Bio-Inspired and Nanotechnology-Enabled Drug Delivery for Diabetes and Brain Cancer

by

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Abstract

Nanotechnology-enabled drug delivery systems, inspired by biological processes, were developed to overcome current treatment challenges facing diabetes and cancer patients. Two different systems were developed. The first system, an implantable closed-loop insulin delivery device, utilizes pH-responsive nanoparticles embedded within a glucose-sensing matrix to mimic the physiological delivery of insulin. To prolong implant lifetime we developed a formulation of highly concentrated insulin, up to 80 mg/mL, which maintained excellent insulin stability for up to 30 days under physiological conditions. Moreover, a novel device structure was designed comprising of a microporous membrane that hindered leukocyte migration to the functional implant surface and minimized host inflammatory response. Combining the thermostable insulin formulation and the new device design, long-term closed-loop glycemic control was achieved in Type 1 diabetic rats. The second system is a nanoparticle platform capable of delivering imaging agents and drugs across the blood-brain barrier by mimicking low-density lipoprotein uptake in the brain. We developed this system based on a polysorbate 80-containing terpolymer for improving chemotherapy of metastatic breast cancer disease in the central nervous system. Two nanoparticle formulations were developed: doxorubicin-loaded terpolymer nanoparticles and docetaxel-loaded terpolymer-lipid nanoparticles. Both nanoparticle formulations exhibited high
drug loading efficiency, colloidal stability and low systemic toxicity. Parenteral administration of the nanoparticle formulations enabled delivery of blood-brain barrier-impermeable compounds to the brain in healthy mice. Systemic administration of the nanoparticles loaded with clinically relevant doses of drug in brain-tumor bearing mice resulted in drug delivery across the blood-brain barrier to brain metastases of breast cancer and significantly enhanced therapeutic efficacy while minimizing side effects compared to free drug. The nanotechnologies and drug formulations developed in this work present significant progress towards improving therapies for the clinical management of Type 1 diabetes and brain metastases of breast cancer.
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Figure 42. Stability of DTX-NP at 37 ºC in pH 7.4 PBS and FBS. a) Nanoparticle size and b) nanoparticle zeta potential over a 72 hour period.

Figure 43. Uptake of fluorescence dye-loaded DTX-NP by triple negative human breast cancer cell MDA-MB-231-luc. a) Fluorescence microscopy image at 2 hours following treatment. Bright field, cell nuclei stained with Hoechst 33342 (blue), DTX-NP stained with Nile Red (red) and overlays are shown. b) Kinetics of dye-loaded DTX-NP uptake in MDA-MB-231-luc over a 2 hour period (I₀: the fluorescence intensity of cells incubated with medium; I: the fluorescence intensity of cells incubated with NR-DTX-NP). c) Cytotoxicity of Blank NP, Free DTX and DTX NP against MDA-MB-231 cells after 24 hours of incubation. From the data fitting corresponding IC₅₀ values were obtained (DTX-NP: IC₅₀= 80.7±1.2 µg/L; Free DTX: IC₅₀= 93.9 ± 3.5 µg/L). Data presented as mean ± SD (n=3).

Figure 44. Laser scanning confocal microscopic images of healthy SCID mice brain sections following treatment with free Hoechst 33342, Hoechst 33342 loaded within FTIC labeled amphiphilic polymer based NP (without PS 80), or Hoechst 33342 loaded within FITC labeled amphiphilic polymer based NP (with PS 80). Mice were treated for 2 hours. Texas Red-dextran was administered to the mice i.v. 15 minutes before euthanasia. Hoechst 33342 and Hoechst 33342-labeled cell nuclei appear blue. Texas Red-dextran appears as red. Arrows: indicating
representative nuclei and DTX-NP located away from blood vessels. Scale bar = 40 µm for all images. ................................................................. 156

Figure 45. a-b) Whole body imaging of live mice with brain tumor: a) fluorescence images showing tumor accumulation of HF 750- and FA-labeled DTX-NPs, b) bioluminescence image of luciferase expressing-tumor cells. c) Ex vivo bioluminescence image of tumor cells (iii) and fluorescence image of NP (i: HF 750 filter; ii: FITC filter) in brain. d) Representative fluorescence images of NP in different organs. e) Confocal microscopic images of brain sections taken from mice bearing brain metastases 2 hour after i.v. injection of saline (top panel) or FA-labeled DTX NP (green, bottom panel). Texas red-dextran (red) was administered to the mice i.v. 15 minutes before euthanasia. Hoechst 33342-stained cell nuclei shown in blue (T: brain tumor area; N: brain area without tumor). Arrows point to representative FA-labeled DTX-NP away from blood vessels and accumulated into tumor areas. Scale bar = 50 µm for all images. .......... 158

Figure 46. Quantitative results for different tissue distributions for NIR-DTX-NP. Fluorescence intensity in the major organs as a function of time after intravenous injection of NIR-DTX-NP. Data normalized to respective organs of saline-treated mice. Data are presented as mean ± SD (n=3). ................................................................................................................................. 159

Figure 47. Pharmacokinetics of DTX-NPs and Taxotere® in brain and whole blood. a) The concentration of DTX in healthy brains and tumor-bearing brains at 15 min after i.v. injection. b) Illustration of a semi-PBPK model for DTX disposition. All parameters are defined in the main text. Concentration profiles of DTX in brain (c) and in blood (d) after i.v. injection of DTX-NP or Taxotere® (symbols are measured values, lines are fitted data). DTX concentration in the blood of Taxotere® treated mice at 24 hours was below the detection limit. Therefore a value of zero was used for PK modeling (Red Arrow). All measured data in a, c, d are the mean ± SD (n=3). ................................................................................................................................. 162

Figure 48. Particle size distribution of micelles in Taxotere® in DDIW determined by dynamic light scattering (DLS). The number average particle size: Diameter = 7.8 ± 0.3 nm; Polydispersity Index = 0.18 ± 0.11. Data presented as mean ± SD (n=3). ......................................................... 163

Figure 49. a) Comparison of DTX concentration (determined by LC-MS/MS) and ex vivo NP fluorescence intensity versus time after a single i.v. injection of HF750-labeled DTX-NPs (20
mg/kg DTX) in the brain (b) and whole blood (c) of tumor-bearing mice. d) Fitted pharmacokinetic parameters of HF750-labeled DTX-NP and DTX-NP in whole blood and brain. All data represent the mean ± SD (n=3).

Figure 50. Inhibition of brain tumor growth and animal survival. a) Treatment and imaging schedule for tumor-bearing mice injected with saline (n=6), blank NP (n=7), Taxotere® (20 mg/kg DTX, n=8), or DTX-NP (20 mg/kg DTX, n=9). b) In vivo images of brain tumor bioluminescence over a 28 day period. c) Fold increase in the total tumor radiance. d) Body weight and e) Kaplan–Meier survival curve of tumor-bearing mice following treatment. f) Effect of treatment on median survival time and tumor growth delay of tumor bearing mice. All data presented as mean ± SD.

Figure 51. H&E stained sections of lungs, liver, kidneys and hearts of tumor-bearing mice treated with saline, Blank-NP, DTX-NP at a dose of 2×20 mg/kg, or an equivalent dose of Taxotere®. Organs were collected at survival end point. All images were scanned at 20x magnification.

Figure 52. Blood glucose concentration in normal and STZ-diabetic rats with implanted glucose-responsive insulin delivery devices (60 µm membrane device filled with 50 mg/mL insulin) after glucose challenge (i.p. administration of 1g/kg dextrose) at t=0. Error bars represent standard deviation (n=3). Measurements were taken 5 and 10 days following device implantation.

Figure 53. Cellular uptake of fluoresceinamine-labeled ter-polymer NP loaded with dox at 4 h into MDA-MB-231-luc cells. Top row: cells incubated with nanoparticles. Bottom row: cells incubated with nanoparticles and RAP. Scale bar represents 100 µm.
<table>
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<td>hydrogen nuclear magnetic resonance spectroscopy</td>
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<td>ABC transporter</td>
<td>ATP-binding cassette transporter</td>
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<td>Apo-E</td>
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<td>(3-Aminopropyl)triethoxysilane</td>
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<td>blood-brain barrier</td>
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<td>BCRP</td>
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<td>BMBC</td>
<td>brain metastases of breast cancer</td>
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FA  fluoresceinamine isomer I
FBR  foreign body response
FBS  fetal bovine serum
FDA  United States Food and Drug Administration
FITC  fluorescein isothiocyanate
FTIR  Fourier transform infrared spectroscopy
FOV  field of view
Gd  gadolinium
GDM  gestational diabetes mellitus
GDP  guanosine-5’-triphosphate
GFP  green fluorescent protein
GIP  gastric inhibitory polypeptide
Gln  glutamine
GLP  glucagon-like peptide
GLUT  glucose transporter
Gly  glycine
GOX  glucose oxidase
H&E  hematoxylin and eosin
HbA1c  glycated hemoglobin
HBEGF  heparin binding EGF like growth factor
<table>
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<td>HEPES</td>
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<tr>
<td>k&lt;sub&gt;2,1&lt;/sub&gt;</td>
<td>rate constant from compartment 2 to compartment 1</td>
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<td>KPS</td>
<td>potassium persulfate</td>
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<td>LC</td>
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<td>LC-MS</td>
<td>liquid chromatography-mass spectrometry</td>
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<td>LDL</td>
<td>low density lipoprotein</td>
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<td>LDLR</td>
<td>low density lipoprotein receptor</td>
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<tr>
<td>LRP1</td>
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<td>MAA</td>
<td>methacrylic acid</td>
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<tr>
<td>MBA</td>
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<tr>
<td>MDR</td>
<td>multidrug resistance</td>
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<td>MnO₂</td>
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<tr>
<td>mPEG</td>
<td>poly(ethylene glycol) methyl ether</td>
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<td>MPS</td>
<td>mononuclear phagocytic system</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
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<td>MRP1</td>
<td>multidrug resistance protein 1</td>
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<td>NIPAM</td>
<td>N-isopropylacrylamide</td>
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<td>NIR</td>
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<td>NO</td>
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<td>NP</td>
<td>nanoparticle</td>
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<tr>
<td>NPC</td>
<td>4-nitrophenyl chloroformate</td>
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<td>NPH</td>
<td>neutral protamine hagedorn</td>
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<tr>
<td>NR</td>
<td>nile red fluorescent dye</td>
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<td>OD</td>
<td>outside diameter</td>
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<td>PBA</td>
<td>phenylboronic acid</td>
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<tr>
<td>PBPK</td>
<td>physiologically based pharmacokinetic modelling</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PCL</td>
<td>poly$(\varepsilon$-caprolactone)</td>
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<td>poly-L-lysine</td>
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<td>polymer lipid nanoparticle</td>
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<td>PMAA</td>
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<tr>
<td>PMAA-PS 80-g-St</td>
<td>polymethacrylic acid, polysorbate 80 grafted starch</td>
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<td>PNIPAM-MAA</td>
<td>poly(N-isopropylacrylamide-co-methacrylic acid)</td>
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<td>progesterone receptor</td>
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<tr>
<td>Pro</td>
<td>proline</td>
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<td>polysorbate 80</td>
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<td>receptor associated protein</td>
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<td>relative centrifugal force</td>
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<td>reactive oxygen species</td>
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<td>s.c.</td>
<td>subcutaneous</td>
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<td>SCID</td>
<td>severe combined immunodeficiency</td>
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<td>SD</td>
<td>standard deviation</td>
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<td>sodium dodecyl sulphate</td>
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<td>Description</td>
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<tr>
<td>S.E.M.</td>
<td>standard error of the mean</td>
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<tr>
<td>SEM</td>
<td>scanning electron microscope</td>
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<td>solid lipid nanoparticle</td>
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<td>self-monitoring of blood glucose</td>
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<td>t_{1/2}</td>
<td>half life</td>
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<td>Time at maximum concentration</td>
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<td>TOF-SIMS</td>
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<td>TUNEL</td>
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</tr>
<tr>
<td>USP</td>
<td>United States Pharmacopeia</td>
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UV  ultraviolet
UV-Vis  ultraviolet-visible spectrophotometry
V  volume
v/v  volume by volume
VEGF  vascular endothelial growth factor
VFA  variable-flip-angle
w/v  weight by volume
w/w  weight by weight
WBRT  whole brain radiation therapy
XPS  X-ray photoelectron spectroscopy
1.1 Introduction

Drug delivery systems have found increasing applications in the clinic to deliver therapeutic agents to the right place at the right time and at the right levels, with the ultimate goal of improving therapeutic outcomes. The past two decades has witnessed the explosive growth of nanotechnologies, engineered structures with unique properties and capabilities derived from their nano- to submicron scale (a few tens to a few hundred nanometers) dimensions, in almost every aspect of our lives. The application of nanotechnology for drug delivery provides us with new materials capable of directly interacting with the physiological environment in ways that were not previously possible; and promises exciting possibilities in the way we treat diseases. In this thesis we develop new drug delivery systems inspired by normal biological processes and realized through the use of nanotechnology to improve therapy in two disease areas: Type 1 diabetes (T1D) and brain metastases of breast cancer.

1.1.1 Rational: Nanotechnology-Enabled Implantable Closed-Loop Insulin Delivery System for the Treatment of Type 1 Diabetes

Diabetes mellitus is a group of metabolic disorders characterized by elevated blood glucose concentration which affects an estimated 350 million people worldwide\(^1\). Chronic hyperglycemia is associated with a number of microvascular and macrovascular complications in diabetes patients\(^2\). Insulin therapy, the administration of exogenous insulin, is required to maintain normal glycemic levels in all T1D patients and about 20\% of Type 2 diabetes patients\(^3\). Optimal insulin therapy requires frequent daily measurements of blood glucose concentration and administration of the correct insulin dose to lower and control blood glucose levels. Correct insulin dosing is patient specific and dependent on day-to-day activities including carbohydrate intake and level of physical activity. Currently the burden of predicting daily insulin requirement falls to the patient\(^3\), which may result in deadly consequences in the case of improper dosing. Furthermore, insulin therapy requires strict patient compliance despite frequent and painful finger pricks for blood glucose measurement and needle injections for insulin administration. Therefore efforts have been made in the past decades to develop glucose-responsive insulin delivery systems with
the goal of recapitulating the homeostatic capability of the human pancreas, known as an
“artificial pancreas”4-6.

Electro-mechanical artificial pancreas systems are commercially available and are comprised of a
digital continuous glucose sensor, an electro-mechanical drug pump and a controlling algorithm7. These systems have been shown to lessen the burden of predicting insulin dose, improve patient
compliance, quality of life, and improve control of blood glucose levels8,9. Despite these advances, artificial pancreas systems are currently limited by the performance of their components, namely the continuous glucose monitor (CGM) and the continuous subcutaneous insulin infusion (CSII) pump. CGMs currently require frequent recalibration and have a limited lifetime (<7 days) of efficacy, while insulin infusion pumps must be monitored for catheter occlusion and replaced after a few days of operation. These systems must be worn externally with constant use of subcutaneous catheters which severely limits patient quality of life, and have high equipment costs10. A second type of artificial pancreas system are so called chemically-driven artificial pancreases, which rely on glucose-specific chemical or enzymatic reactions to drive glucose-responsive insulin delivery5. Several variants of this system have been proposed based on the glucose-reacting moiety including glucose oxidase (GOX)11,12, concanavalin A (ConA)13,14, and phenylboronic acid (PBA)15,16. Of these variants, GOX-based systems are the most promising due to the enzyme’s specific interaction with glucose and its ability to operate in physiological conditions.

Inspired by the physiological regulation of blood glucose levels, our lab has previously
developed a GOX-based bioinorganic glucose-responsive hydrogel membrane capable of self-
regulated insulin delivery17. This membrane is composed of a cross-linked albumin matrix
embedded with pH-responsive poly(N-isopropyl acrylamide-co-methacrylic acid) nanoparticles (p(NIPAM-MAA) NPs), GOX, catalase, and MnO₂ nanoparticles. In hyperglycemic conditions, GOX reacts with glucose to produce gluconic acid, lowering the pH of the internal membrane microenvironment. As a result, the pH-sensitive p(NIPAM-MAA) NPs shrink to increase insulin permeability through the membrane and into the body18,19. The novel use of MnO₂ nanoparticles within our system, in combination with catalase, served to remove hydrogen peroxide byproduct and regenerate oxygen within the membrane microenvironment, a necessary reactant to propagate the chemical reaction. Implantation of this material in the intraperitoneal cavity of diabetic rats successfully reproduced the normal physiological response to hyperglycemia and
further maintained normal blood glucose levels for up to 4 days. Premature device failure is attributed to depletion of the insulin reservoir and degradation of the glucose-responsive membrane that regulates insulin release.

Building upon previous results, in this thesis we propose to improve device performance and extend device lifetime to achieve long-term maintenance of normal glycemia in type 1 diabetic rats. To combat insulin depletion and loss of insulin bioactivity within the device reservoir, we proposed a new poloxamer gel formulation of highly concentrated insulin that maintains excellent insulin stability for up to 30 days under physiological conditions. Current insulin formulations have low concentration and poor stability under physiological conditions owing to physical denaturation and chemical degradation. Therefore these formulations are unsuited for long-term use in insulin implants. To minimize the host inflammatory response to the implant and delay in vivo degradation of the glucose-responsive membrane, we propose to impede leukocyte migration to the functional implant surface in a passive manner using a microporous membrane barrier. With these improvements, we aim to prolong the functional lifetime of the chemically-driven closed-loop insulin delivery system to 30 days following implantation in diabetic rats.

1.1.2 Rational: Nanoparticle-Mediated Drug Delivery to Brain Metastases of Breast Cancer

Brain metastases are one of the most difficult malignancies to treat and have poor patient prognosis. The mean 1-year survival of patients with brain metastases from breast cancer is estimated at 20% while fewer than 2% of patients survive for more than 2 years. Brain metastases are estimated to occur in 20-40% of all breast cancer patients, with the vast majority (78%) presenting with multiple lesions. While surgical resection may be possible for primary brain tumors and brain metastases with up to three lesions, whole brain radiotherapy (WBRT) is preferred for patients with multiple brain metastases due to a high risk of surgical complications including intracranial infection or brain edema. Given the significant neurotoxicity associated with WBRT and short median survival (4 to 5 months), alternative noninvasive methods including chemotherapy are desired for the treatment of brain tumors and brain metastases.

Unfortunately, malignancies in the central nervous system (CNS) are notably resistant to systemic chemotherapy as many chemotherapeutic agents are incapable of efficiently crossing
the blood-brain barrier (BBB). This barrier at the capillary-CNS interface is comprised of tight junction-expressing endothelial cells which interact with pericytes and astrocytes to regulate the entry of nutrients and others substances into the brain. The action of drug efflux pumps such as P-glycoprotein (P-gp) or breast cancer resistance protein (BCRP) at the BBB further reduce accumulation of therapeutic agents to the brain. The vast majority of CNS drugs currently on the market must be administered at very high doses resulting in severe side effects in the peripheral organs.

Various approaches have been investigated to enhance drug delivery to the brain including both invasive and non-invasive means. Invasive approaches, including intrathecal cerebrospinal fluid delivery, intraventricular administration, and intratumoral injection are expensive, associated with a high degree of patient discomfort, and have a high risk of complication. Other approaches aim to transiently disrupt the BBB (e.g. administration of hyperosmotic solutions or vasoactive compounds) to enable entry of circulating drugs into the brain, however these methods are non-specific and allows other compounds to enter the brain resulting in neurological toxicity, aphasia and hemiparesis. Non-invasive CNS drug delivery approaches using retro-metabolic pro-drugs or specific drug-antibody conjugates targeting endocytic receptors on the BBB have been developed to improve treatment outcomes; however these approaches are susceptible to loss of drug activity after modification and require complicated and expensive preparation procedures. To mitigate this problem, nanoparticle drug carriers with a variety of targeting moieties have been investigated.

Surface modified nanoparticle carrier systems for non-invasive CNS drug delivery offer several advantages over competing technologies including high drug loading capacity, improved pharmacokinetics, the ability to protect cargo from metabolism, and the ability to provide controlled drug delivery. Various targeting moieties have been studied to enhance nanoparticle transport across the BBB via receptor-mediated pathways, including those targeting the insulin receptor, transferrin receptor, low density lipoprotein (LDL) receptor, or glutathione receptor. Certain surfactants, including polysorbate 80 (PS 80) have also been employed to coat nanoparticles for brain targeted drug delivery. Given its low cost and approved use in many injectable pharmaceutical products, PS 80 offers tremendous potential as compared to antibodies.
Recently cell-penetrating peptide-linked dendrigraft poly-L-lysine nanoparticles and arginylglycylaspartic acid (RGD)-conjugated polymer micelles have been developed and shown to exhibit antitumor efficacy in U87MG human glioblastoma xenografts. Other groups have also demonstrated the use of transferrin receptors and LDL receptors to shuttle chemotherapy drugs to glioblastomas. To our knowledge, the studies of nanoparticles for drug delivery across the BBB have been limited so far to primary brain tumors such as glioblastomas. Given the vast anatomical differences between brain metastases and primary glioblastomas, delivering drugs to multiple brain metastasis lesions remains very challenging.

To improve chemotherapy treatment of metastatic breast cancer disease, in this thesis we develop a polysorbate 80-containing terpolymer nanocarrier system capable of delivering imaging agents and drugs to brain metastases of breast cancer by mimicking low-density lipoprotein uptake in the brain. We propose to optimize this system for the efficient loading of two model first-line chemotherapy drugs, the anionic hydrophilic drug doxorubicin (Dox) and the hydrophobic drug docetaxel (DTX), and demonstrate delivery of both drugs to brain metastases of breast cancer in a mouse model.

1.2 Goals of This Work

The overall goal for this work is to design novel bio-inspired drug delivery systems based on functional polymeric nanoparticles in combination with nano-/micro-technologies to improve the management of diabetes mellitus and treatment of brain metastases of breast cancer (BMBC). Two different drug delivery systems have been developed:

1. Closed-loop insulin delivery implants based on glucose-responsive polymer nanoparticles
2. Polymeric nanoparticles for the delivery of therapeutics and imaging agents to BMBC

1.2.1 Closed-Loop Insulin Delivery Implants Based on Glucose-Responsive Polymer Nanoparticles

Previously a chemically driven closed-loop insulin delivery system was developed based on pH-responsive poly(NIPAM/MAA) nanoparticles embedded within a glucose-oxidase containing bio-inorganic membrane. A high concentration yet stable formulation of insulin and a biologically inert implant design was developed to provide long-term maintenance of euglycemia in T1D following subcutaneous implantation.
Hypothesis:
The functional lifetime of a chemically-driven, fully implantable closed-loop insulin delivery device for the treatment of T1D can be prolonged by increasing the concentration and stability of loaded insulin and improving the biocompatibility of the device following subcutaneous implantation.

The objectives of this project are as follows:

1. Develop a highly concentrated poloxamer gel insulin formulation that exhibits excellent insulin stability for up to 30 days under physiological conditions.
2. Evaluate sustained drug release kinetics of the poloxamer gel insulin formulation from a diffusion-controlled membrane-reservoir drug delivery device.
3. Develop a subcutaneous pH-responsive poly(NIPAM/MAA) nanoparticle-enabled closed loop insulin delivery device and demonstrate its ability to restore and maintain euglycemia in a T1D rat model.
4. Evaluate the effect of limiting cell migration to the immunogenic implant surface on the immune response following subcutaneous implantation in rats.
5. Evaluate the effect of improved implant biocompatibility and poloxamer gel insulin formulation on the in vivo functional lifetime and efficacy of the pH-responsive poly(NIPAM/MAA) nanoparticle-enabled closed loop insulin delivery device in a diabetic rat model.

1.2.2 Polymeric Nanoparticles for the Delivery of Therapeutics and Imaging Agents to BMBC

A novel nanocarrier systems based on poly(methacrylic acid)-polysorbate 80-grafted-starch (PMAA-PS 80-g-St) were developed to enhance delivery of various chemotherapy drugs to BMBC following intravenous administration.

Hypothesis:
A nanoparticle system based on PMAA-PS 80-g-St can deliver drugs and contrast agents across the blood-brain barrier to brain metastases of breast cancer following intravenous administration and improve therapeutic efficacy compared to treatment with an equivalent dose of free drug.
The objectives of this project are as follows:

(1) Develop a PMAA-PS 80-g-St polymer nanoparticle formulation of doxorubicin (Dox-NP) with high drug loading efficiency and content. Optimize the size, stability and composition of Dox-NP to permit long circulation \textit{in vivo} with minimal toxicity.

(2) Develop an alkyl chain-grafted PMAA-PS 80-g-St polymer lipid nanoparticle formulation of docetaxel with high drug loading efficiency and content. Optimize the size, stability and composition of DTX-NP to permit long circulation \textit{in vivo} with minimal toxicity.

(3) Evaluate \textit{in vitro} drug release kinetics and cytotoxicity of the nanoparticle formulations against triple-negative breast cancer cells. Demonstrate the ability of PS 80-containing terpolymer-based NP to delivery cargos into the brain of healthy mice. Evaluate the role of covalently linked PS 80 in facilitating extravasation and accumulation of the nanoparticles in the brain.

(4) Evaluate the drug biodistribution following intravenous administration of Dox-NP and DTX-NP to mice with BMBC. Evaluate therapeutic efficacy of Dox-NP and DTX-NP formulations in mice with BMBC compared to an equivalent dose of free drug.

1.3 Synopsis

Chapter 2 presents the background information and a comprehensive review of literature in the fields pertinent to the scope of this thesis.

Chapter 3 describes the development of a poloxamer gel formulation of highly concentrated insulin, up to 80 mg/mL (2160 IU/mL), which exhibits excellent insulin stability for up to 30 days under simulated physiological conditions of continuous shear at 37°C. This formulation overcomes traditional limitations of rapid macromolecule degradation at physiological conditions and its exceptionally high insulin concentration enabled development of an implantable sustained-release device with reduced size requirements for long-term efficacy in a T1D rat model.

In Chapter 4, a chemically-driven closed-loop insulin delivery device was developed to regulate blood glucose levels in diabetic rats. Rapid glucose-dependent drug release from an insulin reservoir is demonstrated using a variable-porosity diffusion barrier composed of an albumin
matrix with embedded pH-responsive PNIPAM-MAA nanoparticles, and supporting enzymes and catalysts. Implant biocompatibility was improved by rational design of implant geometry to impede leukocyte migration to the immunogenic albumin matrix. In combination with the high concentration thermostable insulin formulation developed in Chapter 3, these improvements prolonged in vivo efficacy duration of the implant by 3-fold.

Chapter 5 describes the development of PMAA-PS 80-g-St polymer nanoparticles and investigates their use as delivery vehicles for drugs and imaging agents to the brain. Nanoparticle accumulation within the brain and penetration through the BBB was evaluated using MRI, fluorescence imaging and fluorescence microscopy. Entry into the brain is attributed to the presence of PS 80 on the particle surface, which was confirmed using TOF-SIMS analysis. The nanoparticles were further used to encapsulate and deliver doxorubicin to brain metastases of triple negative breast cancer in an experimental mouse model, resulting in increased tumor apoptosis and inhibited tumor growth compared to free doxorubicin.

In Chapter 6, amphiphilic dodecyl-modified PMAA-PS 80-g-St polymer lipid nanoparticles were developed to efficiently load hydrophobic drugs (i.e. docetaxel) for delivery into the brain. Nanoparticle accumulation within brain metastases of breast cancer was evaluated using fluorescence imaging and fluorescence microscopy. We further demonstrate improved drug pharmacokinetics and efficacy in a mouse model of brain metastases of triple negative breast cancer following treatment with docetaxel-loaded nanoparticles compared to Taxotere®, the commercial formulation of docetaxel.

A summary of the thesis follows in Chapter 7, identifying the main outcomes of the work in relation to the overall goal of these studies. Future considerations evolving from the results of this thesis are also included here.

Supplementary material is presented in the Appendices (Chapter 8).


Chapter 2
General Background

2.1 Diabetes

2.1.1 Normal Glucose Metabolism

The body is subjected to widely varying states of glucose availability and glucose requirements, including the fed, fasting, resting and exercise states (Figure 1). Circulating glucose is derived from carbohydrate ingestion in the fed state and glucose synthesis in the fasted state, predominantly via hepatic glycogenolysis and gluconeogenesis. Renal gluconeogenesis substantially contributes to the systemic glucose pool only during periods of starvation. When transitioning from a state of rest to a state of exercise, a rapid shift in glucose metabolism must occur in response to the sudden increases in energy demands in muscle. Plasma glucose concentration is a function of glucose entering the circulation balanced by glucose removal from the circulation. Normal glucose regulation allows the body to maintain glucose homeostasis both within and between these varying states. Perturbation to physiological homeostasis results in either hyperglycemia, defined as abnormally high plasma glucose concentration, or hypoglycemia which is defined as an abnormally low plasma glucose concentration. Quantitatively, hypoglycemia is a measured plasma glucose concentration ≤70 mg/dL (or equivalently ≤3.9 mM)\(^56\). Similarly, hyperglycemia is a measured plasma glucose concentration ≥100 mg/dL when fasting or ≥140 mg/dL when fed (or equivalently ≥5.6 mM and 7.8 mM respectively)\(^56\). Physiologically, blood glucose concentrations are monitored by cells in the pancreas’s Islets of Langerhans and are regulated through a negative feedback loop by pancreatic endocrine hormones, primarily through the actions of insulin and glucagon.

2.1.1.1 Hormone Regulation During Hyperglycemia

Insulin is a potent regulator of glucose metabolism. Elevated plasma glucose concentrations stimulate insulin secretion from pancreatic \(\beta\)-cells. The glycemic threshold for insulin secretion is 3.3 mM, below which no insulin is released and above which insulin is released at increasing amounts with higher glucose levels\(^57\). Insulin secretion is pulsatile with an oscillation period of 3 to 6 minutes, and is biphasic in which pre-synthesized stores of insulin are rapidly released in
response to hyperglycemia and a sustained slow release of newly formed vesicles is triggered independently of glucose. Insulin helps control hyperglycemia in three ways (Figure 2). First, insulin simultaneously suppresses hepatic glucose production and promotes hepatic glycogenesis. Secondly, insulin stimulates adsorption of plasma glucose into adipose tissue and striated muscle (i.e. skeletal muscle and cardiac muscle). In adipocytes, insulin further stimulates lipid synthesis and exerts an antilipolytic effect leading to a marked reduction in plasma free fatty acid concentration, which in turn escalates glucose uptake in muscle and further suppresses hepatic glucose production. Finally, insulin suppresses glucagon release from pancreatic α-cells which, as will soon be discussed, counteracts insulin action by stimulating hepatic glucose release into the blood. Thus insulin secretion is triggered by hyperglycemia and causes a drop in blood glucose concentration through a variety of mechanisms. Other actions of insulin include promotion of triglyceride storage in adipose tissue, protein synthesis in liver and muscle, and proliferation of cell growth. While glucose is the most potent stimulus of insulin secretion, other stimuli also induce insulin release including some amino acids (arginine, leucine, lysine), incretin hormones GLP-1 and GIP released from the gut, and parasympathetic stimulation via the vagus nerve.

Figure 1. Profile of blood glucose and insulin concentration with high-starch and high-sucrose diets. (Reproduced with permission from Daly et al.)
Figure 2. Normal regulation of blood glucose concentration. (Adapted from https://sites.google.com/a/bcssd.com/painter/anatomy-physiology/08-endocrine-system).

Glucose transporters (GLUTs) are a family of twelve membrane proteins that mediate the transport of glucose across the plasma membrane. Each glucose transporter plays a specific role in glucose metabolism dictated by its substrate specificity, transport kinetics, and tissue expression. Glucose transporter type 4 (GLUT4), the major insulin-stimulated glucose transporter, is expressed predominantly in adipose tissue and striated muscle and is largely responsible for insulin-stimulated glucose transport. In these tissues, insulin activation of the insulin receptor induces translocation of GLUT4-containing vesicles to and fusion with the plasma membrane, thus permitting glucose flux into the cell. Glucose transport via GLUT4 into muscle and adipose tissue is the rate-controlling step in insulin-mediated glucose disposal. In the fasted state these tissues dispose of 25% of all glucose in the body. Roughly 80-85% of insulin-mediated glucose disposal occurs in muscle, with only 4-5% occurring in adipocytes. Glucose transporters (i.e. GLUT 1-3) which are non-responsive to insulin are expressed within insulin-independent tissues including the brain, kidneys, liver and the intestine, where they dispose of 75% of all glucose in the fasted state.
Amylin is a peptide hormone that contributes to glycemic regulation by inhibiting glucose appearance in the plasma. Amylin acts to slow gastric emptying, a key determinant of postprandial glucose concentration, and further inhibits digestive secretions, promotes satiety and inhibits glucagon secretion from pancreatic α-cells. Importantly, amylin exerts its actions primarily through the CNS, specifically the area postrema in the dorsal vagal complex of the brain stem, and may be over-ridden during hypoglycemia. It is co-secreted with insulin from pancreatic β-cells in response to hyperglycemia.

Glucagon-like peptide-1 (GLP-1) is an incretin that is secreted by ileal L cells in response to carbohydrates, proteins and lipids in the lumen of the small intestine. GLP-1 stimulates insulin secretion from pancreatic β-cells during hyperglycemia, but not during hypoglycemia. GLP-1 also inhibits glucagon secretion from pancreatic α-cells, and acid secretion and gastric emptying in the stomach. Finally, GLP-1 increases insulin sensitivity in both α-cells and β-cells in the pancreas.

Gastric inhibitory peptide (GIP) is an incretin that is synthesized by K cells in the mucosa of the duodenum and jejunum. It stimulates insulin secretion and regulates fat metabolism, but does not inhibit glucagon secretion or gastric emptying.

2.1.1.2 Hormone Counter-Regulation During Hypoglycemia

Hypoglycemia in healthy individuals is rare due to robust glucose counter-regulation mechanisms. Suppression of endogenous insulin secretion and activation of glucagon secretion are the primary mechanisms that protect the body against hypoglycemia. Decreased insulin secretion alleviates both its suppressive effect on hepatic glucose production and glucagon release from pancreatic α-cells, and its stimulatory effect of glucose utilization in muscle and adipose tissue. This occurs as a direct result of hypoglycemia on pancreatic β-cells, typically when blood glucose concentration drops below 4.6 mM. Glucagon secretion by pancreatic α-cells sustains plasma glucose concentration during fasting conditions by stimulating hepatic glucose production via glycogenolysis or gluconeogenesis resulting in an increase in blood glucose concentration. During hyperglycemia, paracrine signaling from pancreatic β-cells, through insulin and co-secreted Zn\(^{2+}\) and γ-aminobutyric acid mediators, and somatostatin-producing δ-cells in the pancreas, stomach and intestine suppress glucagon release by α-cells. Glucagon release is triggered when blood glucose levels fall below 3.8 mM and is thought to
be a result of direct effects of hypoglycemia on pancreatic α-cells, decreased intra-islet insulin[^63], and to occur in response to hypoglycemia-triggered release of norepinephrine, acetylcholine and epinephrine. The glucagon response accounts for approximately 40% of glucose recovery from hypoglycemia[^64].

### 2.1.2 Pathogenesis and Classification of Diabetes Mellitus

Altered communication among tissues and loss of the ability of tissues to respond to metabolic states play a critical role in the altered glucose homeostasis, leading to the development of diabetes. Diabetes can be classified into one of four broad categories based on pathogenesis: Type 1 diabetes mellitus (T1D) or Type 2 diabetes mellitus (T2D), gestational diabetes mellitus (GDM), and other types of diabetes.

Type 1 diabetes is an autoimmune disorder that results from macrophage- and T lymphocyte-mediated destruction of insulin-producing pancreatic β-cells, leading to insufficient production of endogenous insulin[^58]. T1D is likely initiated by exposure of a genetically susceptible individual to environmental factors such as chemical, dietary or infectious agents. Preclinical β-cell autoimmunity precedes the diagnosis of T1D by up to 9 to 13 years[^58]. Hyperglycemia occurs when 80% to 90% of the pancreatic β-cells are destroyed due to an absolute deficiency of endogenous insulin[^58]. T1D affects over 700,000 people, roughly 1 in every 600 children, in the United States and accounts for roughly 5-10% of all cases of diabetes[^56].

Type 2 diabetes is characterized by insulin resistance and a relative lack of insulin secretion, with progressively lower insulin secretion over time. Cells are unable to effectively utilize insulin leading to hyperglycemia. T2D has a strong genetic predisposition, however the genetic cause is currently not well defined. Obesity is thought to be a primary cause of T2D in people who are genetically predisposed to the disease. T2D affects more than 15 million adults in the United states and accounts for roughly 90-95% of all cases of diabetes[^56]. T2D is a polygenic disease with a strong heritable component, which is also heavily influenced by environmental factors, especially diet and physical activity. Gestational diabetes is characterized by glucose intolerance during pregnancy. It is similar to T2D in that it involves a combination of inadequate insulin secretion and insulin resistance. GDM complicates roughly 7% of all pregnancies and may improve or disappear after delivery.
2.1.3 Defective Glucose Regulation in Diabetes

Normal regulatory responses to hyperglycemia and counter-regulatory responses to hypoglycemia have been described above as it applies to healthy individuals. However, fundamental defects in glucose regulation and counter regulation are detectable in individuals with type 1 and type 2 diabetes long before clinical symptoms become apparent

Autoimmune destruction of pancreatic β-cells in T1D results in complete deficiency in insulin and amylin regulation, leading to an unchecked rise in glucose concentrations following carbohydrate ingestion, poorly regulated hepatic glucose production, abnormal gastric emptying, and unresponsive glucagon release from pancreatic α-cells during hypoglycemia.

In T2D, peripheral insulin resistance coupled with progressive β-cell failure results in marked decrease in availability of insulin and amylin. Postprandial GLP-1 levels are also significantly reduced in patients with T2D. Deficient amylin and GLP-1 regulation results in accelerated gastric emptying. Deficient insulin, amylin and GLP-1 results in inadequate suppression of postprandial glucagon secretion, hypergucagonemia and elevated hepatic glucose production. The combined effects of peripheral insulin resistance, accelerated gastric emptying and elevated hepatic glucose production exacerbates the occurrence of hyperglycemia. Notably, GIP levels remain normal in people with T2D.

2.1.4 Complications Associated with Diabetes

Acute, life-threatening consequences of uncontrolled diabetes include diabetic ketoacidosis due to sustained production of ketone bodies by the liver, and hyperosmolar non-ketotic state in which water is osmotically drawn out of cells into the blood due to elevated blood glucose levels. Insulin therapy treatment of diabetes may additionally result in hypoglycemia, as discussed previously, due to overdose or incorrectly timed administration of exogenous insulin, excessive exercise or insufficient carbohydrate intake. All three acute complications may result in diabetic coma or death.

