.1), they usually lack specificity as they will cross any cell membrane. While the internalization pathways of most CPPs are not well-defined, internalization is initialized via interactions of the cationic CPPs with the phospholipids of the cell membrane [318].

**Table 4.1** Common cell-penetrating peptides.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Origin</th>
<th>Charge$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAT (48-60)[319, 320]</td>
<td>GRKKRRQRRPPQ</td>
<td>Derived from HIV type 1</td>
<td>+8</td>
</tr>
<tr>
<td>Penetratin[321]</td>
<td>RQIKIWFQNRRMKWKK</td>
<td>Antennapedia homeodomain</td>
<td>+7</td>
</tr>
<tr>
<td>Transportan/TP10 [322, 323]</td>
<td>GWTLNS/AGYLLGKINLKA LAALAKKIL$^a$</td>
<td>Neuropeptide galanin-mastoparan fusion</td>
<td>+4</td>
</tr>
<tr>
<td>VP22[324]</td>
<td>NAKTRHERRRKLAIER</td>
<td>Herpes simplex virus</td>
<td>+7</td>
</tr>
<tr>
<td>Polyarginine[325]</td>
<td>$R_n^a$, n=8-9</td>
<td>Engineered for positive charge</td>
<td>+8 or +9</td>
</tr>
<tr>
<td>Pep-1[326]</td>
<td>KETWWETWWTEWSQP KKKRKV$^b$</td>
<td>Fusion of NLS from simian Virus 40 and reverse transcriptase of HIV-1</td>
<td>+3</td>
</tr>
<tr>
<td>CADY[327]</td>
<td>GLWRALWRLLSLWRLL WRA$^b$</td>
<td>Derived from PPTG1 peptide, addition of W and charged amino acids</td>
<td>+5</td>
</tr>
</tbody>
</table>

$^a$ C-terminal amide. $^b$ C-terminal cysteamide. $^c$ pH 7.4.

To achieve greater specificity of CPPs, one of three strategies is typically employed: (1) triggering CPP deprotection at the tumor site; (2) local delivery of the CPP to the tumor site; or (3) conjugation to cell-targeting ligands.

Using the first strategy, Sun *et al.* synthesized a polyarginine CPP, used for siRNA complexation and siRNA release, sandwiched between a hydrophobic poly(caprolactone) (PCL) block and a hydrophilic PEG block [328]. The PEG corona was conjugated to the CPP through 2-propionic-3-methylmaleic anhydride linkers, which are cleavable under the acidic environment of the tumor site, deshielding the CPP and facilitating cellular uptake. *In vitro* experiments revealed 60% PEG cleavage under acidic conditions and ~70% knockdown of cyclin-dependent kinase 4 (CDK4) in adenocarcinoma cells. *In vivo* experiments in a mouse model of
adenocarcinoma resulted in significantly delayed tumor growth compared to scrambled controls over 21 days and 50% knockdown of CDK4.

For the second strategy of local CPP delivery, Kanazawa et al. used an intranasal delivery route to carry siRNA directly to the brain in a mouse model of brain cancer using a CPP-nanoparticle formulation [329]. PCL nanoparticles were conjugated to a TAT CPP and hydrophilic PEG. The authors were able to use the TAT peptide for siRNA complexation and delivery. In vitro experiments demonstrated significant nanoparticle uptake, minimal cytotoxicity, and 70% knockdown of Raf-1, a gene associated with cell proliferation and apoptosis, and ultimately resulted in significantly lengthened survival in an in vivo rat model of malignant glioma.

For the third strategy, conjugating CPPs to cell targeting peptides can increase their specificity. Fang et al. conjugated the TAT CPP to A1, a peptide with high affinity for vascular endothelial growth factor receptor-1 (VEGFR1) and demonstrated selective delivery to tumor cells overexpressing VEGFR [330]. Similarly, R9 can be fused to a cyclic arginine-glycine-aspartic acid (cRGD) peptide for targeting [331]. However, this hybrid strategy has not yet been reported for delivery of a synthetic polymeric formulation of siRNA.

Although stimuli-responsive nanoparticles or local delivery routes can offer improvements in activity, cationic peptides and polymers may be limited by non-selectivity and cytotoxicity. Therefore, strategies that avoid reliance on cationic charges should be considered such as antibodies or ligands for receptor-mediated endocytosis or aptamer-mediated uptake. While in these strategies protein corona formation [332, 333] may hinder cell uptake, avoiding the toxicity and non-specificity of cationic charges offers a significant advantage. The protein corona can be at least partially overcome by, for example, functionalizing the polymeric nanoparticles with a PEG corona to reduce opsonisation and protein accumulation on the surface of the NPs [334, 335].

4.2.5.2 Receptor-mediated cell uptake via small molecule ligands or antibodies

Targeting ligands can be attached to polymeric delivery vehicles to increase the specificity of cellular uptake. These specifically bind receptors overexpressed on cancer cell
membranes, facilitating receptor-mediated endocytosis of the nanoparticle [336]. Interestingly, the MMP-7 responsive nanoparticle, previously discussed [313], was conjugated to folate ligands [337]. In this case, PEG cleavage was triggered by MMP-7 at the tumor site, exposing folate-conjugated nanoparticles for receptor-mediated endocytosis. *In vitro* experiments, including MMP-7 pre-treatment and folate ligand competition assays, revealed that knockdown was dependent on both MMP-7 activity and folate receptor binding. Under optimal conditions, the formulation achieved greater than 50% luciferase protein knockdown with no detectible cytotoxicity in a luciferase positive breast cancer cell line [337].

Antibodies can also be conjugated to nanoparticle formulations for targeted siRNA delivery, triggering internalization via a receptor-mediated endocytosis pathway [338]. Palanca-Wessels *et al.* synthesized a nanoparticle in which siRNA was encapsulated and to which anti-human epidermal growth factor receptor 2 (HER2) antibodies were conjugated for cellular internalization [339]. Delivery of siRNAs against a variety of chemotherapy resistance-associated mRNAs resulted in 80% knockdown *in vitro* and 70% knockdown of a target gene *in vivo* in a mouse model of ovarian cancer. However, the authors noted a slight immune response in some of the streptavidin-containing control groups [339, 340].

### 4.2.5.3 Aptamers for highly specific cellular uptake

Nucleic acid aptamers are relatively short strands of DNA or RNA that are identified by screening from a large random sequence pool that tightly bind to specific receptors [341]. Preclinical studies using soluble aptamer-siRNA chimeras have been very successful in mouse models of cancer [341]. Building on this strategy for use in a nanoparticle formulation, Subramanian *et al.* synthesized an aptamer-PEI-siRNA polyplex stabilized with sodium citrate for targeting and knockdown of epithelial cell adhesion molecule (EpCAM) [342]. Using an anti-EpCAM aptamer to target breast cancer and retinoblastoma cell lines, the delivery of anti-EpCAM siRNA resulted in an approximately 50% reduction in EpCAM expression leading to 80-90% reduction in cell proliferation *in vitro* in both cell lines (Figure 4.4) [342]. Aptamers are advantageous as targeting ligands for several reasons, including their low molar mass, low immunogenicity, and high specificity for cellular antigens [343]. The use of aptamers is a promising strategy for cell binding and internalization of polymeric nanoparticles.
Figure 4.4 A) Uptake of EpCam aptamer-conjugated complexes. The scrambled aptamers (EpApt) and the corresponding conjugates (PEI-ScrApt-siEp) show no uptake whereas the active aptamer (EpApt) shows uptake and the polymer conjugate (PEI-EpApt-siEp) shows enhanced uptake compared to the aptamer alone. B) mRNA levels following treatment. Significant EpCAM mRNA decrease is seen in two cell lines when the anti-EpCAM siRNA is transfected (Lipo-siEp) or delivered by the polymer conjugate (PEI-EpApt-siEp). C) mRNA knockdown leads to a decrease in cell proliferation. Figure reproduced from Suramanian et al. [344] with open access permissions.

4.2.6 Escaping the endolysosomal pathway

Regardless of the cell surface target, the majority of nanoparticle formulations enter the cell through the endolysosomal pathway where rapid acidification of the endolysosomes results in pH ranges from pH 6.5 to 5.5 in the endosomes and pH 5.5 to 4.0 in the lysosomes [345]. SiRNAs will eventually degrade under the acidic conditions and enzymatic activity in the lysosomes
[346], and thus endosomal escape agents must be used. These agents function by the “proton sponge” effect, pore formation or membrane destabilization [319, 347-349].

4.2.6.1 Amines for endolysosome escape

To achieve the “proton sponge” effect (Figure 4.5), nanoparticle delivery systems are functionalized with groups that are protonated at acidic pH, causing an influx of chloride anions, followed by osmotic swelling and endosomal lysis [350], enabling contents within the endosome to be expelled into the cytosol. Functional groups that are commonly used for endosomal escape include primary or secondary amines, such as linear or branched PEI [351-353], guanidines [354-356], lysines [357-359], and imidazoles, such as histidine [360]. Polymer synthesis can be designed for facile incorporation of each of these functionalities through monomer modification or post-polymerization modification.

![Figure 4.5 Nanoparticle escape from the endolysosomal pathway via the “proton sponge” effect. Nanoparticles enter the early endosome, which matures into late endosome and then lysosome. Nanoparticles bearing: (A) primary amines, such as lysine, (B) guanidines, such as in arginine, or (C) imidazoles, such as in histidine, promote the “proton sponge” effect where an influx of chloride ions along with water leads to an eventual rupture of the endosome. The contents of the endosome are then freed into the cytosol for intracellular delivery of siRNA to RNAi machinery.](image-url)

[97]
Many of the cationic polymers and peptides already discussed contain amine moieties that, in addition to crossing the cellular membrane, will lead to the “proton sponge” effect and endosomal escape. For example, Cheng et al. synthesized mPEG-poly(ε-caprolactone)-graft-poly(2-(dimethylamino) ethyl methacrylate by ring opening polymerization of the caprolactone, followed by atom-transfer radical-polymerization of 2-(dimethylamino) ethyl methacrylate (pDMAEMA) [361]. DMAEMA contains a tertiary amine that aids endosomal escape through proton sponge effects [362]. The authors hypothesize that the nanoparticles showed significant uptake and efficacy in MDA-MB-231 cells in vitro and in vivo due, in part, to endosomal escape caused by protonation of the pDMAEMA. However, the presence of the cationic pDMAEMA caused significant cytotoxicity when treating cells with blank nanoparticles at increasing concentrations, demonstrating that the vehicle itself needs to be safe for efficacy [361].

Oligoarginines are known as cell-penetrating peptides, but are also active in the endosome for endosomal escape due to the bidentate hydrogen binding of the guanidine side group of arginine to the negatively charged endosomal membrane [150]. Zhao et al. synthesized a monomethoxy poly(ethylene glycol)-block-poly(d,l-lactide)-block-poly(arginine) (mPEG3000-PLA2000-R15) for the delivery of siRNA targeting epidermal growth factor receptor (EGFR) [363]. The addition of the polyarginine peptide led to a significant increase in the zeta potential, and delivery of anti-EGFR siRNA, resulting in 60% protein reduction in vitro in MCF7 cells. In vivo data revealed significant volume reduction in xenografted MCF-7 tumors following nanoparticle-siRNA treatment (Figure 4.6A). More recently, Nam et al. incorporated an arginine grafted bio-reducible poly(cystamine bisacrylamide-diaminohexane) onto a poly(amido amine) to deliver VEGF siRNA to three cancer cell lines [364]. The authors reported 70-80% knockdown of VEGF with minimal cytotoxicity using this system in vitro.

A major concern when using primary or secondary amines with pKₐs greater than physiological pH is the off-target effects and cytotoxicity of cationic delivery vehicles. Imidazole groups are attractive functionalities to incorporate into polymers because they have pKₐs of ~6.0 [365]. Therefore, these groups will be neutral outside of the cell, and only become protonated once inside of the endosomes, reducing off-target effects and cytotoxicity. Ghosn et al. used imidazole acetic acid to modify 20-30% of the amino groups of chitosan [366]. The authors reported the delivery of siRNA targeting glyceraldehyde 3-phosphate dehydrogenase (GAPDH)
and demonstrated 90% protein knockdown of lung A549 cells in vitro using the 30% imidazole-substituted chitosan. This system did not demonstrate significant cytotoxicity in vitro.

Furthermore, Han et al. formed nanoparticles from imidazole-modified urocanic acid-modified galactosylated trimethyl chitosan [367]. While the non-imidazole modified nanoparticles delivered siRNA with strong uptake in hepatocellular carcinoma QGY-7703 cells, they were mainly localized and trapped in the lysosomes after 4 h in vitro (Figure 4.6C). The imidazole-modified nanoparticles exhibited a diffuse fluorescence in the cytosol, indicative of endosomal escape. Furthermore, the imidazole modified nanoparticles outperformed Lipofectamine 2000, a commercially available transfection reagent, in vitro, with negligible cytotoxicity. Using this formulation, imidazole-modified nanoparticles demonstrated significantly more tumor inhibition in vivo compared to non-imidazole nanoparticles due to their enhanced endosomal escape. Thus, incorporation of imidazoles in nanoparticle formulations is promising as it results in a stimulus-responsive nanoparticle where endosomal escape is only activated in the acidic organelles.

4.2.6.2 Pore-forming peptides for endosomal escape

An emerging alternative to cationic charges for endosomal escape is the use of pH-switchable groups or peptides, such as endosomolytic peptides [368-370]. Endosomolytic peptides destabilize membranes when a critical concentration of the peptide is located near a membrane, followed by interaction with the negatively charged phospholipid bilayer and pore formation. The resultant pore then destabilizes and disrupts the membrane [371]. Examples of this strategy for siRNA delivery using polymeric delivery vehicles are still rare, but there is some strong proof-of-concept work in the field of gene delivery. For example, Cheng et al. synthesized virus-inspired polymers for endosomal release (VIPER) by grafting ‘caged’ melittin, a membranolytic peptide, through a disulfide bridge to a polymer containing a hydrophilic cationic block for therapeutic loading and a pH-sensitive block for the triggered display of the melittin peptide [372]. The authors demonstrated nanoparticle disassembly at pH 5.7, enhanced hemolytic activity at acidic pH compared to neutral pH, and greater transfection in vitro and in vivo relative to commercially available reagents (Figure 4.6B). Although this system was used to deliver DNA, the same strategy could be used for the delivery of siRNA.
Melittin is a non-specifically membranolytic peptide, and thus it needed to be shielded by additional chemical modifications until reaching the acidic pH of the endosomes. An alternative that has not yet been explored for polymeric nanoparticle systems, but has been successful for siRNA delivery in other forms, is a class of peptides called ‘fusogenic’ peptides (Table 4.2) [373]. These peptides do not interact with cells until they reach the endosomal pH, where they adopt a pore-forming α-helical structure. Although these peptides have not yet been used with synthetic polymer systems, they have shown efficacy in cationic lipid [374] and oligo(amino acid)-based systems [375] and should be promising for use with polymeric delivery vehicles.

**Figure 4.6** A) Relative volume changes of xenografted MCF-7 tumors when injected intravenously with micelle/siRNA complexes targeting EGFR at 1 mg / kg for 5 injections. Micelleplexes contained an R15 peptide for cancer cell uptake, and endosomal escape. Reprinted from Zhao et al. [115] with permission from Elsevier. B) Luciferase activity from excised A549 tumor tissues of mice treated with polyplexes containing no membranolytic
peptide, mellitin, (CP), polymer grafted with melittin (VIPER), and branced PEI (bPEI). Data are shown as mean ± SD (n = 4; student’s t test, *p < 0.05, **p < 0.01). Reproduced from Cheng et al. [124] with permissions. C) Confocal laser scanning microscopy images of QGY-7703 cells treated with TAMRA-siRNA loaded micelles (red) for 4 h and stained with Lysotracker (green) and Hoechst 33258 (blue). Bar represents 20 um. Adapted from Han et al. [119] with permission from Elsevier.