Chronic hyperglycemia is associated with a number of long-term complications which develop gradually. Possible complications include cardiovascular disease (including coronary artery disease, atherosclerosis, stroke or myocardial infarction); peripheral neuropathy leading to loss of sensation in the limbs, diabetic foot ulcers, or amputation; autonomic neuropathy causing...
nausea, vomiting, diarrhea, constipation or erectile dysfunction; nephropathy leading to end-stage kidney disease; retinopathy potentially leading to blindness, cataracts or glaucoma; hypertension and cerebrovascular disease leading to stroke or vascular dementia. Abnormalities of lipoprotein metabolism are often found in people with diabetes. Recent evidence suggests that T2D is also linked to Alzheimer’s disease.

2.1.5 Diagnosis

Diabetes mellitus is a group of metabolic disorders characterized by elevated blood glucose levels resulting from insufficient insulin secretion, resistance to the action of insulin, or both. The clinical manifestation of these disorders is hyperglycemia. Diabetes is clinically defined by fasting or post-prandial hyperglycemia, or abnormally increased glucose excursion in response to a defined glucose load. Diabetes is diagnosed based on any one of the following metrics:

- Fasting (8-hours) plasma glucose ≥ 126 mg/dL (7.0 mmol/L)
- Symptoms of diabetes plus casual plasma glucose concentration ≥ 200 mg/dL (11.1 mmol/L)
- 2-hour postload glucose (75 g glucose dissolved in water) ≥ 200 mg/dL (11.1 mmol/L) during an oral glucose tolerance test

The American Diabetes Association recommends using the fasting glucose test as the principle tool for the diagnosis of diabetes mellitus in non-pregnant adults. Normal plasma glucose concentrations are defined as one of the following:

- Fasting (8-hours) plasma glucose ≤ 100 mg/dL (5.6 mmol/L)
- Postload glucose (75 g glucose dissolved in water) < 140 mg/dL (7.8 mmol/L)

In addition, normal plasma glucose levels must be above the hypoglycemic threshold defined by plasma glucose concentrations < 70 mg/dL (3.9 mmol/L).

2.1.6 Clinical Management of Type 1 Diabetes

2.1.6.1 Assessment of Glycemic Control

Patient self-monitoring of blood glucose (SMBG) and hemoglobin A1C testing are two primary techniques for determining whether glycemic targets are being achieved and assessing the
patient’s response to therapy. SMBG results are useful for preventing hypoglycemia, adjusting medication dosing and guiding medical nutrition therapy and physical activity. SMBG frequency and timing are dictated by the patient’s specific needs and goals.

Real-time continuous glucose monitoring (CGM) measures interstitial glucose, which correlates well with plasma glucose. However these devices require calibration with SMBG and are not approved as a sole agent to monitor glucose. SMBG is still required for making acute treatment decisions. Frequent use of CGM has been shown to lower A1C in adults over 25 years of age, however there was no significant A1C benefits in those below 25 years of age. Studies have also not shown consistent reductions in severe hypoglycemia.

The hemoglobin A1C test measures the percentage of glycated hemoglobin and reflects the average blood glucose concentration over the past two to three months. It determines whether patients’ glycemic targets have been reached and maintained and has strong predictive value for diabetes complications. A1C testing should be performed at least two times a year, with increased frequency depending on the clinical situation, the treatment regimen and the clinician’s judgement.

2.1.6.2 Insulin Therapy

Patients with T1D must follow a daily management regimen which includes blood glucose monitoring, monitoring of carbohydrate intake and physical activities, and administration of exogenous insulin to lower and control blood glucose concentrations. Insulin dose and dosing schedule will depend on specific patient requirements including basal needs, carbohydrate intake and level of physical activity, as well as target glycemic levels. These decisions must be determined by the patient based on self-blood glucose monitoring and in consultation with a physician. Several insulin formulations with varying drug kinetics, including different onset time, peak time, and duration of action, are commercially available (Table 1). These insulin variants, each exhibiting different pharmacokinetics, may be used in combinations to achieve improved patient-specific glycemic control.
Table 1. Commercial insulin formulations

<table>
<thead>
<tr>
<th>Compound</th>
<th>Product</th>
<th>Manufacturer</th>
<th>Onset Time*</th>
<th>Peak Time*</th>
<th>Duration*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin lispro</td>
<td>Humalog</td>
<td>Eli Lilly</td>
<td>15 min</td>
<td>30-90 min</td>
<td>3-5 h</td>
</tr>
<tr>
<td>Insulin aspart</td>
<td>NovoLog</td>
<td>Novo Nordisk</td>
<td>15 min</td>
<td>30-90 min</td>
<td>3-5 h</td>
</tr>
<tr>
<td>Insulin glulisine</td>
<td>Apidra</td>
<td>Sanofi</td>
<td>15 min</td>
<td>30-90 min</td>
<td>3-5 h</td>
</tr>
<tr>
<td>Insulin lispro</td>
<td>Humalong U-200</td>
<td>Eli Lilly</td>
<td>15 min</td>
<td>60 min</td>
<td>3-5 h</td>
</tr>
<tr>
<td>Regular insulin</td>
<td>Humulin R, Novolin R</td>
<td>Eli Lilly, Novo Nordisk</td>
<td>30-60 min</td>
<td>2-3 h</td>
<td>5-8 h</td>
</tr>
<tr>
<td>Regular insulin</td>
<td>Humulin R U-500</td>
<td>Eli Lilly</td>
<td>15 min</td>
<td>4-8 h</td>
<td>13-24 h</td>
</tr>
<tr>
<td>Neutral protamine Hagedorn (NPH)</td>
<td>Humulin N, Novolin N</td>
<td>Eli Lilly, Novo Nordisk</td>
<td>2-4 h</td>
<td>4-10 h</td>
<td>10-16 h</td>
</tr>
<tr>
<td>Insulin glargine</td>
<td>Lantus</td>
<td>Sanofi</td>
<td>2-4 h</td>
<td>No peak</td>
<td>20-24 h</td>
</tr>
<tr>
<td>Insulin glargine 300 U/mL</td>
<td>Toujeo</td>
<td>Sanofi</td>
<td>6h</td>
<td>No peak</td>
<td>24 h</td>
</tr>
<tr>
<td>Insulin detemir</td>
<td>Levemir</td>
<td>Novo Nordisk</td>
<td>3-4 h</td>
<td>No peak</td>
<td>24 h</td>
</tr>
<tr>
<td>Insulin degludec</td>
<td>Tresiba</td>
<td>Novo Nordisk</td>
<td>3-4 h</td>
<td>No peak</td>
<td>&gt; 24 h</td>
</tr>
<tr>
<td>Rapid-acting (Inhalable)</td>
<td>Technosphere insulin</td>
<td>Afrezza, Sanofi</td>
<td>15 min</td>
<td>30 min</td>
<td>3 h</td>
</tr>
</tbody>
</table>

* data adapted from Gilroy and Cengiz$^{66,67}$.

In T1D, the daily insulin requirements generally range from 0.5-1 units/kg/day. The basal insulin requirement, approximately 30-50% of the total dose$^{68}$, is fulfilled using intermediate or long-acting insulins while the balance is fulfilled using rapid or short-acting insulins in bolus doses administered before meals. Insulin is administered with (1) insulin syringes or insulin pens that inject insulin under the skin, or (2) external insulin pumps that connect an external insulin reservoir to a catheter inserted below the skin of the abdomen. Typical insulin therapy regimens include$^3$:

1. **Conventional therapy**: Two daily injections of mixed insulin (rapid/short-acting and intermediate-acting insulin) administered before breakfast and evening meal.

2. **Conventional therapy with split night-time dose**: One injection of mixed-insulin before breakfast, 1 injection of rapid/short-acting insulin before evening meal and 1 injection of intermediate-acting insulin before bedtime snack. This regimen reduces fasting hyperglycemia associated with the long interval between the evening meal and breakfast, and additionally manages the dawn phenomenon.

3. **Intensive insulin therapy**: Rapid/short-acting insulin injections before meals, excluding snacks, with additional intermediate- or long-acting insulin injections once or twice a day.
4. **Intensive therapy with a continuous subcutaneous insulin infusion (CSII or insulin pump):** Rapid-acting insulin is delivered continuously delivered with the help of a continuous insulin infusion pump. A bolus dose of insulin is programmed into the pump to be given before meals and snacks. Blood glucose testing must be performed > 4 times/day to monitor carbohydrate intake and to adjust insulin doses.

Self-blood glucose testing is typically performed before each meal and the bedtime snack to aid optimization of insulin dosing. Blood glucose testing at 2-3 am is useful for evaluating nocturnal hypoglycemia and fasting hyperglycemia. Self-blood glucose testing is performed with the use of blood glucose testing meters, many of which record measurement dates, times and test results.

As food intake influences insulin dosing requirements, patients are often taught to monitor carbohydrate intake from various foods in order to better predict insulin requirements.

Despite optimal insulin treatment, roughly 0.3% of T1D patients experience large and frequent fluctuations in their blood glucose levels, a form of severe diabetes termed ‘brittle’ or ‘labile’ diabetes69. These patients experience recurring episodes of hyperglycemia and hypoglycemia, hypoglycemic unawareness in which hypoglycemia presents without warning symptoms and diabetic ketoacidosis, a life-threatening complication that results in elevated levels of ketones in the blood.

### 2.1.7 Economic Cost of Diabetes

In 2012, the American Diabetes Association estimated that the direct medical cost of diagnosed diabetes in the United States was $173 billion. An additional $69 billion in costs was estimated to be a result of lost productivity10. The total cost of diabetes in 2012 increased by 41% from the 2007 estimate of $174 billion. Therefore diabetes imposes a substantial and growing burden on the economy in the United States.

### 2.2 Emerging Technologies for the Treatment and Management of Type 1 Diabetes

Patients with T1D require lifelong treatment with exogenous insulin to maintain euglycemia and for survival. Blood glucose targets are difficult to achieve using conventional insulin therapy due to errors in predicating insulin requirements. In addition, use of needles and lancing devices for frequent blood glucose testing and insulin administration results in poor compliance and quality
of life in some patient populations. As a result, efforts to restore glucose regulation via islet transplantation or closed-loop insulin delivery systems have been actively pursued in the past decade. Despite intensive efforts and promising initial results, to date none of these solutions are suitable for wide-spread clinical use in the home setting.

2.2.1 Pancreas & Islet Transplantation

Endogenous insulin secretion can be restored through whole pancreas or islet transplantation in patients with T1D. These procedures can result in insulin independence and glucose stability and are the definitive cure for T1D.

Pancreas transplantation has been shown to improve or stabilize diabetic retinopathy, improve motor neuropathies and improve cardiovascular function, carotid intimal medial thickness, blood pressure and lipid metabolism\textsuperscript{70}. Unfortunately, the impact on overall patient survival is uncertain\textsuperscript{70}. In T1D patients with end stage renal failure, simultaneous pancreas and kidney transplantation results in lower short-term survival in the perioperative period up to 18 to 24 months, but those with successful pancreas grafts after 12 months have similar or improved long-term survival compared to living or deceased donor kidney transplantation. A retrospective cohort study of patients with diabetes and preserved kidney function who received a pancreas transplantation suggested that overall survival was significantly worse compared to the survival of wait-listed patients receiving conventional therapy\textsuperscript{71}. Long-term survival of patients receiving whole pancreas grafts decline with time, with 9-year median survival for pancreas transplants and less than 10% survival at 21 years\textsuperscript{72}. Pancreas transplantation necessitates life-long treatment with immunosuppressive drugs to prevent rejection, and is associated with significant perioperative risks including graft thrombosis, hemorrhage, pancreatitis, infection, peripancreatic abscesses and duodenal stump leakage.

Islet allotransplantation involves the extraction, isolation and purification of islets isolated from pancreata of deceased organ donors and subsequent infusion into the recipient’s liver via a percutaneous transhepatic catheter, resulting in insulin independence and stable, near-normal glycemic control with reduced or eliminated hypoglycemia\textsuperscript{73}. Compared to whole-organ pancreas transplantation, islet transplantation is less invasive for the recipient. However the procedure is still associated with considerable risk as patients are at risk for complications including portal vein thrombosis, bleeding and portal hypertension.
The Edmonton Protocol was the first demonstration of this approach that yielded high success rates, in which all seven patients enrolled in the study achieved insulin independence at 1 year\textsuperscript{73}. While these results were unprecedented at the time, challenges were encountered in its reproducibility and follow-up at 5 years revealed graft function loss, with 90\% of patients returning to insulin therapy\textsuperscript{74}. Modifications to the original protocol, including changes to the preparation and transport of harvested islets\textsuperscript{75}, and the use of immunosuppressive and inflammatory drugs\textsuperscript{76} prior to islet transplantation have resulted in improved efficacy and safety outcomes\textsuperscript{77,78}.

The two limiting factors that prevent widespread use of islet transplantation are the limited availability of donor organs from which islets can be harvested, and the need for life-long immunosuppressive therapy. Current research directions explore the use of alternative islet sources such as living pancreas donors, xenotransplantation\textsuperscript{79}, stem cell-derived β-cells\textsuperscript{80}, transdifferentiation of liver, bile duct and exocrine pancreatic cells into β-cells\textsuperscript{81}. Immuno-isolation techniques which enclose islets within a semi-permeable immune-protective capsule to protect the islets from the host immune system while simultaneously allowing exchange of nutrients and insulin represents an attractive strategy to prolong graft survival without immunosuppressive therapy\textsuperscript{82}. Methods of islet microencapsulation using different biocompatible materials and processing strategies are being explored for this application\textsuperscript{83-87}. Other research directions include development of improved islet selection criteria\textsuperscript{88}, use of different scaffolding materials\textsuperscript{89}, and implantation at different sites (e.g., kidney capsule or subcutaneous tissue)\textsuperscript{90,91}.

### 2.2.2 Closed-Loop Insulin Therapy

Closed-loop drug delivery systems, also known as artificial pancreas or smart insulin therapies, are alternative technologies that replicate the pancreas’s endocrine function. They provide real-time regulation of blood glucose levels through glucose-dependent insulin delivery (Figure 3). These systems are based on feedback coupling between a continuous glucose sensor, which monitors blood or plasma glucose concentrations, and an insulin delivery system to provide autonomous glucose-dependent insulin delivery. This form of insulin therapy offers patients improved glycemic control, improved compliance and improved quality of life over conventional insulin therapy. Importantly, these systems are able to provide autonomous around-the-clock glucose regulation with no patient intervention. Various closed-loop insulin delivery systems
have been developed over the past decades, with the most common approaches make use of electromechanical components or responsive polymers.

Figure 3. Operation of closed-loop insulin delivery systems. Adapted with permission from Gordijo et al.\textsuperscript{20}

2.2.2.1 Electromechanical Systems

Electro-mechanical closed-loop drug delivery systems that are comprised of a digital continuous glucose sensor, an electro-mechanical drug pump and a controlling algorithm (Figure 4) are perhaps the most mature technology available for diabetes patients\textsuperscript{7}. These systems make use of previously developed technologies including continuous glucose monitors (CGM) and electromechanical insulin infusion pumps. Commercially available CGMs typically measure the glucose concentration of interstitial fluid in the subcutaneous tissue every 5 minutes to provide a detailed history of glucose control throughout the day (Table 2). Insulin infusion pumps are portable insulin delivery devices that administer programmed doses of insulin \textit{via} a catheter placed in the abdominal subcutaneous tissue. Insulin is typically provided as a combination of basal insulin, a low background dose of insulin needed throughout the day, and larger bolus doses of insulin to compensate for meals and correct for high glucose levels. When operated in an open-loop fashion, the use of these devices provide better health outcomes and reduced the difficulty of treatment compared to traditional insulin therapy, however patients are still required to manually observe glucose concentration, calculate the desired insulin dose and operate the insulin infusion pump. Control systems, typically consisting of feedback or feedforward algorithms, make it
possible to couple CGMs and insulin infusion pumps to provide fully-automated closed-loop insulin delivery without patient intervention. The prospect of closing the loop between the CGM and insulin pump offers tremendous potential to further improve insulin therapy by removing the burden of patient intervention resulting in less patient error, improved patient compliance and better quality of life.

Figure 4. Components of an electromechanical artificial pancreas system. Adapted from reference 92.

Electro-mechanical closed-loop systems have been developed and have shown promise in a number of clinical settings, including critical care and out-patient settings, however its widespread adoption is currently limited by several factors including poor accuracy of CGMs due to differences between blood and interstitial fluid glucose concentration, poor long-term stability of CGMs resulting in the need for frequent calibration and replacement, delayed drug action associated with subcutaneous insulin infusion, and inadequate control algorithms which provide correct insulin dosing following meals. Current devices on the market also tend to be expensive, bulky, and can lead to lipohypertrophy, due to repeated insulin injections at the same site, or increased risk of infection and contact dermatitis due to frequent transcutaneous placement of the needle or cannula.

To overcome these delays, many artificial pancreas systems implement control algorithms with feedforward capabilities known as “meal announcement” to predict bolus insulin requirements.
prior to food ingestion. Several different control algorithms have recently been developed to better predict insulin requirements and improve insulin dosing accuracy\textsuperscript{96,97}. Intraperitoneal glucose sensing and insulin delivery has also been explored to reduce diffusion lag associated with subcutaneous systems, however these systems are considerably more invasive compared to subcutaneous systems\textsuperscript{95}.

Table 2. Commercial CGM systems.

<table>
<thead>
<tr>
<th>CGM system</th>
<th>Sensor Life</th>
<th>Sensor Canula Size</th>
<th>Calibration frequency</th>
<th>Measurement frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbot Freestyle Navigator</td>
<td>5 days</td>
<td>5 mm</td>
<td>10, 12, 24, and 72 hrs after insertion</td>
<td>Every 1-2 min.</td>
</tr>
<tr>
<td>Dexcom SEVEN Plus</td>
<td>7 days</td>
<td>13 mm</td>
<td>Every 12 hrs</td>
<td>Every 5 min.</td>
</tr>
<tr>
<td>Guardian REAL-Time CGM</td>
<td>3 days</td>
<td>14 mm</td>
<td>2 and within 6 hrs after insertion, every 12 hrs afterwards</td>
<td>Every 5 min.</td>
</tr>
<tr>
<td>MiniMED Paradigm REAL-Time System</td>
<td>3 days</td>
<td>14 mm</td>
<td>2 and within 6 hrs after insertion, every 12 hrs afterwards</td>
<td>Every 5 min.</td>
</tr>
</tbody>
</table>

2.2.2.2 Glucose-Responsive Polymer Systems

The use of “smart” or environmentally responsive polymers for drug delivery is an expanding area of research\textsuperscript{7}. These polymer systems are typically designed to modulate drug release in response to various physiological stimuli (\textit{e.g.}, pH, proteins, enzymes, or disease-related analytes) or external stimuli (\textit{e.g.}, heat, light, ultrasound, electric fields or magnetic fields). Responsive polymer systems that are sensitive to glucose concentrations in the blood or interstitial fluid may be used to recapitulate the natural dynamics of insulin release. Glucose-binding molecules, including GOX, phenylboronic acid or concanavalin A, are typically incorporated into responsive polymers or hydrogels to impart glucose-sensitivity to polymer systems.

2.2.2.2.1 Phenylboronic Acid

Phenylboronic acids (PBAs) are synthetic glucose-sensing moieties developed by Kataoka \textit{et al.}\textsuperscript{98,99}. These boronic acids that contain a phenyl substituent and two hydroxyl groups attached to a boron (Figure 5). PBAs are known to bind reversibly with \textit{cis}-1,2 or \textit{cis}-1,3 diols such as glucose to form hydrophilic phenylborate which contains a stabilized negative charge on the boronic acid (Figure 5)\textsuperscript{100,101}. Meaningful binding between boronic acid and diols are generally believed to occur at pH above the pKa of the boronic acid, however Yan \textit{et al.}\textsuperscript{100} has shown that
the optimal pH for binding is related to both the pKa values of the boronic acid and the diol such that \( pH_{\text{optimal}} = \frac{pK_{a\text{acid}} + pK_{a\text{dial}}}{2} \). Experimentally, PBA has a pK\(_a\) of 8.2-8.6 and typically binds glucose (\( pK_{a\text{glucose}} = 12 \)) at around pH of 9.0\(^{100,102}\). To optimize glucose binding at physiological pH, recent studies have designed fluoro-, nitro-, or sulfo-containing PBA compounds to provide electron-withdrawing character to PBA, resulting in a PBA derivatives with lower pKa\(^{15,101,103,104}\). A variety of studies have used PBA to develop different glucose-responsive insulin delivery systems including glucose-sensitive polymer membranes, capsules and bulk hydrogels, polymeric nanoparticles, and insulin derivatives.

![Complexation between glucose and (alkylamido)phenylboronic acid in aqueous solution. Reproduced with permission from Ravaine et al.\(^5\).](image)

PBA incorporated into polymer membranes, capsules or bulk polymer serve as a diffusion barrier to insulin. Glucose-induced swelling of PBA may be used to facilitate release of insulin from these systems. Kitano et al.\(^{105}\) incorporated a phenylboronic acid moiety into poly(N-vinyl-2-pyrrolidone) (poly(NVP-co-PBA)). Poly(vinyl alcohol) (PVA) forms a reversible borate/diol complex with PBA and was used to form a highly viscous gel complex with poly(NVP-co-PBA). In the presence of glucose, which competes with PVA-borate complexes to form glucose-borate complexes, PVA interaction with poly(NVP-co-PBA) is reduced resulting in a phase transition of the polymer from a gel state to a sol state with significantly reduced viscosity. By entrapping insulin within a PVA/poly(NVP-co-PBA) complex gel capsule, glucose-mediated swelling of the capsule allows for more rapid diffusion of insulin from the system. Glucose-dependent swelling of PBA has also been used to develop a variety of different glucose sensors\(^{106,107}\).

PBA has also found use as a glucose-sensing moiety in glucose-sensitive nano- and micro-particles loaded with insulin. Ma et al.\(^{102}\) developed glucose-responsive micelles composed of
PBA-containing poly(ethylene glycol)-block-poly(acrylic acid-co-acrylamidophenylboronic acid) complexed with poly(acrylic acid-co-acrylglucosamine). At pH 6.8, the neutral-charged hydrophobic block copolymers form the micelle’s hydrophobic core, which is stabilized by a hydrophilic poly(ethylene glycol) corona. In the presence of glucose, the negatively charged tetrahedral form of PBA is stabilized resulting in increased polymer hydrophilicity, destabilization of the micelle polymer core, and subsequent release of encapsulated insulin. This reaction occurs under physiological conditions due to a decrease of apparent pKa of PBA as a result of complexation with glycopolymer. Other groups have developed and optimized PBA-based glucose-responsive microgels based on glucose-triggered polymer swelling for insulin delivery\textsuperscript{108-110}, as well as for glucose-sensing applications\textsuperscript{16,111}.

Recently PBA has been used to develop glucose-responsive insulin analogs by covalent attachment of insulin to both a dodecanoic fatty acid chain and a phenylboronic acid moieties\textsuperscript{101}. This insulin analog provided glucose-responsive insulin activity for up to 13 hours in diabetic mice and restored normal blood glucose levels within 3 hours following repeated intraperitoneal glucose challenges. The extent to which the insulin analog elicited hypoglycemia was also reduced compared to native insulin. Although the mechanism of glucose-responsive insulin activity remains to be determined, the authors hypothesize that it is related to glucose-mediated binding to hydrophobic domains in serum proteins, such as albumin, or reversible binding to immobilized diols, such as those present on glycoproteins, glycosylated proteins, proteoglycans, or glycosaminoglycans.

Among glucose-responsive moieties, PBA exhibits greater stability compared to protein-based systems, such as GOX and concanavalin A, which are prone to unfolding and denaturation. While much progress has been made on developing glucose-responsive drug delivery systems based on PBA, the nature of boronic acid interaction with diols dictates that PBA binding to glucose is non-specific. Boronic acids readily react with other compounds commonly present in the body including saccharides (including glucose and fructose), catechol, carbohydrates, and glycoproteins\textsuperscript{100}.

2.2.2.2 Concanavalin A

Lectins are a family of carbohydrate binding proteins that interact with glycoproteins and glycolipids on the cell membrane to regulate cell adhesion or hormone regulation. Concanavalin
A is a lectin that exhibits reversible and strong binding affinity for non-reducing α-D-glucose, α-D-mannose, N-acetyl-D-glucosamine, polysaccharide and glycopolymers with unmodified hydroxyl groups at C-3, C-4 and C-6 in the pyranose ring\textsuperscript{112}.

Glucose-responsive hydrogels can be synthesized by crosslinking saccharide-bearing polymers (e.g., poly(2-glucosyloxyethyl methacrylate)) with Con A and an additional crosslinking agent. In these systems, Con A acts as an additional crosslinker in the absence of monosaccharides. In the presence of glucose, competitive binding between glucose and polymer-bound saccharides for Con A will dictate the degree of hydrogel crosslinking and therefore the hydrogel volume. Thus, cross-linking density decreases and the gel swelling ratio increases progressively as free glucose concentration is increased. Using this method, Yin et al. developed a series of insulin-loaded ConA-based microgels which demonstrated glucose-dependent insulin release\textsuperscript{13,112,113}. Similarly, Brownlee et al. developed glucose-responsive insulin complexes by covalent modification of insulin with various oligosaccharides followed by complexation with concanavalin A\textsuperscript{114,115}. The sugar-insulin/lectin complexes served as a glucose-sensitive insulin reservoir from which glycosylated insulin, which retained its biological activity, could be displaced by glucose. Other groups have also employed the use of concanavalin A to develop bulk hydrogels which exhibit glucose-triggered insulin release\textsuperscript{116,117}. Sato et al. used a layer-by-layer approach to develop insulin-loaded Con A/glycogen microcapsules without the use of a second crosslinker\textsuperscript{14}. The use of concanavalin A for the development of glucose-responsive systems is limited by its non-specificity for glucose. In addition, concanavalin A is a protein and is therefore susceptible to denaturation and unfolding.

### 2.2.2.2.3 Glucose Oxidase

β-D-glucose:oxgen 1-oxidoreductase (GOX) is a flavoprotein that catalyzes oxidation of β-D-glucose at its first hydroxyl group in the presence of oxygen to produce hydrogen peroxide and D-glucono-δ-lactone which is partially hydrolyzed to gluconic acid (Figure 6). At pH 7, glucose exists in solution in cyclic hemiacetal form as 63.6% β-D-glucopyranose and 36.4% α-D-glucopyranose. The GOX binds specifically to β-D-glucopyranose and does not act on α-D-glucose. It is able to oxidize all of the glucose in solution because the equilibrium between the α and β anomers is driven towards the β side as it is consumed in the reaction.
Typically pH-sensitive polymers, including polymers which contain acidic groups (e.g., -COOH, -SO$_3$H) or basic groups (-NH$_2$), can be rendered glucose-responsive by immobilizing GOX within the polymer matrix.$^{12,17,19,20,118,119}$ Accumulation of gluconic acid within the polymer matrix alters the protonation state of the pH-sensitive polymer leading swelling/collapse of hydrogels or dissociation of polymer films. GOX activity requires the presence of molecular oxygen, which is depleted during the enzymatic reaction, and is inhibited by hydrogen peroxide, which accumulates during the oxidation reaction. Catalase, which catalyzes the decomposition of hydrogen peroxide to water and oxygen, is often embedded alongside GOX to prevent accumulation of hydrogen peroxide and to regenerate oxygen. Compared to concanavalin A and PBA, GOX activity is highly specific for glucose and is therefore an ideal candidate in the development of glucose-responsive closed-loop insulin delivery systems. However, since it is a protein, GOX is susceptible to unfolding and denaturation.

![Figure 6. Enzyme-catalyzed conversion of glucose to gluconic acid. GOX catalyzes the oxidation of β-D-glucose into D-glucono-1,5-lactone and hydrogen peroxide. D-glucono-1,5-lactone then hydrolyzes to gluconic acid. Catalase converts hydrogen peroxide into water and oxygen, which is required to continue the cycle. Adapted with permission from Aikten et al.$^{120}$](image)

### 2.2.3 Closed-Loop Dual Hormone Therapy

Intensive insulin therapy has been recommended for controlling hyperglycemia; however, unfortunately it frequently results in fluctuations in glycemia, especially hypoglycemia$^2$. Hypoglycemia is the most serious acute complication associated with insulin therapy in diabetic patients and the limiting factor of intensive insulin treatment. Patients are particularly susceptible to episodes of nocturnal hypoglycemia due to delayed action of exogenously administered insulin before sleep. Furthermore, adherence to tight blood glucose targets are often relaxed for operators of motor vehicles to mitigate the risk of hypoglycemic episodes while driving, resulting in long-term complications associated with hyperglycemia$^2$. The problem of
hypoglycemia is particularly difficult in T1D because counter-regulation of hypoglycemia is impaired. In the normal response to hypoglycemia, insulin secretion is suppressed and counter-regulatory hormones, including glucagon, is released to restore normal blood glucose levels. However in T1D, hypoglycemia cannot decrease insulin release to the blood because insulin is supplied exogenously which cannot be adjusted in real-time. Moreover, glucagon release from pancreatic α-cells, which is normally triggered by decreased endogenous insulin release, is impaired in T1D. To restore counter-regulation to T1D patients and prevent hypoglycemia, closed-loop delivery of glucagon has been previously proposed. Many systems currently being investigated deliver both glucagon and insulin in a closed-loop fashion in so called dual hormone closed-loop systems that mimic both insulin and glucagon regulation in pancreatic islets. These systems operate by delivering insulin alone during hyperglycemia, with the safety of glucagon delivery during episodes of hypoglycemia. In combining the opposite actions of insulin and glucagon in a glucose-responsive manner, tighter glycemic control may be realized.

Recently electromechanical systems have been used for closed-loop glucagon delivery to counter-regulate closed-loop insulin delivery systems, with the goal of providing better glycemic control. Such dual-hormone closed-loop systems have demonstrated superior performance in a number of diabetes patient populations, however these systems suffer from several challenges including poor stability of glucagon formulations, short system lifetime, poor accuracy, high treatment costs and poor patient compliance in some patient populations including juvenile and adolescent T1D populations and in the adult T1D population where use of CGMs interfere with patient lifestyle.

2.3 Insulin Formulation and Design

2.3.1 Insulin Structure & Stability

Insulin is a peptide hormone responsible for the regulation of glucose concentration in the blood. Its discovery in 1921 transformed the treatment of diabetes and remains the cornerstone for insulin therapy in all T1D and 30% of type 2 diabetes patients today. Human insulin is a 5808 Da alpha-helical peptide composed of 51 amino acids. It is composed of two polypeptide chains. The A-chain contains two alpha helix sections joined by a ribbon which enables these helices to lie alongside one another. The B-chain wraps around the A-chain which consists of a larger alpha-helix section and two smaller glycine residues at 20 and 23 that allow it to fold onto itself.
The tertiary structure of insulin is stabilized by three disulfide bridges, two between the A- and B-chains and one within the A-chain. The exterior of this molecule is mainly polar, whereas the interior is mainly non-polar. A third polypeptide chain, the so called C-peptide, is a short 31 amino acid chain that connects insulin’s A and B chain in the proinsulin molecule. C-peptide is cleaved from the proinsulin molecule to produce insulin.

Insulin is stored in the body as a hexamer through complexation with two central zinc ions (due to interactions with the hydrophobic surfaces), however this form of insulin is in equilibrium with dimeric and monomeric forms of insulin. Only the monomeric form of insulin is bioactive. The hexamer quaternary structure facilitates long-term insulin stability, which serves as a way to keep the highly reactive insulin protected, yet readily available.

Under unfavorable conditions, insulin is prone to physical (i.e. denaturation and fibrillation) and chemical (i.e. hydrolysis and deamidation) instability, especially at high insulin concentrations and when subjected to agitation-induced shear forces\textsuperscript{121} or elevated temperatures. Insulin aggregation proceeds via monomer interaction with hydrophobic surfaces (e.g., air bubbles) in which the monomeric species partially unfolds upon adsorption and either returns to its native conformation or, in combination with other unfolded species, initiates nucleation of a stable intermediate aggregate that may go on to interact with native molecules to cause formation of fibrillary interdigitated beta-sheets, resulting in loss of insulin activity\textsuperscript{122}. Injection of this form can cause amyloidosis and thus prevents the storage of insulin for long periods. Non-enzymatic degradation of insulin is due to hydrolysis and deamidation of asparagine residues leading to loss of insulin activity. In the absence of zinc, insulin stability decreases with increasing concentration due to greater intermolecular interaction and more rapid aggregation\textsuperscript{122-124}. Therefore commercial formulations of insulin typically include zinc to facilitate hexamer formation and to minimize insulin instability.

2.3.2 Commercial Insulin Formulations

Extensive efforts have been taken to develop and commercialize insulin analogs with varying pharmacokinetics, including different onset time, peak time, and duration of action (Table 1)\textsuperscript{125}. These insulin variants may be used in combination to achieve improved patient-specific glycemic control. Native unmodified insulin, formulated as Humulin R and Novalin R, has an onset time of 30-60 min, a peak time of 2-3 h, and a duration of action up to 5-8 h.
Fast-acting insulins with shorter onset time, peak time and duration of action are achieved by introducing modifications to the insulin structure to inhibit dimer and hexamer formation and improve absorption. For example, the position of the B29 lysine residue and the B28 proline residue is switched in the fast-acting insulin analog Insulin Lispro. Similarly, Insulin aspart has an amino acid sequence in which the B28 proline is substituted with an aspartic acid residue, resulting in increased charge repulsion that prevents formation of hexamers. Insulin glulisine differs from native human insulin in that the amino acid asparagine at position B3 is replaced with a lysine, and the lysine in position B29 is replaced with glutamic acid.

Intermediate-acting and long-acting insulin analogs are achieved using a variety of mechanisms. Insulin glargine, an intermediate-acting insulin analog, has a substitution of glycine for asparagine at position N21 and two arginines added to the carboxy terminal of the B chain. These modifications shift the isoelectric point from pH 5.4 to pH 6.7, making this analog less soluble at physiological pH. Therefore, upon injection of Insulin glargine into the neutral subcutaneous space, higher-order aggregates form resulting in a slow, peakless dissolution and absorption of insulin. Neutral protamine Hagedorn (NPH) insulin is an intermediate-acting insulin formulation composed of a suspension of crystalline zinc insulin complexed with protamine, a positively charged polypeptide. Slow dissociation of insulin monomer from this complex results in slower onset time, peak time and duration of action. The long-acting insulin analog Insulin detemir is a result of covalent attachment of a saturated 14-carbon alkyl (fatty acid) segment to the ε-amine of B29 lysine which facilitates insulin binding and sequestration by circulating serum albumin. Slow dissociation from this complex results in prolonged onset time and duration of action, with no peak. Insulin degludec is a modified insulin with a deletion of threonine at position B30 and a conjugated hexadecanedioic acid via a gamma-L-glutamyl spacer at lysine B29. These modifications allow for insulin degludec to exist as a dihexamer in solution and formation of multi-hexamers after subcutaneous injection resulting in a subcutaneous depot from which insulin is slowly released into the circulation.

2.4 Foreign Body Response to Implants

2.4.1 Immune Response

A major concern with implanted medical devices is the threat of exacerbated inflammation in the surrounding tissue. The body’s inflammatory response to the implant, whether acute or
chronic in nature, compromises implant function via cellular degradation and/or isolation. Inflammation is coordinated by the body’s innate immune system (i.e. anatomic and physiological barriers, activation of the complement system, and inflammation) as well as the adaptive immune system\textsuperscript{131}.

Inflammation initiated by tissue injury at the site of implantation is mediated by the release of eicosanoids and cytokines by the injured cells\textsuperscript{132}. Eicosanoids including prostaglandins and leukotrienes cause blood vessel dilation and leukocyte recruitment, respectively. Exudation of fluid, proteins and cells from the vasculature into the implantation site leads to adsorption of blood and/or interstitial fluid proteins onto the implant surface, release of cytokines (interleukins (responsible for communication between leukocytes), chemokines (chemotaxis and recruitment of cells), and interferons (anti-viral effects)), and recruitment and differentiation of blood borne monocytes into macrophages of various phenotypes\textsuperscript{131}. The presence of immune cells at the site of implantation promotes removal (or isolation) of foreign materials and healing of damaged tissue via the coagulation cascade. The normal wound healing process around a foreign material is altered in patients with diabetes, including decreased re-epithelialization, decreased growth factor expression, impaired angiogenesis and formation of dysfunctional provisional matrix, and impaired inflammatory cell infiltration\textsuperscript{133}.

2.4.2 Methods for Overcoming FBR

Implantation of a foreign object into the body initiates a host response under normal physiological conditions as the first step of tissue repair. Modern implants are designed to improve implant integration by promoting tissue regeneration while avoiding chronic inflammation and foreign body reactions that may result in the loss of implant function. Directing these processes requires in-depth knowledge of the immunological process and an understanding of the intended implant function.

Medical implants including scaffolds for tissue integration and regenerative medicine, dental implants, and internal fixation for bone fractures seek to induce and accelerate the foreign body response leading to faster tissue healing and thorough implant integration. However for biosensing and drug delivery applications, the focus of this thesis, the foreign body reaction can have detrimental effects on both the structural integrity of the implant and its functionality over time following implantation. This section summarizes a number of approaches that have been
developed for modulating the immune response around implanted biomaterials in an effort to reduce long-term inflammatory effects that may compromise the useful life of the implant.

2.4.2.1 Non-Biofouling Surface Coatings

The type, confluence, and conformation of adsorbed serum proteins depend on the terminal chemistry of the biomaterial. As this initial layer of adsorbed proteins provides binding sites for protein-specific receptors (integrins, pattern recognition receptors) on poly-morphonuclear leukocytes, monocytes and macrophages\textsuperscript{134}, several strategies that modulate the inflammatory response to a foreign object do so by treating implant surfaces with passive coatings that resist the adsorption of proteins and adhesion of cells, thereby reducing inflammatory processes like opsonization and phagocytosis\textsuperscript{131}. This can be achieved by coating the bulk material with non-fouling materials or through chemical surface modification using terminal functional groups present on the bulk material surface.

In addition to surface chemistry, surface topography also plays an important role in dictating the foreign-body reaction to an implant\textsuperscript{135,136}. In their natural environment, cells respond to ECM components in the nanometer scale in terms of adhesion, proliferation, migration, and gene expression\textsuperscript{134}. The morphology of implant surfaces has been demonstrated to affect the function of adsorbed fibroblasts, epithelial cells, endothelial cells, and macrophages, including macrophage cellular morphology, macrophage cytokine secretion, and macrophage adhesion independent of biomaterial surface chemistry\textsuperscript{134}.