Table 4.2 Commonly used fusogenic peptides.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Origin</th>
<th>Sequence</th>
<th>Charge (pH 7.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA2 [376, 377]</td>
<td>Derived from influenza hemagglutinin (HA) proteins of influenza viral capsid</td>
<td>GLF GAI AGF IEN GWE GMI DGW YG</td>
<td>-3</td>
</tr>
<tr>
<td>INF-7 [378]</td>
<td>Derivative of HA2; glutamic-acid enriched for improved endosomal escape</td>
<td>GLF EAI EGF IEN GWE GMI DGW YGC</td>
<td>-5</td>
</tr>
<tr>
<td>GALA [379]</td>
<td>Synthetic peptide with EALA repeats; E for pH-sensitivity and ALA for hydrophobicity</td>
<td>WEA ALA EAL AEA LAE HLA EAL AEA LEA LAA</td>
<td>-6</td>
</tr>
</tbody>
</table>

4.2.7 Releasing the siRNA

One of the most common methods of delivering siRNA is by cationic vehicles that complex the anionic siRNA, and then the siRNA is released via spontaneous dissociation [380]. In order to increase extracellular stability, many vehicles incorporate covalently conjugated siRNA. The siRNA must be released from the polymer vehicle in the cytosol in order to be effective [381, 382], and release of the siRNA from covalent conjugation requires a specific mechanism. As shown in Figure 4.7, there are many cues available for the release of siRNA that can be exploited including: acidic pH, enzymatic activity, reducing conditions, and the presence of specific bioactive molecules.
Figure 4.7 Cleavage strategies for siRNA release from a nanoparticle system. (A) Acid sensitive groups (ie. maleic acid anhydride, hydrazone, thiolmaleiamide, etc.) are cleaved under the acidic endosomal conditions. (B) Disulfide bonds are cleaved by high glutathione levels in the cytosol. (C) Phenylborate associates strongly with the terminal diols of siRNA but is displaced by the high diol (ATP) concentration in the cytosol. (D) The Dicer enzyme cleaves Dicer-substrate siRNA.

4.2.7.1 Acid-triggered siRNA Release

The acidification in the endolysosomal pathway can be exploited to trigger the cleavage of the siRNA from the polymeric vehicle in conjunction with endosomal escape strategies previously discussed. Takemoto et al. developed an siRNA conjugate nanoparticle designed to be destabilized under acidic conditions to promote endosomal escape and siRNA cleavage, all based on one acid-sensitive maleic acid amide (MAA) linkage [383]. Under acidic conditions, the MAA groups of the polyplex are cleaved, releasing the siRNA and revealing primary amines that destabilize the endosomes. Using this system, delivery of various siRNAs to ovarian cancer and adenocarcinoma cells resulted in 50-70% gene knockdown in vitro; however, no in vivo experiments have been reported using this system.

Another strategy is to encapsulate the siRNA within an acid-degradable polymer shell. Hong et al. used this strategy to deliver siRNA in a core-shell nanoparticle platform, with acid-cleavable diamines crosslinking the PCL shell [384]. The shell degrades within the acidic lysosomes, releasing the siRNA. This system achieved 40% knockdown of GFP in vitro using
GFP+ breast cancer cells, while demonstrating significantly less cytotoxicity than traditionally used transfection reagents. However, it is unclear how the siRNA escapes from the endolysosomal pathway, so it is possible that incorporating a specific endosomal escape strategy would further increase potency. A better strategy would be to use a sequential siRNA release mechanism that occurs after endosomal escape of the nanoparticle, taking advantage of bioactive molecules and enzymes in the cytosol.

4.2.7.2 Glutathione-triggered siRNA release

Once inside the cytosol, a diverse array of enzymes and bioactive molecules are available for triggered siRNA release. One of the most commonly exploited biomolecules for siRNA release is glutathione (GSH), which reduces disulfide bonds often used to immobilize siRNA to a polymeric carrier [385-387]. GSH is found at high concentrations (5-10 mM) in the cytosol of most mammalian cells, which is significantly higher than concentrations typically found in the blood (0.05 mM) [388]. Namgung et al. took a unique approach and conjugated the sense and antisense strands of the siRNA to separate polymer backbones through disulfide bonds, and then annealed them together, yielding a system where the siRNA is tethered on both ends within the core of the nanoparticle and effectively acts as a crosslinker [389]. The backbone consists of chitosan polymers, and together the chitosan and crosslinked siRNAs form the core of the nanoparticle, while peptide aptamers targeting prostate cancer cells form the corona. Although this system was able to affect gene knockdown to 50% in vitro, the concentration of siRNA required was significantly higher (200-400 nM) than typical siRNA concentrations of <50 nM. The high doses required may reflect the slow kinetics of siRNA release when it is tethered on both ends. This system requires further optimization before it will be a useful in vivo delivery strategy [389].

Instead of attaching the siRNA via a disulfide bond, it can be complexed to a positively charged pendant group that is cleaved from the polymer backbone in the cytosol. For example, Li et al. designed a copolymer of PEG and poly(L-lysine) that was grafted to polyethyleneimine through reducible disulfide bonds and to which siRNA was complexed non-covalently [390]. Anti-HER2 was conjugated to this nanoparticle for selective uptake by HER2 overexpressing cells while the proton buffering capacity of PEI enhanced endosomal escape. Once in the
reducing conditions of the cytosol, the disulfide bonds were cleaved, releasing PEI and its siRNA cargo. This formulation led to an 80% knockdown of XIAP, a gene associated with apoptosis, in vitro in a HER2+ ovarian cancer cell line. In vivo studies in a subcutaneous model of ovarian cancer revealed an 80% increase in apoptosis following treatment, as well as significantly delayed tumor growth and longer survival. Notably, 80% of the animals in the targeted siRNA formulation group were alive after 45 days whereas none remained alive in the control groups [390].

Another strategy is to ‘cage’ the siRNA within disulfide crosslinked polymer constructs, which will degrade and release the siRNA within the cytosol. Yoon et al. used a hyaluronic acid scaffold conjugated to both pDMAEMA and a crosslinker which, with the addition of siRNA and a redox reagent, formed a crosslinked hyaluronic acid nanoparticle encapsulating siRNA with cationic pDMAEMA for siRNA complexation [391]. Overexpressed CD44 receptors for hyaluronic acid on the cell surface promoted internalization of the nanoparticle, and once inside the cytosol the disulfide bonds were cleaved by GSH, releasing siRNA. This system demonstrated efficient cell uptake and RFP knockdown in vitro in an RFP+ melanoma cell line, although significant cytotoxicity in CD44+ cells was also observed, potentially due to the toxicity of pDMAEMA. In vivo studies demonstrated reduced RFP levels in melanoma tumors [391].

4.2.7.3 ATP-triggered siRNA release

Adenosine triphosphate (ATP) can be used to trigger release in the cytosol as well. This strategy was employed by Naito et al., who synthesized a polyion complex micelle for ATP-triggered release of siRNA [392]. In this study, some of the lysine residues of PEG-b-poly(lysine) were modified with phenylboronic acid, which binds strongly to siRNA but can be displaced by an excess of other diols (ie. ATP) in solution. Although the authors were able to demonstrate ATP-triggered siRNA release, the in vitro studies are limited, with the supporting information showing 30% gene knockdown against polo-like kinase 1 (PLK1) in renal carcinoma cells at 500 nM. The concentration used is very high for siRNA where typically sub-50 nM concentrations are used [392]. Therefore, although this approach is interesting, more work is required to prove its utility in vitro and in vivo.
4.2.7.4 Dicer-mediated cleavage of siRNA

One key enzyme particularly important in siRNA trafficking is Dicer, which cuts longer siRNAs (27-30 base pairs) into 21 base pair siRNAs and traffics them into the RNA-induced silencing complex (RISC) [393]. In addition to providing a mechanism for cleavage from a polymer vehicle, Dicer-substrate siRNAs have been reported to be 10-100 fold more potent than non-Dicer substrates [259]. Thus, Dicer-substrate siRNAs are one of the most promising strategies for siRNA release. However, some cancer tissues have been shown to have less Dicer expression than normal tissues [394, 395], so the target tissue must be carefully considered when choosing to use Dicer-substrate siRNAs.

Chan et al. developed a polymeric micellar system composed of a poly(lactide-co-2-methyl, 2-carboxytrimethylene carbonate) backbone with grafted PEG to deliver both Dicer-substrate siRNAs as well as targeting antibodies [137]. SiRNA-modified with DBCO and anti-HER2 antibody-modified with maleimide were conjugated to the terminal ends of PEG-azide and PEG-furan, respectively, through click conjugation reactions. The nanoparticles carrying both Dicer-substrate siRNA and anti-HER2 antibodies effectively knocked down luciferase expression by approximately 80% in vitro in luciferin-positive ovarian cancer cells [137]. This study demonstrates the proof-of-concept of covalently bound siRNA for effective knockdown, taking advantage of the siRNA duplex, with stable siRNAs and the sense strand covalently immobilized, leaving the anti-sense available to Dicer for facilitated processing.

Dicer-substrate siRNA has also shown promise in vivo. Liu et al. synthesized a dendrimer platform for siRNA delivery comprising poly(amidoamine) dendrimers which are capable of complexing both siRNA and anionic targeting peptides [396]. Interestingly, when using this system to deliver siRNA against heat shock protein 27 in human prostate cells in vitro, no significant gene knockdown was observed using a conventional 21-mer siRNA. However, when using Dicer-substrate siRNA, significant gene silencing (50%) was observed. Delivery of this Dicer-substrate formulation with a targeting peptide to a prostate cancer xenograft model resulted in significantly slower tumor growth compared to controls [396].

Overall, although there are many effective ways to release siRNA from the polymer delivery vehicle, one of the most promising strategies is Dicer cleavage. Not only does it provide
a specific enzymatic mechanism for siRNA release, it also increases the potency of siRNA. Examples of Dicer-siRNA in the literature are still rare, and should be considered for future work in the field.

4.2.8 Combination Therapies

Importantly, the combination of sequence-specific siRNA with cytotoxic chemotherapeutics offers interesting advantages, and there are multiple examples of combination therapies already in the literature. For example, Sun et al. used biodegradable triblock poly(ethylene glycol)-b-poly(ε-caprolactone)-b-poly(2-aminoethylethylene phosphate) micelles to deliver both paclitaxel and siRNA targeting polo-like kinase 1 (Plk1) to MDA-MB-435 breast cancer cells [397]. Delivering siRNA with this system resulted in ~70% reduction of Plk1 protein levels, and provided a synergistic reduction in cell proliferation when delivered with paclitaxel relative to each therapeutic alone. Interestingly, when the authors delivered paclitaxel along with control siRNA in vivo, they required a thousand-fold higher paclitaxel concentration to observe similar tumor reduction relative to delivering paclitaxel with siRNA targeting Plk1 (Figure 4.8A). Although siRNA targets can be chosen to act synergistically with chemotherapeutics when delivered simultaneously, sequential delivery can sensitize the cells to the chemotherapeutic. For example, Zhang et al. first delivered siRNA targeting Bcl-2, an anti-apoptotic siRNA, to multidrug resistant (MDR) cells, which then sensitized the cells to doxorubicin treatment, resulting in reduced MDR cells [398]. This siRNA delivery system reduced Bcl-2 protein levels to ~30% while reducing the IC\textsubscript{50} of doxorubicin by 2.5-fold when compared to delivering scrambled siRNA (Figure 4.8B). The combination of chemotherapeutics and siRNA adds more complexity; however, the combination may require lower doses of both therapeutics, thereby reducing toxicity from the vehicle or drugs.
Figure 4.8 A) Dose-response study of paclitaxel delivered by \textsuperscript{paclitaxel}micelleplex\textsubscript{siNonsense} (control siRNA) on inhibition of MDA-MB-435s xenograft tumor growth. Paclitaxel doses were 10 to 1000-fold increase (10x to 1000x) compared to those used in \textsuperscript{paclitaxel}micelleplex\textsubscript{siPlk1} (siRNA targeting PLK-1). Comparable results to \textsuperscript{paclitaxel}micelleplex\textsubscript{siPlk} were only achieved with an 1000-fold more paclitaxel dose when using a control siRNA. Reprinted with permission from Sun \textit{et al.} [397]. Copyright 2011 American Chemical Society. B) Using a PEI-graphene oxide NPs, relative viability of HeLa cells after being treated with either (1) Bcl-2 siRNA or (2) scrambled siRNA for 48 hrs followed by incubation with PEI-graphene oxide NPs loaded with doxorubicin for 24 hrs. The Bcl-2 knockdown sensitized the cells to doxorubicin treatment. Reproduced from Zhang \textit{et al.} [398] with permissions.

4.2.9 Current Clinical Status, Outlook, and Conclusions

As demonstrated by this review, many siRNA-nanoparticle formulations have shown \textit{in vitro} success, but have not been translated \textit{in vivo} or to the clinical setting. There are, however, some siRNA-nanoparticle formulations that are being tested in clinical trials, as summarized in Table 4.3. More complete summaries of the current clinical status of siRNA therapeutics can be found in recent reviews published by Barata \textit{et al.} [399] and Kim \textit{et al.} [71]. The majority of delivery systems in current clinical trials are lipid-based or composed of cationic polymers.
Table 4.3 Selected siRNA-nanoparticle formulations in clinical trials.

<table>
<thead>
<tr>
<th>Name</th>
<th>Formulation Type</th>
<th>Cancer Type</th>
<th>Size (Zeta Potential)</th>
<th>siRNA Target</th>
<th>Clinical Trial Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>CALAA-01</td>
<td>Cyclodextrin-based Polymer</td>
<td>Solid</td>
<td>70 nm (+10 mV)[400]</td>
<td>RRM2[401]</td>
<td>Phase I (NCT00689065)</td>
</tr>
<tr>
<td>Atu027</td>
<td>Liposome</td>
<td>Solid</td>
<td>102 nm (+38.9 mV)[402]</td>
<td>PKN3[402]</td>
<td>Phase I (NCT00938574)</td>
</tr>
<tr>
<td>TKM080301</td>
<td>SNALP (Stable Nucleic Acid Lipid Particle)</td>
<td>Liver</td>
<td>N/A</td>
<td>PLK1[403]</td>
<td>Phase I (NCT01437007)</td>
</tr>
<tr>
<td>ALN-VSP</td>
<td>Lipid Nanoparticle</td>
<td>Liver</td>
<td>80-100 nm (+6 mV)</td>
<td>KSP, VEGF[404]</td>
<td>Phase I (NCT00882180)</td>
</tr>
</tbody>
</table>

RRM2 – ribonucleotide reductase M2, PKN3 – protein kinase N3, PLK1 – polo-like kinase 1, KSP – Kinesin spindle protein, VEGF – Vascular Endothelial Factor

Although cationic vehicles work well for siRNA delivery in vitro, in vivo they result in nonspecific uptake, cellular toxicity and elicit an immune response. With a neutral or negatively charged polymer system, these off-target effects are avoided; however, a cell uptake strategy, such as an antibody or aptamer, is now required. An interesting strategy would have a nanoparticle that responds to the tumor microenvironment by shielding the antibody or aptamer with a polymer that is cleaved by specific cues such as pH or MMPs. To achieve maximum potency, we suggest incorporating a chemically stabilized Dicer-substrate siRNA and a fusogenic peptide for greatest siRNA release. This strategy should minimize toxicity while taking advantage of the tumor microenvironment and maximizing cellular uptake, endosomal escape, and potency.

Overall, polymeric vehicles offer a multitude of functionalities for guided siRNA delivery. While the biggest challenge to the field remains localizing the nanoparticles at the tumor site, once there, siRNA stability, cell uptake, and endosomal escape are the key issues. To increase clinical efficacy, we suggest the following: modified siRNA for greater stability and potency, highly specific cell uptake mechanisms, improved endosomal escape agents, and sophisticated siRNA release mechanisms. Recent advances in these strategies demonstrate the
promise of polymeric vehicles for the delivery of potent biomolecules, such as siRNA, and their translation to the clinic.

4.3 Introduction

Breast cancer is the most frequently diagnosed cancer among women internationally, accounting for 23% of total cancer cases.[8] Breast cancers that overexpress human epidermal growth factor receptor 2 (HER2) exhibit aggressive tumor phenotypes with poor prognosis.[405-407] Chemotherapeutic drugs commonly used for breast cancer lack specificity, acting on both cancer cells and other fast replicating cells. This leads to dose-limiting side effects such as cardiotoxicity.[14] Ribonucleic acid interference (RNAi) therapy is a promising alternative cancer treatment which uses small interfering RNAs (siRNAs) to selectively degrade messenger RNAs (mRNAs). Unfortunately, due to the body’s natural defenses, siRNA is degraded rapidly, resulting in poor cell uptake and specificity. Thus, there is a need to use a vehicle to enhance the circulation of siRNA in the blood, and to target siRNAs towards cancer tissue.