2.4.2.2 Size-Exclusive Semi-Permeable Membranes

Size-exclusive semi-permeable membranes function as a barrier to antibodies and cells, while allowing the exchange of nutrients to and from the implanted cells\textsuperscript{82,85-87}. Typically these membrane are constructed from Ca-crosslinked alginate hydrogels, however other polymer compositions have been explored including alginate-PLL-alginate, alginate-PLO-alginate, and alginate-cellulose sulfate-poly(methylene-co-guanidine)\textsuperscript{137}. These materials have been employed to prevent immunogenic destruction of implanted islet cells in diabetic animals\textsuperscript{82,85-87}. 
2.4.2.3 Immobilization and Delivery of Anti-Inflammatory Drugs and bioactive compounds

Steroidal (i.e. glucocorticoids) and non-steroidal anti-inflammatory drugs have long been used to control inflammation and repair (fibrosis) in tissue\textsuperscript{128,129}. However, long-term systemic use of these drugs is not desirable due to major side effects that can develop with time. Local immobilization or delivery of these drugs may therefore be advantageous to their long-term application. Glucocorticoids inhibit inflammatory cell activation by abrogating the synthesis of inflammatory mediators including several cytokines and chemokines, prostaglandins, leukotrienes, proteolytic enzymes, free oxygen radicals and nitric oxide\textsuperscript{134,138}. Simultaneously, they promote resolution of inflammation and of adaptive immune response by enhancing anti-inflammatory cytokine release and suppressing cellular immunity in favor of humeral immunity and tolerance.
2.5 Breast Cancer

2.5.1 Epidemiology

Cancer affects a global demographic irrespective of sex and race, and is the leading cause of death in both low and middle income countries. There were 12.6 million new cases of cancer in 2008 worldwide and 7.5 million deaths from previously diagnosed cases\(^\text{139}\). In 2012, these numbers rose to 14.1 million new cases and 8.2 million deaths\(^\text{140}\). The adoption of lifestyle behaviors that are known to increase cancer risk including smoking, poor diet, physical inactivity have and will continue to increase the global incidence of cancer, with a projected 20 million new cancer cases annually expected as early as 2025\(^\text{140,141}\).

Breast cancer is the most prevalent form of cancer among women, particularly in developing countries, and is also the leading cause of death among women with cancer. In 2012, there were an estimated 1.6 million new cases of breast cancer and 521,900 deaths due to breast cancer\(^\text{141}\). Improved breast cancer screening techniques and more effective treatment strategies have resulted in a gradual decline of mortality in most developed countries since 1990, however this disease is still the second highest cause of death in women, second only to lung cancer\(^\text{141}\).

2.5.2 Cancer Pathogenesis

Cancer is fundamentally a disease of continuous and uncontrolled cell growth, with the potential to metastasize to distal sites of the body\(^\text{142,143}\). For breast cancer, as in other cancers, tumorigenesis is a multistep process whereby accumulation of successive random genetic mutations, which provide a cell with greater growth advantage, transform normal human cells into a highly malignant phenotype that is optimized for continued mitosis in a particular tissue environment. These mutations typically affect regulatory circuits that govern normal cell proliferation and homeostasis and may be broadly classified as oncogenes which promote cell growth and replication, and tumor suppressor genes that inhibit cell division and survival. Genetic alterations vary in severity and may consist of single point mutations, deletions and insertions within the promoter region or coding sequence of a gene, deletion or gain of a portion of a chromosome via genomic amplification or translocation, and gain or loss of an entire chromosome through errors in mitosis. Mutations that lead to cancer are not corrected by cellular machinery and fail to induce cell apoptosis resulting in survival and propagation of the mutation.
to daughter cells. Thus the mutations self-amplify and compound resulting in a tumor cell genome that contains numerous alterations at multiple sites.

More than a hundred distinct types of cancer and subtypes of tumors can be found within specific organs. Hanahan and Weinberg have proposed that the evolutionary process which gives rise to the malignant phenotype in all human cancer cells requires, at minimum, six essential rate-limiting alterations in cell physiology\textsuperscript{142,143}. The first prerequisite is an alteration that gives rise to acquired mitogenic growth signal autonomy to reduce tumor cell dependence on their microenvironment. This may be achieved through the development of autocrine growth factor stimulation, overexpression of growth factor cell surface receptors, alteration of down-stream signal transduction machinery to enhance growth factor signaling to the nucleus, and induction of local stromal cells to release paracrine growth factors. The second requirement is to override anti-proliferative mechanisms that maintain cell quiescence\textsuperscript{142}; often through loss of function mutations to signaling effectors that prevent cell cycle transition through the G1 phase, downregulation of integrin and adhesion molecules that transduce antigrowth signals, or disruption of molecular machinery that directs terminal differentiation. Third, cancer cells evolve mechanism for evading apoptosis, which is normally activated under abnormal intracellular or extracellular conditions including DNA damage, signaling imbalance provoked by oncogene action, survival factor insufficiency, or hypoxia.

While the first three alterations uncouple cell growth from its environment, on their own this set of mutations does not ensure expansive tumor growth due to the finite replicative potential inherent to each cell\textsuperscript{143}. The finite number of cell replication cycles is attributed to the inability of DNA polymerases to completely replicate the 3’ ends of chromosomal DNA during the S phase, resulting in progressive shortening of telomeres located at chromosome ends. Erosion of telomeres ultimately leads to end-to-end fusion of unprotected chromosome ends, resulting in cell death\textsuperscript{143}. Thus the fourth essential mutation provides cancer cells with limitless replicative potential through evolution of a telomere maintenance mechanism, including upregulation of telomerases. The fifth alteration essential for cancer progression is the ability to induce angiogenesis by enhanced production and release of pro-angiogenic factors and down-regulation of anti-angiogenic soluble factors. The ability to recruit blood vessels to solid tumors is essential to support sustained tumor growth as the growing cell population requires access to nutrients, oxygen and waste removal. The final alteration essential for malignant tumor progression is the
ability of some cancer cells to undergo tissue invasion and metastasis\textsuperscript{143,144}. Metastasis is a multistep process requiring tissue invasion, intravasation, embolization and transit, extravasation, and colonization of a distant tissue. As carcinomas progress to higher pathological grades of malignancy, reflected by local invasion of surrounding tissue followed by distant metastasis, the associated cancer cells develop alterations in cell shape and their ability to adhere to other cells and extracellular matrices. The best characterized alteration involves downregulation or mutational inactivation of E-cadherin, a cell-to-cell adhesion protein responsible for assembly of epithelial cell sheets and maintenance of quiescence among the constituent cells\textsuperscript{144}. Conversely, adhesion molecules normally associated with cell motility and migration, such as N-cadherin, are often upregulated in many invasive carcinoma cells. Metastasis is clinically important because metastatic disease is currently incurable\textsuperscript{21}.

### 2.5.3 Breast Cancer Classification

Despite the shared functional hallmarks among cancer cells, the cancer cell population itself is heterogeneous due to variable selective pressures of the tumor microenvironment and the random development of genetic mutations, causing divergence of cancer cell genotype and phenotype as the tumor grows. As such, breast cancer is a highly heterogeneous disease can be further classified based on the breast cancer molecular subtype to aid development of a personalized treatment plan that is effective against the patient’s particular disease.

**Table 3. Common molecular subtypes of breast cancer.**

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Characteristic</th>
<th>Prevalence</th>
<th>Treatment &amp; outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>• ER+ or PR+ &lt;br&gt; • HER2- &lt;br&gt; • Low Ki67 &lt;br&gt; • &lt;15% contain p53 gene mutations</td>
<td>30-70%</td>
<td>• Treatment includes hormone therapy. &lt;br&gt; • Best prognosis with high survival rates and low recurrence rates</td>
</tr>
<tr>
<td>Luminal B</td>
<td>• ER+ or PR+ &lt;br&gt; • HER2+ or HER2- with high Ki67 &lt;br&gt; • 30% contain p53 gene mutations</td>
<td>10-20%</td>
<td>• Treatment includes hormone therapy. &lt;br&gt; • High survival rates, however poorer prognosis compared to Luminal A tumors.</td>
</tr>
<tr>
<td>HER2+</td>
<td>• ER-, PR-, HER2+ &lt;br&gt; • 75% contain p53 gene mutation</td>
<td>5-15%</td>
<td>• Anti-HER2 drugs such as trastuzumab &lt;br&gt; • High survival rate</td>
</tr>
<tr>
<td>Triple negative/basal-like</td>
<td>• ER-, PR-, HER2- &lt;br&gt; • Most basal-like tumors contain p53 gene mutations &lt;br&gt; • Most BRCA1-related breast cancers are both triple negative and basal-like &lt;br&gt; • Typically aggressive</td>
<td>15-20%</td>
<td>• Combination of surgery, radiation therapy and chemotherapy. &lt;br&gt; • No targeted therapies. &lt;br&gt; • Poorer prognosis compared to ER+ subtypes.</td>
</tr>
</tbody>
</table>
At this time, molecular subtypes are used mostly in research settings and are not included in pathology reports. Prognosis and treatment decisions are still guided mainly by tumor stage, tumor grade, hormone receptor status and HER2 status.

2.5.4 Pathophysiology & Tumor Microenvironment

Much of what is known about the tumor microenvironment stem from the study of carcinomas, which represent roughly 80% of all breast cancers. The normal breast duct consists of a luminal layer of epithelial cells surrounded by myoepithelial cells, together which constitute the parenchyma. The parenchyma is distinct from the surrounding stroma, which is composed of mesenchymal cells (e.g., endothelial cells, immune cells, fibroblasts and adipocytes). Tumors arise when mutant cancer cells recruit and subvert normal cells to fuel and sustain rapid cancer cell expansion, resulting in a complex tissue composed of heterogeneous populations of cancer and normal cells supported by an evolving tumor-associated stroma and parenchyma.

Breast tumors progress through defined stages, typically starting with epithelial hyperproliferation followed by successive progression to ductal carcinoma in situ (DCIS), invasive ductal carcinoma (IDC), and finally metastatic carcinomas.

2.5.4.1 Stroma Cells in the Tumor Microenvironment

Tumor-associated stromal cells are active participants in tumorigenesis that serve as intracellular paracrine signaling partners for cancer cells and as primary effectors of the tumor microenvironment. Fibroblasts, the most abundant cells in connective tissues, are responsible for the production and remodeling of extracellular matrices upon which the tissues form. Within the tumor microenvironment, cancer cells secrete a number of soluble factors to recruit and induce activation of fibroblasts, similar to those present at chronic wound sites. Cancer associated fibroblasts and myofibroblasts in the tissue stroma in turn promote tumor onset by inducing pre-cancerous and cancerous epithelial cell growth by increasing Estradiol (E2) levels, sustain tumor growth through release of paracrine survival factors and, through secretion of extracellular matrix components, are implicated in the formation of the desmoplastic stroma that characterizes many advanced carcinomas. Cancer-associated fibroblasts are also implicated in inducing resistance to various chemotherapies by overexpression and secretion of collagen, which hinders
drug uptake in tumors, directly inducing tamoxifen resistance in various cancers by inducing mitochondrial dysfunction or epithelial morphogenesis in breast cancer cells.

Cancer associated fibroblasts signal recruitment of a variety of bone marrow-derived immune cells, including macrophages, neutrophils, mast cells and myeloid progenitors to tumor margins. These cells stimulate cancer cell proliferation, facilitate tissue invasion and support metastatic dissemination via production of proinvasive matrix-degrading enzymes including metalloproteinases, cysteine cathepsin proteases and heparanase within the tumor periphery. The peri-tumoral inflammatory cells also help to trigger and sustain angiogenesis in previously quiescent tissue. Vascular progenitor cells also migrate into neoplastic lesions and differentiate into neovascular-associated pericytes or endothelial cells. Pericytes are known to provide paracrine support signals to the surrounding endothelium. They also collaborate with endothelial cells to synthesize the vascular basement membrane which anchors both pericytes and endothelial cells, and further supports vessel walls to withstand the hydrostatic pressure of blood flow.

2.5.4.2 Hypoxia

As tumors grow beyond the diffusion limit of nutrients and oxygen, roughly 2 mm³ in volume, areas of hypoxia and acidosis develop within the tumor mass. As a result, tumors contain interspersed regions of well oxygenated (pO₂ > 2.5 mmHg) and hypoxic (pO₂ ≤ 2.5 mmHg) tissue. As previously discussed, cancer cells are able to adapt to thrive in areas of low oxygen concentrations that would otherwise induce cell death. One such adaption, known as the Warburg effect, involves switching of cellular metabolism from high efficiency aerobic oxidative phosphorylation via the Krebs cycle, a metabolic process that requires oxygen, to low efficiency anaerobic glycolysis for cellular chemical energy production. The Warburg effect enhances cancer cell survival in the hypoxic tumor microenvironment by decreasing oxygen requirements in regions distal from blood vessels, and by limiting the production of cytotoxic reactive oxygen species (ROS) that are the by-product of electron transport in the mitochondria during the Kreb cycle.

2.5.4.3 Angiogenesis

As a means to acquire nutrients, oxygen and to remove metabolic waste and carbon dioxide, tumors induce formation of new blood vessels via chronically activated angiogenesis and an
unbalanced mix of proangiogenic signals. Neovasculature produced under such conditions are aberrant. Vascular wall structures are composed of an irregular mosaic of cancer and endothelial cells marked with widened inter-endothelial cell junctions and numerous endothelial fenestrations, leading to distorted and enlarged vessels, leaky vessel walls, micro-hemorrhaging and abnormal levels of endothelial cell proliferation and apoptosis. The tumor vascular network lacks the normal hierarchial arrangement of arterioles, capillaries and venules, and is characterized by convoluted and excessive microvessel branching. This neovascular structure results in blood flow that is sluggish with unstable rheology, leading to heterogeneous tumor perfusion with hypoxia and acidity in low-flow regions. Production of lactic acid in the tumor microenvironment, coupled with insufficient blood supply and poor lymphatic drainage results in an acidic solid tumor microenvironment, typically ranging between pH 6.9 to 5.7.

2.5.5 Invasion and Metastasis

Metastasis is estimated to cause 90% of all human cancer deaths. Metastases are a result of cancer cells that undergo a successive sequence of distinct events known as the invasion-metastasis cascade. This multi-step process includes carcinoma epithelial to mesenchymal transition (EMT) and local invasion, followed by intravasation into local blood or lymphatic vessels, transit of cancer cells to a distant site, subsequent extravasation of cancer cells from the lumen into the parenchyma of distant tissues, mesenchymal to epithelial transition (MET), formation of micro-metastases, and finally colonization or growth if micro-metastatic lesions into macroscopic tumors within the distant microenvironment.

Carcinoma cells can acquire the ability to invade surrounding tissue, resist apoptosis and to disseminate by co-opting a process normally used during embryonic morphogenesis and wound healing known as the EMT. Cancer cells which undergo EMT are characterized by loss of adherens junctions, conversion from a polygonal/epithelial cell morphology to a spindly/fibroblastic morphology, express matrix-degrading enzymes, increase motility, and possess higher resistance to apoptosis. EMT in cancer cells may be induced from environmental stimuli including heterotypic interactions with adjacent tumor-associated stromal cells. The EMT program can be activated transiently or stably, and to various degrees, by carcinoma cells during the course of invasion and metastasis.
Macrophages at the tumor periphery help foster local tissue invasion by production and secretion of matrix-degrading enzymes such as metalloproteinases and cysteine cathepsin proteases. Tumor-associated macrophages also engage in paracrine signaling with cancer cells by supplying cancer cells with epidermal growth factor in response to IL-4 and CSF-1 mediated stimulation. These interactions facilitate intravasation of cancer cells into the circulatory system and dissemination to distal sites in the body.

2.5.6 Breast Cancer Metastasis to the Brain

2.5.6.1 Epidemiology & Pathogenesis

Breast cancer involving the CNS is traditionally viewed as a late stage complication of progressive metastatic disease. Symptomatic metastasis of breast cancer to the brain occurs in roughly 10-20% of patients with metastatic breast cancer, however results from autopsy studies suggest that between 20% and 40% of all patients with metastatic cancer have brain metastases. A retrospective survey of 219 women with brain metastases showed that 78% of the women presented with multiple intracerebral metastases, 14% had a solitary intracerebral metastasis, and 8% had leptomeningeal metastases. The onset of brain metastasis typically occurs 2 to 3 years after initial diagnosis of breast cancer and, in most cases, is accompanied by systemic metastases (e.g. lung, liver, lymph node and bone). Development of brain metastases in breast cancer patients is also more common in younger patients (<40-50 years) and in those who present with high grade primary tumors.

Mounting evidence indicates that primary breast cancer subtype is an important predictor of breast cancer brain metastasis. A recent retrospective analysis of primary breast cancer tumors from 349 patients, 55 patients of whom developed brain metastases, 254 patients who remained metastasis-free and 40 patients who developed extracranial metastases, showed that development of brain metastases was predicted by primary tumors which were positive for epidermal growth factor receptor (EGFR), overexpressed Her-2, were estrogen receptor negative (ER-) or were cytokeratin 5/6 positive. Genetic profiling of breast cancer brain metastasis cells further implicate cyclooxygenase-2 (COX-2), epidermal growth factor receptor (EGFR) ligand HBEGF, and the α2,6-sialyltransferase ST6GALNAC5 as mediators of cancer cell passage through the BBB.
The molecular basis through which these markers increase metastasis of breast cancer to the brain is largely unknown, however several recent studies provide some insight. HBEGF induces cancer cell motility and invasiveness while COX-2 mediates production of prostaglandin during inflammation and is known to increase vascular permeability, including the BBB\textsuperscript{153}. Both COX-2 and EGFR ligands have been previously linked to breast cancer metastasis to the lungs and the brain. In contrast, ST6GALNAC5 is a specific mediator of breast cancer cell infiltration through the BBB. Expression of ST6GALNAC5 by breast cancer cells, which is normally restricted to the brain, catalyzes sialylation of surface cell adhesion molecules to enhance cell adhesion to brain endothelial cells\textsuperscript{153}. Following breast cancer cell adhesion to brain endothelial cells, tumor cells grow along pre-existing brain vessels to form brain micrometastases prior to vascular associated parenchymal invasion\textsuperscript{154}. During this phase, endothelial BBB markers GLUT-1 and ZO-1, and BBB integrity remain unaltered for up to 14 days\textsuperscript{154}.

Subsequent tumor invasion and growth is mediated by several mechanisms. Early growth of breast cancer metastasis in the brain elicits a brain inflammatory response with extensive reactive gliosis surrounding the tumors and recruitment of large numbers of glial cells within the inner tumor mass\textsuperscript{155}. The glial cells secrete a number of soluble factors which induce increased proliferation of the metastatic cells\textsuperscript{155}. Glial cells also play a role in estrogen-mediated tumor growth. A recent study demonstrated that overiectomy decreased the frequency of MRI-detectable brain metastasis lesions from triple-negative breast cancer cells by 56% compared to overiectomized mice supplemented with exogenous 17-\(\beta\)-estradiol. \textit{In vitro} studies indicate that 17-\(\beta\)-estradiol-treated ER+ astrocytes increased proliferation, migration and invasion of MDA-MB-231BR cells, suggesting that estrogens act through ER+ astrocytes in the brain microenvironment to promote brain metastasis of triple negative breast cancer cells\textsuperscript{156}.

Recently Palmieri elucidated the role of Her-2 in breast cancer metastasis to the brain\textsuperscript{146}. Injection of MDA-MB-231-BR cells and Her-2 overexpressing MDA-MB-231-BR cells into the left cardiac ventricle of Balb/c nude mice resulted in formation of a similar number of micrometastases (<50 \(\mu\text{m}^2\)), however Her-2-overexpressing clones formed 3-fold greater number of large, clinically detectable, metastases (> 50 \(\mu\text{m}^2\))\textsuperscript{146}. Therefore the authors concluded that Her-2 increases outgrowth of metastatic tumor cells, but had no effect in the initial formation of micrometastases\textsuperscript{146}. The authors also compared the protein expression in three human breast cancer cells lines: a parental MDA-MB-231 cell line, a bone-seeking (MDA-MB-231-BO)
subline and a brain-seeking (MDA-MB-231-BR) subline and identified an increase in EGFR expression in the brain-seeking cells, but not the bone-seeking cells, compared to the parental MDA-MB-231 cells. Thus EGFR is thought to selectively direct breast cancer metastasis to the brain.

2.5.6.2 Blood Brain Barrier

The BBB is a physical barrier that regulates the passage of molecules from the bloodstream to the tissue of the CNS. This barrier primarily consists of capillary endothelial cells, pericytes, astrocytes and neurons, which together form the neurovascular unit (Figure 7). Unlike the endothelial cell lining of normal blood vessels, brain microvascular endothelial cells lack fenestrations and express tight junction associated proteins (e.g., including occluding, claudin-1, claudin-5 and junctional adhesion molecules) between adjacent cells resulting in a barrier that is characterized with low permeability to ions and small molecules and virtually impermeable to macromolecules. Brain endothelial cells further express a number of ATP-binding cassette transporters, including P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance protein (MDR1), on the luminal membrane to actively remove unwanted substrate from the CNS. Cytochromes P450 is also expressed in adult human BBB which acts as a metabolic barrier to regulate influx of cholesterol, retinoid and drugs into the brain. Under normal conditions, the BBB is able to abolish all paracellular diffusional pathways into the brain with the exception of the smallest lipophilic molecules. By restricting diffusion of molecules into the brain, the BBB is able to allow selective entry of certain molecules (e.g., glucose, low-density lipoproteins, transferrin, electrolytes, vitamins, amino acids) into brain via active molecular transporters.

The barrier to breast cancer metastasis to the brain is distinct from other organs. Capillaries in bone marrow and the liver are fenestrated, while those in the lung are backed by a basement membrane similar to the BBB. However the BBB possess the additional barriers comprised of tight junctions and astrocyte foot processes. These differences are thought to result in protracted progression of breast cancer metastasis to the brain as compared to other sites such as the lungs. These same barriers which hinder brain metastasis, however, are also responsible for preventing entry of chemotherapy drugs into the brain.
2.5.6.3 Blood-Brain Tumor Barrier

Metastasis of breast cancer to the brain arises from extravasation of circulating breast tumor cells into the brain parenchyma or the leptomeninges, followed by metastatic tumor growth. Tumor growths subvert normal endothelial cell function and disrupt the structure and microenvironment of the BBB leading to formation of the blood-brain tumor barrier (BTB) that is structurally and functionally distinct from the BBB (Figure 7). Brain tumor capillaries are composed of three distinct microvessel populations including continuous and non-fenestrated capillaries, continuous and fenestrated capillaries, and capillaries that contain inter-endothelial gaps which may be as large as 1 µm\(^{159,160}\). The presence of fenestra on the microvessel walls allow passage of small molecules into the brain while discontinuous capillaries additionally allow entry of macromolecules\(^{160}\).

A recent study investigated the passive permeability of the BTB in brain metastases of triple negative and Her2+ breast cancer established in mouse models\(^{161}\). The findings support the
notion that the BTB is compromised in comparison to the healthy BBB, with >89% of the lesions exhibiting increased permeability. However the authors note that the increase in permeability varied significantly both within different areas of a tumor and between different tumors, and that the changes in permeability were intermediate at 1.5-3.2 fold increase in 37-76% of metastases. While the uptake of paclitaxel and doxorubicin, two first-line chemotherapy drugs, was generally greater in tumor-bearing brains compared to normal brains, therapeutic drug concentrations were only achieved in ~10% of the metastases\textsuperscript{161}. Similar findings were observed in the BTB of glioblastomas in which large portions of the BTB more closely resemble the intact BBB, especially in lower grade diffuse gliomas\textsuperscript{159}. Therefore despite increased permeability to chemotherapy drugs, the BTB remains a significant barrier to drug accumulation within brain metastases of breast cancer. Increase in BTB permeability was correlated with lower basement membrane collagen IV expression and high pericyte desmin protein expression, but not with tumor size, vascular density or P-gp efflux transporter expression\textsuperscript{162}.

2.6 Breast Cancer Therapy

Breast cancer is a highly heterogeneous disease in terms of different histological subtypes, clinical presentations, and treatment sensitivity profiles. Therefore the correct treatment strategy must be devised by considering the tumor status (\textit{i.e.} tumor grade, receptor status), patient health, and previous therapeutic interventions\textsuperscript{163}. Breast cancer treatment strategies are often multimodal, integrating radiation or chemotherapy prior to (neoadjuvant) or following (adjuvant) surgical resection of the tumor mass.

2.6.1 Local Therapy

Local treatments for breast cancer include both surgical resection of the tumor mass and radiation therapy to induce tumor cell apoptosis. Surgical resection may comprise of either lumpectomy, the removal of tumor tissue and is breast-conserving, or mastectomy, the removal of the affected breast\textsuperscript{164,165}. Recent trends have shifted towards lumpectomy from mastectomy, especially in younger women with breast cancer. However this practice substantially increases the risk of local recurrence\textsuperscript{166}.

This increase risk of reoccurrence may be minimized when lumpectomy is combined with radiation therapy, which can reduce residual disease at the primary tumor site by as much as 3-
Focal exposure to radiation during radiation therapy reduces the exposure of normal tissue to radiation, however the risk of radiation injury to major tissues and organs such as the heart, lungs, and brachial plexus are present. The effectiveness of radiation therapy is also highly dependent on tumor vasculatation and local oxygen concentrations because oxygen proximal to DNA acts to stabilize DNA lesions caused by radiation, thus enhancing the mechanism of cell kill.

2.6.2 Systemic Therapy

Systemic therapies such as surgical resection and radiation therapy are not effective against metastatic disease. While metastatic breast cancer is currently incurable, systemic chemotherapy has a major role in its clinical management, both for delaying progression and for palliative care. Chemotherapy is often applied in combination with surgical resection of a tumor, in either a neoadjuvant or adjuvant regimen. The goal of neoadjuvant chemotherapy is to reduce the tumor bulk in an effort to reduce the extent of local surgery. Adjuvant chemotherapy is used to kill any remaining cancer cells at the resection site following surgical, or to treat systemic disease. Breast cancer chemotherapy can make use of a number of different agents from alkylation, antimetabolite, hormonal, or antibiotic drug classes. These agents may be administered individually, in sequence, or in combination. The prescribed drug regimen is dependent on tumor receptor status, previous treatment history, and the health of the patient.

2.6.2.1 Doxorubicin

Doxorubicin (Dox) (Figure 8) is a hydrophilic anthracycline antitumor antibiotic commonly used as a first-line chemotherapy agent, often in combination with other drugs, for various types of cancer including hematological malignancies, carcinomas and sarcomas. Its widespread use in the treatment of breast cancer has contributed to the decreased mortality rates in the past decades. Dox is commercially available as injections under the trade name Adriamycin® and Rubex®, however two PEGylated liposomal formulations with reduced side effects are also available as Doxil® and Caelyx®.

The broad-spectrum anticancer activity of Dox is attributed to several mechanism including (1) DNA intercalation by its planar three-ring structure leading to inhibition of macromolecular synthesis, (2) stabilization of the topoisomerase II complex after it has broken the DNA chain for replication, thus preventing resealing of the DNA double helix and stopping cell replication,
(3) increase production of cytotoxic quinone free radicals. Treatment of breast cancer with Dox chemotherapy is typically limited by serious systemic side effects including myelosuppression and congestive heart failure\textsuperscript{171,172}. Dox-induced cardiomyopathy appears to be cumulative, limiting the maximum lifetime dose of the drug in humans to 450 mg/m\textsuperscript{2} \textsuperscript{172}.

![Chemical structure of doxorubicin](image)

**Figure 8. Chemical structure of doxorubicin**

### 2.6.2.2 Docetaxel

Docetaxel (DTX) (Figure 9) is a semi-synthetic member of the taxoid class of chemotherapy agents. Its potent anti-mitotic effects stem from DTX stabilization of GDP-bound tubulin in the microtubules, thus inhibiting the process of microtubule depolymerization and cell division. DTX has also been found to lead to phosphorylation of oncoprotein bcl-2, which blocks cancer cell apoptosis\textsuperscript{173}. DTX is a first-line chemotherapy drug commonly used for the treatment of breast, lung, prostate, gastric, head and neck, and ovarian cancer. It is effective in the treatment of patients with locally advanced and metastatic breast cancer, with objective response rates between 54-69\% and 53-82\% when used as a first-line monotherapy or combination therapy, respectively, at a dose of 100 mg/m\textsuperscript{2} \textsuperscript{174}.

Docetaxel is a hydrophobic compound that is difficult to formulate. For this reason, the commercial formulation of DTX contains a high concentration of polysorbate 80 (1040 mg/mL) required for drug solubilization prior to administration. DTX is commercially available under the trade name Taxotere\textsuperscript{\textregistered} and Zydus\textsuperscript{\textregistered}.
2.6.3 Therapies for Brain Metastases

2.6.3.1 Clinical Treatment of Brain Metastases

Corticosteroids may offer symptomatic relief by within hours by reducing capillary permeability leading to decreased cerebral edema surrounding brain metastases\textsuperscript{22}. Surgical resection of metastases offers immediate decompression of large, symptomatic lesions and can improve quality of life and significantly prolong survival time. At present, up to three lesions may be removed surgically with a similar risk to that of a single lesion\textsuperscript{21,22}. However outcomes and applicability of this treatment option are also dependent on tumor size and location in the brain. Adjuvant radiotherapy is often administered following surgical resection as this combined approach has been shown to prolong median survival.

Whole brain radiation therapy (WBRT) is an alternative treatment that can improve quality of life and provide a median survival of 4 to 5 months\textsuperscript{22}. WBRT has been shown to reduce seizures and headaches in 75-85\% of patients, however motor loss is less effectively treated. Stereotactic radiosurgery (SRS) has emerged as an alternative treatment for the treatment of single or multiple lesions, and may be used in combination with neurosurgery or WBRT. SRS may provide nearly equivalent outcomes compared to surgery for single brain lesions. However the risk of neurotoxicity and local failure following SRS increases with size, therefore SRS is typically reserved for lesions with a diameter of 3 cm or less\textsuperscript{22}. Other factors which influence the use of SRS include accessibility of the lesion, symptoms, and functional status of the patient.

The mean 1-year survival of patients with brain metastases from breast cancer is estimated at 20\% while fewer than 2\% of patients survive for more than 2 years\textsuperscript{21}. The mean patient survival,
which typically ranges from 2 to 16 months, is dependent on disease involvement in the CNS, extent of extra-cranial metastatic disease, and the treatment applied. Currently the management of extracranial disease is the limiting factor in patient survival\textsuperscript{175}. However control of intracranial disease will become a more influential predictive factor as systemic therapies improve, as was the case for patients with Her-2 positive breast cancer metastases treated with the monoclonal antibody trastuzumab. For these patients the median survival was 13 months and nearly half of the patients died as a result of progressive CNS disease\textsuperscript{175}.

Chemotherapy has generally not been useful in the treatment of most epithelial cancers that metastasize to the brain due to limitations on drug delivery imposed by the blood-brain tumor barrier\textsuperscript{22,29}. It has been suggested that more than 98\% of CNS drugs fail to enter clinical trials due to poor brain penetration\textsuperscript{176}. However the BTB is frequently dysfunctional within brain metastases as previously discussed. Therefore many chemotherapeutic agents may in fact penetrate the BTB to some degree\textsuperscript{177}, however recent evidence suggests that the BTB still prevents the accumulation of therapeutic concentrations of drug in brain metastases of breast cancer\textsuperscript{161}. In most cases, chemotherapy is reserved for patients whose CNS disease has progressed despite WBRT or SRS. Selection of a chemotherapy drug is guided by the agent’s activity against breast cancer, with preference given to drugs that have been reported to enter the CNS\textsuperscript{22}.

### 2.6.3.2 Alternative Therapies

Various approaches have been investigated to enhance drug delivery to the brain including invasive and non-invasive means\textsuperscript{30,178,179}. Invasive approaches are expensive and associated with a high degree of patient discomfort and a high risk of complications. These approaches include intrathecal cerebrospinal fluid delivery (\textit{i.e.} into the lumbar subarachnoid space), intraventricular administration (\textit{i.e.} into the lateral ventricle), intratumoral injection, intracavitary injection (\textit{i.e.} into the resected tumor cavity)\textsuperscript{160}. These methods directly bypass the BTB, but are limited by local drug diffusion resulting in insufficient drug delivery, infections, neurotoxicity and catheter obstruction. A newer method, such as convection-enhanced delivery into the tumor parenchyma, overcomes drug diffusion limitations by supplementing drug delivery with convection. This method establishes a pressurized extracellular bulk flow at the cannula tip resulting in homogeneous distribution of drug at significant distances away from the cannula tip.
Disruption of the BBB is an alternative method to achieve drug delivery to the brain. This may be accomplished with the use of a hyperosmotic solution of mannitol to cause shrinkage of endothelial cells and subsequent opening of tight junctions for several hours. Other methods for inducing BBB disruption include the administration of vasoactive compounds including bradykinin, interleukin-2, and leukotriene C₄ which act to increase permeability of the BBB at the intercellular junction¹⁶⁰. During this time, circulating drugs may diffuse from the intra-arterial blood vessel lumen into the brain. However this method is limited due to uncontrolled influx of molecules and fluid into the entire brain, resulting in neurological toxicity, aphasia and hemiparesis³³. Inhibition of drug efflux transporters at the BBB is an alternative method for achieving therapeutic efficacy with drugs that are able to passively enter the brain. This method can increase drug penetration into the brain without compromising the integrity of the endothelial layer and tight junctions, and therefore avoids the cytotoxic side effects associated with BBB disruption¹⁶⁰.

Over the past decade, work by Steeg and coworkers has focused on identifying BBB-permeable small molecule drugs for prevention of brain metastases of breast cancer (prophylactic treatments). In a series of studies, they demonstrated that a number of these compounds successfully minimized the number of brain metastases developed following cardiac injection of brain-seeking triple negative breast cancer cells (MDA-MB-231-BR) or HER2-expressing MDA-MB0231-BR cells in a rodent model by early treatment (3-4 days following inoculation) with a variety of drugs including lapatinib¹⁸⁰,¹⁸¹, vorinostat¹⁸²,¹⁸³, pazopanib¹⁸⁴-¹⁸⁶, a Plk1 inhibitor GSK461364A¹⁸⁷, and a BBB-permeable taxane TPI-287¹⁸⁸.

Antibody drug conjugates represent an emerging and ever popular field of study in which therapeutic drug molecules are chemically bound to monoclonal antibodies via labile linkers to selectively target drugs to a specific site in the body. This strategy may be used to transport BBB-impermeable drugs into the CNS via receptor-mediated endocytosis following engagement of the antibody-drug conjugate with certain molecular transporters found on the luminal surface of brain microvessel endothelial cells, including the transferrin, insulin, low density lipoprotein receptors¹⁸⁹-¹⁹¹. The first demonstration of this principle utilized a monoclonal anti-transferrin receptor antibody, OX-26, to deliver BBB-impermeable methotrexate into the brain parenchyma¹⁹²,¹⁹³. More recent efforts have revealed that anti-transferrin antibodies which bind with high affinity to the transferrin receptor remain associated with the BBB, while those with
lower binding affinity are released from the BBB and improve brain exposure of conjugated drugs\textsuperscript{194}. Work by Niewoehner et al. further demonstrated that bivalent engagement of the transferrin receptor, as previously explored, interferes with normal transcytosis and induces lysosomal sorting and degradation of the antibody-drug conjugate, whereas monovalent binding to the receptor leads to successful transcytosis and increases brain exposure by over an order of magnitude\textsuperscript{195}.

While administration of BBB-permeable small molecule drugs and antibody-drug conjugates are promising, these strategies are relatively inefficient in terms of drug delivery efficiency due to rapid clearance of small molecules, metabolism of unprotected drugs in the blood, and low targeting moiety/payload efficiency common to drug-antibody conjugates\textsuperscript{196,197}. Nanoparticle drug delivery systems have been investigated to mitigate some of these challenges because they have exceptionally high loading capacity (e.g., $\geq 10,000$ drug molecules per liposome\textsuperscript{34}), are capable of protecting drug cargo, and may be designed with improved pharmacokinetics\textsuperscript{198}.

2.6.4 Nanoparticle Systems in Cancer Therapy

Classic chemotherapy is constrained by several factors including dose-limiting systemic toxicity, unfavorable biodistribution and pharmacokinetics leading to low drug accumulation within tumors, drug metabolism, and inherent or acquired drug resistance by cancer cells. Over the past two decades, nanoparticle drug carriers have emerged as an exciting field in drug delivery. These systems offer an alternative drug delivery method that promises to alleviate many of the challenges faced by classical chemotherapy drugs. By repackaging drug molecules within nanometer-sized vesicles or matrices composed of polymers, lipids, or inorganic materials, it is possible to tune the drug release profiles, tumor-accumulation, and pharmacokinetics of a drug to improve therapeutic outcomes. In addition, these engineered systems may be further customized by changing particle size, material, surface charge, and inclusion of various targeting moieties or enzymatic/environmental reactive groups. Co-delivery of multiple drugs and imaging agents for combination therapy or theranostic applications is also possible with the use of nanocarrier systems. Therefore the high degree of customizability offered by nanoparticles facilitates the rational design of drug delivery systems with unique capabilities.
2.6.4.1 Nanoparticle Design Parameters for Cancer Chemotherapy

Nanoparticle drug delivery systems are composed of three major constituents: the particle core, a surface which interacts with the physiological environment, and a payload. The payload is typically a chemotherapy drug which is typically, but not always, contained within the nanoparticle core.

The core serves three major functions: to provide structure to the nanoparticle, to efficiently load the payload, and to dictate drug release kinetics from the nanoparticle itself\textsuperscript{198}. Nanoparticle structure is dictated by the nanoparticle size and nanoparticle composition. The core must be of a suitable size for parenteral administration. Particles which are too small, typically less than 10 nm in diameter are rapidly eliminated from the circulation by the renal system\textsuperscript{198,199}. Particles which are too large may cause embolus formation following injection. The nanoparticle core may consist of a polymer or lipid matrix, as is the case for polymer nanogels, solid-lipid nanoparticles or micelles; or water surrounded by a lipid or polymer bilayer membrane in the case of liposomes\textsuperscript{198}. The core materials must be safe for injection, biodegradable, and their metabolic products must not be toxic. The loading capacity, the weight-by-weight percentage of drug contained within the nanoparticle, and loading efficiency, the percent of total drug encapsulated or conjugated to the nanoparticle during synthesis, must be as high as possible. Finally, the core is responsible for releasing the payload at a release rate suitable for the intended application\textsuperscript{199}. In most cases, dose dumping should be avoided as this typically leads to release of drug from the nanoparticle system before they accumulate at the tumor site.