Nanoparticles (NPs) have been established as a suitable delivery vehicle for siRNA due to protection from serum nucleases and avoiding rapid clearance of the siRNA.[65] A proof of concept siRNA delivery system was investigated in vitro and comprised of immuno-NPs of poly(δ, l-lactide-co-2-methyl-2-carboxytrimethylene carbonate)-g-poly(ethylene glycol)-X (poly(LA-co-TMCC)-g-PEG-X), where x was either a furan or an azide.[137] The advantages of using this polymer includes monodisperse and stable NPs that harbor a bifunctional surface permitting the modification of targeting agents or therapeutics. [135-137] Delivery of siRNA targeting luciferase, a model target, by immuno-NPs proved to be as efficient as lipid transfection agents, the standard in the field.[363] Additionally, chemical modifications of the siRNA have been demonstrated to enhance serum stability, increase potency, and reduce immunostimulation in vivo.[408] Combining the two and translating the siRNA to an essential target in cancer cells has the potential to provide a durable and potent siRNA delivery system.

A clinically relevant siRNA target must be chosen based on maximizing cancer cell death while minimizing off target effects such as systemic toxicity. Conventional siRNA therapeutics rely on targeting proteins that are overexpressed in cancers, or expressed solely in cancers.[409] A major advantage with using our immuno-NP system is that essential protein targets may be specifically targeted at the tumor site due to active targeting. Acting targeting of NPs involves
modification of the NPs with a targeting agent, such as Trastuzumab (Anti-HER2, Herceptin), for the selective uptake in HER2 overexpressing cells. A particularly interesting siRNA target is eukaryotic translation initiation factor 3 subunit B (eIF3B) which is required for protein translation and was determined to be essential in 72 breast, pancreatic and ovarian cancer cell lines.[410] Additionally, overexpression of eIF3B is associated with advanced stage, grade, and poor prognosis in patients with bladder and prostate cancer.[411] siRNA targeting eIF3B in glioblastoma and colon cancer cells were demonstrated to significantly knockdown the eIF3B mRNA and protein levels, induce G1 cell cycle arrest, and apoptosis while decreasing cancer cell proliferation. [412, 413] Furthermore, depletion of eIF3B in bladder tumor models in vivo inhibited global protein synthesis, cell migration, and subcutaneous tumor growth.[411] In this work, the NP-mediated, targeted delivery of siRNA targeting eIF3B (si3B) against HER2 overexpressing breast cancer cells, MDA-MB-21/H2N, is investigated. First, we validated a siRNA sequence for the potent knockdown of eIF3B at the mRNA and protein levels. Then we investigated the durability of the knockdown on cancer cell viability. Different cycloaddition strategies were explored between the alkyne modified siRNA and the azide modified NPs. The si3B modified NPs were further investigated for cell uptake, serum stability, and transfection into HER2 overexpressing breast cancer cells. Lastly, the use of amphiphilic endosomal escape peptides were explored for the efficient escape of the siRNA into the cytosol.

4.4 Materials and Methods

Synthesis of benzyl protected 2-methyl-2-carboxy-trimethylene carbonate (TMCC-Bn) was carried out as previously reported.[130, 414] 1-[3,5-bis(trifluoromethyl)phenyl]-3-[(1R,2R)-(-)-2(dimethylamino) cyclohexyl] thiourea and 3,6-dimethyl-1,4-dioxane-2,5-dione (D,L-lactide) were used as received. (Strem Chemicals, Newburyport, MA) Boc-NH-PEG-O-C₃H₆-CO-NHS (10 kDa) (Rapp Polymere, Tübingen, Germany) was used as received and modified using published protocols for azide-terminated ends.[136, 137] Dibenzylcyclooctyne-poly(ethyleneglycol)₄-succinimidyl ester (DBCO-PEG₄-NHS) was used as received(Click Chemistry Tools, AZ, USA) Hydroxybenzotriazole, N, N’-diisopropyl carbodiimide, and N, N’-diisopropylethylamine (TRC, Toronto, ON) were used as received. Sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) was purchased from Pierce (Rockford, IL). Trastuzumab was purchased through Hoffmann-La Roche Limited (Mississauga,
ON). Alexa fluor 647-succinimidy ester (Invitrogen, Eugene, OR) was used as received. Lipofectamine 2000 Reagent, Presto Blue, SYBR green PCR Master Mix, SYBR Gold Nucleic Acid Stain, TRIzol Reagent, Turbo DNA Free, 7-Aminoactinomycin D and Opti-Mem Reduced Serum, were used as received (Life Technologies Inc.) BCA Protein Assay Reagent and 5x Lane Marker Reducing Sample Buffer was used as received. (Pierce, Rockford, IL) SuperSignal West Dura Chemiluminescent Substrate, 0.2 µm nitrocellulose membrane and Ribonuclease A (DNase and protease free) were used as received for the western blots. (Fischer Scientific Company, Ottawa, ON) β-Actin (13E5) rabbit mAb (HRP conjugated) #5125, and Anti-rabbit IgG, HRP linked antibody #7074, and RIP buffer (10X) #9806 were used as received. (Cell Signaling Technology, Inc, Danvers, MA) Anti-eIF3B antibody (ab50709) used as received. (Abcam Inc, Toronto ON) iScript Select cDNA synthesis was used as received. (Bio-Rad Laboratories, Mississauga, ON) Fluor 488-alkyne, Fluor 488-dibenzylecyclooctyne (Fluor-488-DBCO), 11-azido-3,6,9-trioxaundecan-1-amine, and all solvents and reagents were received as received from Sigma-Aldrich, unless otherwise noted.

4.4.1 Polymer Synthesis and Characterization

poly(D,L-lactide-co-2-methyl-2-carboxytrimethylenecarbonate)\textsubscript{12K}-grafted-poly(ethylene glycol)\textsubscript{10K}-azide (P(LA-co-TMCC)-g-PEG-N\textsubscript{3}) was synthesized following previously established protocols and as described in Chapter 3.3.3.[231] Briefly, a ring opening polymerization of D,L-lactide and benzyl ester protected 2-methyl-2-carboxytrimethylenecarbonate (TMCC-Bn) catalyzed by 1-[3,5-bis(trifluoromethyl)phenyl]-3-[(1R,2R)-(−)-2(dimethylamino) cyclohexyl] thiourea and initiated by a pyrenebutanol was then followed by benzyl deprotection and afforded P(LA-co-TMCC) with a number average molecular weight of 12,104 g/mol with 137 LA units, and 14 TMCC units per copolymer (Figure S 2.1A). 10,000 g/mol PEG chains are grafted onto the backbone by carbodiimide chemistry as described in Chapter 3.3.3. There were ~2.2 PEGs/backbone as determined by H\textsuperscript{1} NMR (Figure S 2.1B).

4.4.2 siRNA Synthesis and Characterization

All non-targeting, fluoro modified and unmodified alkyne, and fluoro modified and modified dibenzylecyclooctyne siRNAs were synthesized and characterized by the Damha lab. (McGill
University). siRNAs were synthesized and characterized as previously described.[137] The siRNA sequences can be found in Table 4.7.

### 4.4.3 siRNA Sequences Targeting eIF3B (si3B)

At the time of experimenting (2012), there were no known siRNA sequences targeting eIF3B. eIF3B siRNA (si3B) sequences were obtained using Integrated DNA Technologies RNAi Design Sci Tool (http://www.idtdna.com/Scitools/Applications/RNAi/RNAi.aspx) finding the most theoretically optimized siRNA sequences against the eIF3B mRNA (accession number NM_003751). Four siRNA sequences were used as received (Integrated DNA Technologies, Inc, Coralville, IA) and are listed in Table 4.4.

**Table 4.4** Optimized siRNA sequences targeting eIF3B obtained from IDT DNA with the corresponding antisense position.

<table>
<thead>
<tr>
<th>siRNA #</th>
<th>Sequence a</th>
<th>AntiSense Position b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5’ GAA GCA GAUGGA AUC GAU UCG GUG A 3’ 642</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3’ UC CUU CGU CUA CCU UAG CUA AGC CAC U 5’</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5’ GAG GCA GAA UGC AGA GAU CAG UAC A 3’ 1002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3’ UU CUC CGU CUU ACG UCU CUA GUC AUG U 5’</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5’ GCA GAA GAA CGG AGA CUA CUU GU GUG T 3’ 1664</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3’ AC CGU CUU CUU GCC UCU GAU GAA CAC A 5’</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5’ GAG AGA AAU UCA AGC AAA UUC AGA G 3’ 1192</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3’ CC CUC UCU UUA AGU UCG UUU AAG UCU C 5’</td>
<td></td>
</tr>
</tbody>
</table>

a DNA (bolded), RNA (Uppercase)
bStarting position on eIF3B’s mRNA sequence that the antisense (bottom strand of each siRNA) is complimentary.

### 4.4.4 Cell Culture Maintenance

MDA-MB-231 and MDA-MB-231/H2Ns human breast cancer cells were maintained in RPMI media containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified incubator with 5% CO₂ atmosphere. Cells were passaged every week once confluency was achieved.
4.4.5 Anti-Cancer Activity of si3Bs

Breast cancer cells were seeded into 96-well flat-bottomed tissue culture plates at a density of 3,000 cells per well, and allowed to adhere for 24 hours. Lipofectamine 2000 (0.2 µL / well) was complexed with siRNAs for 15 minutes before addition to media. A vehicle control was included with no siRNA complexed. Treatments were incubated with cells for 6 hours, then replenished with fresh media and incubated for an additional 66 hours. The Presto Blue (Life Technology) assay was used as per the manufacturer’s protocol and as a proxy for cell viability calculated as follows:

\[
\text{Relative Viability (\%)} = \frac{\text{Fluorescence Intensity}_{\text{Treated}}}{\text{Fluorescence Intensity}_{\text{Untreated}}} \times 100\%
\]

To test the durability of the anti-cancer activity, Presto Blue was applied to the treated cells every 48 h post-transfection for up to 10 days. After the anti-cancer activity of the siRNAs was measured, cells were rinsed with fresh media, and incubated in fresh media for another 48 h.

4.4.6 Quantitative Polymerase Chain Reaction (qPCR)

MDA-MB-231s and MDA-MB-231-H2Ns were seeded in 12 well plates at a density of 100,000 cells / well. siRNAs were complexed with Lipofectamine 2000 (0.3 mL / well) for 15 minutes before adding to cell media. Non-targeting siRNA was used as a negative control and added to cells at the highest concentration that si3B was tested (1 nM) The cell media was replaced with fresh after 6 h. RNA was isolated at 72 h post transfection using TRIzol reagent as per manufacturer’s protocol. Free DNA remaining in the RNA isolate was removed by treating each sample with Turbo DNA Free as per manufacturer’s protocol. cDNA synthesis was completed using the iScript Select cDNA synthesis kit as per manufacturer’s protocol. Finally, the qPCR was accomplished using SYBR green PCR Master Mix and MicroChemi 4.2 (FroggaBio Inc., Toronto ON). Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) mRNA levels were used to normalize the mRNA levels in each sample. GAPDH and eIF3B forward and reverse primers were obtained from NCBI Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and can be found in Table 4.5. Fold expression was averaged from three biological repeats and calculated using the following equations:
\[
\Delta C_t = C_{t(GAPDH)} - C_{t(eIF3B)} 
\]  
(5)

\[
\Delta \Delta C_t = \Delta C_t(\text{treated cells}) - \Delta C_t(\text{control cells}) 
\]  
(6)

\[
Expression\ Level = 2^{-\Delta \Delta C_t} 
\]  
(7)

Where \(C_t(X)\) is the cycle threshold of X mRNA, \(\Delta C_t\) is the difference in the cycle thresholds of the GAPDH and eIF3B within a sample, and \(\Delta \Delta C_t\) is the difference in the difference of cycle thresholds between treated cells and control cells.

**Table 4.5** Forward, reverse primers, product lengths used for GAPDH and eIF3B.

<table>
<thead>
<tr>
<th></th>
<th>Forward Primer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reverse Primer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Product Length&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>TGCACCACCAACTGCTTAGC</td>
<td>GGCATGGACTGTGGTCATGAG</td>
<td>87</td>
</tr>
<tr>
<td>eIF3B</td>
<td>AGTACCGGAAAAATGCCAGG</td>
<td>CACAGTGCTCCAGGTCACTC</td>
<td>184</td>
</tr>
</tbody>
</table>

<sup>a</sup>GAPDH (accession number NM_002046) and eIF3B (accession number NM_003751) primers were received from Primer Blast results.

### 4.4.7 Western Blot

Breast cancer cells were transfected as described in Section 4.4.6. At 72 h post transfection, cells were lysed using RIPA buffer as per manufacturer’s protocol. Isolated protein was determined using the bicinchoninic acid (BCA) assay as per manufacturer’s protocol. Protein extracts were stored in a -20 °C freezer until further use. Sodium dodecyl sulphate polyacrylamide (SDS-PAGE) were prepared in-lab to ensure that the running gel is at 8 %. 4 µg of protein was diluted with 5x reducing loading buffer, heated in boiling water for 5 minutes, and loaded on top of the gels. The gels were run at 120 V (constant voltage) for 1.25 h. Afterwards the gel was soaked in standard transfer buffer for 5 minutes, and transferred to a nitrocellulose membrane at 20 V (constant voltage) for 1 h. The nitrocellulose membrane was then blocked with 5 % BSA overnight at 4 °C. Primary antibodies used were anti-GAPDH diluted to 1:50,000, and anti-eIF3B diluted to 0.25 µg / mL. Primary antibodies were applied to the membranes for 2 h at room temperature. The GAPDH antibody was horse radish peroxidase conjugated, so Supersignal West Dura was applied to those membranes for visualization of protein bands. A secondary antibody was applied to the anti-eIF3B membrane using a 1:1000 dilution. Supersignal West Dura was applied to the membranes for visualization of protein bands. Chemiluminescence was visualized using the MicroChemi 4.2 system (FroggaBio Inc., Toronto
ON). Relative protein levels were averaged from three biological repeats and calculated using the following equation:

\[
\text{Relative eIF3B Protein Level} \ (\%) = \frac{B.I.\text{eIF3B}_{\text{siRNA treated}}}{B.I.\text{eIF3B}_{\text{GAPDH untreated control}}} \times 100 \%
\]  

(8)

Where B.I.\text{X} represents the band intensity of X protein.

### 4.4.8 Cell Cycle Analysis

MDA-MB-231-H2N cells were transfected as described in Section 4.4.6. The concentration of the si3Bs and non-targeting sequences were 1 nM. At 72 h post transfection, cells were harvested using accutase and diluted to \(10^6\) cells / mL. Cells were centrifuged (1500 rpm for 5 minutes) and washed once with ice cold PBS. Cells were then added drop wise into 70 % ethanol in a BSA pre-coated 5 mL round-bottom 12x75 mm tube. The cells were stored at 4 °C for 24 h and then centrifuged and washed twice with ice cold PBS. The cells were treated with triton-X (0.1%), RNAse (0.5 mg/mL) and 7-actinomycinD (7-AAD, 20 µg/mL) for 30 minutes at 37 °C. Cells were placed on ice before measuring fluorescence in a flow cytometer. Cell debris and doublets were gated out using the FSH-A vs FSH-H, and 10,000 events were collected. The FL-3 channel (ex/em 640/>670 nm) was set in a linear range, and cell cycle analysis was completed using the FlowJo software (Flowjo, OR, USA). The results were expressed as proportion of cells in the G1, S, and G2 phases relative to DMSO control, and averaged from three biological repeats.

### 4.4.9 Trastuzumab-DBCO-647 (Tras-DBCO) Preparation

Trastuzumab (50 nmole), Alexa fluor 647-succinimidyl ester (50 nmole), and DBCO-PEG4-NHS (500 nmole) were added to 0.1 bicarbonate buffer (pH 8.3) and allowed to react for 2 h. Tras-DBCO was purified by fast protein liquid chromatography (FPLC) using a superdex 200 column at 2 mL/min while monitoring absorbance at 647 nm. Purified Tras-DBCO was collected and concentration using a Vivaspin 6 (30 kDa cutoff). Alexa Fluor 647 was quantified using fluorescence with ex/em 640/680 against a standard curve, and DBCO was quantified by measuring absorbance at 309 nm (\(\varepsilon = 12,000 \text{ M}^{-1}\text{cm}^{-1}\)). Purified Tras-DBCO contained 6.2 DBCO and 0.5 Alexa Fluor 647 per antibody.
4.4.10 Vitamin E Succinate modified Decaarginine (VR10) Synthesis

VR10 and similar vitamin E succinate modified peptides were synthesized and purified as described in section Chapter 2.3.1.