The nanoparticle surface is responsible for interaction with the physiological environment\textsuperscript{200}. It dictates many essential nanoparticle properties including colloidal stability and biocompatibility after injection. The surface charge of a nanoparticle is a critical factor that dictates both stability, typically due to electrostatic repulsion of other nanoparticles to prevent aggregation, and biocompatibility through recruitment of complement proteins\textsuperscript{198}. Both the magnitude and change of the nanoparticle are important in dictating toxicity and biocompatibility of the formulation. Cationic nanoparticles induce significantly more hemolysis compared to anionic nanoparticles\textsuperscript{201}. Furthermore, a cationic charge reduces the nanoparticle’s circulation time due to electrostatic interaction with anionic tissues. Thus anionic nanoparticle formulations are preferred for drug delivery applications. The chemical structure of the nanoparticle surface further modulates
protein-nanoparticle interaction. Modification of the surface with hydrophilic polymers such as poly(ethylene glycol) (PEG) may reduce adsorption of plasma proteins onto the nanoparticle surface\textsuperscript{198}.

### 2.6.4.2 Nanoparticle Structure and Materials

Nanoparticle with a variety of different structures have been devised and, for drug delivery applications, are typically constructed using biocompatible and biodegradable polymers, lipids or a combination of both materials.

Liposomes are small vesicles of phospholipid/lipid bilayers which surround a central aqueous core. These structures may be composed of a single lipid bilayer (unilamellar liposomes) or several lipid bilayers (multilamellar liposomes). Surface modification may be accomplished by incorporating phospholipids conjugated with PEG or targeting moieties into the vesicle bilayer. Both hydrophilic and lipophilic drugs can be loaded into liposomes. Typically hydrophilic are loaded into the aqueous liposome core while lipophilic drugs may partition into the phospholipid/lipid bilayers. Liposomes are perhaps the most widely studied nanoparticle systems due to their excellent \textit{in vivo} stability, low systemic toxicity and long history of use in the clinic since 1995 when Doxil\textsuperscript{®}, a liposomal formulation of doxorubicin, became the first nanomedicine to be approved by the FDA (1995)\textsuperscript{202}. Limitations of the liposomal formulation include the inability to provide sufficient drug release rate, despite long blood circulation time and high tumor accumulation, resulting in low bioavailability of doxorubicin at the tumor site\textsuperscript{203}.

Normal-phase (oil-in-water) micelles, typical in biomedical applications, are supramolecular assemblies of amphiphilic molecules, typically phospholipids or block copolymers, dispersed in an aqueous medium. These structures assemble when the surfactant concentration exceeds the critical micelle concentration. Under such conditions, micelle assembly is driven by the hydrophobic effect in which hydrophobic tails cluster together and are sequestered away from surrounding water molecules, leaving the hydrophilic head regions in contact with the aqueous environment. These particles are therefore composed of a hydrophobic core into which lipophilic compounds may be loaded. Micelles tend to be smaller in size compared to other nanoparticles, typically between 2 and 20 nm. Micelles constructed from block copolymers may be larger due to the increased size of their constituent molecules.
Solid lipid nanoparticles are composed of a solid lipid core matrix stabilized with the use of surfactants. Typically SLNs are constructed using physiological lipids such as fatty acids, mono-, di- or triglycerides, glycerine mixtures and/or waxes. These structures may efficiently incorporate hydrophobic drugs within their solid lipid core and provide sustained release of the encapsulated drug. Disadvantages of SLN include their low encapsulation efficiency for hydrophilic compounds and the use of high temperatures for SLN preparation, which can degrade heat-labile drugs. Encapsulation of hydrophilic compounds may be improved by including amphiphilic polymer into the solid lipid core to form complexes with charged, water-soluble molecules. These structures are typically known as polymer-lipid hybrid nanoparticles (PLN).

Polymer nanoparticles include both polymer-based liposomes (i.e. polymersomes), as previously discussed, and nano-sized hydrogels (nanogels). Nanogels are physically or chemically cross-linked networks of hydrophilic polymer. They may be synthesized from both natural (e.g., chitosan, gelatin, albumin) or synthetic polymers (e.g., poly(lactic acid) (PLA), poly(D,L-lactide-co-glycolide) (PLGA), poly(ε-caprolactone) (PCL), poly(alkyl-acyanoacrylate)). The cross-linked polymer structure contains numerous functional groups that can be used for loading of both hydrophilic and lipophilic drugs, or for further chemical modification with various functional moieties (e.g., targeting ligands, stimuli-responsive elements, imaging agents) resulting in highly customizable nanocarriers suitable for targeted or controlled drug delivery.

2.6.4.3 Targeting Tumor Tissue with Nanoparticles

2.6.4.3.1 Passive Tumor Targeting

The tumor vasculature must constantly adapt to support continuous growth of cancer cells, as previously discussed. The resulting tumor neovasculature is often tortuous, highly branched, lacking of a proper basement membrane and replete with fenestrations and poorly formed inter-endothelial cell junctions. These defects in blood vessel structure result in a tumor vasculature that is highly permeable, leading to accumulation of cells, molecules and even nanoparticles in the interstitial tumor space. In addition, the rate of clearance of nanoparticles from the tumor is impaired due to poor tumor lymphatic drainage, promoting the enhanced retention of nanoparticles in tumor tissue. Taken together, the enhance permeability of nanoparticles in tumor tissue and reduced clearance rates result in an Enhanced Permeability and Retention (EPR) effect.
for nanoparticles (Figure 10)\textsuperscript{205-207}. This effect provides a passive mechanism by which nanoparticles selectively accumulate within tumor tissue for targeted drug delivery.

![Diagram of passive tumor targeting](image)

Figure 10. Passive tumor targeting is achieved by extravasation of nanoparticles through fenestrations present in the tumor vasculature and ineffective lymphatic drainage (EPR effect). Inset: active cellular targeting may promote cell-specific recognition, binding and internalization following passive accumulation within the tumor microenvironment. Adapted with permission from the publisher\textsuperscript{207}.

To take advantage of the EPR effect, nanoparticles must possess several critical properties. The first such property is a proper particle size. Particles which are smaller than 10 nm are rapidly cleared through the kidneys following parenteral administration, preventing adequate time for tumor accumulation. Conversely, particles which are larger than 400 nm do not benefit from the EPR effect as these particles are too large to take advantage of the leaky vasculature\textsuperscript{199}. Thus, the optimal particle size range for passive tumor targeting by the EPR effect is between 10 and 400 nm. A second critical parameter is the ability of nanoparticles to remain in the circulation for an extended period of time in order to take advantage of the EPR effect\textsuperscript{208}. Foreign materials, including nanoparticle drug delivery systems, are promptly removed from the circulation by the complement system via cells of the mononuclear phagocyte system (MPS), after recognition of immunoglobulins and complement proteins on the nanoparticle surface. The gold standard for
prolonging nanoparticle circulation is to impart these systems with a “stealth” coat of poly(ethylene glycol) (PEG). PEGylation resists nanoparticle opsonization by masking any surface charges that would otherwise promote opsonin binding, by enhancing steric repulsion between the nanoparticle surface and blood components, and by forming an inert and impenetrable polymer layer over the nanoparticle surface. Furthermore, the nanoparticle surface must be anionic or neutral to avoid unfavorable ionic interactions with anionic lumen of the vasculature\textsuperscript{205}. EPR effect-mediated passive tumor uptake is optimized with circulations times of at least 6 hours\textsuperscript{205,206}.

2.6.4.3.2 Active Tumor Targeting

Active targeting is the directed accumulation of nanoparticles to specific tissues or to specific cell types through the conjugation of recognition ligand to the nanoparticle surface\textsuperscript{209}. These ligands are selected based on their ability to recognize a particular cell-surface marker with high affinity and high specificity. This ligand-target recognition facilitates drug delivery to a specific site, with the additional benefit of reducing drug exposure to non-target sites. In active nanoparticle targeting to breast cancer, the target is typically a specific cancer cell or tumor-associated vascular endothelial cell. Targeting moieties may take the form of antibodies, antibody fragments, proteins, aptamers, peptides, or small molecules. However the presentation of an active targeting ligand on the nanoparticle surface presents somewhat of a conundrum in that this biologically active molecule may act as an opsonin or promote nanoparticle opsonization, ultimately promoting clearance by the MPS system\textsuperscript{210}. Furthermore, as nanoparticles extravasate through the tumor tissue, active tumor targeting enhances nanoparticle binding to their target cell surface and simultaneously clears the interstitial fluid of free nanoparticles\textsuperscript{211}. This effect limits the diffusion distance of nanoparticles through a solid tumor and may in fact lead to poor tumor penetration\textsuperscript{212}. Thus the net benefits of active nanoparticle targeting may not be apparent in every situation.

2.6.4.4 Nanoparticle Systems for Brain Cancer

More than 98\% of chemotherapy drugs are unable to penetrate through the BBB\textsuperscript{176} and a vast number of these are also substrates of efflux transporters at the BBB\textsuperscript{25}. Therefore most chemotherapy drugs are unable to accumulate within the CNS at therapeutic concentrations leading to treatment failure\textsuperscript{161,213}. To overcome these challenges, chemotherapy drugs may be
loaded into BBB-penetrating nanoparticle drug carrier systems to improve drug bioavailability in the brain, leading to improved brain cancer therapy. In general, nanoparticle carrier systems may be engineered for non-invasive transport into the CNS by surface modification with targeting moieties that preferentially bind to certain receptors or transporters expressed on the luminal membrane of brain capillary endothelial cells for selective transcytosis across the BBB (Figure 11)\textsuperscript{25,214}. Common targeting moieties include antibodies or substrates that selectively bind insulin\textsuperscript{215,216}, transferrin\textsuperscript{217-219}, EGF, low density lipoprotein (LDL)\textsuperscript{40,220,221}, glutathione receptor\textsuperscript{222}, thiamine, folate, glycoside, and lactoferrin receptors\textsuperscript{25}. Cell penetrating peptides\textsuperscript{223}, cationic polymers\textsuperscript{204,219,220}, surfactants\textsuperscript{224} and targeting moieties to CD44\textsuperscript{177} have also been employed to coat nanoparticles for targeted drug delivery into the brain\textsuperscript{26,42,43,225,226}.

Work by Pardridge and co-workers first demonstrated that OX-26, a monoclonal antibody that binds to rat transferrin receptors found on brain endothelial cells, can be bound to daunomycin-loaded pegylated liposomes to facilitate transcytosis across the BBB. They identified further identified the optimal OX-26 content per liposome (~30) to maximize nanoparticle accumulation in the brain and showed that conjugation of PEG-liposomes to OX-26 greatly enhanced the carrying capacity of each OX-26 mAb molecule by up to 4 logarithmic orders in magnitude\textsuperscript{34}. Other transferrin receptor targeted moieties including transferrin\textsuperscript{227-229} and 8D3 anti-mouse transferrin receptor mAb\textsuperscript{219,230,231} have also shown promise in delivering nanoparticles and BBB-impermeable drugs into the brain.

Studies by Kreuter and co-workers have demonstrated that surfactant coatings, typically polysorbate 80 or poloxamer 188 \textit{(i.e.} Pluronic F-68), are able to facilitate nanoparticle passage through the BBB and delivery of therapeutics in the CNS\textsuperscript{23,232}. Passage through the BBB is facilitated by adsorption of endogenous apolipoproteins \textit{(e.g.,} apolipoprotein E) onto the surfactant coating followed by transcytosis across brain microvessel endothelial cells \textit{via} low-density lipoprotein receptor (LRP1)-mediated endocytosis\textsuperscript{40,233}. PLGA and poly(butyl cyanoacrylate) nanoparticles coated with these surfactants have been used for drug delivery in the treatment of glioblastomas\textsuperscript{50,233,234} and other CNS diseases\textsuperscript{235}. 
Figure 11. Schematic illustrating the main molecular trafficking routes across the BBB. Adapted with permission from Abbott et al. \textsuperscript{214}.
Chapter 3

Thermostable Poloxamer Gel Formulation of Highly Concentrated Insulin Enables Long-Term Sustained Drug Release from Micro-fabricated Implantable Devices

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To be submitted.

All work in this manuscript was performed by J. Li with assistance from the co-authors.
3.1 Abstract

Development of stable high concentration formulations of protein and peptide drugs is a major challenge due to increased susceptibility to oxidation, deamidation, aggregation and precipitation at higher drug concentrations. Numerous drug delivery applications including implantable, transdermal, portable/wearable and controlled release dosage forms would benefit immensely from such formulations due to longer efficacy durations and reduced size requirements. We report a poloxamer gel formulation of highly concentrated insulin, up to 80 mg/mL (2160 IU/mL), which exhibits excellent insulin stability for up to 30 days under conditions of continuous shear at 37°C. Insulin stability was found to be improved with increased poloxamer concentration, as evidenced by reduced degradation products and preserved secondary structure. In contrast, insulin stability was found to be independent of insulin concentration. Enhanced insulin stability is attributed to both the surfactant properties of poloxamer and increased formulation viscosity. The poloxamer gel formulations further demonstrated linear insulin release profiles from a microfabricated diffusion-controlled membrane-reservoir drug delivery device with no observable burst or lag effects. Insulin release kinetics are found to be dependent on both membrane porosity and formulation poloxamer concentration. Subcutaneous implantation of the devices into diabetic rats resulted in sustained insulin delivery and maintenance of normal blood glucose levels for the duration of drug release. These findings suggest that poloxamer gel formulations may be useful for enhancing the long-term stability of highly concentrated biological macromolecules under physiological conditions and are suitable for long-term and implantable drug delivery applications.

3.2 Introduction

Insulin is a peptide hormone responsible for the regulation of glucose concentration in the blood. Its discovery in 1921 transformed the treatment of diabetes and remains the cornerstone for insulin therapy in all Type 1 Diabetes (T1D) and 30% of Type 2 Diabetes (T2D) patients today. A number of formulations have been developed for injection using a syringe or metered insulin pen devices, and more recently insulin pumps, however these formulations are of relatively low insulin concentration and therefore unsuited for long-term implantable insulin delivery systems given the large reservoir volume required. Formulation of a highly concentrated stable formulation of insulin is also required for development of smaller and more portable insulin
pumps and transdermal delivery systems. To date, the highest concentration insulin formulation on the market is Humulin U-500 from Eli Lilly with a concentration of 500 units per mL.

Development of high concentration formulations of insulin is limited by its physical (*i.e.* denaturation and fibrillation) and chemical (*i.e.* hydrolysis and deamidation) instability at high concentrations, especially at elevated temperatures or under the influence of agitation induced shear forces\textsuperscript{121}. Insulin aggregation proceeds *via* monomer interaction with hydrophobic surfaces (*e.g.*, air bubbles) in which the monomeric species partially unfolds upon adsorption and either returns to its native conformation or, in combination with other unfolded species, initiates nucleation of a stable intermediate aggregate that may go on to interact with native molecules to form fibrillary interdigitated beta-sheets, resulting in loss of insulin activity\textsuperscript{122}. Non-enzyme degradation of insulin typically results from hydrolysis and deamidation of asparagine residues. In the absence of zinc, insulin stability decreases with increasing concentration due to greater intermolecular interaction and more rapid aggregation\textsuperscript{122-124}.

Zinc ions are commonly used to stabilize insulin formulations by facilitating self-association of insulin monomers and dimers into more stable hexamers\textsuperscript{122,124,236,237}, however hexamer formation delays the onset of action of insulin which is undesirable for many controlled drug delivery systems, including the artificial pancreas. Thus commercial insulin analogues marketed for use in pumps are designed to prevent hexamer formation to ensure rapid onset of action. Increased insulin stability has also been realized through amino acid substitution or chemical modification of native insulin to induce self-assembly of more stable higher-order structures or to introduce additional disulfide bonds to stabilize the insulin monomer\textsuperscript{238}, which typically results in altered onset and action times of insulin. Use of antimicrobial phenolic compounds such as phenol or meta-cresol is also able to increase stability of native insulin, however their use has been linked to a number of detrimental side effects including cancer\textsuperscript{236,239}. Cyclodextrins and surfactants such as lecithins, polysorbates and poloxamers (Pluronic\textsuperscript{®}) may also preserve insulin stability by binding to hydrophobic interfaces to inhibit their interaction with insulin\textsuperscript{240}. Various formulations utilize polygeline, methyl cellulose (MC) or hydroxypropylmethylcellulose (HPMC) additives to increase solution viscosity with the goal of reducing flow-induced shear stresses. The above strategies have proven successful at improving insulin stability in dilute formulations, however maintaining long-term stability of insulin in concentrated formulations remains a challenge.
Here we develop a poloxamer 407 (Pluronic® F-127) gel formulation of highly concentrated insulin (80 mg/mL or 2160 IU/mL) that is suitable for long-term sustained insulin release due to preserved insulin stability for more than 30 days under conditions of physical shear at physiological temperature (37°C). Poloxamers are amphiphilic block copolymers of hydrophilic ethylene oxide and hydrophobic propylene oxide. Concentrated poloxamer formulations possess a number of unique material properties that are ideal for stabilizing insulin including excellent tolerance by the body and temperature-dependent sol-gel transition which is convenient for easy handling, loading and injection into devices at low temperatures. Interestingly, the viscosity of poloxamer solutions is dependent on polymer concentration and can form viscous gels at high concentrations when heated above the sol-gel transition temperature. Therefore enhanced insulin stability in our formulation is achieved using Pluronic® F-127 to shield insulin from hydrophobic interfaces/residues while simultaneously retarding shear forces through enhanced viscosity.

Previous studies have investigated the use of low concentrations of poloxamer as additives for stabilizing insulin solutions by occupation of hydrophobic surfaces, however these studies of dilute poloxamer solutions did not explore the role of viscosity enhancement on insulin stability. Other studies utilized the thermal-triggered gelation properties of high concentration poloxamer solutions to form subcutaneous in situ gel depots for sustained insulin release or adhesive gel formulations for transdermal, rectal, and buccal delivery of insulin, however the structural stability of insulin in these formulations at physiological temperatures were largely not investigated. To date, the role of viscosity enhancement in poloxamer insulin formulations to reduce interfacial shear forces and enhance insulin stability have not been studied. Furthermore, there have been no attempts to develop thermally stable formulations of highly concentrated insulin suitable for long-term implantable systems using poloxamer solutions or other methods.

In this study, we examine the effect of poloxamer concentration on the formation of high concentration insulin gels (40 – 80 mg/mL) and long-term structural stability of insulin when subjected to continuous shear at physiological temperatures. We further developed a sustained release membrane-reservoir implant loaded with the thermostable insulin gel formulation and demonstrate linear in vitro insulin release kinetics over a 30-day period. Finally, we demonstrate long-term maintenance of normal blood glucose levels in diabetic rats following subcutaneous implantation of the sustained-release insulin delivery device.
3.3 Materials and Methods

3.3.1 Materials

All chemicals were used without further purification. USP grade human recombinant insulin (27 U·mg⁻¹) was purchased from Wisent (Quebec, Canada). Pluronic® F-127 (PF-127) was provided by BASF Corporation (Ludwigshafen, Germany). Acetonitrile (HPLC grade) and distilled deionized (DDI) water (HPLC grade) was purchased from Caledon Labs (ON, Canada). Trifluoroacetic acid (TFA) was purchased from Sigma Aldrich (ON, Canada).

Nalgene Pharma Grade Silicone Tubing (8600-0020, 1/16” ID x 1/8” OD) was purchased from Thermo Fisher Scientific (MA, USA). Ethylene-vinyl acetate copolymer resin (EVAC, ELVAX-40W) was provided by DuPont (DE, USA). Polydimethylsiloxane (PDMS, Sylgard 184) was purchased from Dow Corning Corporation (MI, USA). S1805 photoresist, SU-8 photoresist and SU-8 developer (1-Methoxy-2-propyl acetate) were purchased from Microchem (MA, USA). Distilled and deionized (DDI) water was obtained from a Milli-Q water purifier (Milli-Pore Inc., MA, USA).

3.3.2 Insulin Formulation

Formulations with varying concentrations of human recombinant insulin and Pluronic F-127 were prepared in a glass vial according to Table 1. In a typical preparation, insulin was dissolved in 800 µL DDI water. 1M NaOH was added dropwise until the insulin was completely solubilized. Solution pH was then adjusted to 8.0 by dropwise addition of 1M HCl and topped up to 1000 µL with DDI water. The solution was agitated to re-solubilize any formed aggregates. Pluronic F-127 was then added to the solution and allowed to dissolve for 24 h at 4°C. The formulation was gently stirred for 30 minutes at 4°C and used immediately. Turbidity visually was checked to ensure complete dissolution of insulin. Final Pluronic F-127 concentrations were 0, 15, 20, or 25% w/w. Final insulin concentrations were 40, 60, or 80 mg/mL.
Table 4. Composition of poloxamer gel insulin formulations.

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<th>Insulin (mg)</th>
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### 3.3.3 Insulin Stability

Insulin formulations were transferred into glass vials, sealed and placed in a 37°C oven to induce formation of a transparent gel. The vials were then placed on an orbital shaker (90 rev/min) and maintained at 37°C in an oven for a duration of 30 days. Aliquots for stability analysis were taken at predetermined time points by temporarily removing the gels from the oven and cooling them to 4°C. The aliquots were diluted in pH 7.4 phosphate buffered saline (PBS) to an insulin concentration of 0.5 mg/mL and stored at 4°C for subsequent analysis. The vials containing the insulin gel formulations were then returned to the oven at 37°C for 30 min to induce gel formation and subsequently placed on the rocking plate at 37°C.

### 3.3.4 RP-HPLC Analysis

Insulin and its degradation products were assayed using a reversed-phase high performance liquid chromatography (RP-HPLC) system (Waters, MA, USA) equipped with a binary pump, autosampler and UV detector (2899 Photodiode Detector Array, Waters, MA, USA). Insulin was separated from its degradation products on a C-18 column (Gemini-NX 3u C18 110Å 50×2 mm, Phenomenex, CA, USA). For all separations 40 μL was injected on to the column running at a flow rate of 0.5 mL/min. Mobile phase A was 0.1% (v/v) TFA in water and mobile phase B was 0.1% (v/v) TFA in acetonitrile. A linear gradient from 15%B to 65%B over 10 minutes, followed by a linear gradient from 65%B to 15%B over 10 minutes was used for all separations. The column was washed using 85%A and 15%B for 15 minutes between samples. Elution times of insulin and its degradation products was monitored at 215 nm wavelength. The column was maintained at 37°C throughout the experiment.
3.3.5 Circular Dichroism Analysis

Circular dichroism (CD) spectroscopy in the far ultraviolet wavelength region, from 200 nm to 260 nm, was used to assess the secondary structure of insulin. The CD spectra were measured using a circular dichroism spectropolarimeter (Jasco J-810, MD, USA) equipped with a Peltier temperature controller operated at 25°C. Samples were diluted to an insulin concentration of 31.25 µg/mL and transferred to a quartz cuvette with a 1 cm path length for far-UV measurements. Samples were scanned at 1 nm intervals between 200 nm and 260 nm using an 8 s response time. Measurement were repeated 3 times and averaged. Spectra deconvolution to predict protein secondary structure was performed using the CDSSTR variable selection method implemented using CDPro software (Colorado State University).

3.3.6 Preparation of Implantable Sustained-Release Device

A silicone microporous membrane with carefully controlled geometry was prepared using a self-release soft-lithography method described in Chapter 4. Briefly, a microporous membrane replica mold was constructed using standard microfabrication methods (Chapter 4, Figure 18). A 60 µm layer of SU-8 50 was spin coated onto a 4 inch silicon wafer substrate and baked at 65°C for 1 min followed by 95°C for 5 min. Arrays of 60 µm tall free-standing pillars were formed by selective photo polymerization of the SU-8 layer using photolithography followed by a 1 min bake at 65°C and a 5 min bake at 95°C. Uncrosslinked SU-8 was removed using SU-8 developer. The replica mold was rinsed with isopropyl alcohol, DDI water, and gently dried under nitrogen. A final bake was performed at 170°C for 4 h. Separate replica molds were fabricated with 3068 µm², 7854 µm² and 49286 µm² permeation areas, which corresponds to membrane porosities of 0.31%, 0.79% and 4.91% respectively. After cooling to room temperature, the molds were coated with a 1 µm thick sacrificial layer of Shipley S1805 positive photoresist and baked at 120°C for 90s. Degassed PDMS pre-polymer mix (1:10 ratio of polymer to cross-linker) was then spin-coated onto the mold and cured at 120°C for 5 min resulting in a polymerized 100 µm thick PDMS membrane adhered to the mold surface. Self-release of the microporous PDMS membrane was achieved by scoring the wafer edges and soaking the entire mold in acetone. The PDMS membrane was transferred onto a sheet of aluminum foil for subsequent handling. Membrane geometry was verified using a Dektak Profilometer (Bruker, USA) and under SEM.
To form implantable membrane-reservoir devices, individual porous membranes were bonded to 3 cm long sections of pharmaceutical grade silicone tubing at 120°C after treatment with oxygen plasma. The devices were oxidized for 5 min (5 W Harrick Plasma Cleaner/Sterilizer PDC-3XG) and placed in a 0.1 M APTES anhydrous ethanol solution for 12 h. The silanized tubing was extensively rinsed with ethanol, dried under nitrogen at room temperature, and incubated in a 4-nitrophenyl chloroformate-activated poly(ethylene glycol) methyl ether (mPEG, 20 kDa) solution (0.01 M activated PEG in pH 7.4 PBS) for 48 h at 4°C to ensure modification of all device surfaces. Devices were extensively washed with DDI water, sealed on the back end with EVAC copolymer by dip-coating in a 20% EVAC dichloromethane solution, dried under nitrogen and stored in pH 7.4 PBS at 4°C prior to use.

3.3.7 In Vitro Insulin Release

To evaluate insulin release kinetics from the devices, individual devices were placed on ice and filled with 60 μL of insulin formulation. The devices were then heated to 37°C to allow for gelation and subsequently incubated in 4 mL of pH 7.4 PBS release medium. Vials were placed on a rotating plate and maintained at 37°C for the duration of the experiment. Insulin release from each device was measured intermittently using UV absorbance at a wavelength of 276 nm.

3.3.8 Animal Model

All in vivo procedures strictly complied with the ethical and legal requirements under Ontario’s Animals for Research Act and the Federal Canadian Council on Animal Care guidelines for the care and use of laboratory animals and were approved by the University Animal Care Committee of the University of Toronto.

Male Sprague Dawley rats (300-350 g, Charles River Laboratories, NC, USA) were used to establish a T1D model for in vivo efficacy studies. Streptozotocin, prepared in a sterile saline solution, was administered to healthy rats via intraperitoneal injection at a dose of 65 mg·kg⁻¹. Animals were allowed three days of resting to confirm the diabetic state. The rats were housed individually on a reverse light-cycle and were given ad libitum access to food and water.
3.3.9 *In vivo* Efficacy

The ability of the sustained insulin delivery implants to maintain normal blood glucose levels was evaluated in STZ-induced diabetic adult male Sprague-Dawley rats. A one 0.5 cm midline incision was made in the lower abdomen followed by blunt dissection to create subcutaneous pockets for device implantation. The rats were separated into 2 groups, with each animal receiving either a microporous membrane-capped device (0.79% porosity) loaded with 80 mg·mL⁻¹ insulin gel formulation (25% Pluronic F-127), or an identical device loaded with saline. Blood glucose levels were measured pre-surgery and post-surgery to ensure that the rats recovered properly and did not suffer from severe hypoglycemia. Blood glucose levels were measured regularly over the course of 34 days using a glucose meter (OneTouch® Ultra®, LifeScan Inc., USA). Rat weight and health was monitored regularly for the duration of the study.

3.4 Results and Discussion

3.4.1 Properties of Poloxamer Gel Formulation of Native Insulin

Commercial formulations of insulin are typically prepared at acidic or neutral conditions. The present formulations were developed at slightly basic conditions (pH 8.0) to minimize hydrolytic deamidation commonly encountered in acidic insulin preparations and to mitigate poor insulin solubility at neutral pH. Formulations composed of 25% w/w Pluronic F-127 were capable of completely solubilizing insulin at drug concentrations up to 80 mg/mL. These formulations remained transparent and formed viscous gels at 37°C. Further addition of insulin above 80 mg/mL or Pluronic F-127 above 25 wt.% resulted in incomplete dissolution of one or both components. Compared to previous studies which reported Pluronic F-127 solubility limits in excess of 30%, the lower solubility limit observed in the current formulations is attributed to the high insulin content which reduces the availability of free water in the system.

3.4.2 Effect of Time, Poloxamer Content and Insulin Concentration on Insulin Degradation

Separation between insulin and its breakdown products was achieved using RP-HPLC. Insulin from freshly prepared formulations eluted as a well-defined peak at ~11.8 minutes retention time with no detectable contaminants (Figure 12). All insulin formulations exhibited time-dependent
degradation under conditions of continuous shear at 37°C as indicated by decreased insulin peak height and concurrent appearance of additional chromatogram peaks at 10.4, 10.7, 10.8, 10.9 and 13.2 minutes retention time (Figure 12, Figure 13a). These new peaks correspond to known insulin degradation products produced by deamidation and other mechanisms\textsuperscript{254}. Close inspection of the chromatograms reveals an additional peak or shoulder at ~12.1 minutes retention time, however this peak is not well resolved and therefore was not quantified. For this reason, peak height was used to quantify the various eluting species.

Insulin was rapidly degraded in the absence of Pluronic F-127 (0% PF-127, 80 mg/mL insulin) as indicated by near-complete disappearance of the primary insulin peak within 10 days (Figure 12a). In contrast, insulin degradation and formation of degradation products was substantially delayed in formulations with increasing poloxamer content (Figure 12b-d, Figure 13b). The best performing formulation, composed of 80 mg/mL insulin and 25 wt. % PF-127, exhibited complete retention of the insulin peak at day 10, and 55% loss after 30 days (Figure 12d, Figure 13a). This formulation also produced the least amount of degradation products at day 30 compared to the other formulations (Figure 12d, Figure 13c). These results demonstrate that poloxamer concentration within the present formulation plays a critical role in preserving insulin stability and limiting the formation of insulin degradation products. The presence of poloxamer is thought to shield insulin interaction with hydrophobic surfaces and unfolded insulin molecules. The use of high concentration poloxamer within the formulation additionally results in the formation of a viscous gel at physiological temperatures, which serves to retard convection in the presence of agitation and additionally reduce the rate of insulin diffusion within the formulation. Both effects serve to minimize interaction between insulin molecules and hydrophobic domains resulting in higher insulin stability. Insulin degradation over time is inevitable under the experimental conditions, however rate at which this process proceeds may be considerably reduced with the use of poloxamer gels.

The insulin peak height among formulations with different insulin concentrations (40, 60 and 80 mg/mL) were not significantly different suggesting that insulin concentration did not affect insulin stability Figure 13d). This observation may be a result of decreased interaction between insulin molecules due to the high viscosity of poloxamer gel despite high insulin concentrations. While insulin concentration is typically strongly correlated with the rate of insulin degradation in
solution formulations, these two parameters appear to be independent in poloxamer gel formulations.

a) **Effect of Time**
   (0% PF-127, 80 mg/mL insulin)

b) **Effect of Time**
   (15% PF-127, 80 mg/mL insulin)

c) **Effect of Time**
   (20% PF-127, 80 mg/mL insulin)

d) **Effect of Time**
   (25% PF-127, 80 mg/mL insulin)
Figure 12. RP-HPLC chromatographs showing elution of insulin and its degradation products as a function of incubation time. Formulations were subjected to continuous shear at 37°C. Results shown for 80 mg/mL insulin formulations with a) 0% (solution), b) 15 wt. %, c) 20 wt. % and d) 25 wt. % PF-127. Right inset: higher magnification chromatographs showing insulin degradation products.

Figure 13. Quantitation of insulin and its degradation peaks in RP-HPLC chromatographs. Insulin formulations were subjected to continuous shear at 37°C. a) Effect of time on retained insulin content for 80 mg/mL insulin, 25% w/w PF-127 formulations. Results normalized to initial insulin concentration. b) Effect of poloxamer content on retained insulin content for 80mg/mL insulin formulations after 30 days incubation. Results normalized to initial insulin concentration. * p<0.05, n=3 for all groups. c) Effect of poloxamer content on the formation of
insulin degradation products after 30 days incubation of 80 mg/mL insulin formulation. Peak results normalized to their respective solution formulation (0% PF-127) values. d) Effect of insulin concentration on retained insulin content after 30 days in the formulation containing 25% w/w PF-127 after 30 days incubation. Results normalized to initial insulin concentration. Error bars represent standard deviation (SD) for n=3 experiments. * p<0.05.

3.4.3 Effect of Time, Poloxamer Content and Insulin Concentration on Insulin Structure

Circular dichroism was used to monitor the secondary structure and conformational change of insulin due to agitation at 37°C. The far-UV CD spectra of freshly prepared insulin revealed two characteristic negative bands at 208 and 222 nm that are indicative of the alpha-helical secondary structure of native insulin (Figure 14). Deconvolution of the CD spectra revealed that the secondary structure of native insulin is composed of 40% alpha helix, 16% beta-strand, 15% turns and 30% unordered. These results are in agreement with previously reported values.

Under conditions of shear and elevated temperature, insulin from all formulations exhibited time-dependent loss of alpha-helical conformation characterized by disappearance of the peaks at 222 nm and 208 nm (Figure 14). Loss of helical conformation occurred most rapidly in solution insulin formulations without Pluronic F-127 (Figure 14). In contrast, changes in the secondary structure was both delayed and less pronounced as Pluronic F-127 content in the formulations increased from 15% to 25% w/w (Figure 15a). By day 30, formulations with 25 wt. % poloxamer maintained 32% alpha-helix content compared to only 18% alpha-helix in formulations without poloxamer. At the concentrations tested, there was no observable effect of insulin concentration on loss of secondary structure (Figure 15b).

The loss of secondary structure in insulin is due to partial protein unfolding and occurs after extensive losses in the protein’s tertiary and 3D structure. Loss of insulin’s alpha-helical structure is associated with loss of biological activity. In addition, partial unfolding of an insulin molecule further exposes its hydrophobic interior to the environment leading to further interaction and unfolding of additional insulin molecules. In this manner, insulin unfolding and degradation is solution formulations is often an exponential process as exemplified by the rapid degradation of insulin in the solution formulation (0% Pluronic F-127) by day 10 (Figure 14a). In
contrast, these results demonstrate that insulin unfolding in the presence of high poloxamer concentration is a gradual process (Figure 14b), presumably due to poloxamer shielding of exposed hydrophobic surfaces of unfolded protein and minimized protein-protein interaction through increased viscosity.

Figure 14. Circular dichroism spectra and relative percentage of secondary structure showing the effect of incubation time on insulin structure. a) solution formulation (0% PF-127). b) 25 wt.% insulin gel formulation.
Figure 15. Circular dichroism spectra and relative percentage of secondary structure showing the effect of a) poloxamer content and b) insulin concentration on insulin secondary structure. Formulations were subjected to continuous shear at 37°C for 30 days.

3.4.4 Fabrication of an Implantable Membrane-Reservoir Device

To demonstrate the suitability of the poloxamer gel insulin formulation for sustained drug delivery, an implantable membrane-reservoir system was developed using microfabrication methods described in a subsequent section (Chapter 4)\textsuperscript{253}. These devices feature a tubular insulin reservoir sealed at one end with EVAC polymer and capped at the other end with a microfabricated microporous membrane that serves as a rate-limiting barrier to insulin diffusion out of the reservoir. The microporous membranes with a porosity of 0.31%, 0.79% or 4.91% were 100 µm thick and featured nontortuous pores which traversed the entire thickness of the
membrane. In addition, the membrane surfaces were topographically smooth to minimize in vivo inflammation and tissue growth and membrane geometry (number, size and organization of the pores, as well as membrane thickness) is highly reproducible. The reservoir chamber and microporous were constructed from biocompatible silicone and surface-modified with 20 kDa PEG to minimize surface adsorption of protein. Both construction materials, PEGylated silicone and EVAC are non resorbable biocompatible materials that elicit minimal inflammatory response when implanted into the body.\textsuperscript{253}

\subsection*{3.4.5 In Vitro Release Kinetics}

\textit{In vitro} insulin release was measured from implantable devices loaded with insulin formulations containing 0\%, 15\%, 20\% and 25\% poloxamer (Figure 16). Insulin release profiles of all formulations were linear with time until reaching a late stage, with no observed burst release or lag time. Insulin release rates from poloxamer-containing formulations were significantly lower compared to the solution formulation. In addition, the rate of insulin release decreased as poloxamer content increased from 15 wt.% to 25 wt.%. This may be due to increased viscosity of the gel formulations. Linear insulin release kinetics was observed for roughly 15 days.

The insulin release rate and duration of sustained drug delivery can also be altered by microporous membrane porosity. Devices fitted with a 4.9\% porosity membrane exhibited rapid insulin release over 72 hours (Figure 16b). Decreasing membrane porosity to 0.79\% and 0.31\% decreased the rate of insulin release and significantly prolonged the duration of linear drug release to 15 and >30 days respectively. These results demonstrate that the poloxamer gel formulations is suitable for long-term sustained release drug delivery applications and that insulin release kinetics from the devices may be tuned by optimizing both formulation viscosity and membrane porosity.
3.4.6 \textit{In Vivo} Efficacy

To assess the performance of the present formulation in maintaining long-term efficacy, membrane-reservoir devices filled with insulin gel formulation (80 mg/mL insulin, 25 wt.% PF-127) were implanted into the abdominal subcutaneous tissue of STZ-induced diabetic rats. Fed-state blood glucose levels were measured over a 34-day period. C-peptide levels were consistently below 150 pM over the duration of the experiment.

Healthy rats maintained normal blood glucose levels of \(~160\) mg/dL while diabetic rats experienced severe hyperglycemia with blood glucose levels above 500 mg/dL (Figure 17). Implantation of the devices into the diabetic rats immediately restored normal blood glucose levels. Efficacy was maintained for roughly 17 days which correlates well with the \textit{in vitro} duration of linear drug release (Figure 17). In general, blood glucose levels following implantation bordered the low range of physiological glycemia at \(~80\) mg/dL. Occasional episodes of elevated glycemia occurred due to meals. A gradual return to hyperglycemia after 17
days results from decreased insulin delivery rates due to depletion of the drug reservoir. Return to hyperglycemic levels comparable to pre-implantation indicates that the rats did not regain pancreatic function over the duration of the experiment. These results demonstrate the suitability of the present insulin gel formulation for sustained delivery of insulin in the body and preservation of insulin stability at physiological conditions, resulting in long-term maintenance of normal blood glucose levels in diabetic rats.

Figure 17. Blood glucose levels of diabetic rats implanted with sustained insulin delivery devices (0.8% membrane porosity) filled with insulin gel formulation (80 mg/mL insulin, 25% wt. PF-127). Healthy and diabetic rats served as controls.