4.4.11 Nanoparticle Formation and Encapsulation

6 mg of P(LA-co-TMCC)-g-PEG-N\textsubscript{3} was dissolved in 100 uL of methanol:acetonitrile solution (1:1), and then diluted with 2 mL of dH\textsubscript{2}O. Prepared NPs were filtered through a 0.45 µm PES filter, methanol and acetonitrile was removed by rotary evaporation and aliquots were frozen until required. VR10 and similar peptides were encapsulated in NPs by dissolving 1.2 mg of peptide in the methanol:acetonitrile solution before dilution in dH\textsubscript{2}O. Diameters and zeta potentials of the NPs were measured using a Malvern Zetasizer Nano ZS (4 mW, 633 nm laser) at a concentration of 1 mg/mL.

4.4.12 CuAAC vs SPAAC for si3B Conjugation

Copper catalyzed alkyne-azide cycloaddition (CuAAC) and strain promoted alkyne-azide cycloaddition (SPAAC) were compared using either an alkyne modified si3B or DBCO modified si3B respectively. NPs (42 µM of the azide functionality), alkyne-si3B (2 µM), Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, 104 µM), copper sulfate (CuSO\textsubscript{4}, 52 µM), and sodium ascorbate (520 µM) were suspended in PBS and allowed to react on a shaker for 2 h. Similarly, NPs (42 µM of the azide functionality) and DBCO-si3B (2 µM) was suspended in PBS and allowed to react on a shaker for 2 h. Unreacted si3B was by FPLC using the superdex 200 column while monitoring absorbance at 547 nm for Cy3. Purified si3B modified NPs were collected and concentrated using a Vivaspin 6 (30 kDa MWCO) centrifugal concentrator. si3B modified NPs were quantified by fluorescence against a standard curve using ex/em 540 nm / 580 nm. Three repeats were used for the CuAAC and the SPAAC reactions. Conjugation yields were calculated as follows:

\[
\text{Conjugation Yield (\%)} = \frac{\text{Mole } si3B_{\text{Purified NPs}}}{\text{Mole } si3B_{\text{Initial}}} \times 100\%
\]
4.4.13 Serum Stability of si3B Conjugates

PEG_{10K-N_3} (500 nmole) was mixed with either unmodified, F1, F2, or F3 DBCO si3B (500 nmole, F1, F2, and F3 siRNA sequences found in Table 4.7) and allowed to reacted for 4 h at 25 °C. The reacted solutions were diluted to 10% FBS and incubated at 37 °C on a shaker. At predetermined time points, aliquots were taken from the samples exposed to FBS, added to 10x glycerol loading buffer and frozen at -20 °C for later use. 25 nmole of si3B was loaded per well in a polyacrylamide gel (12.5%) and the gels were run at 250 V for 45 minutes. The cy3 fluorophore on the si3B was visualized with a Typhoon Trio system exciting at 488 and using a 580-nm band-pass filter (580 BP 30). The amount of si3B conjugated to PEG relative to time 0 was calculated as follows:

\[
\text{Relative si3B Bound (\%)} = \frac{\text{Fluorescence Intensity of si3B}_{\text{Time x}}}{\text{Fluorescence Intensity of si3B}_{\text{Time 0}}} \times 100\%
\]

Where Time x represents the time that the aliquot was taken.

An identical study was setup using NPs, where si3B conjugated NPs (SNPs) were prepared as described in the previous section. Here, the SNPs were incubated with either PBS, 10% FBS, or 50% FBS and si3B quantified after running through a polyacrylamide gel as described above for up to 24 h.

4.4.14 Transfection of si3B modified immunonanoparticles (SINP)

MDA-MB-231/H2N breast cancer cells were seeded in a 96-well plate at a density of 3,000 cells / well the day before transfection. NPs (60 µM), si3B-DBCO (1.3 µM) and Tras-DBCO (0.9 µM) were suspended in PBS and allowed to react on a shaker for 2 h at 37 °C. si3B-DBCO was also reacted with NPs (SNPs) without the Tras-DBCO as a targeting control. Identical conditions were used for the VR10 encapsulated NPs (VR10-NPs) or VR10 encapsulated immunonanoparticles (VR10-SINPs). SNPs and SINPs were diluted in full cell media (10% FBS) to a concentration of 20 nM and incubated with cells for 24 h. As a positive control, si3B was transfected with Lipofectamine 2000 as described in Section 4.4.5. Cell viability was measured 48 h later as described previously.
4.4.15 Complexation/Conjugation of siRNA to VR10-NPs

siRNA or DBCO-siRNA was added to NPs or VR10-NPs at various polymer:siRNA mole ratios and placed on a shaker at 37 °C for 15 minutes. Afterwards, 25 pmole of siRNA was loaded on top of polyacrylamide gels (12.5%) and the gels were run at 250 v for 45 minutes. The cy3 fluorophore on the si3B was visualized with a Typhoon Trio system exciting at 488 and using a 580-nm band-pass filter (580 BP 30).

4.4.16 Cell Uptake and Lysosome Colocalization of Cy3 Labeled NPs and VR10-NPs

DBCO-Cy3 (50 µM) was reacted with 500 µM of either NPs (CNP), immunonanoparticles (CINPs), VR10-NPs (VR10-CNP), or VR10 encapsulated immunonanoparticles (VR10-CINPs) for 2 h at 37 °C. MDA-MB-231/H2N cells were seeded in 12-well plates at a density of 100,000 cells/well and allowed to adhere overnight. For the lysosome colocalization studies, cell lysosomes were labeled with Alexa Fluor 647-dextran (10,000 g/mol) overnight at 50 µg/mL prior to incubation with NPs. The Cy3 labeled NPs were then diluted in cell media to a final concentration of 500 nM. Treatments included 2, 6, and 24 h for live cell fluorescent microscopy, and 24 h for flow cytometry. The cells were washed thrice with PBS and incubated with Hoescht 33342 nuclei dye (Molecular Probes, Inc., Eugene, OR, USA). Live cell fluorescent microscopy was completed on an Olympus FV1000 confocal microscope through a 40x lens. Excitation and emission wavelengths are as follows: Hoescht 33342 (ex/em: 405/460 nm) and Cy3 (ex/em 545/580 nm). Cell fluorescence was quantified using a BD Accuri C6 flow cytometer with excitation wavelength of 488 nm and emission filters of 585(30) nm (Cy3, FL-2 channel). Cell debris and doublets were gated out using FSH-A vs FSH-H, and at least 10,000 events were collected. The mean fluorescence intensity in the FL-2 channel for three biological repeats was used to measure the amount of Cy3 uptake.

4.5 Results and Discussion

4.5.1 si3B Exhibits Potent and Durable Anti-Cancer Activity

siRNAs used for anti-cancer treatment must exhibit potencies in the low nanomolar range in order to reduce undesirable effects, such as off-target knockdown.[67] In order to obtain a potent siRNA sequence targeting eIF3B, an siRNA sequence optimization algorithm was
employed provided by Integrated DNA Technologies RNAi Design Sci Tool. Here, the
algorithm searches the complementary strand of the messenger RNA sequence of eIF3B for an
optimal 27-mer sequence that is most suitable to be a dicer substrate. Importantly, this algorithm
also checks that the chosen siRNA sequence does not match off-target mRNAs, in order to have
a selective si3B sequence. As the algorithm is based off of a library of siRNAs and
generalizations are applied for optimal siRNA knockdown, four si3B sequences were chosen in
order to test the anti-cancer activity against a breast cancer cell line. The top 4 sequences from
the RNAi design Sci Tool targeted the eIF3B mRNA at various positions ranging from 642 to
1192 of the antisense strand. (Table 4.4) It is expected that successful knockdown of an essential
protein such as eIF3B would result in decreased cell viability, therefore a metabolic assay was
used as a proxy to determine the anti-cancer activity of the si3B sequences. Fortunately, all four
si3B sequences exhibited potent anti-cancer activity against MDA-MB-231 cells across the
concentrations tested (3-33 nM) (Figure 4.9). There were no significant differences in the anti-
cancer activity among the 4 si3B sequences at each concentration tested. Importantly, the vehicle
control, Lipofectamine 2000, and transfection of a non-targeted siRNA (negative control)
exhibited minimal toxicity against the breast cancer cells. si3B-1 was chosen as the desired
siRNA sequence as the RNAi design Sci Tool determined this sequence would produce the least
number of off-target effects.

![Figure 4.9](image-url)

**Figure 4.9** Anti-cancer activity of different siRNA sequences targeting eIF3B (si3B) in MDA-
MB-231 cells. Proliferation was measured 4 days post transfection relative to the no treatment
group. Lipo2000 represents the vehicle control, Lipofectamine 2000, with no siRNA complexed. (n=4, mean ± standard deviation, N.S. = p > 0.05, ** p< 0.05 determined by one way ANOVA and Bonferroni post-hoc test)

Two of the attractive features of siRNA knockdown are potency and durable effects on breast cancer cells. Moreover, several groups have shown that fluoro-modified siRNA elicits long-lasting knockdown due to the nuclease stability relative to unmodified siRNA.[70] Importantly, the position and quantity of the fluoro-modified siRNA have significant impact on the potency and durability of knockdown.[415] Therefore, an optimal fluoro-modified siRNA sequence pattern was chosen as determined from a library of differently patterned fluoro-modified siRNA sequences.[408] Fluoro modified si3B-1 (F si3B) was compared to unmodified si3B in terms of anti-cancer potency on MDA-MB-231/H2N and MDA-MB-231 cells. The dose response curves of both siRNA indicated vastly different anti-cancer potency in both cell types (Figure 4.10). For example, MDA-MB-231/H2N cells incubated with unmodified si3B at 0.01 nM exhibited a relative viability of 16.1% ± 5.1%, whereas the F si3B treated cells exhibited a relative viability of 78.5% ± 9.1% at 4 days post transfection. Importantly, non-targeting siRNA treated cells, serving as a negative control, incubated at the highest concentrations tested exhibited minimal toxicity (0.1-10 nM), indicating that the transfection agent is non-toxic and that the siRNA sequence must be specific to eIF3B. The dose response curves of unmodified si3B and F si3B were evaluated to obtain an inhibitory concentration to reduce relative viability to 50% (IC$_{50}$). (Table 4.6) Interestingly, the IC$_{50}$ of F si3B was significantly more relative to the IC$_{50}$ of unmodified si3B in both breast cancer cells. For example, the IC$_{50}$ of unmodified si3B and F si3B was 2.7 pM ± 0.8 pM and 41.7 pM ± 10.1 pM, respectively, against the MDA-MB-231/H2N cells. The MDA-MB-231 cells were less sensitive to the siRNAs as the IC$_{50}$ of unmodified si3B and F si3B was 19.0 pM ± 6.1 pM and 168.1 pM ± 64.3 pM, respectively. In general, the IC$_{50}$ of F si3B was 9-15-fold greater than the IC$_{50}$ of unmodified si3B, indicating that F si3B is less potent than unmodified si3B.
**Figure 4.10** Dose response curves of siRNA targeting eIF3B (si3B) on the anti-cancer activity against breast cancer cells. Relative viability is normalized to untreated control cells. Dose response curves were obtained from treatment of si3B and non-targeting siRNA control against MDA-MB-231/H2N (A), and MDA-MB-231 (B) cells. Green represents the fluoro modified non-targeting siRNA control, red represent the fluoro modified si3B, and blue represents the unmodified si3B. (n=3, mean ± standard deviation)

**Table 4.6** Inhibitory concentration to affect the viability of cells to 50% (IC\(_{50}\)) of the siRNAs targeting eIF3B (si3B) in breast cancer cells in vitro.

<table>
<thead>
<tr>
<th>IC(_{50})</th>
<th>MDA-MB-231/H2Ns</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>si3B</td>
<td>2.7 pM ± 0.8 pM</td>
<td>19.0 pM ± 6.1 pM</td>
</tr>
<tr>
<td>F si3B</td>
<td>41.7 pM ± 10.1 pM</td>
<td>168.1 pM ± 64.3 pM</td>
</tr>
</tbody>
</table>

F si3B was compared to unmodified si3B in terms of duration of anti-cancer activity in MDA-MB-231 and MDA-MB-231/H2N cells (Figure 4.11). Surprisingly, unmodified si3B exhibited significantly more potent and durable anti-cancer activity at 0.01 nM and 0.1 nM relative to F si3B in MDA-MB-231/H2N cells. The difference is diminished when the cells were treated at 1 nM for both unmodified si3B and F si3B, and the durability of the knockdown is maintained until 10 days post-transfection. The unmodified si3B and F si3B exhibited less potent anti-cancer activity against MDA-MB-231, similar to the difference in the IC\(_{50}\) between the cell lines. For example, incubation of MDA-MB-231/H2N cells with F si3B at 1 nM resulted in a relative viability of 20.9% ± 6.8% at 4 days post-transfection, while F si3B treated MDA-MB-231 cells exhibited a relative viability of 48.2% ± 4.6% in similar conditions. Importantly, incubation of non-targeting siRNA (negative control) in identical conditions did not induce
significant toxicities in both breast cancer cells. Interestingly, F si3B did not exhibit more potent and durable anti-cancer activity relative to unmodified si3B. The fluoro modifications in F si3B may have impacted siRNA loading into the dicer enzyme, as observed in the past.[408] Secondly, the optimized fluoro-modified pattern determined from the library of siRNAs targeting a model protein may not be as easily transferable to other sequences, which paves the way for a more generalized approach to fluoro-modified siRNA patterns.[69] However, the superior nuclease stability of the fluoro-modified siRNA would be beneficial for use with NPs.

Figure 4.11 Durability of the anti-cancer activity of siRNA targeting eIF3B (si3B) on breast cancer cells. Relative viability is normalized to untreated control cells. MDA-MB-231/H2Ns were treated with si3B and non-targeting siRNA control at 0.01 nM (A), 0.1 nM (B) and 1 nM (C). MDA-MB-231 cells were treated with si3B and non-targeting siRNA control at 1 nM (D). Green represents the fluoro modified non-targeting siRNA control, red represent the fluoro modified si3B, and blue represents the unmodified si3B. (n=3, mean ± standard deviation, N.S. > 0.05, * p< 0.05, **<0.01, ***<0.001 determined by one way ANOVA and Bonferroni post-hoc test)
4.5.2 si3B is Potent at eIF3B Knockdown at the mRNA and Protein Level

siRNAs are an attractive therapeutic as they can induce potent but selective knockdown of the targeted protein. To verify the knockdown of eIF3B mRNA, quantitative polymerase chain reaction (qPCR) was used to measure the level of eIF3B mRNA normalized to a housekeeping mRNA, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Figure 4.12). eIF3B mRNA was measured after incubation with si3B at four concentrations, ranging from non-toxic levels (below the IC50) to above the IC50 of the si3Bs. Both unmodified si3B and F si3B elicited eIF3B knockdown at concentrations above 0.1 nM in both cell types. For example, unmodified si3B and F si3B reduced the relative eIF3B mRNA levels to 6.2% ± 4.3% and 8.8% ± 3.1% in MDA-MB-231/H2N cells when incubated at 1 nM. Importantly, the non-targeting siRNA incubated at 1 nM did not significantly reduce the relative eIF3B mRNA levels. Next, eIF3B knockdown was verified at the protein level using western blot normalized to β-actin protein levels (Figure 4.13). Following similar trends to the mRNA knockdown, both unmodified si3B and F si3B elicited significant effects on eIF3B protein levels in both breast cancer cells. For example, unmodified si3B and F si3B decreased the relative eIF3B protein levels to 9.6% ± 6.3% and 7.4% ± 3.6% in the MDA-MB-231/H2N cells, respectively. As expected, the non-targeting siRNA control elicited minimal eIF3B knockdown. The eIF3B mRNA and protein knockdown corroborate the anti-cancer activity results in the previous section. For example, the nearly complete knockdown of the eIF3B mRNA and protein levels at 1 nM in MDA-MB-231/H2N led to less than 10% viability, relative to control. These results suggest that the anti-cancer activity of both unmodified si3B and F si3B hinges on the selective knockdown of eIF3B at the mRNA and protein levels.
Figure 4.12 siRNA targeting eIF3B (si3B) effectively decreases the relative levels of eIF3B mRNA. si3Bs and non-targeting siRNA controls were incubated with MDA-MB-231/H2Ns (A), or with MDA-MB-231s and mRNA was measured 3 days post-transfection. mRNA levels were evaluated by qPCR and normalized to the GAPDH mRNA levels in each sample. Green represents the fluoro modified non-targeting siRNA control, red represents the fluoro modified si3B, and blue represents the unmodified si3B. The non-targeting control siRNA was tested at the highest concentration of si3B used. (n=3, mean ± standard deviation)

(A)  

(B)  

Figure 4.13 siRNA targeting eIF3B (si3B) effectively decreases the relative levels of eIF3B protein. si3Bs and non-targeting siRNA controls were incubated with MDA-MB-231/H2Ns (A), or with MDA-MB-231s and eIF3B protein levels were measured 3 days post-transfection normalized to β-actin protein levels. Green represents the fluoro modified non-targeting siRNA control, red represents the fluoro modified si3B, and blue represents the unmodified si3B. The non-targeting control siRNA was tested at the highest concentration of si3B used. (n=3, mean ± standard deviation)
4.5.3 si3B induces G1 Cell Cycle Arrest

To gain a deeper understanding of the effects of eIF3B knockdown on the breast cancer cells, cell cycle arrest of the transfected cells was evaluated. Treatment of MDA-MB-231/H2N cells with si3B or F si3B resulted in a G1 cell cycle arrest (Figure 4.14A). For example, incubation with si3B or F si3B resulted in a significantly increased proportion of cells in the G1 cell cycle to 142.6% ± 1.9% and 141.9% ± 4.8%, respectively, relative to no treatment control group (Figure 4.14B). This led to a reduction of cells in the S phase to 56.2% ± 8.3% and 66.3% ± 4.3% for si3B and F si3B, respectively. Importantly, the transfection control and transfection of a nontargeting siRNA did not significantly change the proportion of cells in each cell cycle relative to no treatment control. As the eIF3B knockdown has been verified at the mRNA and protein levels, the absence of the eIF3B protein results in cells not progressing past the G1 cell cycle checkpoints and halting at the G1 cell cycle phase. With a reduction of cells in the S phase following si3B treatment, the treated cells cannot synthesize the necessary protein and DNA required for mitosis, which aids in the understanding of the dose-dependent anti-cancer activity of si3B.[411, 416] The G1 cell cycle arrest is expected after si3B treatment as eIF3B is primarily involved as a scaffolding protein for the eukaryotic transcription initiation factor 3 (eIF3) that is required for the translation of mRNA.[413]

**Figure 4.14** si3B and F si3B treatment induces G1 cell cycle arrest in MDA-MB-231/H2N. MDA-MB-231/H2Ns were incubated with si3Bs or controls, and cell cycle analysis performed 3 days post-transfection. Cntl represents untreated control cells, Veh Cntl represents cells treated with lipofectamine 2000, Neg Cntl represents cells treated with fluoro modified non-
targeting siRNA, si3B represents the unmodified si3B and F si3B represents the fluoro modified si3B. (A) Proportion of treated cells in each cell cycle phase relative to the respective proportion of cells in each cell cycle of untreated control cells. (B) Representative histograms of the cell cycle analysis or control cells (Cntl), cells treated with fluoro modified scrambled siRNA (Neg. Cntl), si3B and F si3B.

4.5.4 SPAAC is the Superior Conjugation Method for siRNA-NPs

Typical siRNA delivery strategies rely on electrostatic interaction of the siRNA with a polycationic polymer; yet a detrimental burst release is commonly observed when administering the formulation in vivo.[417] Here we employed a covalent attachment of the siRNA to the NPs either through copper catalyzed azide-alkyne cycloaddition (CuAAC), or strain promoted azide-alkyne cycloaddition. Two siRNAs were synthesized either with an alkyne or a dibenzylcyclooctyne (DBCO) for the CuAAC and SPAAC reactions, respectively, while the NPs are functionalized with azides. The CuAAC and SPAAC conjugation strategies were evaluated at various incubation times of 2-24 h. At all incubation times tested, the SPAAC conjugation method was superior to the CuAAC method in terms of conjugation yield and siRNA per NPs (Figure 4.15). For instance, the conjugation yield of the SPAAC reaction at 24 h was 3.1% ± 0.3%, while the yield of the CuAAC reaction was only 0.5% ± 0.1%. This led to a similar trend with siRNAs per NP, where the SPAAC reaction resulted in 4.0 ± 0.4 siRNAs per NP, while the CuAAC resulted in ~0.3 siRNA per NP. As the CuAAC reaction relies on the coordination of the alkyne, accessible azides on the NPs, copper catalyst, and the accelerating ligand, THPTA, it is likely that all of these components coordinating on the NP surface hinders the conjugation yield.[418] On the other hand, the SPAAC reaction relies on the coordination of the strained alkyne from DBCO and an accessible azide on the NP surface.[138] Moreover, the DBCO moiety on the siRNA is relatively more hydrophobic, compared to an alkyne, permitting enhanced interaction with the hydrophobic core of the NPs and increasing the likelihood to react with an azide within the PEG corona of the NP. Previously, the CuAAC conjugation method was used to modify NPs with an alkyne functionalized siRNA, resulting in approximately ~120 siRNA per NP.[137] However, more siRNA was used in order to prepare these formulations. Modifying the surface of the NPs with fewer siRNA that are strongly electronegatively charged may be beneficial in regards to NP interaction with the negatively charged plasma membrane of cancer cells.[419] Therefore, using a lower ratio of siRNA to NPs, it is clear that the SPAAC reaction is superior to the CuAAC reaction in terms of covalently attaching siRNA to NPs.
Figure 4.15 Strain promoted alkyne-azide cycloaddition (SPAAC) is superior to copper catalyzed alkyne-azide cycloaddition (CuAAC) for enhanced conjugation yields leading to more siRNAs per nanoparticle (NP). (A) Conjugation yields of reacting si3B with NPs either through CuAAC (green) or SPAAC (blue) after purification. (B) Quantified siRNA per NP after purification using either CuAAC or SPAAC. (n=3, mean ± standard deviation, *** p< 0.001, determined by one way ANOVA and Bonferroni post-hoc test)

4.5.5 siRNA with a Fully Fluoro modified Sense Strand exhibit Superior Nuclease Stability

The pattern of the fluoro modified RNA in a siRNA strand has dramatic effects on both siRNA potency and nuclease stability.[69] Moreover, the intracellular nuclease stability of the siRNA has been directly linked to both siRNA potency and duration of the knockdown.[70]

Three fluoro modified si3Bs were prepared with either partially or fully modifying the sense strand of the siRNA. (Table 4.7) First, the unmodified si3B and the three fluoro modified si3Bs were evaluated for nuclease stability by reacting with a poly(ethylene glycol), as a proxy for siRNA-NP conjugates, and testing the stability against 10% fetal bovine serum (FBS), mimicking in vitro conditions (Figure S 4.2). Only the F3 si3B-PEG conjugate demonstrated enhanced nuclease stability relative to the unmodified si3B-PEG conjugate. For instance, 68.2% ± 1.1% of F3 si3B-PEG remained intact upon incubation with 10% FBS over 24 h, whereas 60.7% ± 3.4% of the unmodified si3B-PEG was intact. The long 10 kDa PEG may provide steric protection against nucleases in these si3B-PEG conjugates. Interestingly, both the F1 and F2 si3B-PEG conjugates exhibited rapid degradation and reduced nuclease stability relative to the unmodified si3B-PEG conjugate at all time points tested, making F1 and F2 si3B unsuitable for NP delivery. Potentially, the last 4 RNA nucleotides of the sense strand of the fluoro modified
si3B are a crucial point for nuclease degradation, supported by the mechanism that the dicer enzyme cleaves 27 nucleotide long siRNA.[420]

**Table 4.7** Sequences of siRNA targeting eIF3B and the position of the fluoro modifications. Legend for modifications: 1 RNA (uppercase), 2’F-RNA (bold), 2’F-ANA (italic underlined), Cy3 = cyanine 3, and DBCO = dibenzylcyclooctyne. The upper sequence is the sense strand while the bottom sequence is the antisense strand.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-targeting Control</td>
<td>5’ Cy5 GCU UGA UUU CUG AAA UUA AUU CUG C DBCO 3’</td>
</tr>
<tr>
<td></td>
<td>AC CGA ACU AAA GAC UUU AAU UAA GAC G 5’</td>
</tr>
<tr>
<td>Unmodified</td>
<td>5’ Cy3-GAA GCA GAU GGA AUC GAU UCG GUG A-DBCO 3’</td>
</tr>
<tr>
<td></td>
<td>3’ UC CUU CGU CUA CCU UAG CUA AGC CAC U 5’</td>
</tr>
<tr>
<td>F1</td>
<td>5’ Cy3-GAA GCA GAU GGA AUC GAU UCG GUG A-DBCO 3’</td>
</tr>
<tr>
<td></td>
<td>3’ UC CUU CGU CUA CCU UAG CUA AGC CAC U 5’</td>
</tr>
<tr>
<td>F2</td>
<td>5’ Cy3-GAA GCA GAU GGA AUC GAU UCG GUG A-DBCO 3’</td>
</tr>
<tr>
<td></td>
<td>3’ UC CUU CGU CUA CCU UAG CUA AGC CAC U 5’</td>
</tr>
<tr>
<td>F3</td>
<td>5’ Cy3-GAA GCA GAU GGA AUC GAU UCG GUG A-DBCO 3’</td>
</tr>
<tr>
<td></td>
<td>3’ UC CUU CGU CUA CCU UAG CUA AGC CAC U 5’</td>
</tr>
</tbody>
</table>

The F3 si3B-PEG demonstrated enhanced nuclease stability relative to the unmodified si3B-PEG and was used further to prepare siRNA modified NPs. Unmodified si3B and fluoro modified si3B was conjugated onto NPs and evaluated for nuclease stability in PBS, 10% FBS, and 50% FBS (Figure 4.16). Importantly, both unmodified and fluoro modified si3B-NPs demonstrated suitable stability in PBS alone where more than 80% of the initial siRNA remained bound to the NPs after 24 h. Unmodified si3B-NPs exhibited rapid degradation where only 63.8% ± 5.4% of the initial si3B remained bound to NPs upon exposure to 10% FBS for 6 h, whereas fluoro modified si3B-NPs retained 93.9% ± 2.9% of the initial si3B under identical conditions. This trend is accelerated when incubated in 50% FBS for 6 h, where unmodified si3B-NPs retained only 25.1% ± 9.3% of the initial siRNA, while the fluoro modified si3B-NPs retained 87.0% ± 10.2%. It is expected that the unmodified si3B-NPs would degrade more rapidly in vivo as the serum and nuclease concentrations are much higher.[421] After the si3B is cleaved from the NPs, the released siRNA will either be degraded completely in circulation or cleared out through the liver and kidneys.[422] This will be significant for the improved delivery
of fluoro modified si3B-NPs as more fluoro modified si3B remain covalently attached to the NPs, while potentially delivering increasing amounts of intact siRNA to the tumor.

**Figure 4.16** Fluoro modified siRNA targeting eIF3B (si3B) conjugated to nanoparticles (NPs) exhibits superior nuclease stability relative to the unmodified siRNA counterpart. Unmodified si3B (A) and fluoro modified si3B (B) conjugated NPs were exposed to PBS solution (blue), 10% fetal bovine serum (FBS) (red) or 50% FBS (green) at 37 °C. At the indicated time points, the si3B conjugated NPs were purified and si3B quantified by fluorescence normalized to an internal standard. The amount of siRNA conjugated to NPs was normalized to the siRNA quantified at time 0. (n=3, mean ± standard deviation)

4.5.6 In Vitro Anti-Cancer Activity of SNPs and SINPs

The enhanced stability of the fluoro modified siRNA-NPs (SNPs) prompted the evaluation of the anti-cancer activity against MDA-MB-231/H2N cells. The anti-cancer activity of si3B and trastuzumab modified immunonanoparticles (SINPs) were also compared to elucidate the benefits of active targeting of the human epidermal growth factor receptor 2 (HER2), which is overexpressed in the MDA-MB-231/H2N cells. Unfortunately, both SNPs and SINPs incubated at 25 nM (~595 x above the IC$_{50}$ of si3B) did not result in significant breast cancer cell death (Figure 4.17). As a positive control, the F si3B transfected with lipofectamine 2000 resulted in significant breast cancer death, while the vehicle control was relatively nontoxic. A successful transfection of si3B in breast cancer cells has been established to result in cancer cell death, offering an easy readout where the successful delivery of si3B by SNP or SINPs should result in similar cancer cell death. The absence of cell death is a strong indication of unsuccessful delivery of si3B into the cytoplasm, the location of the siRNA machinery.
required for mRNA knockdown. It is expected that both SNPs and SINPs are shuttled through the endo/lysosomal pathway, and potentially trapped in the lysosomes where degradative enzymes and lowered pH destroy the siRNA, prompting the use of endosomal escape peptides.[151]

**Figure 4.17** Fluoro modified siRNA targeting eIF3B conjugated to immunonanoparticles (F3SINP) do not kill MDA-MB-231/H2N breast cancer cells in vitro. Following a 6 h transfection, proliferation was measured 6 days later. F3 si3B represents the F3 siRNA sequence targeting eIF3B transfected with Lipofectamine 2000, Lipo Cntl represents the lipofectamine 2000 transfection control, F3 SNP represents the F3 si3B modified NPs, NP Cntl represents the blank NP control, and Cntl represents the untreated control group. (n=3, mean ± standard deviation, N.S. = p > 0.05, *** p< 0.001, determined by one way ANOVA and Bonferroni post-hoc test)

4.5.7 Vitamin E Decaarginine Enhances Cancer Cell Uptake

The unsuccessful transfection of SNPs or SINPs prompted the use of endosomal escape peptides in the NPs to promote the escape of si3B into the cytoplasm. Typically, peptides containing 8-24 cationic arginines are used to bind electrostatically with siRNA but also with the inner endosomal membrane forcing either a pore to form, or to destabilize the endosomes.[359] Therefore a decaarginine was employed to be encapsulated in NPs; however decaarginine is water soluble and not a suitable peptide for NP encapsulation. Here, decarginine was modified with a hydrophobic vitamin E succinate at its N-terminus (VR10), and used for encapsulation in NPs (VR10-NPs) (Table 4.8). The VR10-NPs exhibited slightly smaller diameters relative to standard nanoparticles (40 vs 64 nm), while exhibiting a more neutral zeta potential (Table 4.8). Presumably, the negative charges of the acids located in the polymer backbone of the nanoparticles are neutralized by the cationic arginine in VR10. Both VR10-NPs and standard
NPs exhibited similar critical micelle concentration (CMC) and VR10-NPs encapsulated approximately 282 µg of peptide per 1 mg of NP mass.

Table 4.8 Physicochemical characteristics of NPs, and VR10-NPs.

<table>
<thead>
<tr>
<th>Name</th>
<th>Size (d.nm) [PDI]¹</th>
<th>Zeta Potential (mV)¹</th>
<th>Peptide (ug) / Backbone+PEG (mg)²</th>
<th>CMC (µM) [µg/mL]³</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>64 ± 4 [0.10 ± 0.07]</td>
<td>-22.9 ± 3.4</td>
<td></td>
<td>0.66 ± 0.10 [ 11.7 ]</td>
</tr>
<tr>
<td>VR10-NP</td>
<td>40 ±4 [0.11 ± 0.06]</td>
<td>+4.3 ± 1.2</td>
<td>282 ± 38</td>
<td>0.88 ± 0.12 [20.6]</td>
</tr>
</tbody>
</table>

1. Dynamic light scattering (DLS)
2. Amino acid analysis
3. Pyrene method

Cationic peptides incorporated into other NPs systems have been demonstrated to induce increased cell uptake as well as endosomal escape.[350] The cell uptake of the NPs was compared by confocal fluorescent microscopy in MDA-MB-231/H2N cells. Here the NPs were labelled with a Cy5 dye and VR10 encapsulated NPs were compared to the blank NPs, as well as comparing the benefits of active targeting using trastuzumab modified immunonanoparticles. At all time points tested, VR10 encapsulated Cy5-NPs (VR10-CNPs) and VR10 encapsulated Cy5-immunonanoparticles (VR10-CINPs) exhibited superior cell uptake relative to Cy5-NPs (CNPs) and Cy5-immunonanoparticles (CINPs) (Figure 4.18). Moreover, at 24 h both VR10-CNPs and VR10-CINPs demonstrate diffuse cytoplasmic fluorescence, indicative of endosomal escape.[423] Interestingly, the active targeting benefits of VR10-CINPs are diminished as cells treated with either VR10-CNPs or VR10-CINPs qualitatively exhibit similar fluorescence in the HER2-overexpressing MDA-MB-231/H2N cells. A quantitative comparison of the cell uptake was evaluated through flow cytometry after 24 h of incubation with the MDA-MB-231/H2N cells (Figure 4.19). Both VR10-CNPs and VR10-CINPs exhibited superior cell uptake relative to CNPs and CINPs. For example, the VR10-CNP and VR10-CINP treated cells exhibited a mean fluorescence intensity (MFI) of 70,569 ± 4,374 and 63,885 ± 1,879, respectively, while CINP treated cells exhibited an MFI of 7004 ± 698, or approximately ~10 less uptake. These results indicate that VR10 greatly improves NP-cell interaction and uptake as determined from confocal microscopy and flow cytometry.
Figure 4.18 Encapsulating vitamin E-decaarginine (VR10) in NPs (VR10-CNPs) or immunonanoparticles (VR10-CINP) increases breast cancer cell uptake relative to the NPs (CNPs) or immunonanoparticles (CINPs). NPs were pre-labeled with a Cyanine 3 dye (Cy3) and MDA-MB-231/H2Ns were treated with Cy3 modified NPs (500 nM) for the indicated incubation periods. Live cells were imaged by confocal fluorescent microscopy. Nuclei are shown in blue, and the Cy3 dye is shown in green.
Figure 4.19 Vitamin E decaarginine (VR10) significantly increases cell uptake of nanoparticles (VR10-CNP) and immunonanoparticles (VR10-CINP) relative to nanoparticles (CNP) or immunonanoparticles (CINP). NPs were prelabeled with a cyanine 3 dye (Cy3) and MDA-MB-231/H2N breast cancer cells were incubated with NPs for 24 hrs before Cy3 fluorescence was measured by flow cytometry. Cells were incubated with Cy3 at a concentration of 500 nM, and Cntl represents untreated control group. (n=3, mean ± standard deviation, *** p< 0.001, determined by one way ANOVA and Bonferroni post-hoc test)

4.5.8 VR10-NPs Improve siRNA Complexation/Covalent Attachment

Cationic peptides containing arginine allow for the electrostatic binding with siRNA, thus providing a means to deliver siRNA. The siRNA complexation onto the NPs was evaluated either with blank NPs or VR10-NPs. Here, alkyne modified siRNA (siRNA) was compared to DBCO-siRNA to ascertain the benefits of covalent attachment afforded from the SPAAC reaction. Without a copper catalyst, alkyne modified siRNA react extremely slowly with azides, and thus are not expected to covalently bind during these reaction times. VR10-NPs were able to complex with alkyne modified siRNA and covalently attached to DBCO-siRNA (Figure 4.20).