3.5 Conclusions

A poloxamer gel formulation of highly concentrated insulin (up to 80 mg/mL) demonstrated excellent insulin stability under conditions of continuous shear at 37°C. Increased poloxamer concentration was found to minimize the formation of insulin degradation products and preserve its alpha helical secondary structure in a dose-dependent manner. Enhanced insulin stability is attributed to both the surfactant properties of poloxamer and increased formulation viscosity. The poloxamer gel formulations further demonstrated linear insulin release profiles from a microfabricated diffusion-controlled membrane-reservoir drug delivery device with no
observable burst or lag effects. Subcutaneous implantation of the devices into diabetic rats resulted in sustained insulin delivery and maintenance of normal blood glucose levels for 17 days. These findings suggest that poloxamer gel formulations may be useful for enhancing the long-term stability of highly concentrated biological macromolecules under physiological conditions and are suitable for diffusion-controlled drug delivery applications.

3.6 Acknowledgments

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Chapter 4

Microfabricated Microporous Membranes Reduce the Host Immune Response and Prolong the Functional Lifetime of a Closed-Loop Insulin Delivery Implant in a Type 1 Diabetic Rat Model

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4.1 Abstract

Implantation of a medical implant within the body inevitably triggers a host inflammatory response that negatively impacts its function and longevity. Nevertheless, the degree and severity of the response may be reduced by selecting appropriate materials, implant geometry, surface topography and surface treatment. Here we demonstrate a strategy to improve the biocompatibility of a chemically-driven closed-loop insulin delivery implant. A microfabricated microporous, poly(ethylene glycol)-grafted polydimethylsiloxane membrane was placed on top of the glucose-responsive insulin release plug of the implant. Implant biocompatibility was assessed in healthy rats while implant function was evaluated in a type 1 diabetic rat model. The microporous membrane with a small distance to the plug provided a geometric barrier to inflammatory cell migration and prevented leukocyte-mediated degradation of the plug for at least 30 days. Membrane-protected devices elicited a significantly milder inflammatory response and formation of a well-defined fibrous capsule at the device opening compared to unprotected devices. The glucose-responsiveness was nearly unchanged, although the insulin release rate decreased with decreasing pore size. The microporous membrane-improved biocompatibility prolonged in vivo efficacy of the implant by ~3-fold. This work suggests the importance of implant design in modulating inflammatory response and thereby extending the duration of the implant.

4.2 Introduction

Implantable medical devices improve patient care and quality of life in many aspects of medicine including cardiology, orthopedics, neurology and ophthalmology. Proper implant function is strongly dependent on the interaction between the body and the implanted material that contacts the host tissue. As such, the use of biologically inert materials in pacemakers, cardiovascular stents, and catheters has resulted in prolonged device lifetimes and reduced incidence of complications.

In spite of these achievements, long-term implantable sensors, macromolecular drug-delivery systems, and combination closed-loop drug delivery systems remain elusive as these systems often feature active components made from non-biocompatible materials that must contact and interact unimpeded with the host tissue. Examples of such materials include metallic or semiconducting sensing electrodes, and stimuli-responsive polymers,
enzymes, allogenic or transgenic cells or tissues. As these components cannot simply be masked using biologically inert materials, these devices often fail shortly following implantation due to biocompatibility issues, namely fibrous encapsulation and cell-mediated degradation. Innovative strategies have thus been extensively explored to overcome the foreign body response (FBR) whilst simultaneously enabling efficient mass transport to and from these devices, which are both vital to prolonging implant lifetime and viability in the clinic and technologically challenging to achieve.

Active strategies to mitigate the FBR rely on the sustained local release of anti-inflammatory agents (e.g., dexamethasone or nitric oxide (NO)) or pro-angiogenic mediators (e.g., vascular endothelial growth factor (VEGF)) from the implant surface. The continuous release of dexamethasone or NO has been shown to reduce inflammatory cell recruitment and minimize fibrous capsule formation around implants; however it is difficult to provide a steady supply of the agents throughout the device lifetime. Other coatings are also conceivable, like sirolimus or paclitaxel coatings, which are already used in interventional cardiology as so called drug eluting stents. Nevertheless, contraindications with the patients’ medical condition must also be carefully considered with the use of bioactive compounds, as is the case for dexamethasone and T1D mellitus in which dexamethasone may exacerbate the underlying condition and increase the risk of both diabetes-related complications and the onset of type 2 diabetes. Similarly, sirolimus has been associated with insulin resistance and the onset of type 2 diabetes in kidney transplant recipients.

Passive strategies for limiting the FBR involve alteration of the implant surface topography and chemistry to minimize serum protein adsorption, subsequent leukocyte adhesion, and downstream inflammatory responses. These strategies make use of non-fouling self-assembled monolayers, polymer brushes, or hydrogel coatings made from hydrophilic polymers such as polyethylene glycol (PEG), alginates, poly(2-hydroxyethyl methacrylate), poly(N-isopropyl acrylamide), poly(acrylamide), and phosphoryl choline-based polymers. Hydrogels are of particular interest to sensory and drug-delivery applications as they may be optimized to permit selective diffusion of biomolecules, drugs, and analytes. Despite these advances, surface coatings that completely eliminate protein adsorption over the lifetime of a device have yet to be attained. These coatings are also prone to cellular overgrowth, infiltration and degradation, and may reduce molecular transport to and from the implant surface.
As both fibrous encapsulation and cellular degradation of implanted materials proceed *via* recruitment of immune cells to an active implant surface, we hypothesize that the biocompatibility of an implant can be improved by limiting cell migration to the implant surface using a microporous membrane placed between the active implant surface and the host tissue. Incorporation of the microporous membrane serves as a barrier to surface-bound cell migration by altering the implant geometry without significantly impeding the diffusion of analytes and drugs from the device.

We demonstrate this approach on a chemically driven closed-loop insulin delivery system that features a glucose-responsive hydrogel plug responsible for the self-regulated release of insulin from the implant reservoir in the presence of glucose\textsuperscript{5,17,20}. Closed-loop insulin delivery systems offer diabetic patients improved glycemic control, compliance and quality of life over conventional insulin therapy\textsuperscript{4,265,281}. To date a number of such systems have demonstrated short-term success (~1 week) in maintaining normal blood-glucose levels in diabetic rats\textsuperscript{20,269}, however a long-term use has not yet been achieved due to premature device failure attributed to poor biocompatibility including fibrous encapsulation of the implant and/or cell-mediated degradation of the glucose-responsive hydrogel plug.

In this paper, we report on a novel microfabrication process for producing polymeric microporous membranes with strictly defined geometries. We examine the effect of membrane geometry and surface hydrophobicity on the insulin permeability through the membrane, and the insulin release rate out of glucose-responsive insulin devices. The effect of membrane geometry on implant biocompatibility is examined *in vivo*. Finally, we demonstrate that the improved implant biocompatibility resulting from the use of the microporous membrane translated to prolonged device efficacy and functional lifetime in in a streptozotocin (STZ)-induced diabetic rat model.

4.3 Materials and Methods

Bovine serum albumin (BSA), catalase (13 mg/ml), 3-aminopropyl triethoxysilane (APTES), glutaraldehyde, n-octyl-β-glucopyranoside, poly(ethylene glycol) methyl ether (MW 20,000), and streptozotocin (STZ) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Glucose oxidase (GOX; 230 U mg\textsuperscript{-1}) was purchased from Calzyme (USA). Human recombinant insulin (27 U mg\textsuperscript{-1}) was purchased from Wisent (Canada). Nalgene 50 silicone tubing with a 1.6 mm ID
and 3.2 mm OD was purchased by Nalgene (USA). Ethylene-vinyl acetate copolymer resin (ELVAX-40W; EVAC) was provided by E. I. Du Pont (USA). Polydimethylsiloxane (PDMS, Sylgard 184) was purchased from Dow Corning Corporation (USA). S1805 photoresist, SU-8 photoresist and SU-8 developer (1-Methoxy-2-propyl acetate) were purchased from Microchem (USA). Distilled and deionized (DDI) water were obtained from a Milli-Q water purifier (Milli-Pore Inc.; USA).

4.3.1 Preparation of Poly(N-isopropyl Acrylamide-co-Methacrylic Acid) Nanoparticles

Poly(N-isopropyl acrylamide-co-methacrylic acid) (poly(NIPAM/MAA)) nanoparticles (380 ± 110 nm in pH 7.4 PBS and 157 ± 50 nm in pH 4.8 PBS; NIPAAM:MAA NPs) were prepared as previously described by our group\(^1\) using a one-pot aqueous dispersion polymerization process. NIPAM, MAA and N,N’-Methylene-bis-acrylamide (BIS), were dissolved in DDI water at a molar ratio of 1:1:0.068, giving a total concentration of 135 mM. Sodium dodecyl sulphate (SDS) at a concentration of 0.4 mM was added to the solution as a stabilizer. After purging N\(_2\), and potassium persulfate (2.1 mM) was added to the mixture to initiate polymerization. The reaction was carried out at 70ºC under a N\(_2\) atmosphere and stirring at 200 rpm for 4 h. The obtained nanoparticles were purified by membrane dialysis (Molecular weight cutoff 12,000 to 14,000, Fisher Scientific) against DDI water. Particle size distribution was determined by laser light scattering at room temperature.

4.3.2 Preparation of Nano-MnO\(_2\) Powder

Nano-MnO\(_2\) powder (80 ± 30 nm) was prepared via sonochemical reduction of permanganate with manganese ions as previously described by our group\(^1\). In brief, manganese acetate aqueous solution (1 mL, 0.07 mM) was injected into an ultra-sonicated potassium permanganate aqueous solution (5 mL, 0.05 mM). Ultra-sonication was achieved using ultrasonic processor probe operating at 50 Hz (Heischer UP100H, Germany). The reaction was allowed to proceed for 30 s under ultrasonic conditions. Solid nanoparticles were isolated by ultra-centrifugation, thoroughly washed with DDI water, and freeze-dried overnight.
4.3.3 Preparation of Insulin Formulation

A concentrated insulin stock solution (100 mg mL$^{-1}$ human insulin in 50 mM HEPES buffer, pH ~7.0) was prepared as follows for *in vitro* use: human insulin (100 mg) and n-octyl-β-D-glucopyranoside (3.65 mg) were dissolved with 0.1 m NaOH aqueous solution (600 μL). 12.6 mg of HEPES was added to the solution. A transparent solution was obtained by adjusting the solution pH to approximately 7.8 with the slow addition of 0.1 M HCl. The volume was topped up to 1 mL by the addition of DDIW. The solution was diluted in HEPES buffer as required and stored at 4°C. A concentrated formulation of insulin (80 mg/mL) was prepared as previously described in Chapter 3 for use in long-term efficacy studies.

4.3.4 Preparation of the Microporous Membrane

A microporous membrane replica mold was constructed using standard microfabrication methods (Figure 18). A 60 μm thick layer of SU-8 50 was spin coated onto a silicon wafer substrate and soft-baked at 65°C and 95°C for 1 and 5 min respectively. Arrays of 60 μm tall free-standing pillars were formed by selective photo polymerization of the SU-8 layer using photolithography followed by a post-exposure bake at 65°C and 95°C for 1 and 5 min respectively. Uncrosslinked SU-8 was subsequently removed using SU-8 developer. The replica mold was rinsed with isopropyl alcohol, DDI water, and dried under nitrogen. A final hard-bake procedure was performed at 170°C for 4 h. Separate replica molds were fabricated with 10 μm, 20 μm, and 60 μm post diameters.

Microporous PDMS membranes were replica molded from the SU-8 templates (Figure 18). To enable self-release of the membrane from the template, the replica mold was first coated with a 1 μm thick sacrificial layer of Shipley S1805 positive photoresist. Following a 90 s bake at 120°C, PDMS pre-polymer mix (1:10 ratio of polymer to cross-linker) was spin-coated onto the mold and cured at 120°C for 5 min. After scoring the wafer edges, release of the microporous PDMS membrane was achieved by soaking the entire mold in acetone. The floating PDMS membrane was transferred onto a sheet of aluminum foil for subsequent handling. Individual porous membranes were bonded to silicone tubing at 120°C after treatment with oxygen plasma. Membrane geometry was verified using a Dektak Profilometer (Bruker, USA) and under SEM.
Figure 18. a) Schematic illustrating the process for fabricating microporous PDMS membranes. b) SEM images of micro-fabricated porous membranes (10 µm pore diameter membrane shown). Pores traverse the width of the membrane as evidenced by the absence of charging.

4.3.5 Preparation of the Glucose-Responsive Device

Three centimeter sections of silicone tubing, with or without a microporous membrane, were oxidized for 5 min (5 W Harrick Plasma Cleaner/Sterilizer PDC-3XG) and silanized in a 0.1 M APTES anhydrous ethanol solution for 12 h (Figure 19). The silanized tubing was extensively rinsed with ethanol and dried under nitrogen at room temperature. Successful surface modification was verified using XPS elemental analysis (ThermoFisher Scientific ThetaProbe X-ray photoelectron spectrometer, East Grinstead, UK).

Synthesis of the glucose-responsive hydrogel plug was performed as previously described by our group\(^\text{17}\). All reagents and device components were sterilized prior to use. Devices were
assembled and stored in a sterile environment. 28 mg of BSA, 3 mg of GOX and 0.86 mg of catalase was dissolved in 200 μL of pH 4.8 phosphate buffered saline (PBS) solution containing 6 mg of dispersed MnO₂ nanoparticles. 85 μL of 200 mg/mL NIPAM:MAA nanoparticles dispersed in DDI water was added to the mixture and stirred for 10 min at 37°C. Upon the addition of 15 μL of glutaraldehyde crosslinker solution, 2.5 μL of the mixture was transferred into the silanized tubing to form a glucose-responsive hydrogel plug that spans the entire tube cross-section. Three different device configurations, based on the relative position of the hydrogel plug, were produced (Figure 19a): (1) bare device: an unprotected device in which the glucose-responsive plug is placed at the tip of tube, (2) gap device: a membrane protected device in which a gap separates the glucose-responsive plug and the porous PDMS membrane, and (3) no-gap device: a membrane-protected device in which the glucose-responsive plug is placed in contact with the porous PDMS membrane. Glucose-responsive hydrogel devices were stored pH 7.4 PBS at 4°C. The open end of the tubing was sealed with EVAC solution (8% in dichloromethane (w/v)) and allowed to dry in air to form a solid round cap over the tubing end.

Poly(ethylene glycol) methyl ether (mPEG, 20 kDa) was activated with 4-nitrophenyl chloroformate (NPC) as described elsewhere. Silanized devices were incubated in and filled with activated PEG solution (0.01 M activated PEG in pH 7.4 PBS) for 48 h at 4°C to ensure modification of all device surfaces (Figure 19b). Devices were extensively washed with DDI water and kept in pH 7.4 PBS at 4°C prior to use.
Figure 19. a) Schematic of an insulin-delivery device. Inset: Device configurations for ‘bare, ‘gap’ and ‘no-gap’ devices. b) Chemical reaction scheme for silicone silanization, crosslinking of the glucose-responsive hydrogel plug, and pegylation. c) Mechanism of glucose-responsiveness for poly(NIPAM/MAA) nanoparticles (top) and glucose-sensitive insulin release from devices (bottom). Plug porosity is increased at hyperglycemic glucose levels in response to enzymatic oxidation of glucose to gluconic acid, leading to higher insulin release rates.
4.3.6 *In Vitro* Insulin Release Measurements

To quantify device responsiveness *in vitro*, individual devices were filled with 50 μL of 25 mg/mL insulin solution and incubated in 3 mL of release medium (pH 7.4 PBS buffer with 100 mg/dL glucose) for 4 h. The glucose concentration of the release medium was increased from 100 mg/dL (euglycemic conditions) to 400 mg/dL (hyperglycemic conditions) after 2 h. Insulin release from each device was measured intermittently using UV absorbance at a wavelength of 276 nm. All experiments were conducted at 37°C under mixing conditions. The rate of insulin release was determined from the slope of these curves. The glucose-responsiveness of the insulin delivery device was defined as the ratio of the rate of insulin release at hyperglycemic glucose levels (400 mg/dL) to the rate of insulin release at normal glucose levels (100 mg/dL).

Circular dichroism (CD) spectroscopy was used to determine the secondary protein structure of the device-released insulin following the *in vitro* release study to determine the stability of the released insulin. The resulting spectrum was compared to that of freshly prepared insulin solution.

4.3.7 *In Vivo* Biocompatibility

All *in vivo* procedures strictly complied with the ethical and legal requirements under Ontario’s Animals for Research Act and the Federal Canadian Council on Animal Care guidelines for the care and use of laboratory animals and were approved by the University Animal Care Committee of the University of Toronto.

The effect of the microporous membrane on device biocompatibility was evaluated in 300-350 g adult male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC, USA). Implants were filled with saline prior to implantation and stored in sterile saline. Devices were implanted subcutaneously in the periscapular region of the dorsum. Implantation at this site compared to the abdominal area ensured minimal contamination. Four separate 0.5 cm incisions were made in the skin. Subcutaneous pockets were created at the site of each incision using blunt dissection. Each subcutaneous pocket received one of the following implants: device with a 10, 20, or 60 μm diameter protective membrane, or an unprotected device (no porous protective membrane). The wounds were closed with absorbable sutures.
Rats were sacrificed at 3, 7, 14, and 30 days post-surgery. The implants were removed with the surrounding tissue and placed in a fixative solution. Samples to be analyzed under eSEM were fixed in universal fix (1.0% glutaraldehyde, 4.0% formaldehyde in 0.1M phosphate buffer) for 24 h, incubated in osmium for 5 min, and cut longitudinally to expose the glucose-responsive plug. Samples for histological analysis were fixed in 10% buffered formalin for 24 h, embedded in paraffin, sectioned into 5 μm-thick slices, and stained with hematoxylin & eosin (H&E), Mason’s Trichrome and CD68 immunohistochemical stain.

The biocompatibility of each implant was evaluated based on degradation of the glucose-responsive plug, fibrous capsule thickness, presence and abundance of inflammatory cells. Degradation of the glucose-responsive plug was analyzed using eSEM. Histomorphometric analysis of inflammation and fibrosis was done by a board-certified veterinary anatomic pathologist. Semiquantitative grading of inflammatory response was done based on the cellular densities of granulocytes, lymphocytes, macrophages, and foreign body giant cells (multinucleated giant cells) from H&E stained tissue samples. Arbitrary grades on a scale of 0-4 were assigned (0=cells absent, 1=low numbers, 2=moderate numbers, 3=large numbers, 4, excessive number of cells with marked expansion of the subcutis and/or extension into the implant). Fibrous capsule thickness measured both at and away from the implant opening was quantified from trichrome-stained tissue samples. The fibrous capsule was defined as the region of dense collagenous connective tissue adjacent to the implant surface. Identification of macrophages was aided by CD68 immunohistochemistry. Fibrous capsule data was expressed as mean values ± standard deviation. Inflammation data was expressed as median values along with the semiquantitative grade range and was analyzed for significance (p<0.05, two-tailed) with the non-parametric Mann-Whitney U test (SPSS 16.0, IBM, USA).

4.3.8 In Vivo Device Lifetime

The effect of the microporous membrane on device lifetime was evaluated in STZ-induced diabetic adult male Sprague-Dawley rats (300-350 g, Charles River Laboratories, Raleigh, NC, USA). A one 0.5 cm midline incision was made in the lower abdomen followed by blunt dissection to create subcutaneous pockets for device implantation. The rats were separated into 3 groups, with each animal receiving one of the following type of implants: Four insulin-filled membrane-protected devices (60 μm pore diameter), three insulin-filled bare devices (no
protective porous membrane), or four saline-filled sham devices which served as control. Blood glucose levels were measured pre-surgery and post-surgery to ensure that the rats recovered properly and did not suffer from severe hypoglycemia.

Rats were housed individually on a reverse light-cycle and were given ad libitum access to food and water. Blood glucose levels were monitored daily using a glucose meter (OneTouch® Ultra®, LifeScan Inc., USA). Similarly, blood insulin and c-peptide levels were measured once daily. Plasma insulin and C-peptide level was determined using an antibody radioimmunoassay kit specific for rat insulin (Linco Research Inc., USA) according to the manufacturer’s instructions. Rat weight and health was monitored regularly for the duration of the study.

4.4 Results

4.4.1 Microporous Membrane Design and Fabrication

Biocompatible polymer membranes featuring micron-sized pores were fabricated using a novel self-releasing microfabrication method outlined in Figure 18. This method enabled construction of thin (below 100 µm), mechanically robust, and topographically smooth membranes with accurate and uniform membrane dimensions including pore diameter, pore pitch and membrane thickness. Membranes with 10, 20, and 60 µm pore diameters were constructed out of PDMS, a biocompatible polymer which can be rendered hydrophobic after modification with PEG to facilitate insulin permeation.

4.4.2 In Vivo Biocompatibility – Degradation of Glucose-responsive Plug

Environmental SEM (eSEM) imaging was used to assess the effect of device configuration (e.g., ‘bare’, ‘gap’, and ‘no-gap’ devices) and membrane geometry (10 µm, 20 µm, and 60 µm pore sizes) on cell accumulation at the active implant surface, and the resulting cell-mediated degradation of the glucose-responsive plug. Devices explanted at day 3, 7, 14 and 30 following subcutaneous implantation in Sprague-Dawley rats were fixed and sectioned longitudinally for eSEM imaging.

The effect of device configuration (‘bare’, ‘gap’, and ‘no-gap’ devices) on cell infiltration and degradation of the glucose-responsive hydrogel plug at day 3 and day 7 is presented in Figure 20. Devices without the protection of the microporous membrane exhibited early signs of cell-mediated degradation at day 3 as evident from the slightly resorbed glucose-responsive plug.
surface. Progression of this process resulted in appreciable cell recruitment at the glucose-responsive plug surface, formation of a loose fibrous matrix, and significant resorption of the glucose-responsive plug by day 7.

Incorporation of the microporous membrane directly in contact with the glucose-responsive plug prevented plug resorption by day 3, however considerable cell infiltration and degradation of the plug was observed by day 7. This may be due to the close proximity of the glucose-responsive plug to the exterior of the device where the albumin plug served as a matrix through which recruited cells were able to migrate across unimpeded.

In contrast, when the glucose-responsive plug and the microporous membrane were separated with a small distance, few infiltrating cells accumulated on the glucose-responsive plug and no signs of cell-mediated degradation were observed at days 3 or 7. Thus the gap between the microporous membrane and the glucose-responsive plug is critical for minimizing cellular migration to the active implant surface and subsequent degradation. The presence of cells on the underside of the microporous membrane (Figure 21 inset) shows the ability of the infiltrating cells to pass through the micron-sized pores. Surprisingly, these cells remained on the underside of the microporous membrane and preferentially aggregated at the device corners where they eventually produced a loose fibrous matrix by day 7 (Figure 20 inset). Photographs of the glucose-responsive hydrogel plug after a 7-day implantation period are also shown in Figure 20. Degradation of the glucose-responsive plug in the bare and no-gap devices is clearly evident, while the device with a gap between the plug and the microporous membrane remains intact.
Figure 20. Scanning electron microscope images (top and middle row) and photographs (bottom row) comparing the effect of device configuration (bare, no-gap, and gap device) on implant degradation at day 3 and day 7.

The influence of microporous membrane pore size on cell migration and cell-mediated degradation of the glucose-responsive plug over a 30 day period is shown in Figure 21. For 10 μm and 20 μm pore sizes, no cells were found residing on the glucose-responsive plug at days 7 and 14. This contrasts with 60 μm pore membranes in which a small number of cells were found on the glucose-responsive plug, however no signs of plug resorption were observed. By day 30, a number of infiltrating cells and some fibrous material was observed on the glucose-responsive plug for all three membrane pore diameters, however the plugs were not blanketed with cells, as is the case of the day 7 bare device shown in Figure 20, and resorption of the plug was not apparent. Infiltrating cells were observed along the inside tube wall as a means of migration towards the glucose-responsive plug.
Figure 21. Scanning electron microscope images comparing the effect of implantation time and membrane pore size on cell migration and accumulation at the glucose-responsive plug.

4.4.3 In Vivo Biocompatibility – Inflammatory Response

Devices with or without microporous protective membranes were implanted subcutaneously in Sprague-Dawley rats for up to 30 days. Figure 22a shows a low magnification microscopic view of the implant site (device with no microporous membrane, 30 days). The rectangular cavity denotes the location of the implanted device beneath the panniculus carnosus muscle. The device is encased by fibrosis and there is enhanced inflammation at the device opening, where the glucose-responsive hydrogel plug contacts the subcutaneous tissue. In comparison, the side walls of the device away from the opening were lined by milder fibrosis and inflammation throughout the 30-day implantation period as evidenced by fewer inflammatory cells. Invariably all devices induced a minimal to mild acute inflammation at day 3 that was characterized by low number of lymphocytes, macrophages, neutrophils and minimal proliferation of fibroblasts and blood vessels (granulation tissue). By day 7, lymphocytes and macrophages were more abundant
admixed with proliferating fibroblasts (fibroplasia). Inflammation on day 14 was typically characterized by lymphocytes, plasma cells, and macrophages with variable amounts of fibrosis. On day 30, inflammation was absent or minimal with well-defined fibrous capsule in membrane-protected devices, except in bare devices as described below. In all implants, compression of the surrounding subcutaneous parenchyma created an additional layer of pseudocapsule.

Quantification of inflammation and fibrosis as a function of presence or absence of microporous membrane, pore size, and implantation period is summarized in Table 5. Briefly, membrane-protected devices elicited a milder inflammatory response compared to bare devices at all time points. By day 30, inflammation was resolved in all membrane-protected groups as evidenced by rare perivascular lymphocytes and macrophages at the implant site, and a well-defined fibrous capsule all around the device including at the device opening. The effect of pore size on fibrous encapsulation of the implant after a 30-day implantation period is depicted in Figure 23. Differences in fibrous capsule thickness were observed between the various membrane pore sizes, with 60 µm and 20 µm pore diameter membranes exhibiting a relatively thicker fibrous capsule compared to those formed around 10 µm pore diameter membranes.

In contrast, bare-devices elicited intense chronic active inflammation characterized by large numbers of lymphocytes, plasma cells, macrophages and neutrophils that markedly expanded the subcutis and extended into both the device opening and reservoir by days 14 and 30. As a result, a well-defined fibrous capsule, as observed in membrane-protected devices, was not evident at the device opening. Fibrous capsule was evident at the side walls away from the device opening.
Figure 22. Histological examination of subcutaneously implanted devices (bare and 60 µm pore diameter membrane devices) retrieved from rats comparing the effect of implantation time. a) H&E stained section showing the implant cavity and surrounding subcutaneous tissue. The inflammation is more pronounced adjacent to the device opening compared to elsewhere. b) Tissue sections adjacent to the device opening for ‘bare’ and ‘60 µm pore diameter gap’ devices retrieved at four different time points (3, 7, 14, and 30 days). Sections stained with Mason’s Trichrome stain. Collagen is stained green. Regions with marked fibroplasia (arrow) and fibrous capsule (C) are labeled. Large numbers of inflammatory cells are admixed with the fibroplasia in the ‘bare’ device at 7, 14, and 30 days. Mature fibrous capsule is evident in the ‘60 µm pore’ device at 14 and 30 days. Scale bar = 500 µm.
Figure 23. Histological examination of subcutaneously implanted devices retrieved from rats after 30 days comparing the effect of microporous membrane pore size (10 µm, 20 µm, and 60 µm pore diameter membranes and bare devices) on tissue compatibility. Tissue sections were stained with H&E (left panels), Mason’s Trichrome stain (center panels), and CD68 (right panels). Labeled are inflammatory cells (arrow), fibrous capsule (C), fibroplasia (F), and the implant cavity. Scale bars represent 100 µm.
Table 5. Inflammation and fibrosis as a function of implant type and implantation period

<table>
<thead>
<tr>
<th>Device Type</th>
<th>Time (day)</th>
<th>Inflammation score&lt;sup&gt;6&lt;/sup&gt;</th>
<th>Fibrous capsule thickness at device opening (µm, Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No membrane</td>
<td>3</td>
<td>1 (1;1)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>4 (2;4)</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>4 (4;4)</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>4 (1;4)</td>
<td>ND*</td>
</tr>
<tr>
<td>Membrane (60 µm pore size)</td>
<td>3</td>
<td>1 (1;1)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1 (1;1)</td>
<td>100/ND</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>1.5 (1;2)</td>
<td>225 ± 75</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1 (0;1)</td>
<td>72 ± 17</td>
</tr>
<tr>
<td>Membrane (20 µm pore size)</td>
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<td>1 (1;1)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1.5 (1;2)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>3 (1;3)</td>
<td>225 ± 176</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0 (0;1)</td>
<td>97 ± 2.5</td>
</tr>
<tr>
<td>Membrane (10 µm pore size)</td>
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</tr>
<tr>
<td></td>
<td>7</td>
<td>2 (1;2)</td>
<td>ND</td>
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<td></td>
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<td>1 (1;1)</td>
<td>126 ± 64</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1 (1;1)</td>
<td>53 ± 23</td>
</tr>
</tbody>
</table>

<sup>6</sup>Median (minimum; maximum) from n=3 specimens; 0=cells absent, 1=low numbers, 2=moderate numbers, 3=large numbers, 4=excessive number of cells with marked expansion of the subcutis and/or extension into the implant. Mann-Whitney U-test (each time point, comparing inflammation around devices with no membrane versus membrane): p≤0.05 (bold).

ND – non-detectable as the fibrous tissue did not develop conspicuously due to active and intense inflammation

*Well-defined fibrous capsule was absent at device opening; but plump fibroblasts (fibroplasia) admixed with inflammatory infiltrates was present.

4.4.4 Effect of Membrane Pore Size on In Vitro Insulin Release and Stability

The effect of microporous membrane pore size on insulin release under normal (100 mg/dL dextrose) and hyperglycemic (400 mg/dL dextrose) conditions was quantified in vitro using
UV/Vis spectroscopy (Figure 24a). The glucose-responsive insulin release rate of the ‘bare’ device was also quantified. Incorporation of the microporous membranes decreased the insulin release rate of the devices by as much as 60% in the case of membranes with 10 µm diameter pores. However all devices maintained an elevated insulin release rate under hyperglycemic conditions compared to euglycemic conditions. The responsiveness of the membrane-protected devices (R_{400/100}), as defined as the ratio of the rate of insulin release at elevated glucose levels to the rate of insulin release at normal glucose levels, are comparable to that of the bare device (Figure 24a) which suggests that incorporation of the microporous membranes did not adversely affect glucose sensitivity.

As devise released insulin may interact with the porous protective membrane surface, CD analysis was performed to verify the structural integrity of the released insulin. Far-UV CD spectral analysis of the released insulin revealed a characteristic double peak consistent with alpha-helical proteins (Figure 24b). The spectrum of device-released insulin was consistent with that of freshly prepared insulin, suggesting that the insulin released from the membrane-protected devices maintained their secondary structure.
Figure 24. a) *In vitro* insulin release from bare devices and membrane-protected devices with 60 µm, 20 µm, and 10 µm pore diameters (n=4). Insulin release rate was measured at two different glucose concentrations (100 mg/dL and 400 mg/dL) over a 4 hour period. Data points represent mean ± SD. Ratio of insulin release in 100 and 400 mg/dL glucose solutions (R400/100) are listed for each device. b) Far-UV spectrum of released insulin measured using circular dichroism (CD) spectroscopy. CD spectrum of the release medium following a 4-hour release study. The CD spectrum of the released insulin matches that of control insulin, demonstrating stability of the device-released insulin immediately after release.

### 4.4.5 *In Vivo* Efficacy

To assess whether the improvements to implant biocompatibility resulting from the use of a microporous protective membrane translated to prolonged device efficacy, gap devices featuring a 60 µm pore diameter PDMS membrane were implanted into the abdominal subcutaneous tissue of STZ-induced diabetic rats. Fed-state blood glucose and plasma insulin levels were measured.
over a 30-day period (Figure 25). Plasma c-peptide levels were consistently below 150 pM over the duration of the study.

Healthy control rats maintained a normal blood glucose level of ~160 mg/dL (Figure 25a). Following STZ administration, diabetic rats maintained an elevated blood glucose level of >400 mg/dL. Subcutaneous implantation of bare devices resulted in normal blood glucose levels for 6 days followed by a sharp return to hyperglycemia. Implantation of 60 µm pore diameter membrane-protected devices regulated blood glucose levels in the diabetic rats over a 21-day period followed by a gradual onset of hyperglycemia. Blood insulin levels in the diabetic rats were significantly elevated immediately following membrane device implantation followed by a steady insulin level of 400-800 pM for 30 days (Figure 25b). Therefore the gradual increase in blood glucose levels over the 30-day period may be attributed to increased insulin requirements due to animal weight gain and/or decreased insulin bioactivity in the reservoir over time.
Figure 25. *In vivo* performance of the closed-loop insulin delivery device implanted subcutaneously in STZ-induced diabetic rats: a) blood glucose over the course of 30 days following implantation (arrow) with saline-filled devices (diabetic control), unprotected insulin-filled devices (bare device), or microporous membrane-protected insulin-filled devices (membrane gap device with 60 μm pore diameter). Blood glucose levels from healthy non-diabetic rats are plotted for comparison. b) Plasma insulin levels over the course of 30 days following implantation (arrow) saline-filled devices (diabetic control) or insulin-filled membrane-protected devices. Data points represent mean ± SD (n>3).
4.5 Discussion

The microfabricated microporous membranes were able to prevent cell-mediated degradation of the glucose-responsive hydrogel plug by hindering the migration of inflammatory cells to the implant surface. Rather than size exclusion, the microporous membrane system achieved this passively through geometric means that capitalized on the inability of the cells to readily migrate over topographically smooth and biologically inert surfaces such as silicone. Thus the use of topographically smooth pegylated silicone in the construction of the microporous membrane and device sidewalls, and the spacing between the microporous membrane and the glucose-responsive plug were critical design features that resulted in delayed cell migration to the immunogenic surface. This was observed in Figure 20 and Figure 21, in which inflammatory cells were unable to migrate across the silicone sidewalls to the glucose-responsive plug, and therefore preferentially settled on the underside of the microporous membrane. Subsequent migration of the inflammatory cells to the glucose-responsive plug was only possible after the generation of a loose provisional matrix along the smooth inner tube surface. As the microporous membrane did not prevent cell penetration due to size exclusion, pore sizes larger than that of an inflammatory cell were permissible and facilitated rapid diffusion of glucose and insulin to and from the implant. Therefore microporous membranes with pore sizes as large as 60 µm in diameter were able to prevent the degradation of the glucose-responsive hydrogel plug for at least 30 days.

By hindering cell migration to the immunogenic implant surface, macrophage activation and subsequent secretion of pro-inflammatory cytokines which serves to propagate the inflammatory response was likely reduced, resulting in a relatively mild FBR. This was observed in Figure 22 and Figure 23 where the microporous membrane-protected devices elicited a significantly milder inflammatory response at the device opening compared to unprotected devices. Resolution of the inflammatory response and formation of a well-defined fibrous capsule all around the devices, including at the device opening, by day 30 suggests that the devices are likely tolerable for a prolonged period of time.

The observed improvements in device biocompatibility can be partly attributed to the fact that the microporous membranes themselves elicit little immune response as they are constructed from a biocompatible polymer known to be well-tolerated by the body and further modified...
with a PEG monolayer. Microfabrication of the membranes enabled accurate control over membrane geometry, low inter-membrane variability, and formation of topographically smooth membrane surfaces to ensure minimal response from the body upon implantation. The capability of this manufacturing process to produce exceedingly thin yet mechanically robust membranes allows for maximized diffusion of glucose and insulin between the body and the glucose-responsive plug. Efficient fabrication and high batch yield was achieved through the development of a novel self-release process (Figure 18a) which avoids manual separation of the microporous-membrane from the replica mold, thereby preventing damage typically incurred during this process to both the membrane and the replica mold, as is required by previously reported methods\textsuperscript{282,283}.

The improvements to implant biocompatibility prolonged in vivo efficacy in a type 1 diabetic rat model by ~3-fold (Figure 25). Membrane-protected ‘gap’ devices with 60 µm diameter pores were used for this experiment because this system exhibited the highest rate of insulin release in vitro (Figure 24) and was expected to represent the worst-case scenario of the membrane-protected device designs in terms of biocompatibility based on SEM and histological analysis. The loss in device efficacy after 21 days may be due to increased insulin requirements due to increased animal weight over time and/or decreased insulin bioactivity in the reservoir over the duration of the experiment as plasma insulin levels remained elevated for at least 30 days. These in vivo results compare favorably with other closed-loop insulin-delivery implants reported in literature\textsuperscript{20,269} and further demonstrate prolonged device lifetime associated with improved biocompatibility of microporous membrane-protected gap implants.

A preliminary glucose-challenge test, conducted to investigate the in vivo response of microporous membrane-protected devices, demonstrated that blood glucose concentrations in diabetic rats implanted with these 60 µm microporous membrane devices closely resembled that of healthy rats receiving the same treatment (Supplementary materials, Chapter 8.1.1).

### 4.6 Conclusions

These findings suggest that improvements to the biocompatibility and functional lifetime of a chemically driven closed-loop insulin delivery implant may be achieved by hindering leukocyte migration to the functional device surface. This is accomplished in a passive manner for up to 30
days with the aid of a topographically smooth and biologically inert microporous membrane that served to alter the geometric design of the implant opening.

4.7 Acknowledgements

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Chapter 5

A Multifunctional Polymeric Nanotheranostic System Delivers Doxorubicin and Imaging Agents Across the Blood-Brain Barrier Targeting Brain Metastases of Breast Cancer

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Author contributions described in section 5.6.

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5.1 Abstract

Metastatic brain cancers, in particular cancers with multiple lesions, are one of the most difficult malignancies to treat owing to their location and aggressiveness. Chemotherapy for brain metastases offers some hope. However, its efficacy is severely limited as most chemotherapeutic agents are incapable of crossing the blood-brain barrier (BBB) efficiently. Thus a multifunctional nanotheranostic system based on poly(methacrylic acid)-polysorbate 80-grafted-starch was designed herein for the delivery of BBB-impermeable imaging and therapeutic agents to brain metastases of breast cancer. *In vivo* magnetic resonance imaging and confocal fluorescence microscopy were used to confirm extravasation of gadolinium and dye-loaded nanoparticles from intact brain microvessels in healthy mice. The targetability of doxorubicin (Dox)-loaded nanoparticles to intra-cranially established brain metastases of breast cancer was evaluated using whole body and *ex vivo* fluorescence imaging of the brain. Coexistence of nanoparticles and Dox in brain metastatic lesions was further confirmed by histological and microscopic examination of dissected brain tissue. Immuno-histochemical staining for caspase-3 and terminal-deoxynucleotidyl transferase dUTP nick end labeling for DNA fragmentation in tumor-bearing brain sections revealed that Dox-loaded nanoparticles selectively induced cancer cell apoptosis 24 hours post injection, while sparing normal brain cells from harm. Such effects were not observed in the mice treated with free Dox. Treatment with Dox-loaded nanoparticles significantly inhibited brain tumor growth compared to free Dox at the same dose as assessed by *in vivo* bioluminescence imaging of the brain metastases. These findings suggest that the multifunctional nanoparticles are promising for the treatment of brain metastases.