For example, when alkyne modified siRNA was incubated with VR10-NPs at a siRNA:NP molar ratio of 12.5:1, 25:1, and 50:1, all of the siRNA was complexed with the NPs whereas blank NPs exhibited no complexed siRNA, as expected. Moreover, all the DBCO-siRNA was complexed/covalently attached at siRNA:NP molar ratios as low as 6.3:1 whereas the blank NPs exhibited a very low number of siRNAs covalently attachment to NPs. Here, the slightly positive surface of VR10-NPs aided in the complexation of alkyne modified siRNA, where this phenomenon is enhanced in the case of DBCO-siRNA as the DBCO moiety offers a way to covalently attach to the NPs. Therefore, the VR10-NPs were further evaluated for anti-cancer
activity with the DBCO-siRNA as the covalent attachment is more suitable for in vivo applications.

**Figure 4.20** Vitamin E decaarginine encapsulated NPs (VR10-NPs) electrostatically bind siRNA, and electrostatically and covalently conjugate with DBCO-siRNA. siRNAs were added to NPs at the molar ratio of polymer:siRNA as indicated on top of the gel. Complexed siRNA do not penetrate the gel and remain on the top, while unreacted, uncomplexed siRNA migrate through the gel. Standard NPs are not able to complex with siRNA, and react with a limited amount of DBCO-siRNA as determined in the polyacrylamide gel.

Enhanced cell uptake with indications of endosomal escape and diffuse cytosolic fluorescence prompted the evaluation of the anti-cancer activity of VR10-SNPs and VR10-SINPs. Unfortunately, both VR10-SNPs and VR10-SINPs did not significantly affect the viability of MDA-MB231/H2N cells (Figure 4.21). The si3B positive control demonstrates strong anti-cancer activity as the viability of si3B treated cells is 35.9% ± 5.2% relative to the no treatment control. Importantly, the lipofectamine 2000 vehicle control did not exhibit significant toxicity in the cancer cells. The failure of VR10-SNPs and VR10-SINPs to kill the breast cancer cells is a strong indication that the NPs are not escaping the endo/lysosomal pathway, and therefore an insufficient amount of siRNA is delivered to the cytosol. To test this hypothesis, time dependent colocalization of both VR10-SNPs and VR10-SINPs with lysosomes were evaluated in the breast cancer cells up to 6 h of incubation (Figure 4.22). There are strong indications that both VR10-SNPs and VR10-SINPs are trapped in lysosomes by 6 h as determined by the colocalization of both NPs with dextran-647, a lysosomal marker. Moreover, the absent diffuse fluorescence of the si3B in the cytoplasm is another indicator that the endosomal escape is a major problem with these formulations. The addition of histidine in the peptide sequence can provide the necessary buffering capacity required to burst the endosomes, as used in other siRNA delivery systems.[366, 424]
**Figure 4.21** Vitamin E-decaarginine (VR10) does not aid in the transfection of si3B nanoparticles (VR10-SNP) or si3B immunonanoparticles (VR10-SINPs) in MDA-MB-231/H2N breast cancer cells in vitro. MDA-MB-231/H2N were incubated with either control treatments or si3B (20 nM) containing media for 6 h, and viability measured 72 h later. si3B represents cells transfected with si3B and Lipofectamine 2000, Lipo Cntl represents cells treated with Lipofectamine 2000 alone, VR10-NP cntl represents NPs encapsulating VR10. (n=3, mean ± standard deviation, N.S. = p > 0.05, *** p< 0.001, determined by one way ANOVA and Bonferroni post-hoc test)

**Figure 4.22** Vitamin E-decaarginine (VR10) encapsulated in si3B NPs (VR10-SNPs) or si3B immunonanoparticles (VR10-SINP) are trapped in lysosomes as determined by colocalization.
with a lysosomal marker. MDA-MB-231/H2N breast cancer cells were prelabeled for lysosomes with Dextran-647 and incubated with NPs for the indicated times. Live cell imaging was obtained from confocal fluorescence microscopy. The nuclei are shown in blue, lysosomes are shown in red, the si3B is shown in green, and colocalization of siRNA and lysosomes are shown in yellow. In each treatment group, the concentration of si3B is 100 nM.

4.5.9 Alternative Endosomal Escape Peptides are Unsuccessful

The insertion of multiple histidines in endosomal escape peptides has been shown to be beneficial as the buffering capacity of the histidine side group falls in the range of the pH range for the endo/lysosomal pathway.[366] Therefore three other peptides were prepared by incorporation of 5-10 histidines as well as changing the hydrophobic anchor from vitamin E succinate to stearic acid (Str), typically used in liposomal formulations.[151, 425] The physicochemical properties of NPs encapsulating the various peptide sequences indicate similar diameters and neutral zeta potential similar to VR10-NPs. (Table 4.9) The various peptide encapsulated NPs were complexed with si3B and evaluated for anti-cancer activity against MDA-MB-231/H2N cells as previously described. Unfortunately, none of the peptide sequences resulted in a successful transfection of NP-mediated delivery of si3B, as determined by an absence of anti-cancer activity. It is possible that the length of the hydrophilic component of the NP backbone is too long (10 kDa) and shields the peptides in a way that inhibits the endosomal escape capabilities of the peptides. Another possibility is that the NP formulation is not suitable to siRNA delivery, which paves the way to exploring other established NP systems for siRNA delivery, such as liposomes.[426]

<table>
<thead>
<tr>
<th>Name</th>
<th>Size (d.nm) [PDI]</th>
<th>Zeta Potential (mV)</th>
<th>siRNA Transfection?</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>64 ± 5 [0.10 ± 0.07]</td>
<td>-22.9 ± 3.4</td>
<td>No</td>
</tr>
<tr>
<td>VR10-NP</td>
<td>40 ± 4 [0.11 ± 0.06]</td>
<td>+4.3 ± 1.2</td>
<td>No</td>
</tr>
<tr>
<td>VH5R10-NP</td>
<td>52 ± 9 [0.15 ± 0.06]</td>
<td>+3.8 ± 0.8</td>
<td>No</td>
</tr>
<tr>
<td>VH5R8-NP</td>
<td>59 ± 8 [0.14 ± 0.08]</td>
<td>+4.6 ± 1.5</td>
<td>No</td>
</tr>
<tr>
<td>Str-H10R5-NP</td>
<td>51 ± 6 [0.14 ± 0.09]</td>
<td>-3.9 ± 1.1</td>
<td>No</td>
</tr>
</tbody>
</table>

4.6 Conclusions

An optimized siRNA targeting eIF3B was chosen and validated for potent, and durable knockdown of eIF3B at the mRNA and protein levels. Moreover, the potency was maintained for up to 10 days in breast cancer cells. Fluoro modified si3B was found to be less potent relative to
unmodified si3B possibly due to inhibited dicer loading. An optimized fluoro modified si3B was evaluated for enhanced nuclease stability, and found to be more stable in serum when conjugated to NPs, relative to the unmodified si3B counterpart. si3B conjugated NPs (SNPs) and immunonanoparticles (SINPs) failed to deliver si3B into the cytoplasm as determined by the absence of anti-cancer activity. An endosomal escape peptide was modified with vitamin E succinate for encapsulation in the NPs, VR10-NPs. VR10-NPs and VR10-immunonanoparticles (VR10-INPs) demonstrated enhanced cell uptake relative to blank NPs, with diffuse cytoplasmic fluorescence. Additionally, the VR10 enabled better siRNA complexation/covalent attachment to the NPs. Unfortunately, VR10-NPs and NPs encapsulating other endosomal escape peptides did not successfully deliver siRNA into the cytoplasm, prompting the use of other established siRNA delivery vehicles, such as liposomes.
4.7 Supplemental Information

Figure S 4.1 $^1$H NMR (CDCl$_3$) of A) P(LA-co-TMCC-Bn) and B) P(LA-co-TMCC)-g-PEG-N$_3$.
(A) P(LA-co-TMCC-Bn): δ 8.25-7.23 (s, CH from pyrene), 7.33 (m, aromatic from TMCC-Bn), 5.15 (m, CH from LA), 4.32 (m, CH$_2$ from TMCC), 1.57 (m, CH$_3$ from LA) ppm. (B) P(LA-co-TMCC)-g-PEG-N$_3$: δ 5.17 (m, CH from LA), 4.30 (m, CH$_2$ from TMCC), 3.64 (bs, CH$_2$ from PEG) 1.57 (m, CH$_3$ from LA) ppm.
Figure S 4.2 Serum stability of Poly(ethyleneglycol) modified small interfering ribonucleic acids (siRNA) in 10% fetal bovine serum (FBS). Purified PEG-si3B was monitored for nuclease stability over the indicated incubation periods, and resolved in a polyacrylamide gel. Bound siRNA at a specific time point was determined relative to the amount of siRNA at time 0. (n=3, mean ± standard deviation)
5 Thesis Discussion

5.1 Elucidating the Intracellular Fate of Amphiphilic Cationic Peptides and Polymers

In chapter 2, we investigated the anti-cancer activity of H₈R₈ modified with various lipids. H₈R₈ is a commonly used cationic peptide in the NP and gene delivery fields, however other cationic peptides that are typically arginine rich have also been used.[330, 349, 423] While H₈R₈ was utilized as a model cationic peptide with endosomal escape capabilities, attributed to the octahistidine, the investigations in Chapter 2 highlighted important implications for delivery vehicles encapsulating similar amphiphilic cationic peptides. These implications include previously unknown synergistic effects where investigators did not take into consideration the intracellular fate and effects of the amphiphilic cationic peptide, and instead attribute the anti-cancer activity solely to the chemotherapeutic. For instance, NPs typically encapsulate stearyl modified Tat or octaarginine (R₈) peptides to introduce cationic charges on the surface of the NPs. The resultant NPs exhibit increased cell uptake with prevalent endosomal escape, and, in some cases, permit the intracellular targeting of the mitochondria.[427, 428] Of note, a NP formulation encapsulating stearyl-octaarginine (Str-R₈), termed MITO-Porter, was rationally designed for the intracellular targeting of the mitochondria.[419] MITO-Porter efficiently penetrates into cancer cells, where the cationic charge of Str-R₈ guided the intracellular targeting to the negatively charged mitochondria. MITO-Porter then fuses with the mitochondrial outer membrane to unload its encapsulated cargo into the mitochondria.[429] MITO-Porter has been used for the efficient delivery of Dox, deoxyribonucleic acids (DNA), siRNA, ROS scavengers and other macromolecules selectively to the mitochondria.[429-432] Specifically, MITO-Porter had been utilized at sub-lethal doses, as determined by a dose-response curve of the delivery vehicle alone, to deliver Dox selectively to the mitochondria.[433] The investigators did not consider the potential synergistic effects of Str-R₈ and doxorubicin, but rather emphasized the anti-cancer activity of Dox on the mitochondria alone. The work presented in Chapter 2 suggests that Str-R₈ would interact with the mitochondria and inhibit its functions, potentially working synergistically with the doxorubicin delivered into the mitochondria. In Chapter 3, the
synergistic effects of co-encapsulated pDox and VES-H₈R₈ reduced the concentration of VES-H₈R₈ required to kill 50% of the MDR breast cancer cells by 3.4-fold, relative to singly-loaded NPs. Likewise, the anti-cancer activity observed with MITO-Porter, co-encapsulating Str-R₈ and Dox, is likely due to synergistic effects against the mitochondria. This advances the nanoparticle field by considering the materials used in NP formulations, especially NPs encapsulating other amphiphilic cationic peptides, and pushes for the careful investigation of each amphiphilic cationic peptide in order to elucidate previously unknown synergistic effects.

While the work in Chapters 2 and 3 focused primarily on cationic amphiphilic peptides, cationic amphiphilic polymers may also exert a similar intracellular fate on the mitochondria. It is established that large linear or branched cationic polymers, such as polyethylenimine (PEI) or poly(L-lysine), affect the mitochondria through impairment of the mitochondrial membrane potential, respiration and membrane integrity.[149, 246, 434] In particular, high molecular weight linear PEI (750 kDa) and branched PEI (25 kDa) have been demonstrated to inhibit mitochondria bioenergetics and form pores in the mitochondria membrane, thereby inducing intrinsic apoptosis.[149, 435] Furthermore, high molecular weight poly(L-lysine) (27.4 kDa) has been demonstrated to rapidly induce intrinsic apoptosis and mitochondrial depolarization relative to low molecular weight poly(L-Lysine) (2.9 kDa).[436] These findings, coupled with the results in Chapters 2 and 3, extend to investigations of amphiphilic cationic polymers like polyethylenimine-graft-polycaprolactone-block-poly(ethylene glycol) (PEI-PCL-PEG).[437] Here, branched PEI (25 kDa) is covalently attached to a block poly(caprolactone), providing amphiphilicity similar to the peptides investigated in Chapter 2. The PEI-PCL-PEG was utilized at sub-lethal doses, as determined from the dose response curves of PEI-PCL-PEG, and potent anti-cancer siRNA was transfected successfully in cancer cells, ascribing the anti-cancer activity solely on the siRNA. In reality, the observed anti-cancer activity is likely a synergistic effect of mitochondria depolarization from the PEI-PCL polymer coupled with the effects of the anti-cancer siRNA. Another notable example is the triblock copolymer of poly(ethylene glycol)₂kDa-poly(lactide)₃kDa-poly(arginine)₁₅ (PEG-PLA-R₁₅).[363] PEG-PLA-R₁₅ was complexed with siRNA targeting epidermal growth factor receptor (EGFR) at sub-lethal concentrations of the delivery vehicle. Delivering siRNA targeting EGFR with the amphiphilic properties of the PEG-PLA-R₁₅ copolymer, may have exerted synergistic anti-cancer effects as investigators did not
consider potential effects on the mitochondria. The results in Chapters 2 and 3 advances the siRNA and gene delivery field by prompting investigators to characterize the intracellular fate and effects of amphiphilic cationic polymers for potential synergistic effects on mRNA and protein knockdown, and anti-cancer activity.

5.2 Significance of Nanoparticle-mediated Anti-Cancer Synergism Against Multi-Drug Resistant Cancers

In Chapter 3, the NP-mediated, synergistic anti-cancer activity of co-encapsulated VES-H₈R₈ and pDox was established against MDR breast cancer cells. The importance of this synergy has implications when sensitive cancer cells die first, permitting the drug-resistant cancer cells to proliferate and potentially metastasize. For instance, similar NPs used in this thesis were chemically modified with a docetaxel binding peptide in order to enhance docetaxel loading in the core of the NPs.[134] The docetaxel loaded NPs were investigated through a preclinical evaluation versus the conventional docetaxel formulation, and the NPs exhibited an increased therapeutic index, while permitting a maximum tolerated dose 1.6-fold higher relative to the conventional docetaxel formulation. While the results are exciting, the prevalence of MDR after administering the docetaxel loaded NPs was not fully investigated. For instance, Pgp has been found to be rapidly overexpressed in tumors after the administration of chemotherapy.[216] The NPs did not contain any component that would inhibit Pgp efflux and reverse MDR. Fortunately, the co-encapsulated VES-H₈R₈ and pDox provides a facile route to combat and reverse the expected increased prevalence of MDR in the docetaxel treated tumors. Importantly, VES-H₈R₈ would be the main active agent to inhibit Pgp efflux, either directly or indirectly as discussed in Chapter 2. The in vivo dosing schedule of docetaxel loaded NPs and the co-encapsulated VES-H₈R₈ and pDox would need to be investigated as both NPs can be administered simultaneously, or sequentially. Specifically, there is precedent for simultaneous administration of docetaxel and doxorubicin as this combination is one of the drug cocktails currently administered in the clinic.[111] Simultaneous administration of both NPs should provide benefits such as rapid cancer cell death, through pDox and docetaxel, and reduction in the prevalence of MDR, through VES-H₈R₈. However, simultaneous administration of both NPs may amplify off target toxicities in the heart and liver, where cardiotoxicity occurs or metabolizing enzymes are oversaturated,
respectively. If these issues arise, then sequential delivery of the NPs would be an alternative. Here, the docetaxel loaded NPs would affect the sensitive cancer cells, and as the tumor becomes more resistant to the docetaxel treatment, administration of the co-encapsulated VES-H₈R₈ and pDox NPs may significantly impact the tumor growth. MDR is an important ongoing issue in cancer treatment and the drug delivery field should place higher emphasis not only on tumor reduction, but also on the reversal of drug resistance. In vivo investigations may include tumors with mixed ratios of drug-sensitive and drug-resistant cancer cells as discussed in Chapter 6.4.

The co-encapsulated VES-H₈R₈ and pDox NPs have the potential to be beneficial in vivo in reducing off target toxicities such as cardiotoxicity. For instance, dexrazoxane is co-administered with Dox in order to reduce the undesired side effects of doxorubicin-induced cardiotoxicity and lower the incidence of heart failure.[438] The mechanism of cardioprotection by dexrazoxane includes inhibition of ROS production by binding to free or loosely bound iron, or iron-bound Dox.[438] Other cardioprotective agents have emerged, such as ubiquinone, vitamin E, and coenzyme Q10, with the common property of delivering anti-oxidants to cardiomyocytes to counteract Dox-induced ROS (Figure 5.1A).[439-441] The investigation of the probable cardioprotective properties of VES-H₈R₈ co-administered with Dox in vivo would be interesting, as VES is an established anti-oxidant, potentially permitting the administration of higher doses of Dox that may lead to better tumor response. Alternatively, the VES-H₈R₈ peptide can be further modified with an antioxidant containing amino acid, such as 2’,6’-dimethyltyrosine (Dmt), to further enhance ROS scavenging (Figure 5.1B).[442] Dmt has been incorporated in Schiller-Szeto tetrapeptides which were designed as mitochondrial targeted antioxidants and demonstrated to scavenge hydrogen peroxide radicals while inhibiting the oxidation of low-density lipoproteins and linoleic acid.[443] Rational instalment of the Dmt residues would have to be elucidated in terms of location, N- or C-termini, and number of Dmt residues for optimal antioxidant properties. Ideally, these newly modified cardioprotectants should exhibit minimal toxicity in cardiomyocytes and other non-cancer cells.

[143]
Figure 5.1 Established antioxidant agents that may be incorporated with the octahistidineoctaarginine(H$_8$R$_8$). A) Coenzyme Q10 that can replace vitamin E succinate on the N-terminus of H$_8$R$_8$. B) 2',6'-dimethyltyrosine (Dmt) that can be incorporated into the peptide sequence and known for its radical scavenging.

5.3 Towards Amphiphilic Peptide-Nanoconjugates as Drug Delivery Systems

Modifying chemotherapeutics with cationic peptides is a strategy commonly employed to increase cell uptake into cancer cells.[444, 445] The cationic charges help these conjugates interact with the negatively charged plasma membrane, and cell uptake can occur through direct translocation, inverted micellar or endocytic pathways.[446] In Chapter 2, VES-H$_8$R$_8$ was compared directly to VES to elucidate the mechanism of action of the observed anti-cancer activity. When dose matched, only VES-H$_8$R$_8$ depolarized the mitochondria, induced ROS, apoptosis, and G1 cell cycle arrest, while free VES was relatively benign. While the rate of degradation of the esters and amides of VES-H$_8$R$_8$ in the cytosol are unknown, it is clear that the cationic charge of the H$_8$R$_8$ peptide enhanced the intracellular uptake of VES. Since VES-H$_8$R$_8$ was synthesized completely with L-amino acids, intracellular degradation may be prevalent. Here, it is unclear if the fully intact VES-H$_8$R$_8$ rapidly interacts with the mitochondria, or if a degraded intermediate is the main active agent in the cytosol. For instance, two cationic Tat peptides were synthesized with all the amino acids harboring either the L- or D-stereochemistry.[171] Peptides synthesized with all D-amino acids are peptidase-stable, indicating that peptide degradation is drastically diminished. The all L-amino acid containing Tat peptide exhibited rapid intracellular degradation within 1 h in the cytosol, while the all D-amino acid counterpart was stable for up to 24 h. In Chapter 2, an all D-amino acid containing VES-H$_8$R$_8$ exhibited similar low-micromolar potencies to the L-amino acid counterpart, suggesting
that the interaction with the mitochondria may be faster than the intracellular degradation of the amides or esters within VES-H₈R₈. Eventually, VES-H₈R₈ would be completely degraded, releasing vitamin E that can then inhibit the Pgp efflux pumps, an established mechanism for both VES and Vitamin E.[191] The depolarization of the mitochondria and inhibition of Pgp efflux using VES-H₈R₈ was exploited in Chapter 3 when co-encapsulated with pDox in NPs to synergistically kill MDR breast cancer cells. The modification of VES with H₈R₈ presents a facile way to co-encapsulate with pDox in NPs, and may potentially satisfy one of the requirements of synergistic nanoformulations in vivo: maintaining synergistic ratios from injection site to tumor site. Although the maintenance of the synergistic ratio was not investigated in vivo, stability data presented in Chapter 3 indicate suitable in vitro stability for up to 96 h. The drug delivery strategy exploited in Chapters 2 and 3 advances the field by introducing a technique to modify drugs with peptides that easily assemble with NPs, and provides novel ways to inhibit intracellular organelles. Due to solid phase peptide synthesis, the VES moiety can be replaced with other potentially more potent Pgp inhibitors or more selective anti-cancer agents, as discussed in Chapter 6.1. This may lead to more potent effects on the mitochondria, due to the amphiphilic nature of the chemotherapeutic modified cationic peptide, and upon the degradation of this peptide, the released chemotherapeutic induces its own mechanism of action.

5.4 Conclusions

A co-encapsulated NP exhibited synergistic anti-cancer activity against MDR breast cancer cells. Although the siRNA work was successful to only a limited extent, the work in this thesis validated the hypothesis, “When compared to each drug alone, or single drug encapsulated NPs, NPs co-encapsulating peptide-modified vitamin E succinate, and a pH-sensitive prodrug of doxorubicin will be more efficacious against multi-drug resistant (MDR) breast cancer cells in vitro.” The peptide-modified vitamin E succinate was rationally chosen from a library of lipid modified cationic H₈R₈-based peptides based primarily on potent anti-cancer activity in the low micromolar range, as well as exhibiting superior cancer-selective potency. Interestingly, a lipid longer than or equal to 12 carbons was required for the potent anti-cancer activity of lipid modified H₈R₈-based peptides. The mechanism of action of VES-H₈R₈ encompasses depolarized
mitochondria in a mPTP-dependant pathway, inhibited bioenergetics, and induction of ROS, apoptosis, and G1 cell cycle arrest. Importantly, VES-H8R8 inhibited Pgp efflux, and thus reversed MDR in breast cancer cells, making it suitable for co-delivery with a chemotherapeutic.

In order to fully exploit the mechanism of action of VES-H8R8 against MDR resistance cells, VES-H8R8 was co-encapsulated with a pH-sensitive prodrug of Dox in NPs. The pH-sensitive prodrug of Dox was synthesized by modification of Dox with a palmitic acid hydrazide (pDox), forming a pH-sensitive hydrazone bond that is degraded in acidic media, such as the pH exposed in the endo/lysosomal pathway. The co-encapsulation of pDox and VES-H8R8 in NPs was successful, and importantly, exhibited suitable stability for up to 96 h. Not only did the co-encapsulated NPs exhibit additive anti-cancer effects on the parental cell line, it also resulted in a synergistic anti-cancer activity against the MDR resistant breast cancer cells. Importantly, the synergistic anti-cancer activity was only observed when pDox and VES-H8R8 were co-encapsulated, while the synergism was lost in the combination of free drugs, or combination of singly-loaded NPs. The synergistic anti-cancer activity of the co-encapsulated NPs required 3.3-fold less pDox, and 3.4-fold less VES-H8R8 to affect 50% of the breast cancer cells, further highlighting the importance of co-encapsulation. The mechanism of the synergistic anti-cancer activity of co-encapsulated NPs involved Pgp efflux inhibition with increased intracellular concentrations of pDox, mitochondrial depolarization, and induction of apoptosis and ROS. To evaluate the translation potential of the co-encapsulated NPs, an in vivo investigation would be required where harsher physiological barriers exist.

The NP-mediated delivery of siRNA against breast cancer cells was pursued. siRNA targeting an essential protein, eIF3B, was validated at the mRNA and protein levels with durable and potent knockdown. The siRNA, along with a targeting agent, were successfully covalently attached to the NPs and siRNA modified NPs exhibited satisfactory nuclease stability, suitable for in vitro and in vivo studies. Initially, the NP-mediated delivery of siRNA was unsuccessful, primarily due to poor cell uptake and endosomal escape, prompting the use of endosomal escape cationic peptides. This resulted in the encapsulation of various vitamin E and stearic acid modified endosomal escape cationic peptides, ultimately leading to enhanced cancer cell uptake. Unfortunately, the NP-mediated delivery of siRNA incorporating these modified endosomal escape peptides did not successfully deliver siRNA into the cytosol, and accumulated mostly in
the lysosomes. These results prompt the use of an established NP delivery vehicle for siRNA, such as cationic liposomes.

5.5 Achievement of Objectives

The majority of this research was motivated by the following hypothesis:

*When compared to each drug alone, or single drug encapsulated nanoparticles (NPs), NPs co-encapsulating peptide-modified vitamin E succinate, and a pH-sensitive prodrug of doxorubicin, will be more efficacious against multi-drug resistant (MDR) breast cancer cells in vitro.*

Herein, a co-encapsulation strategy was developed that is synergistically more efficacious against multi-drug resistant breast cancer cells in vitro. Achievement of the objectives stated in Chapter 1 are summarized below:

1) Investigate the mechanism of cell death induced by vitamin E succinate modified octahistidinooctaarginine (VES-H₈R₈) and similar lipid modified peptides (Chapter 2, currently accepted with revisions in the J. Controlled Release).
   • A library of lipid modified H₈R₈ was synthesized and tested for anti-cancer activity against parental and MDR breast cancer cells
   • Cationic peptides modified with a lipid longer than or equal to 12 carbons was required for potent, low micromolar anti-cancer activity
   • VES-H₈R₈ and stearyl modified H₈R₈ (Str-H₈R₈) were further investigated for the mechanism of action in MDR breast cancer cells
   • The anti-cancer activity of VES-H₈R₈ was similar to Str-H₈R₈ in both cancer cell lines, however VES-H₈R₈ exhibited double the cancer-selective activity relative to Str-H₈R₈
   • The mechanism of action of VES-H₈R₈ included Pgp efflux inhibition and MDR reversal, mitochondrial depolarization in a mPTP-dependent pathway with inhibited bioenergetics, and induction of ROS, apoptosis, and G1 cell cycle arrest
2) Investigate the synergism between vitamin E succinate modified H₈R₈ (VES-H₈R₈) and pH-sensitive prodrug of doxorubicin (pDox) on MDR breast cancer. (Chapter 3, manuscript submitted to Scientific Reports)

- A pH-sensitive prodrug of doxorubicin was synthesized and pDox encapsulated in NPs exhibited satisfactory pH-dependent within 48 h
- VES-H₈R₈ and pDox were successfully co-encapsulated in NPs while exhibiting sufficient stability for up to 96 h, suitable for in vitro studies
- Co-encapsulating VES-H₈R₈ and pDox in NPs exhibited additive anti-cancer effects in the parental breast cancer cell line, while synergistic anti-cancer effects were observed against the MDR breast cancer cell
- The synergistic anti-cancer effects of co-encapsulated NPs required 3.3-fold less pDox and 3.4-fold less VES-H₈R₈ to kill the MDR cancer cells, relative to singly-loaded NPs
- The synergistic anti-cancer effects were only observed in the co-encapsulated formulation, and not when singly-loaded NPs were co-administered
- The mechanism of action of the synergistic anti-cancer activity of co-encapsulated NPs included Pgp efflux inhibition and MDR reversal, increased intracellular pDox concentrations, mitochondrial depolarization, and induction of ROS and apoptosis

Part of this research was additionally motivated by the following hypothesis:

*Targeted NP delivery of eIF3B siRNA (si3B) will effectively knockdown eIF3B mRNA and protein, and induce cell death in human epidermal growth factor receptor 2 (HER2) overexpressing breast cancer cells in vitro.*

3) Design an effective delivery system for si3B (Chapter 4).

- siRNA targeting eIF3B were successfully validated for potent and durable anti-cancer activity, where the eIF3B knockdown was validated at the mRNA and protein level
- DBCO-siRNA and DBCO-Trastuzumab were successfully modified onto NPs which exhibited superior nuclease stability of the siRNA relative to free siRNA for up to 24 h

[148]
siRNA modified NPs failed to successfully transfected breast cancer cells in vitro, while the co-encapsulation of vitamin E succinate modified cationic peptides did not aid the siRNA transfection.

siRNA modified NPs encapsulating vitamin E succinate modified cationic peptides were observed to accumulate in the lysosomes.
6 Recommendations for Future Work

Future work stemming from this thesis includes four categories: (1) using established siRNA delivery vehicles; (2) tailoring amphiphilic peptides for enhanced potency and cancer-selective anti-cancer activity against MDR cancers; (3) rational design of nanoconjugates to enhance and maintain anti-cancer synergism; and (4) investigating in vivo prescreening and heterogeneous models of disease to better represent the current status of NPs in the clinic.

6.1 Tailoring Amphiphilic Peptides for Multi-Drug Resistance Inhibition, Enhancing Cancer-Selectivity and Novel Radical Scavengers

In Chapter 2, amphiphilic cationic peptides modified with a lipid that is greater than or equal to 12 carbons in length exhibited potent anti-cancer selectivity. This suggests that further modification of VES-H₈R₈ would require the final peptide to exhibit a similar hydrophobicity to exert potent anti-cancer activity. The versatility of solid phase peptide synthesis and the facile modifications on the N-terminus or side groups of the peptides presents an exciting route to replace VES or add other inhibitors or chemotherapeutics. Suitable candidates to replace VES may include more potent and selective Pgp inhibitors, such as LY335979.[447] Ideally, VES-H₈R₈ can be tailored either to enhance anti-cancer activity, Pgp efflux inhibition, and cancer-selectivity, or reduce in vivo toxicity. The amphiphilic peptides investigated in Chapter 2 exhibited potent anti-cancer activities in the low micromolar range, while, VES-H₈R₈ exhibited Pgp efflux inhibition and MDR reversal relative to Str-H₈R₈. The Pgp efflux inhibition can be further optimized through two pathways: preserving the VES modification and changing the peptide sequence, or preserving the H₈R₈ peptide and changing the N-terminus with another potentially more potent Pgp efflux inhibitor as discussed later.