5.2 Introduction

Brain metastases are one of the most difficult malignancies to treat and have poor patient prognosis. They are estimated to occur in 10 - 30% of all cancer patients, of which 20-30% presents with solitary brain metastasis and the remainder present with multiple lesions. While surgical resection may be possible for primary brain tumors and brain metastases with up to three lesions, whole brain radiotherapy (WBRT) is preferred for patients with multiple brain metastases due to a high risk of surgical complications including intracranial infection or brain edema. Given the significant neurotoxicity associated with WBRT, alternative non-invasive
methods including chemotherapy are desirable for the treatment of brain tumors and brain metastases\textsuperscript{31}.

Unfortunately, malignancies in the central nervous system (CNS) are notably resistant to systemic chemotherapy as many chemotherapeutic agents are incapable of efficiently crossing the blood-brain barrier (BBB)\textsuperscript{23-27}. This barrier at the capillary-CNS interface is comprised of specialized tight junctions between vascular endothelial cells which interface with associated pericytes and astrocytes to regulate the entry of nutrients and others substances to the brain. The action of drug efflux pumps such as P-glycoprotein (P-gp) or breast cancer resistance protein (BCRP) at the BBB further reduce accumulation of chemotherapeutic agents including doxorubicin (Dox) to the CNS. The vast majority of CNS drugs currently on the market must be administered at very high doses resulting in severe side effects in peripheral organs\textsuperscript{25}.

Various approaches have been investigated to enhance drug delivery to the brain including invasive and non-invasive means\textsuperscript{30-32}. Invasive approaches are expensive and associated with a high degree of patient discomfort and a high risk of complications. Non-invasive CNS drug delivery approaches using retro-metabolic pro-drugs or specific drug-antibody conjugates targeting endocytic receptors on the BBB have been developed to improve treatment outcomes; however these approaches are susceptible to loss of drug activity after modification and require complicated and expensive preparation procedures\textsuperscript{23,27}. To mitigate this problem, nanoparticle drug carriers with a variety of targeting moieties have been investigated\textsuperscript{7-21}.

Surface modified nanoparticulate carrier systems for non-invasive CNS drug delivery offer several advantages such as high drug loading capacity, ability to evade efflux pump action at the brain microvessels, and size-tunability. Various targeting moieties have been studied to enhance nanoparticle transport across the BBB via receptor-mediated pathways, including those targeting the insulin receptor\textsuperscript{36}, transferrin receptor\textsuperscript{37}, low density lipoprotein (LDL) receptor\textsuperscript{39,40}, or glutathione receptor\textsuperscript{41}. Certain surfactants, including polysorbate 80 (PS 80) have also been employed to coat nanoparticles for brain targeted drug delivery\textsuperscript{26,42-44}. Given its low cost and approved use in many injectable pharmaceutical products\textsuperscript{45}, PS 80 offers tremendous potential as compared to antibodies.

In a series of studies, Kreuter and co-workers\textsuperscript{234,289,290} demonstrated a remarkable efficacy of Dox loaded in PS 80-coated nanoparticles for the treatment of intracranial 101/8 glioblastoma
grown in rats. Being a P-gp substrate, free Dox does not penetrate across the BBB in amounts sufficient for effective chemotherapy. Intravenous administration of Dox-loaded poly(butyl cyanoacrylate) nanoparticles coated with PS 80 significantly extended the survival times of rats bearing a single brain tumor\(^{234}\). It has been demonstrated that PS 80 coated nanoparticles recruit apolipoprotein-E (ApoE) in the plasma, mimicking LDL particles which are transported cross the BBB via LDL receptor-mediated transcytosis due to elevated expression of these receptors on brain microvessel endothelium\(^{44}\). Later ApoE or Apolipoprotein-B (ApoB) covalently-modified nanoparticles were prepared for crossing the BBB\(^{291-293}\). However the cost and availability of lipoproteins could limit their future applications.

Recently cell-penetrating peptide-linked dendrigraft poly-L-lysine nanoparticles and arginylglycylaspartic acid (RGD)-conjugated polymer micelles have been developed and shown to exhibit antitumor efficacy in U87MG human glioblastoma xenografts\(^{46,47}\). To our knowledge, the studies of nanoparticles for drug delivery across the BBB have been limited so far to primary brain tumors such as glioblastomas. Given the vast anatomical differences between brain metastases and primary glioblastomas\(^{52}\), delivering drugs to multiple brain metastasis lesions remains very challenging\(^{55}\). In this study, a new multifunctional theranostic nanocarrier system was designed based on a terpolymer containing poly(methacrylic acid) and PS 80 covalently grafted onto starch (PMAA–PS 80-g-St), which was previously developed in our laboratory using a novel one-pot dispersion polymerization method\(^{294-297}\). The PS 80 content in the nanocarrier system was optimized to facilitate brain entry. We first examined BBB-penetration and brain accumulation of the nanoparticles containing gadolinium (Gd) and Hoechst 33342, two BBB-impermeable imaging agents, in healthy mouse brain by using magnetic resonance imaging (MRI) and confocal fluorescence microscopy, respectively. The unique nuclear staining property of Hoechst 33342 was utilized previously to demonstrate the capability of nanoparticles for delivering BBB-impermeable agent to cross the BBB\(^{298,299}\). The accumulation of Dox-loaded nanoparticles in a brain metastasis model of triple (estrogen, progesterone and Her2/neu receptor) negative human breast cancer was assessed by in vivo bioluminescent and fluorescent imaging. Microscopic localization of nanoparticles and Dox in tumor-bearing brain tissue was examined histologically using fluorescence microscopy. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and caspase-3 immunohistochemistry was used to evaluate apoptosis in metastatic lesions following treatment with Dox-loaded nanoparticles. Longitudinal
in vivo bioluminescence imaging of brain tumor-bearing mice was employed to assess the effect of Dox-loaded nanoparticles on tumor growth inhibition as compared to treatment with equivalent doses of free Dox. To our knowledge this work represents for the first time a nanotheranostic system that has been generated to 1) specifically deliver anticancer drugs to multiple lesions of brain metastases with large to micro-sizes and 2) selectively destroyed cancer cells while sparing normal brain cells from damage.

5.3 Materials & Methods

5.3.1 Materials

Soluble corn starch (MW = 11,000 g/mol), methacrylic acid (MAA), sodium thiosulfate (STS), potassium persulfate (KPS), polysorbate 80 (PS 80), sodium dodecyl sulphate (SDS), fluoresceinamine isomer I (FA), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-Hydroxysuccinimide (NHS), gadolinium chloride hexahydrate, diethylenetriaminepenta acetic acid (DTPA), and all other chemicals unless otherwise mentioned were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). HiLyte Fluor™ 750 hydrazide (HF 750) was purchased from AnaSpec (Fremont, CA, USA). DTPA bis-anhydride (DTPA-bis-An) was synthesized in-house using a previously described method.295

5.3.2 Synthesis and Preparation of PMAA-Ps 80-g-St Polymer and Nanoparticles

5.3.2.1 Synthesis of PMAA-PS 80-g-St Polymer

PMAA-PS 80-g-St polymer was synthesized using a method described previously294 with the following feed composition: 1.55 g of starch, 1.55 g of MAA, 1.5 g of PS 80, 0.25 g of SDS, 0.12 g of KPS, 0.20 g of STS. All the chemicals were dissolved in doubly distilled deionized (DDI) water to make a final volume of 200 mL. Briefly, 1.55 g of starch was dissolved in 150 mL of distilled water by heating at 70 °C for 30 min. The solution was purged with N₂ for 30 min to remove any dissolved oxygen. Subsequently, 0.25 g of SDS, 1.5 g of PS 80, 0.12 g of KPS and 0.25 g of STS were added to the starch solution while being stirred. After 10 min, the reaction was started by addition of 1.55 g of nitrogen purged MAA. Opalescence appeared after 5 min and the reaction was continued for 8 hours at 70 °C to ensure complete grafting. The
product was dialyzed (molecular weight cut-off 25,000 g/mol) extensively against warm water for 3 days, against methanol for another 2 days and against water again for another 2 days. The purified polymer was then dried at 50 °C for 24 hours, and stored in a desiccator for future use.

5.3.2.2 Preparation of GD$_{3}^{3+}$ Loaded PMAA-PS 80-g-St Polymer

GD$_{3}^{3+}$ was loaded into the polymer using a method described previously$^{295}$. Briefly, DTPA was conjugated to the starch terpolymer by addition of 1.5 g of DTPA-bis-anhydride to 3 g terpolymer dissolved in 50 mL of dry dimethyl sulphoxide (DMSO). The solution was stirred at room temperature for 24 hours, dialysed against DMSO for 48 hours, and subsequently dialysed against water for another 48 hours. Loading of GD$_{3}^{3+}$ was achieved by dispersing the PMAA-PS 80-g-St-DTPA polymer (0.5 g) in 10 mL of distilled deionized water. The pH was adjusted to 6.5 using 0.1 N NaOH. 10 mL of aqueous solution of gadolinium chloride hexahydrate (10 mg/mL) was then added drop wise while stirring, and the pH of the reaction was kept at 6.5 with the 0.1 N NaOH. After stirring for 1 hour, the product was dialyzed exhaustively against 0.9% NaCl until no free GD$_{3}^{3+}$ was detected in the wash medium using the xylenol orange test. The product was then neutralized and freeze-dried.

5.3.2.3 Preparation of Dye Loaded PMAA-PS 80-g-St Polymer

Covalent linkage of two fluorescence moieties, namely HiLyte Fluor$^\text{TM}$ 750 (HF 750; $\lambda_{\text{ex}}$ = 745 nm, $\lambda_{\text{em}}$ = 820 nm) (0.4 mg) and FA ($\lambda_{\text{ex}}$ = 496, $\lambda_{\text{em}}$ = 520) (0.5 mg), to the polymer (40 mg) was achieved using a method previously described$^{296}$. Briefly, to 2 mL of aqueous solution of purified polymer, 30 mg of EDC and 30 mg NHS were added. After 30 min, 0.4 mg of HF 750 (1.25 mg/mL in DDI water) and/or 0.5 mg of FA was added under stirring. The mixture was protected from light and stirred at room temperature for 24 hours. Finally, the product was neutralized to pH 7.5 using 0.1 N NaOH and purified by extensive dialyzing against DDI water.

5.3.2.4 Preparation of Hoechst 33342-Loaded PMAA-PS 80-g-St Nanoparticles

Hoechst 33342-loaded PMAA-PS 80-g-St nanoparticles were prepared by heating 250 µL of 10 mg/mL Hoechst 33342 solution, 100 µL of 40 mg/mL terpolymer solution, 100 µL of 100 mg/mL PF 68 solution, and 12 mg of ethyl arachidate to 65°C. The mixture was stirred for 20 minutes. Nanoparticles were formed under ultrasonication using a Hielscher UP100H probe
ultrasonicator, (Hielscher USA, Inc., Ringwood JN, USA) for 10 min and suspended in sterile 5% dextrose to a final Hoechst 33342 concentration of 2.5 mg/mL. An analogous control formulation was prepared from PMAA-g-St-lipid polymer without PS 80.

5.3.2.5 Preparation of Self-Assembled Dox-Loaded PMAA-PS 80-g-St Nanoparticles

Self-assembled nanoparticles were prepared by first dissolving 8 mg of PMAA-PS 80-g-St polymer in 1.8 mL of sterile 5% dextrose. The polymer solution was then placed in an ice bath and, while under ultrasonication using a Hielscher UP100H probe ultrasonicator, (Hielscher USA, Inc., Ringwood JN, USA), 200 µL of Dox solution (12 mg/mL in 5% dextrose) was added in small increments to the polymer solution every 30 s. Ultrasonication continued for an additional 10 min. Addition of the Dox resulted in spontaneous formation of nanoparticles. The nanoparticles were then passed through ion exchange resins, Sephadex G50 fine (GE Healthcare, Piscataway, NJ, USA) to remove unbound Dox.

5.3.3 Physicochemical Characterization of the PMAA-PS 80-g-St Polymer and Nanoparticles

Proton magnetic resonance imaging (1H NMR) measurements for PMAA-PS 80-g-St polymer (15 mg/mL in 0.01 M NaOD solution) were obtained using a Varian Mercury 400 MHz spectrometer (Palo Alto, CA, USA). The spectra were obtained over 64 scans using a pulse angle of 25°, a 2 s acquisition time, and a 10 s delay time. All chemical shifts are reported in parts per million (ppm) with the water peak as reference.

The particle size and the ξ-potential of the PMAA-PS 80-g-St nanoparticles were determined with Malvern Zetasizer Nano ZS (Worcestershire, UK). For size measurements, the particles were dispersed in pH of 7.4 phosphate buffered saline (PBS) with an ionic strength of 150 mM. For ξ-potential measurements PBS buffers of pH 7.4 and ionic strength of 10 mM were used. The morphology of the nanoparticles was examined by transmission electron microscopy (TEM). TEM photographs were acquired on a Hitachi H7000 electron microscope (Mississauga, ON, Canada) with an accelerating voltage of 100 kV.
5.3.4 Time-of-Flight-Secondary Ion Mass Spectrometry

TOF-SIMS analysis was carried out on an ION-TOF TOFSIMS IV spectrometer (Munster, Germany). Analysis was performed with a 25 keV Ga\(^+\) primary ion source. Negative secondary ions passing through a reflectron mass spectrometer (Agilent Santa Clara, CA, USA) were detected with a microchannel plate assembly operating at 10 kV post acceleration. The analysis area was 500 × 500 \(\mu\text{m}^2\) to reduce charging effects.

5.3.5 Animal Models

All animal handling and procedures were conducted under an approved protocol from the Animal Care committee at the Ontario Cancer Institute following guidelines set forth by the Canadian Council on Animal Care (CCAC). Eight to ten week old Balb/c mice (Jackson Laboratories, Bar Harbor, ME, USA) were used to evaluate whole-body nanoparticle biodistribution and kinetics in normal (non-tumor bearing) mice. Mice were fed water and food ad libitum, housed with a 12/12 hour light/dark schedule in a temperature and humidity controlled room.

A brain metastatic breast cancer model was established in four to six week old SCID mice (Ontario Cancer Institute, Toronto, Canada) to evaluate nanoparticle and Dox brain tumor accumulation and subsequent tumor cell apoptosis. Luciferase-transfected human breast cancer cells (MDA-MB-231-luc-D3H2LN, obtained from Caliper Life Sciences and checked for mycoplasma contamination) 3×10\(^4\)/mouse were injected intracranially into the cortex (about 3mm depth) by using a stereotactic device. Tumors were monitored for growth with luciferine-induced (15 mg/kg, 10 min post intraperitoneal injection) bioluminescence imaging over the course of the two-week growth period using an Xenogen IVIS spectrum imager (Caliper Life Sciences, Inc., Hopkinton, MA, USA).

A brain metastatic breast cancer model was established in four to six week old NRG-SCID mice (Ontario Cancer Institute, Toronto, Canada) to evaluate inhibition of brain tumor growth following treatment with Dox-loaded NPs, free Dox, or saline. Luciferase-transfected human breast cancer cells (MDA-MB-231-luc-D3H2LN, obtained from Caliper Life Sciences and checked for mycoplasma contamination) 1×10\(^5\)/mouse were injected intracranially into the cortex (about 3 mm depth) by using a stereotactic device. Tumors were monitored for growth with luciferine-induced (15 mg/kg, 10 min post intraperitoneal injection) bioluminescence.
imaging over the course of the two-week growth period using an Xenogen IVIS spectrum imager (Caliper Life Sciences, Inc., Hopkinton, MA, USA).

5.3.6 *In vivo* Magnetic Resonance Imaging

*In vivo* MRI used a 7 Tesla micro-MRI spectrometer (BioSpec USR, Bruker, Ettlingen, DE), fitted with the B-GA12 gradient coil and a 7.2 mm inner diameter quadrature radiofrequency (RF) coil. Mice were anaesthetized by breathing 1.8% isoflurane, and imaged in supine position on a custom slider bed. A 7.2-cm inner diameter linearly polarized cylindrical volume coil was used for RF transmission, and a dedicated murine brain receive-only RF coil was used for MR signal reception. A pneumatic pillow under the thorax/abdomen provided a signal for both physiologic monitoring and respiratory gated imaging (SA Instruments, Stony Brook, NY, USA). Mice were also prepared via tail vein cannulation with a 27 G needle and a precision line (80 µL internal volume), to enable manual contrast injection following baseline scanning. Brain T₁ changes from baseline following contrast agent injection were measured using a respiratory-gated variable-flip-angle (VFA) approach. At each time-point, 3D-FLASH images were acquired sequentially in a vertical plane at flip angles of 2, 10, 20, and 30-degrees, with all other data acquisition parameters held constant (Echo time (TE)/ Repetition time (TR) = 2.6/25 ms, 128 × 128 × 16 matrix over a 16 × 16 × 16 mm field-of-view providing 0.125 × 0.125 × 1 mm spatial resolution, 81.5 kHz readout bandwidth). The acquisition time for each flip angle was approximately 2 minutes. R₁ maps were generated from signal and flip angle data pairs following linearization of the MR signal equation by linear regression (Matlab, The Mathworks, Natick, MA, USA). Quantitative measurements were performed in manually segmented cortical, subcortical, and sagittal sinus sub-regions, which were registered across all time-points, using MIPAV software (National Institutes of Health, Bethesda, MD, USA). Matching multi-slice T₂-weighted 2D-RARE (Rapid acquisition relaxation enhancement) images with RARE factor of 16 were also acquired using the following data acquisition parameters: TE = 72 ms, TR = 4400 ms, readout bandwidth = 50 kHz, scan time = 4 min 24 seconds, FOV = 100 × 100-micron over 16 × 16 mm, and slice thickness of 1 mm (16 slides).

5.3.7 *In Vivo* Whole-Body Fluorescence Imaging

Whole-body *in vivo* nanoparticle biodistribution and tumor accumulation in healthy Balb/c and tumor-bearing SCID mouse brain was examined using Xenogen IVIS spectrum imager (Caliper
Life Sciences, Inc., Hopkinton, MA, USA) at 745 nm excitation and 820 nm emission wavelengths. Mice were anaesthetized with 1.8% isoflurane prior to whole-body in vivo imaging.

5.3.8 *Ex-Vivo* Fluorescence Imaging of the Brain

To examine nanoparticle accumulation in brain tumor-bearing SCID mice, fluorescently labeled nanoparticles (200 µL injection volume; 8 mg/mL polymer; 10 mg/kg Dox for tumor-bearing mice) were injected into the lateral tail vein of the mice. At pre-determined time points the mice were euthanized by CO₂ asphyxiation, and the brain was dissected. NIR fluorescence images of the dissected brains were obtained using the Xenogen IVIS spectrum imager (Caliper Life Sciences, Inc., Hopkinton, MA, USA). The brain fluorescence intensities were then quantified by drawing the region of interest (ROI) using the analysis software package supplied by the manufacturer. The accumulation of the nanoparticles in the brain was evaluated by measuring the ratio of NIR fluorescence intensity of nanoparticle injected tissue to untreated tissue.

5.3.9 Delivery of BBB-Impermeable Dye into Healthy Brain

To examine nanoparticle penetration into healthy brain, Balb/c mice were treated with 200 µL of Hoechst 33342-loaded nanoparticles with PS 80 (PMAA-PS 80-g-St) or without PS 80 (PMAA-g-St) (2.5 mg/mL dye), or free Hoechst 33342 (2.5 mg/mL in saline) via tail vein injection. Mice were euthanized 1 or 2 hours following treatment. Texas red-labeled dextran (10,000 MW, Life Technologies, CA, USA) was administered intravenously 15 minutes prior to euthanasia. The brain was dissected, fixed in 10% formalin for 3 hours, transferred to 30% dextrose solution overnight, embedded in Tissue-Tek OCT resin (Somagen, Torrance, CA, USA) and finally flash frozen. Thaw mounted 20 µm thick frozen sections were obtained on a Leica, model CM3050S cryostat and analyzed using Zeiss LSM700 confocal microscope and fluorescent excitation and emission filters appropriate for detection of the indicated chromophores within the cortex (Dextran, Texas Red: Ex./Em. = 595/615 nm; Hoechst 33342: Ex./Em. = 352/461 nm).

5.3.10 Nanoparticle Microdistribution in Tumor-Bearing Brain Tissue

To investigate nanoparticle accumulation and Dox release in tumor-bearing brain tissue, HF 750-labeled nanoparticles (200 µL injection volume; 8 mg/mL polymer; NPs were loaded with 10 mg Dox per kg mouse bodyweight) were injected into the tail vein of metastatic breast cancer brain tumor-bearing SCID mice. Six hours post treatment, the mice were euthanized and the brain
tissues were dissected, frozen and sliced into 10 µm thick sections. The air-dried samples were imaged using an AMG EVOS FL fluorescence microscope (Thermo Fisher Scientific, Inc., MA, USA). Nanoparticle distribution around the tumor was examined within the QD800 emission window (Ex/Em. = 710/800 nm). Doxorubicin localization was imaged over the GFP spectral window (Ex./Em. = 470/525 nm). Cell nuclei were imaged within the DAPI spectral window (abs/em = 344-357/447-460 nm) after incubation with Hoechst 33342 trihydrochloride, trihydrate (Invitrogen, 1:10,000 dilution) and rinsing with PBS (pH 7.4). Photo contrast corrections and overlays were performed using Adobe Photoshop software.

5.3.11 Apoptosis in Tumor-Bearing Brain Tissue

To investigate brain tumor cell apoptosis following Dox-loaded NP or free Dox treatment, 200 µL of PMAA-PS 80-g-St nanoparticles (8 mg/mL polymer; NPs were loaded with 10 mg Dox per kg mouse bodyweight), or free Dox (10 mg/kg), was administered via lateral tail vein injection into metastatic breast cancer brain tumor-bearing SCID mice. The mice were euthanized 24 hours following treatment. The brain samples were bisected along their midline and post-fixed in 4% paraformaldehyde, 0.1 M PBS overnight at 4°C. Specimens were then removed from fixation, flushed and equilibrated in 30% sucrose at 4°C, embedded in Tissue-Tek OCT resin (Somagen, Torrance, CA, USA) and finally flash frozen. Thaw mounted 10 micron thick frozen sections were obtained on a Leica, model CM3050S cryostat (Concord, ON, Canada). Tumor cell apoptosis was determined by TUNEL reaction (TdT In Situ Apoptosis Detection Kit, R&D Systems, Minneapolis, MN, USA) and immunofluorescent labeling of cleaved caspase-3. The TUNEL reaction product was visualized with streptavidin-biotin-FITC complex at 10X magnification. Apoptotic TUNEL-(+) and Caspase 3+ cells were imaged using a Nikon Eclipse E1000 motorized microscope equipped with a 270° rotating stage, Nomarski contrast optics, and fluorescent excitation and emission filters appropriate for detection of the indicated chromophores (DAPI: Ex./Em. 358/461 nm, FITC: Ex./Em. 470/525 nm) (Tokyo, Japan). Images were captured using a Hamamatsu C4742-95 camera (Hamamatsu, SZK, Japan). Measurement of TUNEL-(+) and caspase-3-(+) cells was performed on >2 visual fields per brain. The number of apoptotic cells within each field was normalized to the tumor area, and this was averaged for each animal for statistical analysis. The number of apoptotic cells for each treatment was taken as the average of the animal normalized apoptotic cell numbers.
5.3.12 Tumor Growth Inhibition Study

Tumor bearing NRG-SCID mice were used to evaluate tumor growth inhibition. Luciferase-trasfected human breast cancer cells (MDA-MB-231-luc-D3H2LN) were injected intracranially into the cortex. The first treatment with free Dox, Dox-loaded NPs (10 mg Dox per kg mouse body weight, 200 µL administered i.v.), or saline (200 µL) was administered on day 0, between one to two weeks following inoculation depending on the tumor size as measured through in vivo bioluminescence imaging. A second treatment was administered two weeks later on day 14. Tumor growth was monitored in vivo using bioluminescence imaging for up to four weeks following the first treatment. The fold increase in average tumor size was obtained by normalizing the tumor radiance over the course of the experiment to the initial tumor radiance at day zero.

5.3.13 Statistics

A commercial statistical software package (SPSS version 13.0, SPSS Institute, Chicago, IL, USA) was used for statistical analysis. Data was presented as a means ± standard deviation (SD and/or SEM), and analyzed using the Student’s t-test. P < 0.05 was considered statistically significant, and all probabilities were two-tailed.

5.4 Results and Discussion

This paper describes the investigation of a multifunctional PMAA-PS 80-g-St nanoparticulate system for the delivery of BBB-impermeable drugs and contrast agents to the brain in healthy mice and to multiple lesions of brain metastases of human breast cancer in a mouse model. The novel nanocarrier system is a single multifunctional platform with multi-modal imaging and drug delivery capabilities. It can effectively co-encapsulate hydrophobic moieties, cationic drug (e.g., Dox) and multiple contrast agents (e.g., MR contrast agent gadolinium, near infrared (NIR) fluorescence probe HF 750), enhance brain tumor accumulation, and improve in vivo biodistribution of drugs. While PS 80-coated nanoparticles have previously been used for the treatment of primary glioblastoma tumors23,234, this paper presents the first application of a multifunctional theranostic system containing covalently bound PS 80 for the diagnosis and treatment of brain metastases of breast cancer with tumor-specific cytotoxicity, a significant advance toward chemotherapy of brain metastases.
5.4.1 Synthesis and Characterization of PMAA-PS 80-g-St Polymer and Nanoparticles

A starch-based polymer was synthesized for the preparation of nanoparticles due to its excellent biocompatibility and biodegradability, and an abundance of reactive functional groups for further modification. Both PMAA and PS 80 are biocompatible and are widely used in Federal Drug Administration (FDA) approved pharmaceuticals. Grafting these two polymers onto the starch backbone imparted the nanocarrier system BBB-penetrating and pH-dependent drug release properties, respectively (Figure 27). This polymer was further modified with a Gd chelating agent DTPA-bis-An and a near infrared NIR fluorescence dye (H₂N-NH-HiLyteFluor™ 750, HF 750) for dual modality in vivo imaging using MRI and fluorescence imaging. The PMAA-PS 80-g-St polymer was characterized by ¹H NMR spectroscopy in 0.01 N NaOD with the peaks corresponding to starch, PMAA, and PS 80 highlighted in Figure 26. The spectrum of PS 80 is also included for comparison. The areas under the peaks at 5.1, 3.70, and 1.66 were used to calculate the molar ratio of starch, PS 80, and MAA in the final product (Table 6).

For studies in healthy mice, FA (λ_ex = 496, λ_em = 520) and Gd conjugation to the DTPA and HF 750 modified PMAA-PS 80-g-St terpolymer resulted in the spontaneous formation of nanoparticles. Hoechst 33342-loaded nanoparticles with or without PS 80 component were formed by the addition of ethyl arachidate under sonication. The Dox-loaded nanoparticle formulation for studies in tumor-bearing mice excluded conjugation with FA because Dox autofluorescence occurs over similar wavelengths and the addition of Dox to the polymer induces nanoparticle self-assembly. The composition, size and surface charge of the nanoparticles are listed in Table 6. All nanoparticles exhibited a negative zeta potential that is favorable for biocompatibility and nanoparticle colloidal stability, especially for particles that rely on electronic repulsion forces for stability. Moreover, the negative surface charge appears important for making nanoparticles non-toxic to the BBB. Transmission electron microscopy (TEM) of the formulated nanoparticles showed spherical particle geometry (Figure 27). Time of flight – secondary ion mass spectrometry (TOF-SIMS) confirmed the presence of covalently linked PS 80 on the nanoparticles surface as evidenced by the characteristic peaks at 255, 265, and 282 m/z in the negative ion mode (Figure 28). These peaks represent the series of oleic and stearic fatty acid side chains of the sorbitan molecule. These peaks were absent from the control
samples lacking PS 80.

\[
\begin{align*}
\text{R} = \text{-CH}_3 \text{OC} \left( \text{CH}_2 \right)_5 \text{CH}_2 \text{CH=CHCH}_2 \left( \text{CH}_2 \right)_6 \text{CH}_3
\end{align*}
\]

Figure 26. \(^1\)H NMR spectra of 1) PS 80, 2) PMAA-PS 80-g-St in 0.1M NaOD.

\[
\begin{align*}
x + y + z + w &= 20
\end{align*}
\]

\[
\begin{align*}
\text{R} &= \text{PS 80}
\end{align*}
\]

Figure 27. Structures of the terpolymer and PS80, and a schematic diagram of the self-assembly of PMAA-PS 80-g-St terpolymer into nanoparticles upon conjugation of the fluorescent
moieties, chelation with Gd$^{3+}$ and complexation with doxorubicin. Inset: TEM image of the self-assembled nanoparticles. Scale bar = 100 nm.

Table 6. Composition and properties of terpolymer nanoparticle formulations loaded with Gd, HF 750, FA or HF 750 and Dox.* Values shown are the means and standard deviations of the mean for n = 3 independent experiments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gd content (wt%)</th>
<th>HF 750 content (µmol/g)</th>
<th>FA content (µmol/g)</th>
<th>Dox content (wt%)</th>
<th>Hoechst 33342 content (mg/mL)</th>
<th>Particle size (nm)</th>
<th>ξ-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMAA-PS 80-g-St NP loaded with Gd, HF 750 and FA</td>
<td>10.2±0.7</td>
<td>5.5±0.1</td>
<td>22.1±0.2</td>
<td>-</td>
<td>-</td>
<td>40.0±5.5</td>
<td>-27.5±5.2</td>
</tr>
<tr>
<td>PMAA-PS 80-g-St NP loaded with Hoechst 33342</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.5</td>
<td>70±6.2</td>
<td>-40±4.5</td>
</tr>
<tr>
<td>PMAA-g-St NP loaded with Hoechst 33342</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.5</td>
<td>70±4.4</td>
<td>-41±3.7</td>
</tr>
<tr>
<td>PMAA-PS 80-g-St NP loaded with HF 750 and Dox</td>
<td>-</td>
<td>4.3±0.01</td>
<td>-</td>
<td>21.1±0.3</td>
<td>-</td>
<td>61.9±5.0</td>
<td>-38.0±1.0</td>
</tr>
</tbody>
</table>

*In all formulations, the feed concentrations of MAA, PS 80 and St were respectively 23.2, 1.1, and 9.2 mmol., that is, at a molar ratio of 1:0.02:0.5. Note that the mole of PS 80 was calculated based on the polymer molecular weight, while St and MAA were from monomer molecular weight.
Figure 28. Negative TOF-SIMS spectra of A) PS 80, B) PMAA-g-St, and C) PMAA-PS 80-g-St, in the m/z range of 0 to 300 atomic mass units. Dotted rectangles show expansion of the y axis by one-hundred fold and the x axis by five-fold for the designated regions. Arrows in the right hand side of the figure indicate PS80 characteristic peaks.

5.4.2 Accumulation of the PMAA-PS 80-g-St Nanoparticles in Healthy Mouse Brain

To investigate whether PMAA-PS 80-g-St nanoparticles are able to cross the BBB and enter the brain, Gd loaded nanoparticles and Hoechst 33342-loaded nanoparticles were administered
intravenously to healthy Balb/c mice for \textit{in vivo} MRI and laser scanning confocal microscopy, respectively. Dox was omitted from these formulations to prevent possible confounding effects of Dox-induced BBB damage on the nanoparticle biodistribution. Similarly, the nanoparticle biodistribution was investigated in healthy Balb/c mice without brain tumors as previous studies have suggested that brain tumor-associated vasculature may be compromised and ‘leaky’\textsuperscript{52}.

\section{5.4.2.1 MRI of Nanoparticle Distribution in Healthy Brain}

Qualitative and quantitative information on nanoparticle brain distribution was inferred from MRI generated longitudinal relaxation rate $R_1 (1/T_1)$ maps with the assumption that the change in $R_1$ relaxation rate, relative to the pre-scan, was dominated by the change in nanoparticle concentration. Examination of the $R_1$ map of the different brain slices at pre- and post-injection of nanoparticles revealed enhancement in certain brain areas such as sagittal sinus, ventricles, and to a lesser extent the cortex and sub-cortical areas (Figure 29a). At 30 minutes post injection, the $R_1$ values were measured at $1.1 \pm 0.1$ s\textsuperscript{-1}, $1.1 \pm 0.1$ s\textsuperscript{-1}, $1.6 \pm 0.2$ s\textsuperscript{-1}, and $1.7 \pm 0.1$ s\textsuperscript{-1} for cortex, sub-cortex, ventricles, and sagittal sinus respectively (Figure 29b). These values decreased to $1 \pm 0.1$ s\textsuperscript{-1}, $0.9 \pm 0.1$ s\textsuperscript{-1}, $1.5 \pm 0.3$ s\textsuperscript{-1}, and $1.1 \pm 0.1$ s\textsuperscript{-1} at 180 minutes post injection. Values shown are the means and standard deviations for $n = 3$ independent experiments.

MRI offers the advantage of imaging brain parenchyma with high resolution and good contrast between different tissues, making this modality particularly useful for monitoring different regions or sub-regions of the brain. However, MRI is susceptible to motion and magnetic field inhomogeneity artifacts and has low inherent sensitivity limiting its application for detecting small quantities of nanoparticles in various brain regions\textsuperscript{302}. For this reason, confocal laser scanning microscopy was used to investigate the ability of the nanoparticles to enter into the healthy mouse brain.
Figure 29. Quantitative MRI of brain distribution: A) $R_1$ maps of Balb/c mice (n = 3) injected with Gd$^{3+}$ loaded PMAA-PS 80-g-St nanoparticles (0.05 mmol/kg Gd$^{3+}$) at baseline (left column) and 30 minutes post injection (center column). An anatomical map is presented in the right column. B) Longitudinal relaxation rates ($R_1$) of sagittal sinus, ventricles, cortex, and sub-cortex for Gd$^{3+}$ loaded PMAA-PS 80-g-St-DTPA polymer overtime. The bottom figure shows $R_1$ values for the different brain regions up to 180 min. The asterisk (*) denotes a significant difference ($p < 0.05$) in $R_1$ values compared to baseline.
5.4.2.2 Delivery of BBB-Impermeable Dye into Healthy Brain

Nanoparticles with and without PS 80, loaded with a BBB-impermeable fluorescent dye (Hoechst 33342) that bind to nucleic acids were administered via intravenous (i.v.) tail vein injection to healthy Balb/c mice and allowed to circulate for up to 2 hours. Laser scanning confocal microscopy was used to examine brain tissue sections in three dimensions for cell nuclei stained with Hoechst 33342 (blue) in relation to brain tissue structures including neuron bodies and brain vasculature (Figure 30). Brain vasculature was labeled with dextran conjugated with Texas Red fluorophore (red). Free Hoechst 33342, or loaded within PS 80-free nanoparticles, was unable to cross the BBB when administered i.v. and thus unable to stain cell nuclei in the brain (Figure 30). In contrast, Hoechst 33342 loaded within PMAA-PS 80-g-St nanoparticles was able to enter into the brain and label cell nuclei located away from brain capillaries. Co-localized non-specific autofluorescence signal observed in both the Hoechst 33342 (blue) and Texas Red (red) filter sets are due to autofluorescing brain tissue components (e.g., lipofuscin) which have broad emission spectrums. These results indicate that PMAA-PS 80-g-St nanoparticles were able to enter the normal brain and further suggest that PS 80 might play a role in nanoparticle transport across the BBB to gain entry into the CNS. Indeed it has been suggested that coating nanoparticles with PS 80 leads to the enhanced adsorption of apolipoprotein-E (Apo-E) from the blood to the particle surface, and that the presence of Apo-E promotes nanoparticle internalization in the brain capillary endothelial cells via members of the LDL receptor family expressed by these cells. Due to presence of PS 80 on the surface of PMAA-PS 80-g-St nanoparticles, as shown by our TOF-SIMS investigations (Figure 28), it is possible that a similar mechanism is also responsible for uptake of the terpolymer nanoparticles by the brain capillary endothelial cells. This uptake mechanism is in agreement with preliminary nanoparticle uptake results in MDA-MB-231 cells (Supplementary material, Chapter 8.2.1).
Figure 30. Laser scanning confocal microscopic images of healthy Balb/c mice sections following treatment with free Hoechst 33342, Hoechst 33342 loaded within PMAA-g-St NPs (without PS 80), or Hoechst 33342 loaded within PMAA-PS 80-g-St terpolymer NPs. Mice were treated for 1 hour or 2 hours. Blood vessels were labeled by i.v. administration of Texas Red-dextran 15 minutes before euthanasia. Hoechst 33342 and Hoechst 33342-labeled cell nuclei appear blue. Texas Red-dextran appears as red. Arrows indicate representative Hoechst 33342 stained nuclei located away from blood vessels. Scale bar = 50 µm.
5.4.3 Accumulation of Dox-Loaded PMAA-PS 80-g-St Nanoparticles in Breast Cancer Brain Metastases

5.4.3.1 Breast Cancer Brain Metastasis Model

Breast cancer metastases to the brain are most prevalent in the triple negative and HER2+ breast cancer subpopulations\textsuperscript{55,304,305}, with the triple-negative subtype being particularly aggressive and unresponsive to targeted therapies due to lack of molecular target\textsuperscript{306,307}. Therefore, we established a metastasis model using MDA-MB-231-luc-D3H2LN triple-negative human breast cancer cell line. These cells overexpress LDL receptors\textsuperscript{40,308}, enabling LDL receptor-mediated cell uptake of the nanoparticles following transport across the BBB. This metastatic brain tumor model was developed in immunodeficient female SCID mice using stereotactic intracranial injection of ~30,000 MDA-MB-231-luc-D3H2LN human breast cancer cells into the primary somatosensory cortex\textsuperscript{309,310}. Brain tumor growth was monitored \textit{in vivo} by bioluminescence imaging (Figure 31a).

Tumor proliferation at the injection site and infiltration into the brain parenchyma was observed over a 2 week period following inoculation. At the injection site, metastatic foci formed along the direction of needle insertion along with neoplastic cell infiltration into brain parenchyma at sites such as the hippocampus. Small neovascular inclusions were also observed at this site between the Cornu Ammonis (CA) 1 and the dente gyrus. Numerous small independent metastatic foci were also observed distal to the injection site both within the cortex and the basal ganglia (data not shown). The stereotactic injection method is highly reproducible and exhibits a metastatic growth pattern similar that described by Saito \textit{et al.}\textsuperscript{309} The aggressive nature of brain metastasis using this breast cancer model is evident from the large number of tumor foci. This resembles breast cancer metastases in humans, which are difficult to treat by standard treatment options (\textit{e.g.} surgical resection). Normally for patients with more than four lesions surgery is not recommended\textsuperscript{311}. Thus this animal model is suitable for testing systemic chemotherapy.

5.4.3.2 \textit{In Vivo} Nanoparticle Distribution in Tumor Bearing Mice.

The tumor accumulation of HF 750-labeled terpolymer nanoparticles loaded with Dox was tracked in brain tumor-bearing mice for up to 6 hours after intravenous injection in the tail vein using near infrared fluorescence imaging (Figure 31b and Figure 32). The nanoparticles localized in the tumor region at 15 minutes post injection and remained there for at least 6 hours (Figure
The *in vivo* finding was confirmed *ex vivo* in the dissected brain (Figure 31c) 6 hours post injection, using the same imaging techniques. The co-localization of both tumor bioluminescence and nanoparticle fluorescence strongly suggests accumulation of nanoparticles in the brain tumor. The distribution of the nanoparticles in whole body was imaged at 15 min, 1 hour and 2 hours by placing the mice on the back. Nanoparticle uptake in major organs such as the liver was examined *ex vivo* at 2 hours post injection. The results are presented in Figure 32. Both *in vivo* and *ex vivo* images indicate that uptake of the nanoparticles by the liver is insignificant; there is even less uptake in the spleen. The relatively stronger fluorescence intensity in the gallbladder and the kidneys at 2 hours suggest the nanoparticles are likely eliminated by the biliary and renal pathways.

### Nanoparticle Whole Body Distribution

*Figure 31.* Brain tumor location and whole-body nanoparticle distribution. The brain metastasis of MDA-MB-231-luc-D3H2LN was established by intracranial injection. a) Bioluminescence of luciferase expressing tumor cells 10 min following i.p. injection of luciferin solution. b) PMAA-PS 80-g-St nanoparticles were labeled with a near infrared dye (HiLyte Fluor 750) and imaged for
up to 6 hours after tail vein injection. c) Bioluminescent image of brain tumor (left) and fluorescence image of nanoparticles (right) in an excised mouse brain.

Figure 32. a) Ventral view of the biodistribution of PMAA-PS 80-g-St nanoparticles labeled with a near infrared dye (HiLyte Fluor 750). b) Organ distribution of PMAA-PS 80-g-St nanoparticles labeled with a near infrared dye (HiLyte Fluor 750) 2 h after tail vein injection.

5.4.3.3 Microdistribution of Nanoparticle in Tumor Bearing Brain Tissue.
Histological analysis of tumor-bearing brain tissue sections from mice treated with Dox-loaded nanoparticles or free Dox as a comparator was performed to examine the microscopic distribution of the nanoparticles and Dox. Fluorescence images were acquired using the appropriate filter sets to visualize NIR HF 750-labeled nanoparticles, Dox, and Hoechst 33342.
stained nuclei, respectively. Brain tumor tissue was identified by areas of hyper-cellularity as evident from Hoechst 33342 stained cell nuclei shown in blue in Figure 33. Mice treated with the Dox-loaded nanoparticle formulation demonstrated nanoparticle and Dox accumulation along the brain tumor periphery (Figure 33). Colocalization of nanoparticles (red) with Dox (green) around the tumor suggests that the nanoparticle delivered Dox to the tumor site and released Dox there. Marked nanoparticle and Dox accumulation around the large tumors may be due to a combination of both LDLR-mediated nanoparticle transport across the BBB and the enhanced permeability and retention (EPR) effect.

In contrast, Dox was absent from the tumor site in mice treated with the free Dox formulation. This result is consistent with previous findings demonstrating the inability of free Dox to penetrate the BBB.\textsuperscript{312} Apparently the administration of Dox-encapsulated PMAA-PS 80-g-St nanoparticles enabled higher Dox concentrations in the brain tumor at 6 hours post treatment compared to free Dox.
Figure 33. Fluorescence images of tumor-bearing mouse brain sections 6 hours following intravenous injection of HF 750-labeled, Dox-loaded nanoparticles (left column) or free Dox (right column). Hoechst 33342-stained cell nuclei shown in blue, HF 750-tagged nanoparticles shown in red, and Dox shown in green. A composite image shows the nanoparticle and Dox distribution around the brain tumor. Tumor identified as areas of hypercellularity (T) compared to normal tissue (N). Colocalization of the nanoparticles (red) and Dox (green) suggests that Dox is delivered by and released from the nanoparticles in the tumor-bearing brain.

5.4.4 Dox loaded PMAA-PS 80-g-St Nanoparticles Inhibit Tumor Growth

5.4.4.1 Dox Loaded PMAA-PS 80-g-St Nanoparticles Selectively Induced Cancer Cell Apoptosis

Brain sections prepared from tumor-bearing SCID mice treated with free Dox or Dox-loaded nanoparticles (10 mg of Dox per kg of mouse bodyweight, administered i.v.) were stained for cleaved caspase-3 and TUNEL nick-end labeling 24 hours post treatment to examine cancer cell apoptosis as an early indication of drug efficacy. Tumors are identified as regions of marked hypercellularity and decreased ground substance with distinct tumor margins in TUNEL-stained sections. Caspases exist as inactive proenzymes in the normal cell which, once activated in the presence of apoptotic stimuli, plays a central role in the initiation and propagation of programmed cell death\textsuperscript{313}. Caspase 3 is responsible for chromatin condensation and DNA fragmentation, and is an early marker of cell apoptosis. The TUNEL reaction is a commonly used method for detecting fragmented DNA that results from apoptotic signaling cascades and is an indicator of late-stage apoptosis.

A marked number of cells expressing activated caspase-3 can be found within and surrounding tumor micro-metastases in mice treated with Dox loaded nanoparticles (Figure 34, Table 7). Such staining of activated caspase-3 is not observed in free Dox-treated mice, indicating that the nanoparticle formulation of Dox is able to induce higher cell apoptosis compared to the free drug 24 hours following treatment. Similarly, a greater number of TUNEL-(+) apoptotic cells were found distributed throughout large metastatic brain tumors (Figure 35, Table 7) and adjacent to micro-metastases (Figure 36, Table 7) of mouse brain treated with Dox loaded nanoparticles compared to free-Dox treatment. In Figure 35 and Figure 36, tumor foci can be identified as areas of hypercellularity and by areas of low background compared to the healthy brain tissue.
The difference in background fluorescence signal is attributed to autofluorescence of the tissue ground substance in the normal brain, and absence of this ground substance within the tumors. Importantly cell apoptosis is not observed in normal brain tissue (Figure 36, left panel). These results suggest that administration of Dox loaded nanoparticles to tumor-bearing mice is able to induce tumor-specific apoptosis around both large brain tumors and micro-metastases within 24 hours of treatment. Induction of tumor cell apoptosis may be attributed to the delivery of Dox rather than the nanocarrier itself as the PMAA-Ps 80-g-St terpolymer NPs were reported non-toxic in several cell lines and found to be non-toxic to MDA-MB-231-luc cells at concentrations as high as 500 µM as determined by the MTT assay (Figure 37).

The observed specific cytotoxicity of the Dox loaded nanoparticles in cancer cells in the brain may stem from (1) the capability of the terpolymer nanoparticles entering the brain and targeting the metastasis lesions (first-stage targeting via LDLR-mediated mechanism and EPR effect), (2) the specificity of binding to MDA-MB-231 cancer cells as a function of LDLR expression following the BBB-permeation (second-stage targeting), and (3) the mechanism of Dox, which induces apoptosis through interference with DNA repair and elevations in ceramide production, preferentially impedes the replication of highly proliferative cells (e.g. metastatic cancer) through DNA intercalation, while exhibiting few effects on non-replicating cell populations such as neurons.

In larger brain metastasis lesions (e.g. larger than 0.25 mm in diameter), the BBB and efflux pump expression may be compromised, allowing systemically delivered therapeutic agent to reach the tumor site and illicit a response to some degree. This disruption of the BBB is less significant in smaller micro-metastases (e.g. smaller than 0.25 mm), preventing drug from reaching the cancer cells. While chemotherapy of large primary lesions in the brain have been reported in the past, the ability of the nanocarrier system to induce apoptosis within micro-metastases in the brain is very important because drug penetration to micro-metastases is much more difficult where the blood vessels are intact. Hence drug delivery via nanoparticle transcytosis across the un-disrupted BBB is critical.
Figure 34. Cells expressing activated caspase-3 in metastatic brain tumors. Tumor-bearing brain sections prepared from mice 24 hours following intravenous injection of HF 750-labeled, Dox-loaded nanoparticles (left) or free Dox (right). Tumor tissue denoted by marked hyper-cellularity (T). Normal brain tissue (N). Caspase 3+ cells shown in brown (arrows).

Table 7. Number of TUNEL-(+) and activated caspase-3 expressing cells per mm² of tumor area. Cell counts presented as average ± SD. n > 3.

<table>
<thead>
<tr>
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<th># Activated caspase-3 expressing cells (per mm²)</th>
<th># TUNEL-(+) cells (per mm²)</th>
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</thead>
<tbody>
<tr>
<td>Dox PMAA-PS 80-g-St NPs  (n=4)</td>
<td>10.5 ± 1.7</td>
<td>43.4 ± 9.6</td>
</tr>
<tr>
<td>Free Dox (n=3)</td>
<td>1.7 ± 0.6</td>
<td>2.9 ± 6.0</td>
</tr>
<tr>
<td>p-value (p)</td>
<td>&lt;0.001</td>
<td>&lt;0.0001</td>
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Figure 35. TUNEL assay for apoptosis in large brain tumors. Tumor-bearing brain sections prepared from mice 24 hours following intravenous injection of HF 750-labeled, Dox-loaded nanoparticles in normal brain (left column) or free Dox (center column) or Dox loaded nanoparticles (right column). First row: Hoechst 33342-stained cell nuclei shown in blue. Tumor tissue denoted by marked hyper-cellularity (T). Normal brain tissue (N). Second row: FITC-labeled TUNEL- (+) cells shown in green (arrows). Tumor tissue denoted in dark green due to absence of ground substance. Third row: A composite image showing TUNEL- (+) cells in relation to tumor tissue.
Figure 36. TUNEL assay for apoptosis around brain tumor micro-metastases. Tumor-bearing brain sections prepared from mice 24 hours following intravenous injection of HF 750-labeled, Dox-loaded nanoparticles (left column) or free Dox (right column). First row: Hoechst 33342-stained cell nuclei shown in blue. Tumor tissue denoted by marked hyper-cellularity (T). Normal brain tissue (N). Second row: FITC-labeled TUNEL-(+) cells shown in green (arrow). Tumor
tissue denoted in dark green due to absence of ground substance. Third row: A composite image showing TUNEL-(+) cells in relation to tumor tissue.

Figure 37. *In vitro* cytotoxicity of MDA-MB-231-luc cells to increasing treatment concentrations of Dox-loaded PMAA-PS 80-g-St NPs, free Dox, PMAA-PS 80-g-St terpolymer and blank PMAA-PS 80-g-St NPs formed from chelation with Gd by MTT assay. Cells were treated for 24 h. Cells with no treatment and incubated with blank nanoparticles were used as control for free drug and drug loaded nanoparticle respectively. Cell viability is expressed as the percent of control for each treatment group. Data points represent the mean ± SEM. n = 4 for each experimental group.

5.4.4.2 Dox Loaded PMAA-PS 80-g-St Nanoparticles Inhibit Brain Tumor Growth

To evaluate the effect of Dox-loaded NPs on tumor growth inhibition, a brain metastasis model was established in immunodeficient female NRG-SCID mice by stereotactic intracranial injection of ~100,000 MDA-MB-231-luc D3H2LN human triple negative breast cancer cells into the cortex (Figure 38). NRG-SCID mice were selected for these experiments because they can tolerate higher doses of Dox required for therapeutic effect compared to SCID mice which lack some mechanisms of DNA repair. At two and four weeks following tumor inoculation, the mice were treated with free Dox, Dox-loaded PS 80-containing NPs (10 mg Dox per kg mouse body
weight, 200 µL administered i.v.), or saline (200 µL) as a control. Tumor growth was monitored in vivo using bioluminescence imaging.

Brain tumor bearing mice treated with saline or free Dox exhibited rapid tumor growth following tumor inoculation (Figure 38). The ineffectiveness of free Dox is in agreement with previous observations as Dox is unable to penetrate through the BBB at appreciable levels. In contrast, brain tumor treated with Dox-loaded PMAA-PS 80-g-St NPs underwent a much slower growth rate indicated by the significantly lower bioluminescence signals. At days 14, 21 and 28, the tumor inhibition rates of Dox-loaded PMAA-PS 80-g-St NPs relative to free Dox are 3.4, 11.7, and 8.8 fold respectively. These results suggest that PMAA-PS 80-g-St NPs are able to deliver Dox across the BBB to intracranial lesions of triple negative breast cancer and inhibit tumor growth.

Note that we did not observe a statistically significant correlation between tumor size and mouse body weight. That is to say that some mice with relatively small tumors experienced significant weight loss while others with relatively large tumors experienced minimal weight loss. This may be a result of the aggressive metastatic nature of the MDA-MB-231-luc D3H2LN breast cancer brain tumor model and the fact that specific locations within the brain are more critical to body weight loss than others. A small metastatic lesion located at a critical region within the brain may be more detrimental to animal health than a large lesion elsewhere. Thus the health and survival of metastatic brain tumor bearing mice may be highly dependent on tumor location rather than size.
Figure 38. Brain tumor growth inhibition in NRG-SCID mice. a) Treatment schedule with saline (200 µL), Dox (10 mg/kg; 200 µL), or Dox-loaded NPs (10 mg/kg Dox; 200 µL). Treatments were administered on day 0 and 14. b) *In vivo* images of brain tumor bioluminescence. c) Fold increase in the average tumor radiance as measured by *in vivo* bioluminescence imaging. Data presented as mean ± SEM (n = 5 for saline; n ≥ 7 for free Dox and Dox-NP). Statistical significance of p < 0.05 denoted by *.

### 5.5 Conclusions

A multifunctional nanotheranostic system based on PMAA-PS 80-g-St terpolymer for the delivery of anticancer drug and imaging agents to the brain and brain metastases of breast cancer
is presented. The ability of the nanocarrier to enter the brain and extravasate from intact brain microvessels of healthy mice was demonstrated by in vivo MRI and ex vivo confocal microscopy. The Dox-loaded nanoparticles accumulated in the intracranial breast cancer brain metastases quickly and released Dox in the metastatic lesions, as detected by histological analysis and fluorescence microscopy, whereas no Dox was detectable in the samples treated with free Dox. A large number of apoptotic cells were detected in both large metastatic lesions and micro-metastases 24 hours following treatment with Dox-loaded nanoparticles, while few were detected in the normal brain tissue, indicating cancer-cell specific cytotoxicity of the treatment. In contrast, few apoptotic cells were observed in the free-Dox control. Treatment with Dox-loaded nanoparticles reduced brain tumor growth in NRG-SCID mice compared to free Dox. These results suggest that PMAA-PS 80-g-St nanoparticles are a promising theranostic system for the delivery of drugs and contrast agents to the brain and for the treatment of brain metastases of breast cancer.

5.6 Author Contributions

A. Shalviri, P. Cai, and J. Li contributed equally to this work. A. Shalviri developed and characterized the terpolymer system. A. Shalviri, P. Cai, and J. Li designed and performed the experiments with healthy mice. J. Li and P. Cai designed and performed the experiments with tumor-bearing mice. J. Li analyzed the data and wrote the manuscript. J.T. Henderson prepared the histological sections. C. He assisted in the preparation of nanoparticles. P. Broderson assisted in terpolymer characterization. W. Foltz collected and analysed the MRI results. P. Prasad assisted in experiments with healthy mice. R. DaCosta and Y. Chen provided technical assistance with tumor-bearing mouse model. X.Y. Wu, J.T. Henderson, and A.M. Rauth, provided technical support, conceptual advice and edited the manuscript. X.Y. Wu supervised the project.

5.7 Acknowledgments

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Chapter 6

Blood-Brain Barrier-Penetrating Amphiphilic Polymer Nanoparticles Deliver Docetaxel for the Treatment of Brain Metastases of Triple Negative Breast Cancer

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Author contributions described in section 6.6.
6.1 Abstract

Brain metastasis is a fatal disease with limited treatment options and very short survival. Although systemic chemotherapy has some effect on peripheral metastases of breast cancer, it is ineffective in treating brain metastasis due largely to the blood-brain barrier (BBB). Here we developed a BBB-penetrating amphiphilic polymer-lipid nanoparticle (NP) system that efficiently delivered anti-mitotic drug docetaxel (DTX) for the treatment of brain metastasis of triple negative breast cancer (TNBC). We evaluated the biodistribution, brain accumulation, pharmacokinetics and efficacy of DTX-NP in a mouse model of brain metastasis of TNBC. Confocal fluorescence microscopy revealed extravasation of dye-loaded NP from intact brain microvessels in healthy mice. DTX-NP were also observed to extravasate from brain microvessels and accumulate in micrometastasis lesions in the brain. Treatment of mice bearing brain metastases with DTX-NP increased the blood circulation time of DTX by 5.5-fold and the AUC\textsubscript{0-24h} in tumor-bearing brain by 5-fold compared to treatment with the clinically used DTX formulation Taxotere\textsuperscript{®}. The kinetics of NP in the brain, determined by ex vivo fluorescence imaging, showed synchronization with DTX kinetics in the brain measured by LC-MS/MS. This result confirmed successful delivery of DTX by the NP into the brain and suggested that ex vivo fluorescence imaging of NP could be an effective and quick means for probing drug disposition in the brain. Treatment with the DTX-NP formulation significantly delayed tumor growth by 11-fold and prolonged median survival of tumor-bearing mice by 94% compared to an equivalent dose of Taxotere\textsuperscript{®}, without inducing histological changes in the major organs.

6.2 Introduction

Brain metastasis of breast cancer (BMBC) occurs in an estimated 15-30% of breast cancer patients\textsuperscript{317-320}. Patients with triple-negative breast cancer (TNBC) or human epidermal growth factor receptor 2 (HER-2) positive breast cancer are at higher risk of developing BMBC compared to patients with other breast cancer subtypes\textsuperscript{146,317-320}. The prognosis of patients with TNBC is particularly poor due to the lack of effective therapies against TNBC and its aggressive biology\textsuperscript{304}. While chemotherapy is routinely used to control peripheral metastasis of breast cancer, it is largely ineffective at treating metastatic lesions in the brain due to poor drug penetration through the blood brain barrier (BBB). The BBB is composed of a collection of endothelial and neuronal cells which operate as a physical, enzymatic and transport barrier at the
The brain-blood interface to regulate entry of molecules into the central nervous system\textsuperscript{25,321}. It is estimated that about 98\% of central nervous system (CNS) drugs fail to enter clinical trials due to poor brain penetration\textsuperscript{176}. While the brain tumor-associated BBB is structurally impaired and more permeable compared to the healthy BBB, it still represents a significant barrier to drug delivery to brain metastases\textsuperscript{161}.

Various invasive approaches have been investigated to enhance drug delivery to the brain by cannula-mediated drug delivery (i.e. intrathecal, intraventricular, or intratumoral injection, and convection-enhanced delivery) or BBB-disruption using hyperosmotic solutions and vasoactive compounds\textsuperscript{160}. The applicability of these methods is limited due to non-specific influx of molecules and fluid into CNS which can potentially lead to neurological toxicity, aphasia and hemiparesis\textsuperscript{33}. Systemic strategies for the treatment of BMBC include identification of novel BBB-permeable drugs suitable for prevention or treatment of BMBC\textsuperscript{177,180,183,184,186-188}, or delivery of clinically established BBB-impermeable chemotherapy drugs across the BBB by conjugation to ligands which bind specific receptors found on the brain endothelial cells, including the transferrin receptor\textsuperscript{25,38,194,195}, low density lipoprotein (LDL) receptor\textsuperscript{40,232}, insulin receptor\textsuperscript{36}, or glutathione receptor\textsuperscript{322}. Binding of the antibody-drug conjugates to their respective receptors enables receptor-mediated transcytosis of the drug across the BBB.

In the past decade, drug-loaded nanocarrier systems with surface conjugated ligands that target the same receptors have also been explored\textsuperscript{323-325}. The nanocarrier systems show advantages over drug-ligand conjugates including their high drug loading capacity, prolonged blood circulation time, sustained drug release, and reduction of enzyme-mediated drug degradation\textsuperscript{25,326}. It has been found that nanoparticles (NP) coated with polysorbate 80 (PS 80), after adsorption of apolipoproteins in the blood circulation, may mimic LDL particles facilitating the entry of NP to the brain via LDL receptor-mediated transcytosis\textsuperscript{40,298}. A number of published work has demonstrated the effectiveness of PS 80-coated NP in the delivery of chemotherapy drugs across the BBB for the treatment of glioblastoma multiforme\textsuperscript{23,44,232,234,327}. We previously developed a PS 80-containing biocompatible polymer system based on poly(methacrylic acid) and maltodextrin\textsuperscript{294,328}, and demonstrated its ability to deliver an ionic drug, such as doxorubicin, across the BBB in healthy mice and to brain metastasis\textsuperscript{326}. Given the low cost of PS 80 and its approved use in many injectable pharmaceutical products, a PS 80-modified NP system offers tremendous potential compared to other BBB-targeting ligands.
In this study, a novel PS 80-based amphiphilic polymer nanocarrier system was developed to encapsulate DTX by self-assembly for the treatment of brain metastases of TNBC. DTX is a widely used anti-mitotic drug for the treatment of locally advanced and metastatic breast cancers\textsuperscript{329-332}, and for the treatment of extracranial TNBC\textsuperscript{333}. However, DTX has not been applied to treat brain metastases as it is subject to P-gp efflux at the BBB and is unable to accumulate in the brain at adequate levels\textsuperscript{334,335}. Furthermore DTX is practically insoluble in water and thus the clinically used product of DTX, Taxotere\textsuperscript{®} is formulated as an alcohol-water mixture with high PS 80 content that is believed to cause hypersensitivity in some patients\textsuperscript{336}. Recently a number of NP formulations of DTX have been developed and shown to increase DTX accumulation and efficacy in glioma xenografts in mice\textsuperscript{337-339}. Nevertheless, no attempt to design DTX NP for treatment of brain metastases of TNBC has been reported, to the best of our knowledge.

To efficiently load and release the poorly water-soluble drug DTX, while maintaining the capability of the nanocarrier crossing the BBB, we have designed and optimized an amphiphilic polymer nanocarrier system for DTX and evaluated its biodistribution, brain accumulation, pharmacokinetics and preclinical efficacy in a severe combined immune deficiency (SCID) mouse model of human TNBC brain metastasis. The present work demonstrates that DTX-loaded NP are able to extravasate from brain microvessels and deliver DTX to brain metastases of TNBC following intravenous administration. The pharmacokinetics of DTX delivered by the NP in the brain was found to synchronize with the kinetics of fluorescence intensity of the NP in the \textit{ex vivo} brain tissue suggesting that DTX entry into the brain was likely facilitated by NP transport across the BBB and that \textit{ex vivo} imaging of NP fluorescence is a good indicator of drug disposition in the brain. Treatment with DTX-NP resulted in significantly extended DTX circulation, markedly higher DTX accumulation in the brain, and prolonged median survival time of brain tumor-bearing mice as compared to Taxotere\textsuperscript{®}.

### 6.3 Materials and Methods

#### 6.3.1 Materials

Maltodextrin (Dextrose Equivalent = 16.5-19.5), methacrylic acid (MAA), sodium thiosulfate (STS), potassium persulfate (KPS), polysorbate 80 (PS 80), sodium dodecyl sulphate (SDS),
fluoresceinamine isomer I (FA), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), dodecylamine, ethyl arachidate, and all other chemicals unless otherwise mentioned were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). HiLyte Fluor™ 750 hydrazide (HF 750) was purchased from AnaSpec (Fremont, CA, USA). Texas red-labeled dextran (MW 70,000 Da) was obtained from Life Technologies (CA, USA). DTX was purchased from LC Laboratories (Woburn, MA, USA). Taxotere® was purchased from Hospira Healthcare Corporation (Saint-Laurent, Québec, Canada). MDA-MB-231-luc-D3H2LN cell line was obtained from Caliper Life Sciences (Hopkinton, MA, USA) and was confirmed to be pathogen free by the supplier using IMPACT Profile I (PCR). These cells were passaged for less than 6 months following resuscitation.

6.3.2 Synthesis of Amphiphilic Polymer

The amphiphilic polymer was synthesized by covalently linking dodecylamine to the terpolymer (Figure 41) of poly(methacrylic acid), polysorbate 80 and starch which was prepared using a method described previously. Purified terpolymer (500 mg), EDC (80 mg), and NHS (80 mg) were dissolved in 5 mL of distilled de-ionized water (DDIW) and allowed to react for 1 hour at room temperature. Between 0.1 to 1 mL of dodecylamine solution (40 mg/mL in dimethyl sulfoxide (DMSO)) was added to the activated terpolymer solution and allowed to react at 37°C for 24 hours. The final product solution was neutralized to pH 7.4 using 0.1 N NaOH, and purified by extensive dialysis (molecular weight cut-off (MWCO) = 12 kDa) against DMSO for 24 hours and DDIW for 48 hours at room temperature. The polymer solution was then lyophilized and stored in a desiccator at 4°C. The molecular weight of the amphiphilic polymer was measured using static light scattering.

6.3.3 Synthesis of Fluorescence Dye-Conjugated Amphiphilic Polymer

For in vivo and ex vivo imaging, two fluorescent dyes, namely HiLyte Fluor™ 750 (HF 750; λ<sub>ex</sub> = 745 nm, λ<sub>em</sub> = 820 nm) and FA (λ<sub>ex</sub> = 496 nm, λ<sub>em</sub> = 520 nm) were conjugated to the amphiphilic polymer using the following method: 500 mg of purified amphiphilic polymer was dissolved in 2 mL of aqueous solution containing 50 mg of EDC and 50 mg of NHS and stirred for 30 min at room temperature, followed by addition of 0.8 mg of HF 750 (1.25 mg/mL in DDIW) or 1.25 mg of FA (5 mg/mL in DMSO). The mixture was protected from light and stirred at room temperature for 24 h. Finally, the product was neutralized to pH 7.5 using 0.1
N NaOH and purified by extensive dialysis (MWCO=12 kDa) against DDI water for 48 hours. The dried dye conjugated-amphiphilic polymer was obtained by lyophilization and stored in a desiccator for future use.

6.3.4 Preparation of DTX-Loaded Amphiphilic Polymer Nanoparticles

To find optimal composition to achieve high DTX loading and good NP properties, amphiphilic polymer with varying amounts of dodecyl contents were used (Table 8). In a typical experiment, 12 mg of ethyl arachidate was added to a 15 mL conical tube, with or without addition of Nile Red (20 µL of 5 mg/mL stock in CHCl₃), and heated to 80°C. Fifty microliters of 100 g/L Pluronic® F-68 (PF68) solution, DTX (200 µL of 10 mg/mL DTX in CHCl₃) and amphiphilic polymer (200 µL of 50 mg/mL in DDIW) were added to the solution and stirred for 20 minutes. The mixture was sonicated for 10 minutes using a Hielscher UP 100H probe ultrasonicator (Ringwood, NJ, USA) at 80% peak. Following sonication, the entire emulsion was quickly transferred into 1 mL of saline (0.9% w/v NaCl) being stirred on ice. The particle size and zeta potential of the DTX-NP were measured with Malvern Zetasizer Nano ZS (Worcestershire, UK). For transmission electron microscopy (TEM), NP dispersed in DDI water were dried onto a carbon coated grid. The TEM images were acquired on a Hitachi H7000 electron microscope (Hitachi Canada, Ltd., Mississauga, Ontario, Canada) with an accelerating voltage of 100 kV.

6.3.5 Determination of Drug Loading, Encapsulation Efficiency, Stability and Drug Release Kinetics

Immediately after formulation, DTX-NP suspension was diluted 4 times by PBS (pH = 7.45, containing 0.5% SDS), transferred to a centrifugal filter (MWCO=30 kDa), and centrifuged for 15 minutes at a RCF of 21,100 ×g. The free drug concentration in the filtrate was assayed at 232 nm using an ultraviolet-visible (UV-Vis) spectrometer (Agilent 8453). The drug loading (% wt drug/wt lipid) and encapsulation efficiency (% wt drug/wt total drug) were then calculated. To determine the stability of the DTX-NP, 200 µL of NP were incubated in 2 mL of buffer (PBS, pH=7.4) or FBS at 37 °C for up to 72 hours. Aliquots were taken at different time intervals and diluted with DDIW for particle size and zeta potential analysis (Malvern Zetasizer Nano ZS, Worcestershire, UK).

To determine in vitro drug release kinetics, DTX-NP suspension (1 mL) or free DTX solution with the same drug concentration (1 mL) was enclosed in a 14 kDa MWCO dialysis tube and
immersed in 200 mL, pH 7.45 PBS (containing 0.5% SDS) at 37°C with continuous magnetic stirring. At selected time intervals, 1 mL of aqueous solution was withdrawn from the release medium and the drug concentration was measured with spectrophotometry. The sample was placed back into the release system after measurement. Each release experiment was repeated 3 times and the mean and standard deviations of triplicates are reported.

6.3.6 In Vitro Cell Uptake of Nanoparticle

To evaluate the cell uptake of NR-DTX-NP, MDA-MB-231 cells were seeded at a density of 30,000 cells per well in 24-well plates and incubated for 24 hours at 37°C under 5% CO₂. Following the incubation, the medium was replaced with fresh medium (1 mL) and 20 µL of Nile red loaded DTX-NP (NR-DTX-NP) were added and incubated at 37°C. At various incubation times, the NP-containing medium was removed and the cells were washed 3 times with cell culture medium. Cell nuclei were stained with Hoechst 33342 (Molecular Probes, Inc. Eugene. OR, USA). The cells were imaged on an AMG EVOSf1 fluorescence microscope (Invitrogen, Carlsbad, CA, USA) and Zeiss LSM700 confocal microscope with the filters for Nile red: Ex./Em. = 530/593 nm, and for Hoechst 33342: Ex./Em. = 360/447 nm. The fluorescence intensity was measured using a microplate fluorescence reader (λ_ex=530 nm, λ_em=630 nm) to quantify the cellular uptake of the NPs.

6.3.7 In Vitro Cytotoxicity in Triple Negative Human Breast Cancer Cells

Cytotoxicity of Blank-NP (no DTX), DTX-NP and free DTX, were evaluated in triple negative human breast cancer cells MDA-MB-231-luc cells. The cells were seeded at a density of 7,000 cells per well in 96-well plates and incubated for 24 hours at 37°C under 5% CO₂. Then the cells were treated for 24 hours with various formulations at different concentrations. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and is expressed as the percent of control for each treatment group. Cells with no treatment and cells incubated with Blank-NP were used as controls for the free drug and DTX-NP, respectively.

6.3.8 Animal Model

All animal handling and procedures were conducted under an approved protocol from the Animal Care Committee at the Ontario Cancer Institute. An animal model for BMBC was
established by injecting luciferase expressing human breast cancer cells (MDA-MB-231-luc-D3H2LN) (5×10⁴ cells/mouse) intracranially into the cortex of four to six week old SCID mice (Ontario Cancer Institute, Toronto, ON, Canada) using a stereotaxic system (SAS-5100, ASI Instruments, Warren, MI, USA). Tumor growth was monitored by luciferin-induced bioluminescence imaging (15 mg/kg luciferin, intraperitoneal injection 10 min prior to imaging) using a Xenogen IVIS spectrum imager (Caliper Life Sciences, Hopkinton, MA, USA). Formation of numerous micro-metastases in the cortical and subcortical regions was confirmed by H&E staining of brain tissue sections (Figure 39).

![Figure 39. H&E staining of brain metastasis of breast cancer, 3 weeks post human breast cancer cell lines MDA-MB-231-luc-D3H2LN (5×10⁴ cells/mouse) injected intracranially into the cortex of four to six week old SCID mice.](image)

6.3.9 Live Animal and Ex Vivo Optical Imaging

Near-infrared (NIR) dye HiLyte Fluor™ 750 (HF 750)- and Fluoresceinamine isomer I (FA)-labeled DTX-NP (200 µL injection volume; 10 mg/mL HF 750- and FA-amphiphilic polymer, 6.7 ± 0.1 µmol of HF 750 and 120 ± 0.2 µmol of FA per gram of polymer; 20 mg/kg DTX for tumor-bearing mice) were injected into the lateral tail vein of tumor-bearing mice. At predetermined time points fluorescence images of the whole body and dissected organs (brain, heart, liver, and spleen) were obtained using the Xenogen IVIS spectrum imager (745 nm excitation, and 820 nm emission wavelengths). NP accumulation within the organs was reported as the ratio of NIR fluorescence intensity of NP-treated tissue to saline-treated tissue.
6.3.10 Confocal Fluorescence Microscopy for Microdistribution of Nanoparticles in Brain Tissue

Delivery of BBB-Impermeable Dye into Healthy Brain. Hoechst 33342-loaded FA-labeled amphiphilic polymer NP were prepared by a suspension-ultrasonication method. A mixture of 250 µL of Hoechst 33342 solution (10 mg/mL), 200 µL of FA-amphiphilic polymer solution (50 mg/mL), 50 µL of PF 68 solution (100 mg/mL), and 12 mg of ethyl arachidate was heated to 80 °C and stirred for 20 minutes. NP were formed under ultrasonication using a Hielscher UP100H probe ultrasonicator, (Hielscher USA, Inc., Ringwood JN, USA) for 10 min and suspended in sterile 5% dextrose to a final Hoechst 33342 concentration of 2.5 mg/mL. An analogous control formulation was prepared from FA-amphiphilic polymer without PS 80. To examine NP penetration into healthy brain, SCID mice were treated with 200 µL of Hoechst 33342-loaded NP with or without PS 80 (2.5 mg/mL dye), or free Hoechst 33342 (2.5 mg/mL in saline) via tail vein injection. Mice were euthanized 2 hours following treatment. Texas red-labeled dextran (100 µL volume, 1% wt. solution) was administered intravenously 15 minutes prior to euthanasia. The brain was dissected, fixed in 10% formalin for 3 hours, transferred to 30% dextrose solution overnight, embedded in Tissue-Tek OCT resin (Somagen, Torrance, CA, USA) and finally flash frozen. Thaw mounted 20 µm thick frozen sections were prepared using a Leica CM3050S cryostat (Leica, Wetzlar, Germany) and analyzed using Zeiss LSM700 confocal microscope (Carl Zeiss, Jena, Germany) using fluorescent excitation and emission filters appropriate for detection of the indicated chromophores (Dextran, Texas Red: Ex./Em. = 595/615 nm; FITC: Ex./Em.=490/520 nm; Hoechst 33342: Ex./Em. = 352/461 nm).

Nanoparticle Microdistribution in Tumor-bearing Brain Tissue. FA-labeled DTX-NP (200 µL injection volume; 10 mg/mL FA-amphiphilic polymer, 120 ± 0.2 μmol of FA per gram of polymer; 20 mg/kg DTX for tumor-bearing mice) were injected into the tail vein of tumor-bearing SCID mice. Two hours later Texas red-labeled dextran (100 µL volume, 1% wt. solution) was administered intravenously 15 minutes prior to euthanasia to label functional brain blood vessels. The brains were processed and examined as described above. NP distribution around the tumor was examined within the FITC emission laser (Ex/Em. = 490/520 nm). Brain blood vessels were imaged over the Texas red spectral wavelengths (Ex./Em. = 595/615 nm). Cell nuclei were imaged within the DAPI spectral laser (Ex./Em.= 352/461 nm) after nuclear
staining with Hoechst 33342 trihydrochloride, trihydrate (Invitrogen, 1:10,000 dilution) and 3x wash with pH 7.4 PBS.

6.3.11 Pharmacokinetic Study of DTX-NP in Brain Tumor-Bearing Mice

Tumor-bearing mice were randomly allocated into two treatment groups and received a single i.v. injection of DTX-NP or Taxotere® at a matched dose of 20 mg DTX/kg. At 0.25, 0.5, 1, 2, 6 and 24 hours after injection, whole blood was collected in heparinized tubes from ketamine anesthetized mice by cardiac puncture. Following perfusion with ice-cold saline via the left ventricle, the tumor-bearing brains were harvested, rinsed in PBS, weighted, snap-frozen and stored at -80°C for further analysis. DTX was extracted from the mouse tissues by protein precipitation. Tissue homogenate was prepared by adding 9 parts DDI water to 1 part whole blood or 3 parts DDI water to 1 part brain before homogenizing at 3600 rpm for 5 minutes using a Precellys 24 homogenizer (VWR, Erlangen, Germany). Tissue homogenate (36 µL) was added to 1.5 mL micro-centrifuge tubes, followed by the addition of 10 µL of methanol and 3.75 µL of 250 ng/mL paclitaxel solution as an internal standard. The mixture was vortexed for 1 min. Methanol/acetonitrile mixture (100.25 µL, 1:1 (v/v)) was then added to the tubes and vortexed for 10 min to precipitate the protein. The samples were centrifuged for 15 min at 21,100×g and the supernatant was transferred to high performance liquid chromatography (HPLC) vials for analysis using liquid chromatography-dual mass spectroscopy (LC-MS/MS) (AB Sciex QStarXL Q-TOF mass spectrometer (AB Sciex, ON, Canada)). DTX transitions were detected by an AB Sciex API4000 triple quadrupole mass spectrometer equipped with an electrospray ionization interface (Advanced Instrumentation for Molecular Structure, Department of Chemistry, University of Toronto). The system was operated in positive ion mode. The multiple reaction monitoring (MRM) transitions monitored for DTX were m/z 544.0/361.1. Results are presented as drug mass per mass of tissue (ng DTX/mg tissue).

6.3.12 Modeling DTX disposition in mouse by a semi-physiologically-based pharmacokinetic (semi-PBPK) model

A semi-physiologically-based pharmacokinetic (semi-PBPK) model was used to model DTX disposition following i.v. treatment with Taxotere® or DTX-NP formulations. This model consists of four compartments that describe the amount of DTX (A) in central (A₁) and peripheral (A₂) compartments as well as the amount in brain blood (A_{Br,B}) and brain tissue (A_{Br})
compartments. C denotes concentrations in the relevant blood or tissue compartment (C = A/V, where V represents the volume of each compartment). The intrinsic metabolic clearance for the brain is denoted as CL\textsubscript{int,met}, which accounts for metabolism of DTX within brain tissue compartment. CL\textsubscript{in} and CL\textsubscript{ef} represent the DTX influx clearance into the brain tissue and the efflux clearance from the brain that is mediated by efflux pumps at the BBB, respectively. All clearance terms (CL) refer to unbound DTX. k\textsubscript{10} is the elimination rate constant from the central compartment, and k\textsubscript{12} and k\textsubscript{21} are the transfer rate constants between the central and peripheral compartments. Q\textsubscript{Br} represents the blood flow to the brain.