Soluble mitochondria-penetrating peptides have been designed with alternating hydrophobic and cationic amino acids such as cyclohexylalanine and arginine, respectively.[56, 448] The cationic amino acids aid in intracellular targeting of the mitochondria, while the alternating hydrophobic and cationic amino acids makes it thermodynamically favorable for
penetration into the mitochondria matrix. Interestingly, the mitochondria-penetrating peptides required a similar hydrophobicity as reported in Chapter 2 for selective mitochondria targeting, prompting the use of the alternating hydrophobic and cationic amino acid scheme with VES-H₈R₈.[27] Specifically, the side group of histidine would be deprotonated under physiological pH, and thus is classified as a hydrophobic amino acid. For example, the peptide sequence may change from VES-H₈R₈ into VES-(HR)₈. The endosomal escape afforded from the histidine was also not fully investigated, therefore the histidine can be replaced with other hydrophobic amino acids, such as cyclohexylalanine, or phenylalanine.[56] A library of peptides with variable peptide lengths, hydrophobicity, and endosomal escape capabilities should be studied in order to fully understand the intracellular fate, anti-cancer activity, and Pgp efflux inhibition while, monitoring for the most optimal cancer-selective activity.

Alternatively, VES can be replaced with a more potent Pgp efflux inhibitor. Ideally the Pgp efflux inhibitor should be hydrophobic, in order to satisfy the hydrophobic threshold established in Chapter 2, and contain a functional group that permits the modification on the peptide, such as an alcohol, acid, alkene/alkyne, or hydrazide. Furthermore, it is unclear whether VES-H₈R₈ affects Pgp efflux directly through competitive inhibition or indirectly through ATP depletion from the mitochondria, suggesting that VES can be replaced with either an established Pgp efflux inhibitor or inhibitors of the mitochondria. For example, saponins such as dioscin have been demonstrated to potently inhibit Pgp efflux and, importantly, harbors six alcohols for modification as well as having the desired hydrophobicity to satisfy the requirements outlined in Chapter 2. Alternatively, curcumin and pentacyclic triterpenoic acids, such as betulinic and ursolic acids, have been shown to inhibit the mitochondria as well as Pgp efflux, and also satisfy the requirements outlined in Chapter 2 (Figure 6.1A). [22, 449, 450] Curcumin and most pentacyclic triterpenoic acids have also been established to exhibit potent and selective anti-cancer activity, and if modified with H₈R₈, have the potential of increasing the cancer-selectivity reported in Chapter 2 (Figure 6.1B). Other notable Pgp and mitochondrial inhibitors include procyanidin, adapalene, gossypol, resveratrol, lonidamine, all-trans-retinoic acid, and parthenolide (Figure 6.1C, D).[450-455] A library of inhibitor modified peptides can be prepared by modifying the N-terminus of the peptide with the suggested inhibitors. The library of inhibitor modified peptides would then be investigated where the ideal candidate would exhibit the most
potent anti-cancer activity and efflux inhibition, while exhibiting the lowest toxicity in non-cancer cells to elucidate cancer selectivity.

The mitochondrial theory of biological aging emphasizes that the accumulation of ROS-induced damage in mitochondria eventually leads to defects in mitochondrial DNA, mitochondrial dysfunction, and declining biogenesis.[456] Targeting of the amphiphilic cationic peptides to aging mitochondria may have some potential applications in reducing the onset of biological aging. Healthy and young mitochondria use natural detoxifying mechanisms, such as radical scavengers, to counteract the induced ROS from functioning mitochondria. Old and less effective mitochondria appear to have delayed detoxifying mechanisms, leading to increased oxidative damage with decreased oxidative capacity.[28] Established radical scavengers, such as coenzyme Q10 or curcumin, modified with cationic peptides may potentially act to scavenge ROS, thus leading to reduced ROS-induced damage.(Figure 5.1A, Figure 6.1A) This novel radical scavenging capacity may reduce the declining physical function and muscle fiber atrophy that comes with aging, that have been linked to ROS-induced damage and apoptosis.[457] A library of established radical scavengers modified with a cationic peptide can be explored that are non-toxic to muscle tissue, yet retain the radical scavenging capacity that may counteract biological aging.
Figure 6.1 Schematic representation for the replacement of vitamin E succinate in vitamin E succinate modified octahistidinoctarginine. A) Curcumin known for its efflux inhibition and radical scavenging. B) Ursolic acid known for its anti-cancer activity specifically in the mitochondria. C) gossypol and D) adapalene known for its efflux inhibition and mitochondrial activity.

6.2 Rational Design of Nanoconjugates for Synergistic Anti-Cancer Activity

The major benefit of synergistic co-encapsulated NPs is that the synergistic ratio of drugs loaded in the NPs can be maintained from injection site to tumor site.[239] However, some synergistic formulations can have different drug leakage rates out of the NPs, calling for a strategy to control and maintain these synergistic ratios.[458] Importantly, drugs leaking out of the NPs at differing rates can reverse the synergism and actually induce antagonistic anti-cancer effects. For example, the CPX-351 formulation exhibits synergistic anti-cancer activity at ratios of 10:1, 5:1, and 1:1 of cytarabine:daunorubicin for the treatment of leukaemia.[239] However if cytarabine leaks out of the NPs, and the resultant encapsulated ratio becomes 1:5 or 1:10 of cytarabine:daunorubicin, then antagonistic anti-cancer activity is exhibited, indicating that more drug is required to kill the cancer cells. Unfortunately, the co-encapsulated VES-H8R8 and pDox NPs in Chapter 3 were not investigated for potentially different drug leakages in in vivo settings.
However if differing drug leakage is prevalent, then solid phase peptide synthesis can provide a rational solution to modify the peptides for increased interaction within the hydrophobic core of the NPs. In Chapter 2, a lysine was inserted in VES-H₈R₈ and then modified with a fluorescein to elucidate potential Pgp efflux inhibition. Here, the fluorescein can be replaced with a chemotherapeutic, such as doxorubicin, with the same pH-sensitive linker used in Chapter 3, the hydrazine bond. The VES-H₈R₈:pDox ratio used in Chapter 3 to demonstrate synergistic anti-cancer activity was 1:1.4, however monitoring the range of synergistic ratios was not performed. If the synergistic drug ratios demand for more than one VES or Dox on the same peptide, a lysine tree can be used to add multiple VES or Dox, according to the range of synergistic ratios (Figure 6.2). A lysine tree would be modified onto resin-bound peptides through standard amide formation, and following base deprotection, the two free amines of lysine permit for the modification of two drugs, or further modification with more lysine. This strategy permits for a controlled ratio of VES:pDox, or other suitable drugs, that would be compatible with the synergistic ratios investigated on anti-cancer activity. Covalently attaching Dox to VES-H₈R₈ guarantees that both VES-H₈R₈ and Dox can be administered in vivo with a desired spatiotemporal distribution from injection site to tumor site. As outlined in the previous section, if the toxicity is amplified with this novel amphiphilic peptide, then multiple VES can be modified on the lysine tree to mitigate cardiotoxicities, typically observed with Dox. These novel amphiphilic peptides would have to be investigated for the potential Pgp efflux inhibition, cancer-selective anti-cancer activity, as well as tolerability in vivo, but are promising for the treatment against MDR breast cancers.
Figure 6.2 Schematic diagram of a rationally designed amphiphilic peptide for synergistic anti-cancer activity. Using the model cationic peptide, octahistidineoctaarginine (H$_8$R$_8$), chemotherapeutics and efflux inhibitors can be modified onto H8R8 through lysine or a lysine tree. The above schematic comprises of an amino acid sequence KH8KR8, where K represents the single letter code for lysine. Depending on the synergistic ratios determined for anti-cancer activity, the ratio of chemotherapeutic to efflux inhibitor can be rationally designed. The red portion of the peptide demonstrates how 4 agents can be modified on a lysine tree at the N-terminus of the peptide, while the blue portion of peptide is demonstrating how 2 agents can be modified in the center of the peptide. These two strategies can be switched, depending on the synergistic ratios observed. Octaarginine is shown in green, while octahistidine is shown in orange.

6.3 Established siRNA Delivery Vehicles

The unsuccessful delivery of si3B by the NPs used in this thesis prompts the use of alternative NPs that have demonstrated successful and efficient siRNA delivery, such as liposomes.[459] While liposomes suffer from in vivo complications, such as accelerated blood clearance, liposomes are the most widely used NP systems currently used in the clinic due to stability, extended circulation in the blood, and facile preparation and scale up.[460-462] Liposomes tailored for siRNA delivery typically include fusogenic lipids to fuse with the endo/lysosomal membranes and expel its contents into the cytosol as well as cationic lipids to interact with siRNA and cellular membranes.[463] A suggested liposomal formulation would comprise of a mixture of cationic lipids, such as dioleoyl phosphatidylethanolamine (DOPE), along with a pegylated lipid, such as N-(carboxylmethoxypolyethyleneglycol-2000)-1,2-
distearoyl-sn-glycero-3-phosphoethanol-amine (DSPE-PEG).[426] The pegylated lipid, DSPE-PEG, has been demonstrated to enhance NP circulation in the blood, relative to non-pegylated NPs. To fully exploit the azide-alkyne reaction optimized in Chapter 4, a commercially available azide modified lipid, such as 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[azido(polyethylene glycol)-2000 (DSPE-PEG-N$_3$), can be easily included in the NP assembly. It’s expected that the amphiphilic, cationic peptides developed in Chapter 2 and co-encapsulated with pDox in the NPs in Chapter 3 should assemble into these liposomal NPs, as similarly structured peptides are currently utilized. The use of liposomal NPs has the potential to fully explore triply-loaded NPs to synergistically kill MDR breast cancers with VES-H$_8$R$_8$, pDox, and si3B.

6.4 In Vivo models of Multi-Drug Resistance and Cancer Stem Cells

MDR significantly impedes the therapeutic success of chemotherapeutic loaded NPs, yet investigations of resistant tumors in animal models are limited. For instance, the majority of highly cited NP containing publications use drug-sensitive, human cancer cells implanted into mice in order to emphasize tumor reduction along with reduced off-target toxicity.[117, 216, 464, 465] The tumors that have been treated with chemotherapeutic loaded NPs are rarely investigated for tumor relapse and subsequent NP treatments, missing an opportunity to explore NP-induced MDR. For instance, increased levels of the Pgp mRNA have been demonstrated to be induced rapidly following the exposure of tumors to chemotherapy, while other studies have linked the therapeutic failure of chemotherapeutics to the overexpression of efflux pumps.[216, 466, 467] While docetaxel loaded in similar NPs used in this thesis have been demonstrated to reduce the breast tumor volume, this treatment may have primed the tumor to become more resistant to subsequent treatments, potentially increasing the rate of tumor metastasis.[134] The co-encapsulated NPs developed in Chapter 3 would provide the necessary efflux inhibition and potential synergistic reduction in tumor volumes following this docetaxel treatment. Investigating the efflux inhibition in vivo focusses on two factors: heterogeneity within the tumor, and dosing schedules. A heterogenous population of drug-sensitive and drug-resistant cancer cells in the tumor would better recapitulate the heterogeneity found within human
tumors. While the proportion of drug resistant cells in a tumor pre- and post-chemotherapy is currently debated, implanting pre-determined ratios of drug sensitive to drug resistant cancer cells would explore the implications of efflux inhibition and synergy in heterogeneous, MDR tumors. Specifically, while conventional in vivo studies use homogeneous tumors, future studies can focus on implanting fixed ratios of drug-sensitive to drug-resistant cancer cells (3:1, 1:1, 1:3, respectively). Dosing schedules would expand these investigations, where simultaneous, or sequential delivery can be explored. The dosing schedule would highlight the importance of synergy and inhibiting efflux either during or after the administration of chemotherapeutic loaded NPs.

Cancer stem cells are emerging as significant targets in the treatment of cancer as they are slower growing and express efflux pumps. Therefore, a cancer stem cell model was established as cancer stem cells are less susceptible to chemotherapeutics and are thought to fuel and propagate tumor growth. This model defines cancer stem cells as a small subset of cancer cells in a tumor with an exclusive ability to self-renew, and are regarded as the main culprits for the heterogenous lineages of cancer cells in a tumor. Moreover, a side population of cancer stem cells, constituting around 2.1-8.7% of the overall cancer stem cell population, has been documented to overexpress efflux pumps. They are labelled as a side population because they were visualized by flow cytometry as a negatively stained population off to the side of the main population, attributed to the high efflux capability. Breast cancer stem cells may be isolated from human breast cancer tumors, identified by cell surface markers of CD44+/CD24−/low with high aldehyde dehydrogenase (ALDH+) phenotypes. In addition to investigating heterogenous tumors suggested in the previous section, incorporation of breast cancer stem cells would provide helpful insights on the impact of cancer stem cell propagation. For example, as few as 200-1000 breast cancer stem cells gave rise to tumors when xenotransplanted into NOD/SCID mice, whereas 50,000 unsorted cells were required for the same effect. It is likely that VES-H₈R₈ would affect the mitochondria of these stem cells, and importantly, inhibit the efflux capability while increasing intracellular concentration of the chemotherapeutic. To this end, investigating the potential synergy and efflux inhibition in tumors harboring drug-resistant cancer cells as well as cancer stem cells should better recapitulate human tumors in the clinical setting.
Appendix A: Abbreviations

$\Delta \Psi_m$ – Mitochondrial membrane potential
17-AAG - 17-(AllylAmino)Geldanamycin
ABC - Adenosine triphosphate-Binding Cassette
ADP - Adenosine DiPhosphate
AGO-2 – Argonateau
Akt – Protein Kinase B
AML - Acute Myeloid Leukemia
Anti-HER2 – Anti-body against Human Epidermal Growth factor Receptor 2
APO B - Apolipoprotein B-100
ASK-1 - apoptosis signal-regulating kinase 1
ATP - Adenosine TriPhosphate
CINPs – Cyanine 5 modified immunonanoparticles
CNPs - Cyanine 5 modified nanoparticles
CMC – Critical Micelle Concentration
CuAAC - copper catalyzed azide-alkyne cycloaddition
DNA – DeoxyNucleic Acid
Dox – Doxorubicin
Dmt - 2’,6’-dimethyltyrosine
EGFR - Epidermal Growth Factor Receptor
eIF3 - eukaryotic transcription initiation factor 3
eIF3B – eukaryotic Translation Initiation Factor 3, subunit B
EPR – Enhanced Permeability and Retention
ETC – Electron Transport Chain
ERK 1/2 – Extracellular- signal-Regulated Kinase units 1 and 2
FBS – Fetal Bovine Serum
FDA – Food and Drug Administration
GAPDH - GlycerAldehyde 3-Phosphate DeHydrogenase
HER2 – Human Epidermal growth factor Receptor 2
HER4 - Human Epidermal growth factor Receptor 4
IC$_{50}$ - inhibitory concentration to reduce relative viability to 50%
IMM – Inner Mitochondrial Membrane
MDR – Multi-Drug Resistance
mPTP – Mitochondrial Permeability Transition Pore
mRNA – messenger RiboNucleic Acid
OXPHOS – OXidative Phosphorylation
P(LA-co-TMCC) - poly(D,L-lactide-co-2-methyl-2-carboxytrimethylene carbonate)
P(LA-co-TMCC)-g-PEG-N$_3$ - poly(D,L-lactide-co-2-methyl-2-carboxytrimethylene carbonate)-graft-poly(ethylene glycol)-azide
pDox – Palmitic Doxorubicin
PEG - poly(ethylene glycol)
PPEG-PLA-R$_{15}$ - poly(ethylene glycol)$_2$ kDa-poly(lactide)$_3$ kDa-poly(arginine)$_{15}$
PEI - Polyethylenimine
PEI-PCL-PEG - Polyethylenimine-graft-polycaprolactone-block-poly(ethylene glycol)
**Pgp** – Permeation Glycoprotein

**Pi** - Phosphate

**PLK1** – Polo-Like Kinase 1

**qPCR** - quantitative polymerase chain reaction

**RES** - Reticulo-Endothelial System

**RISC** - RNA-Induced Silencing Complex

**RNAi** – RiboNucleic Acid Interference

**ROS** – Reactive Oxygen Species

**RRM2** - Ribonucleotide Reductase Regulatory Subunit M2

**si3B** – siRNA targeting eIF3B

**siRNA** – small interfering RiboNucleic Acid

**SPAAC** - strain promoted azide-alkyne cycloaddition

**Str-H₈R₈** – Stearyl modified octaarginineoctahistidine

**Str-R₈** – Stearyl modified octaarginine

**TCA** – tricarboxylic acid

**VEGF** - vascular endothelial growth factor

**VES-H₈Rs** – Vitamin E succinate modified octaarginineoctahistidine

**VR10** – Vitamin E succinate modified decaarginine

**VR10-CNPs** – Cyanine 5 modified, VR10 encapsulated nanoparticles

**VR10-CINPs** - Cyanine 5 modified, VR10 encapsulated immunonanoparticles

**VR10-NPs** - VR10 encapsulated nanoparticles
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