The mass balance equations are presented below:

Central compartment: \[
\frac{dA_1}{dt} = -(k_{10} + k_{12})A_1 + k_{21}A_2 - Q_{Br}C_1 + Q_{Br}C_{Br,B} \quad \text{(Equation 1)}
\]

Peripheral compartment: \[
\frac{dA_2}{dt} = k_{12}A_1 - k_{21}A_2 \quad \text{(Equation 2)}
\]

Brain blood compartment: \[
\frac{dA_{Br,B}}{dt} = Q_{Br}C_1 - Q_{Br}C_{Br,B} + CL\textsubscript{ef}C_{Br} - CL\textsubscript{in}C_{Br,B} \quad \text{(Equation 3)}
\]

Brain tissue compartment: \[
\frac{dA_{Br}}{dt} = CL\textsubscript{in}C_{Br,B} - CL\textsubscript{ef}C_{Br} - CL\textsubscript{int,met}C_{Br} \quad \text{(Equation 4)}
\]

The data from Taxotere\textsuperscript{®} or DTX-NP-treated mice were fitted to the semi-PBPK model using ADAPT5\textsuperscript{®} Maximum Likelihood Estimation-Maximization (MLEM) program (Biomedical Simulation Resource, University of Southern California, Los Angeles, CA, USA) to obtain the micro-rate constants k\textsubscript{10}, k\textsubscript{12} and k\textsubscript{21} and other PK model parameters CL\textsubscript{in}, CL\textsubscript{ef}, CL\textsubscript{int,met} and V\textsubscript{1}.\textsuperscript{346} The area under the curve (AUC\textsubscript{0–24h}) was calculated based on the fitted data by the trapezoidal rule. (AUC\textsubscript{0–\infty}) was calculated by extrapolation of the last concentration over the terminal slope (\(\beta\)). The total clearance was calculated as \(CL = \frac{\text{Dose}_{iv}}{AUC_{tv}}\). The drug half-life (T\textsubscript{1/2}) was calculated by the equation \(T_{1/2} = \frac{\ln(2)}{\beta}\).

6.3.13 Evaluation of Therapeutic Efficacy

To brain tumor-bearing SCID mice, DTX-NPs (20 mg DTX/kg, 200 µL administered i.v.), or an equivalent dose of Taxotere\textsuperscript{®}, or saline (200 µL), or Blank-NP (equivalent NP mass to DTX loaded NP) were administered on day 0, typically between one to two weeks following tumor
inoculation depending on desired initial tumor size. A second identical treatment was administered two weeks later on day 14. This dosing regimen was determined from dose tolerance studies (data not shown). Tumor growth was monitored in vivo using bioluminescence imaging for up to four weeks following the first treatment. The fold increase in total tumor size was obtained by normalizing the tumor radiance (TR) over the course of the experiment to the initial tumor radiance at day 0. Tumor growth delay (TGD) was calculated from the mean survival time of each group according to \[ TGD(\%) = \left( \frac{TR_{treated} - TR_{control}}{TR_{control}} \right) \times 100 \] as previously reported\(^{347}\).

Once the mice exhibited signs of discomfort (i.e., weight loss 20% off, lack of grooming, signs of self-mutilation, resistance to ambulation), defined as endpoint, mice were euthanized by cervical dislocation under 1% isoflurane anesthesia. Immediately after euthanasia, intact hearts, livers, lungs and kidneys were fixed in 10% neutral-buffered formalin, paraffin-embedded and stained with haematoxylin and eosin (H&E) for morphological evaluation, which was conducted by a board-certified veterinary anatomic pathologist.

6.3.14 Statistical Data Analysis

All data are presented as means ± standard deviation (SD). Student's t-test or analysis of variance (ANOVA) followed by Tukey t-test (OriginPro8) were utilized to determine statistical significance between two or more groups, respectively. \( p \)-values < 0.05 were considered statistically significant.

6.4 Results and Discussion

6.4.1 Design, Synthesis and Optimization of DTX-Loaded Amphiphilic Polymer NP

In the present work we developed an amphiphilic polymer-lipid nanocarrier system for delivery of hydrophobic drug DTX to brain metastases of human breast cancer in a mouse model. The nanocarrier is comprised of solid lipid domain of ethyl arachidate stabilized with an amphiphilic copolymer comprised of a maltodextrin, poly(methacrylic acid) (PMAA), PS 80 and n-dodecane (Figure 40a, Figure 41). Maltodextrin serves as the polymer backbone onto which PMAA is grafted to provide abundant carboxylic acid functional groups for further chemical modification with PS 80, dodecylamine and fluorescence probes. PS 80 has been previously reported to
facilitate transport across the BBB$^{40,44,326,348}$. Dodecane renders the polymer amphiphilic to facilitate affinity with the ethyl arachidate domain. DTX was efficiently loaded into the NP through hydrophobic interaction with the dodecyl groups in the amphiphilic polymer and partitioning into the solid lipid domain. All materials used for NP synthesis are biodegradable and generally regarded as safe (GRAS) for injection.

The physicochemical properties of DTX-loaded NP (DTX-NP), including particle size, polydispersity, DTX encapsulation efficiency and DTX loading content, were characterized and summarized in Table 8 as a function of dodecyl content. NP with greater dodecyl content were found to exhibit smaller particle size, narrower size distributions, higher DTX encapsulation efficiency and higher DTX loading content (Table 8), conceivably due to stronger hydrophobic interactions between the amphiphilic polymer, lipid domain and hydrophobic drug. Based on these observations, DTX-NP with 10% dodecyl content was used for all subsequent studies. These NP exhibited an average particle diameter of 100.1±2.6 nm, polydispersity of 0.17, zeta potential of -48 mV, loading efficiency of 98.2±1.0%, and loading content of 6.8±1.4% (Figure 40b,c; Table 8). Transmission electron micrographs of the DTX-NP (Figure 40c) showed spherical NP shape and uniform NP size. The DTX-NP were stable at 37 °C in both pH 7.4 PBS and FBS for at least 72 hours (Figure 42). DTX-NP exhibited a sustained in vitro drug release profile for over 48 hours following a brief lag time (Figure 40d). Half of the encapsulated DTX was released within 30 hours while greater than 85% of the total encapsulated drug was released after 53 hours.
Figure 40. a) Schematic illustration of DTX-NP formation from the amphiphilic terpolymer, recruitment of Apo-E, and proposed EPR/transcytosis mechanism for overcoming the BBB. b-d) Properties of DTX-NPs made from 10% dodecylamine grafted polymer: b) particle size distribution, c) TEM image (scale bar = 100 nm), and d) in vitro release of free DTX and DTX from the DTX-NPs (data presented as means ± SD, n=3).

Figure 41. Chemical synthesis of amphiphilic polymer.
Table 8. Compositions of amphiphilic polymer and their effect on the drug loading efficiency and content, and particle properties of docetaxel-loaded nanoparticle formulations

<table>
<thead>
<tr>
<th>Amphiphilic polymer (mg/mL)</th>
<th>Dodecyl (%)</th>
<th>Ethyl Arachidate (mg/mL)</th>
<th>PF 68 (mg/mL)</th>
<th>Docetaxel (mg/mL)</th>
<th>Size (nm)</th>
<th>Polydispersity Index (PDI)</th>
<th>Zeta potential (mV)</th>
<th>Encapsulation efficiency (%)</th>
<th>Loading Content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0</td>
<td>12</td>
<td>5</td>
<td>2</td>
<td>165.1 ±3.2</td>
<td>0.364±0.04</td>
<td>-51.1±1.5</td>
<td>75.5±2.5</td>
<td>5.3±1.1</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>12</td>
<td>5</td>
<td>2</td>
<td>133.3 ±4.3</td>
<td>0.26±0.02</td>
<td>-49.1±2.4</td>
<td>82.2±1.8</td>
<td>5.74±0.9</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>12</td>
<td>5</td>
<td>2</td>
<td>125.1 ±5.4</td>
<td>0.206±0.05</td>
<td>-50.1±1.7</td>
<td>87.5±2.4</td>
<td>6.09±1.3</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>12</td>
<td>5</td>
<td>2</td>
<td>105.1 ±1.5</td>
<td>0.194±0.06</td>
<td>-49.1±2.8</td>
<td>93.3±1.6</td>
<td>6.46±0.4</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>12</td>
<td>5</td>
<td>2</td>
<td>100.1 ±2.6</td>
<td>0.174±0.02</td>
<td>-48.1±2.4</td>
<td>98.2±1.0</td>
<td>6.78±1.4</td>
</tr>
</tbody>
</table>

1 The molecular weight of the amphiphilic polymer was measured using static light scattering.

2 The content of dodecylamine grafted onto amphiphilic polymer was calculated by molecular weight.

Add data presented as mean ± SD for n=3 independent experiments.

Figure 42. Stability of DTX-NP at 37 °C in pH 7.4 PBS and FBS. a) Nanoparticle size and b) nanoparticle zeta potential over a 72 hour period.
6.4.2 DTX-NP Exhibit Rapid Cell Uptake and Enhance Cytotoxicity in Triple Negative Human Breast Cancer Cells

*In vitro* kinetics of NP uptake by MDA-MB-231-luc breast cancer cells was studied using fluorescence microscopy. The Nile red-loaded NP (NR-DTX-NP) enabled *in vitro* fluorescence detection and quantification of cellular uptake. Rapid NP uptake into the cell cytoplasm was observed, with 50% of the NP being taken up by the cancer cells after 20 minutes (Figure 43a, b). *In vitro* cytotoxicity studies were conducted using the MTT assay. The Blank NPs were found to be non-cytotoxic to the cells at concentrations up to 200 µg/L while DTX-NP exhibited enhanced cytotoxic effect against the MDA-MB-231-luc cells compared to free DTX (DTX-NP: IC\textsubscript{50} = 80.7 ± 1.2 µg/L; Free DTX: IC\textsubscript{50} = 93.9 ± 3.5 µg/L (Figure 43c). This enhanced cytotoxicity may be attributed to the effective uptake of the NP, increased DTX solubility and sustained release of DTX from DTX-NPs in the cytoplasm. The elevated intracellular drug concentration could significantly magnify the anticancer efficacy\textsuperscript{328}. 
Figure 43. Uptake of fluorescence dye-loaded DTX-NP by triple negative human breast cancer cell MDA-MB-231-luc. a) Fluorescence microscopy image at 2 hours following treatment. Bright field, cell nuclei stained with Hoechst 33342 (blue), DTX-NP stained with Nile Red (red) and overlays are shown. b) Kinetics of dye-loaded DTX-NP uptake in MDA-MB-231-luc over a 2 hour period ($I_0$: the fluorescence intensity of cells incubated with medium; $I$: the fluorescence intensity of cells incubated with NR-DTX-NP). c) Cytotoxicity of Blank NP, Free DTX and DTX NP against MDA-MB-231 cells after 24 hours of incubation. From the data fitting corresponding IC$_{50}$ values were obtained (DTX-NP: IC$_{50}$ = 80.7$\pm$1.2 $\mu$g/L; Free DTX: IC$_{50}$ = 93.9$\pm$3.5 $\mu$g/L). Data presented as mean $\pm$ SD (n=3).
6.4.3 PS 80-Containing Amphiphilic Polymer Nanoparticles Deliver BBB-Impermeable Dye into Healthy Brain

To test our hypothesis that only PS 80-containing NPs are able to extravasate brain microvessels and deliver cargos across the BBB, BBB-impermeable fluorescent dye Hoechst 33342 was loaded in FA-labeled NP with or without PS 80. These NP were administered intravenously to healthy SCID mice and allowed to circulate for up to 2 hours. Confocal laser scanning microscopy was used to examine brain tissue sections for cell nuclei stained with Hoechst 33342 (blue) in relation to brain vasculature, labeled with Texas Red-dextran (red) (Figure 44). Intravenous administration of free Hoechst 33342 or Hoechst 33342-loaded NP without PS 80 could only stain nuclei of blood vessel-associated cells (blue), leaving cells away from blood vessels unstained (Figure 44). In addition, PS 80-absent NPs (green) were confined within the blood vessel lumen and were absent from the brain parenchyma. In contrast, the PS 80-containing NP (green) were able to extravasate from the blood vessel lumen and enter the brain parenchyma to and deliver to cells located away from brain capillaries (blue).

These results indicate that the PS-80 containing NP were able to enter the normal brain and further suggests that PS 80 might play a role in NP transport across the BBB to gain entry into the CNS. It has been reported that coating NP with PS 80 leads to enhanced adsorption of apolipoprotein-E (Apo-E) in the blood onto the particle surface, and the presence of Apo-E promotes NP internalization in the brain capillary endothelial cells via members of the LDL receptor family expressed by these cells. Therefore, it is possible that a similar mechanism is also responsible for uptake of the PS 80-containing amphiphilic polymer NP by the brain capillary endothelial cells.
Figure 44. Laser scanning confocal microscopic images of healthy SCID mice brain sections following treatment with free Hoechst 33342, Hoechst 33342 loaded within FTIC labeled amphiphilic polymer based NP (without PS 80), or Hoechst 33342 loaded within FITC labeled amphiphilic polymer based NP (with PS 80). Mice were treated for 2 hours. Texas Red-dextran was administered to the mice i.v. 15 minutes before euthanasia. Hoechst 33342 and Hoechst 33342-labeled cell nuclei appear blue. Texas Red-dextran appears as red. Arrows: indicating representative nuclei and DTX-NP located away from blood vessels. Scale bar = 40 µm for all images.

### 6.4.4 Biodistribution and Brain Tumor Accumulation of DTX-NP

The MDA-MB-231-luc-D3H2LN triple-negative human breast cancer cell line, derived from spontaneous lymph node metastases from mammary fat pad tumors, was selected to establish the brain metastasis model due to its aggressive proliferation and infiltration behavior leading to the formation of multiple metastatic lesions throughout the brain (Figure 39), resembling breast cancer metastases in humans. This tumor model resulted in the formation of numerous smaller sized tumor lesions throughout the brain, including the cortex and subcortex (Figure 39, Figure 45), similar to that found in BMBC models established using the MDA-MB-231-BR cell line. Tumor growth was monitored in vivo using bioluminescence imaging of the tumor cells.
following intraperitoneal administration of luciferin. The brain tumors were allowed to grow for two weeks following intracranial inoculation.

Accumulation of HF 750- and FA-labeled DTX-NP at the brain tumor site is evidenced by the co-localization of fluorescence signal from DTX-NP with the bioluminescence signal of brain tumor cells \textit{in vivo} (Figure 45a, b). The NP remained at the tumor site for at least two hours following treatment. Immediate \textit{ex vivo} fluorescence and bioluminescence imaging of the dissected brain 2 hours after DTX-NP injection confirmed these results (Figure 45c). Laser scanning confocal microscopy of the tumor-bearing brain tissue sections revealed that the FA-labeled DTX-NP (green) were able to extravasate from the blood vessel lumen (red) and accumulate within tumor lesions in the brain (Figure 45e).

NP accumulation in the major organs following a single \textit{i.v.} injection of HF 750-labeled DTX-NP was determined by \textit{ex vivo} fluorescence imaging (Figure 45d, Figure 46). DTX-NP initially accumulated in the kidneys, lungs and the livers, and were cleared from these organs within 6 hours. The NP accumulated in the liver and kidneys for at least 24 hours.
Figure 45. a-b) Whole body imaging of live mice with brain tumor: a) fluorescence images showing tumor accumulation of HF 750- and FA-labeled DTX-NPs, b) bioluminescence image of luciferase expressing-tumor cells. c) \textit{Ex vivo} bioluminescence image of tumor cells (iii) and fluorescence image of NP (i: HF 750 filter; ii: FITC filter) in brain. d) Representative fluorescence images of NP in different organs. e) Confocal microscopic images of brain sections taken from mice bearing brain metastases 2 hour after \textit{i.v.} injection of saline (top panel) or FA-labeled DTX NP (green, bottom panel). Texas red-dextran (red) was administered to the mice \textit{i.v.} 15 minutes before euthanasia. Hoechst 33342-stained cell nuclei shown in blue (T: brain tumor area; N: brain...
area without tumor). Arrows point to representative FA-labeled DTX-NP away from blood vessels and accumulated into tumor areas. Scale bar = 50 µm for all images.

Figure 46. Quantitative results for different tissue distributions for NIR-DTX-NP. Fluorescence intensity in the major organs as a function of time after intravenous injection of NIR-DTX-NP. Data normalized to respective organs of saline-treated mice. Data are presented as mean ± SD (n=3).
6.4.5 DTX-NP Prolong Blood Circulation Time of DTX and Increase its Brain Bioavailability Compared to Taxotere®

The concentration of DTX in the perfused brains of healthy mice was quantified using LC-MS/MS following *i.v.* injection of DTX-NP or Taxotere® (20 mg/kg DTX) (Figure 47a). DTX was found to be present in the brain of healthy mice at low concentrations (<100 ng/g), similar to values reported in previous studies\textsuperscript{350,351}, as early as 15 minutes following *i.v.* injection of Taxotere®. Treatment with an identical dose of DTX-NP resulted in a 3.6-fold higher DTX concentration in the brain of healthy mice 15 minutes after injection. These results demonstrate that DTX encapsulated within the NP were able to cross the intact BBB of healthy mice within 15 minutes after *i.v.* injection leading to enhanced accumulation of DTX in the central nervous system compared Taxotere®.

The mechanism of DTX entry into the healthy brain following treatment with Taxotere® is unclear; though it may be related to the high PS 80 content (80-260 mg/mL PS 80) in this formulation\textsuperscript{352}. We detected micelles in Taxotere® diluted in DDIW by dynamic light scattering with a number average particle size of 7.8 ± 0.3 nm and a polydispersity index of 0.18 ± 0.11 (Figure 48). If the micelles were actually maintained in the blood circulation, they may enter the tumor lesion where the enhanced permeability and retention (EPR) effect may operate. Since clinical treatment with high levels of PS 80 has been associated with unpredictable hypersensitivity reactions\textsuperscript{353}, our DTX-NP formulation may mitigate this problem by significantly reducing PS 80 content to only 10 mg/mL PS 80.

Compared to treatment in healthy mice, intravenous injection of both Taxotere® and DTX-NP to mice with brain tumors resulted in > 2-fold higher DTX concentrations in the tumor-bearing brain 15 minutes after injection (Figure 47a). This phenomenon may be attributed to the compromised BBB structure typical of brain tumor lesions, leading to higher drug permeability into the brain\textsuperscript{354-356}. Previously Lockman *et al.* demonstrated that while the BBB in >89% of BMBC lesions were more permeable to chemotherapy drugs, drug accumulation at cytotoxic concentrations was only achieved in roughly 10% of these lesions\textsuperscript{161}. Therefore it is thought that the compromised BBB remains a significant barrier to drug delivery to brain metastases despite its enhanced permeability. Intravenous injection of DTX-NP to mice with brain tumors with resulted in a 2.7-fold higher DTX concentration in the tumor-bearing brain 15 minutes following treatment compared to treatment with an equivalent dose of Taxotere® (Figure 47a). A time
course study in mice with brain tumors further demonstrated that DTX-NP treatment resulted in markedly higher levels of DTX in the brain at all time points tested compared to treatment with Taxotere® (Figure 47c), indicating that DTX-NP may enhance drug delivery to brain metastases.

Pharmacokinetic modeling was used to quantitatively analyze the influence of formulation (i.e., DTX-NP vs. Taxotere®) on the DTX pharmacokinetics (Figure 47b) with good precision as evidenced by low coefficient of variation (Table 9). Physiological parameters and fitted results are summarized in Table 9 and Table 10, respectively. DTX was not detected in the blood 24 hours after Taxotere® injection; therefore a value of zero was used at this time point for pharmacokinetic modeling.

The blood DTX concentration profiles after a single i.v. dose of Taxotere® or DTX-NP (20 mg/kg DTX) showed a bi-exponential decay of DTX for both formulations (Figure 47d). Taxotere® was quickly eliminated from the blood within 24 hours after injection. In contrast, DTX-NP extended DTX circulation time and significantly increased total drug exposure over time in the blood with a 5.5-fold increase in blood terminal half-life (T_{1/2\text{term}}), a 1.3-fold increase in blood $\text{AUC}_0-24h$, and 29% decrease in total body clearance (CL) compared to Taxotere® (Table 11). The favorable pharmacokinetics obtained by nanoparticle formulation is consistent with other nanoparticle formulations.357

Treatment of brain tumor-bearing mice with DTX-NP resulted in a 3-fold higher $C_{\text{max}}$ and a 5.1-fold higher $AUC_0-24h$ in the brain compare to treatment with an equivalent dose of Taxotere® (Table 11). Transport of PS 80-containing nanoparticles across the BBB is thought to occur via surface adsorption of apolipoprotein E (ApoE) in plasma followed by receptor-mediated transcytosis through brain microvessel endothelial cells348,358. However, increased permeability of the BBB at the tumor site may also play a significant role for large-sized tumor lesions354-356. A redistribution phase was observed between $C_{\text{max}}$ and the terminal elimination phase in the brain after treatment with DTX-NP and Taxotere®. The half-life of redistribution phase (T_{1/2\text{redis}}) in the brain was 3.2-fold longer in the DTX-NP-treated mice compared to the Taxotere® group, suggesting that the nanoparticles likely bypass efflux pumps on the BBB. It should be noted that measured drug concentrations in brain tumor lesions may in fact be much higher than the measured DTX levels, since these levels are averaged over the entire tumor-bearing brain which includes a large amount of non-diseased brain tissue.
Figure 47. Pharmacokinetics of DTX-NPs and Taxotere® in brain and whole blood. a) The concentration of DTX in healthy brains and tumor-bearing brains at 15 min after i.v. injection. b) Illustration of a semi-PBPK model for DTX disposition. All parameters are defined in the main text. Concentration profiles of DTX in brain (c) and in blood (d) after i.v. injection of DTX-NP or Taxotere® (symbols are measured values, lines are fitted data). DTX concentration in the blood of Taxotere® treated mice at 24 hours was below the detection limit. Therefore a value of zero was used for PK modeling (Red Arrow). All measured data in a, c, d are the mean ± SD (n=3).
Figure 48. Particle size distribution of micelles in Taxotere® in DDIW determined by dynamic light scattering (DLS). The number average particle size: Diameter = 7.8 ± 0.3 nm; Polydispersity Index = 0.18 ± 0.11. Data presented as mean ± SD (n=3).

Table 9. Fitted pharmacokinetic parameters for DTX in brain-tumor bearing mice treated intravenously with DTX-NP or Taxotere based on the PBPK model shown in Figure 47b.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DTX-NP</th>
<th>Taxotere®</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Std. Dev.</td>
</tr>
<tr>
<td>$k_{10}$</td>
<td>1.97</td>
<td>0.128</td>
</tr>
<tr>
<td>$k_{12}$</td>
<td>1.82</td>
<td>0.404</td>
</tr>
<tr>
<td>$k_{21}$</td>
<td>0.22</td>
<td>2.03E-02</td>
</tr>
<tr>
<td>$V_1$ (mL)</td>
<td>32.9</td>
<td>15.6</td>
</tr>
<tr>
<td>$CL_{int}$ (mL/h)</td>
<td>7.02E-02</td>
<td>8.25E-03</td>
</tr>
<tr>
<td>$CL_{in}$ (mL/h)</td>
<td>0.255</td>
<td>2.06E-02</td>
</tr>
<tr>
<td>$CL_{ef}$ (mL/h)</td>
<td>6.99E-02</td>
<td>9.29E-03</td>
</tr>
</tbody>
</table>

Table 10. Assigned parameters for PBPK modeling of DTX in brain-tumor bearing mice treated intravenously with DTX-NP or Taxotere®

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Q_{Br}$&lt;sup&gt;a&lt;/sup&gt; (mL/h)</td>
<td>5.34</td>
</tr>
<tr>
<td>$V_{Br}$&lt;sup&gt;a&lt;/sup&gt; (mL)</td>
<td>0.226</td>
</tr>
<tr>
<td>$V_{Br,B}$&lt;sup&gt;a&lt;/sup&gt; (mL)</td>
<td>2.50E-2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Average of values obtained from Davies et al.<sup>359</sup> and Kawahara et al.<sup>360</sup>, and subsequently reported in Chow et al.<sup>361</sup>.

$V_{Br}$ and $V_{Br,B}$ are the volume of the brain and brain blood compartments, respectively.
Table 11. Fitted pharmacokinetic parameters to LC-MS/MS data for DTX-NP and Taxotere® in whole blood and brains of tumor-bearing mice after a single intravenous administration of 20 mg/kg DTX (n=3).

<table>
<thead>
<tr>
<th></th>
<th>DTX-NP</th>
<th>Taxotere®</th>
<th>DTX-NP/Taxotere®</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_{1/2}$ term (h)</td>
<td>6.35</td>
<td>1.16</td>
<td>5.47</td>
</tr>
<tr>
<td>$AUC_{0-24h}$ (ng·h/mL)$^a$</td>
<td>5.95×10³</td>
<td>4.43×10³</td>
<td>1.34</td>
</tr>
<tr>
<td>CL (mL/h)</td>
<td>64.7</td>
<td>90.2</td>
<td>0.71</td>
</tr>
<tr>
<td>$V_1$ (mL)</td>
<td>32.9</td>
<td>25.7</td>
<td>1.28</td>
</tr>
<tr>
<td><strong>Brain:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_{1/2}$ term (h)</td>
<td>31.5</td>
<td>13.7</td>
<td>2.30</td>
</tr>
<tr>
<td>$T_{1/2}$ redis (h)</td>
<td>2.17</td>
<td>0.68</td>
<td>3.24</td>
</tr>
<tr>
<td>$AUC_{0-24h}$ (ng·h/g)$^a$</td>
<td>4.83×10³</td>
<td>0.95×10³</td>
<td>5.09</td>
</tr>
<tr>
<td>$C_{max}$ (ng/g)</td>
<td>0.78×10³</td>
<td>0.25×10³</td>
<td>3.11</td>
</tr>
<tr>
<td>$T_{max}$ (h)</td>
<td>0.64</td>
<td>0.43</td>
<td>1.49</td>
</tr>
<tr>
<td>$AUC_{brain,0-24h}$/AUC_{blood,0-24h}</td>
<td>0.81</td>
<td>0.21</td>
<td>3.86</td>
</tr>
</tbody>
</table>

$^a$ AUC$_{0-24h}$ is area under the curve from time 0 to 24h. The mean (n=3) of all data points were used for calculation.

$T_{1/2}$ term represents the elimination half-life

$T_{1/2}$ redis represents redistribution the half-life in the brain, between $C_{max}$ and the terminal elimination phase.

$T_{max}$ represents the time to reach $C_{max}$

CL is the total body clearance determined according to CL = dose / AUC$_{0-∞}$

$C_{max}$ is the peak concentration of DTX in the brain after administration

$V_1$ is defined as the volume of central compartment

6.4.6 **Ex Vivo** Fluorescence Imaging of Nanoparticles as an Indicator of DTX Distribution Kinetics

To investigate the role of the nanocarrier in drug pharmacokinetics in the brain (Figure 49a) and blood (Figure 49b), we measured the time-dependent fluorescence intensity of the NP carriers by ex vivo fluorescence imaging, and compared it to the kinetics of DTX concentration determined by LC-MS/MS. PK parameters related to the rates of NP and drug disposition were extracted from the fluorescence and drug concentration profiles respectively, and the ratio of these terms (average fluorescence intensity/drug concentration) was calculated to aid comparison between the two detection methods (Figure 49c). The fluorescence signal profile of the NP and
concentration profile of DTX in the brain were found to be similar as indicated by a near-unity of $T_{\text{max}}$, $T_{1/2\text{redist}}$ and $T_{1/2\text{term}}$ NP drug-to-fluorescence ratios. This finding suggests that *ex vivo* fluorescence imaging of the dye-labeled NP carrier is a good indicator of DTX accumulation kinetics in the brain for our DTX-NP formulation and that DTX entry into the brain is likely facilitated by NP transport. In contrast, the pharmacokinetics of circulating NP and blood DTX concentrations were dissimilar with the NP carrier circulating for a longer period of time ($T_{1/2\text{term}}$, DTX/ NP fluorescence = 0.15) than DTX in the blood. This suggests that any DTX released from the NP during circulation is rapidly cleared from the body, presumably due to its small molecular size compared to intact NP. Therefore *ex vivo* NP fluorescence signal is a poor indicator of circulating DTX concentration in the blood.

![Graphs](image)

<table>
<thead>
<tr>
<th></th>
<th>DTX concentration</th>
<th>NP fluorescence</th>
<th>DTX/fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Brain:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>0.64</td>
<td>0.51</td>
<td>1.25</td>
</tr>
<tr>
<td>$T_{1/2\text{redist}}$ (h)</td>
<td>2.2</td>
<td>1.5</td>
<td>1.47</td>
</tr>
<tr>
<td>$T_{1/2\text{term}}$ (h)</td>
<td>31.5</td>
<td>43.2</td>
<td>0.73</td>
</tr>
<tr>
<td><strong>Blood:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$T_{1/2\text{term}}$ (h)</td>
<td>6.35</td>
<td>42</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Figure 49. a) Comparison of DTX concentration (determined by LC-MS/MS) and *ex vivo* NP fluorescence intensity versus time after a single *i.v.* injection of HF750-labeled DTX-NPs (20 mg/kg DTX) in the brain (b) and whole blood (c) of tumor-bearing mice. d) Fitted pharmacokinetic parameters of HF750-labeled DTX-NP and DTX-NP in whole blood and brain. All data represent the mean ± SD (n=3).
6.4.7 DTX-NP Inhibit Growth of Brain Metastases and Extend Survival Compared to Taxotere®

To evaluate therapeutic efficacy of DTX-NP, BMBC-bearing mice were injected with an equivalent dose of DTX-NP (20 mg/kg DTX), Taxotere (20 mg/kg DTX), saline or blank NP on day 0 and day 14 (Figure 50a). The general health, weight, and size of brain tumors, measured using in vivo fluorescence imaging, were monitored over a period of 40 days. Brain tumor-bearing mice treated with saline or blank NP (Figure 50) exhibited the fastest tumor growth, animal weight loss (Figure 50b-d), and the shortest mean survival times at 19 and 18 days, respectively (Figure 50e). Treatment with Taxotere® extended the mean survival time to 20 days with a similar median survival time of 18 days (Figure 50f). This is not surprising given the innate ability of Taxotere® to enter the brain at low concentrations\textsuperscript{350,351}, possibly due to the high PS 80 content of the Taxotere® as discussed above. In contrast, DTX-NP treatment delayed tumor growth by 11-fold (57.9 % vs. 5.3 %), and prolonged the median survival time of tumor-bearing mice by 1.9-fold compared to an equivalent dose of Taxotere®, and 1.7-fold compared to saline control (Figure 50f). The improved therapeutic effect of the DTX-NPs may be due to a combination of the ability of the NP to actively enter the brain through receptor-mediated transcytosis, passive accumulation at sites of impaired blood-tumor vasculature, and sustained release of DTX from the NP which have accumulated at the tumor site\textsuperscript{362}.

6.4.8 DTX-NP Treatment Did Not Induce Histological Changes of Main Organs

To evaluate possible toxicity in the major organs resulting from DTX-NP treatment, histological tissue sections stained with hematoxylin and eosin were prepared from the lungs, liver, kidneys and heart of tumor-bearing mice treated with 20 mg/kg DTX-NP or Taxotere® at the end point. The tissue sections showed no histological abnormalities in any of the major organs as compared to saline and Blank-NP controls (Figure 51). Thus DTX-NP exhibited improved therapeutic efficacy compared to Taxotere® treatment groups without inducing histological changes in the major organs at the administered dosing level and regimen.
Figure 50. Inhibition of brain tumor growth and animal survival. a) Treatment and imaging schedule for tumor-bearing mice injected with saline (n=6), blank NP (n=7), Taxotere® (20
mg/kg DTX, n=8), or DTX-NP (20 mg/kg DTX, n=9). b) *In vivo* images of brain tumor bioluminescence over a 28 day period. c) Fold increase in the total tumor radiance. d) Body weight and e) Kaplan–Meier survival curve of tumor-bearing mice following treatment. f) Effect of treatment on median survival time and tumor growth delay of tumor bearing mice. All data presented as mean ± SD.

![Figure 51.](image)

Figure 51. H&E stained sections of lungs, liver, kidneys and hearts of tumor-bearing mice treated with saline, Blank-NP, DTX-NP at a dose of 2×20 mg/kg, or an equivalent dose of Taxotere®. Organs were collected at survival end point. All images were scanned at 20x magnification.

### 6.5 Conclusions

In summary, we have successfully prepared DTX-loaded amphiphilic polymer NP by a one-step self-assembly method and demonstrated their ability to cross the BBB *via* systemic administration in a BMBC mouse model. The DTX-NPs accumulated within tumor lesions, effectively inhibited tumor growth, and increased median survival compared to an equivalent dose of Taxotere®. Pharmacokinetic modeling showed quantitatively that NP encapsulation of DTX increased drug bioavailability in the brain and prolonged blood circulation compared to
Taxotere®. The similarity between the DTX pharmacokinetics and the kinetics of ex vivo NP
fluorescence intensity in the brain suggested that DTX entry into the brain was likely facilitated
by NP transport across the BBB, and more generally that ex vivo imaging of NP fluorescence is a
potential indicator of drug disposition in the brain. The results of this work suggest that the
developed DTX-NP formulation is a promising NP system for treatment of brain metastases.

6.6 Author Contributions

C. He, P. Cai, and J. Li contributed equally to this work. C. He developed and characterized the
terpolymer system. C. He and L. Lin evaluated in vitro nanoparticle performance. C. He, P. Cai,
and J. Li designed and performed the in vivo experiments. C. He and J. Li evaluated nanoparticle
pharmacokinetics. C. He, J. Li and T. Zhang analyzed the data. J. Li wrote the manuscript. J.T.
Henderson prepared the histological sections. X.Y. Wu, J.T. Henderson, and A.M. Rauth,
provided technical support, conceptual advice and edited the manuscript. X.Y. Wu supervised
the project.

6.7 Acknowledgments

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Matthew Forbes at the Advanced Instrumentation for Molecular Structure (AIMS) Mass
Spectrometry Laboratory (Department of Chemistry, University of Toronto).
Chapter 7
Conclusions and Future Perspectives

7.1 Overall Conclusions and Original Contributions of This Thesis

We have designed two bio-inspired drug delivery systems based on functional polymeric nanoparticles in combination with nano-/micro-technologies to improve the management of diabetes mellitus and treatment of brain metastases of breast cancer (BMBC). The first system, an implantable closed-loop insulin delivery device, we developed using pH-responsive nanoparticles embedded within a glucose-sensing matrix to mimic the physiological delivery of insulin. To prolong implant lifetime, we developed a poloxamer gel formulation of highly concentrated insulin, up to 80 mg/mL, with excellent insulin stability for up to 30 days under physiological conditions. In combination with a novel device design that hinders leukocyte migration to the functional implant surface and minimizes host inflammatory response, long-term closed-loop glycemic control was achieved in Type 1 diabetic rats.

To improve chemotherapy treatment of metastatic breast cancer disease, we developed a polysorbate 80-containing terpolymer nanoparticle system capable of delivering imaging agents and drugs across the BBB by mimicking low-density lipoprotein uptake in the brain. Using this platform, two nanoparticle systems have been developed: a doxorubicin-loaded terpolymer nanoparticle (Dox-NP) and a docetaxel-loaded terpolymer-lipid nanoparticle (DTX-NP) for the treatment of brain metastases from breast cancer. Both nanoparticle systems exhibited high drug loading efficiency, colloidal stability and low toxicity. Systemic administration of the nanoparticles co-loaded with near-infrared fluorescent probe allowed for non-invasive real time monitoring of nanoparticle biodistribution, tumor accumulation and clearance. Systemic administration of the nanoparticles loaded with clinically relevant doses of drug to brain-tumor bearing mice resulted in drug delivery across the BBB to brain metastases of breast cancer and significantly enhanced therapeutic efficacy while minimizing side effects compared to free drug.
The following highlights the significant original contributions of the work presented in this thesis:

1. **Developed a novel poloxamer gel formulation of highly concentrated insulin with excellent physicochemical stability under physiological conditions.** The present formulation represents the highest concentration insulin formulation to be reported in the literature and among commercial formulations. It also represents the greatest insulin when subjected to physiological conditions (agitation at 37°C), as determined by protein tertiary structure and formation of insulin degradation products.

2. **Developed a new self-releasing microfabrication method for manufacturing polymer microporous membranes with carefully controlled dimensions and porosity.** These polymer microporous membranes possess superior and more reproducible geometries and surface topographies compared to those produced by other methods (*i.e.* solvent-cast/particulate leaching, dry-cast, and freezing methods). This method improves upon previously developed microfabrication techniques for manufacturing microporous membranes in that manual separation of the membrane from the mold is avoided, thus enabling formation of thinner membranes, improved fabrication yield and reducing production cost and time.

3. **Demonstrated a novel method for improving implant biocompatibility based on impeding leukocyte migration to immunogenic implant surfaces.** This work is the first to demonstrate that a biologically inert polymer microporous membrane, when placed in a correct configuration, can serve as a geometric barrier to inflammatory cell migration to an immunogenic surface (*i.e.* a glucose-responsive albumin plug) and minimize leukocyte-mediated degradation of an implanted foreign material. This method is different from conventional methods of reducing inflammatory response, which focus on minimizing protein adsorption on implanted surfaces or sustained release of anti-inflammatory agents.

4. **Developed a chemically driven closed-loop insulin delivery implant of long-term maintenance of normoglycemia in diabetic rats.** Improvements upon previous designs developed in our lab resulted in rapid insulin release in hyperglycemic conditions, enabled subcutaneous placement of the implant device, and extended device lifetime and efficacy by 3-fold compared to previous designs. This design provides the longest maintenance of
normoglycemia compared to other chemically-driven closed-loop insulin delivery systems reported in the literature.

5. Developed a multifunctional polymer nanoparticle formulation capable of crossing the BBB to gain entry into the brain. MRI imaging and fluorescence microscopy confirmed distribution of the nanoparticles in the brain following administration in healthy mice. Parenterally administered nanoparticles were non-toxic and demonstrated the ability to deliver BBB-impermeable dye to the brain of normal mice. Entry into the brain is mediated by LDL-receptor mediated endocytosis as a result of PS 80 presence on the particle surface confirmed using TOF-SIMS.

6. Developed a Dox-loaded PMAA-PS 80-g-St nanoparticle system which exhibited high loading efficiency of Dox and the ability to deliver Dox across the BBB to brain metastases of triple-negative breast cancer. This is the first nanoparticle formulations to demonstrate non-invasive delivery of chemotherapy drugs to brain metastases of breast cancer. This system also demonstrates the ability to selectively induced cancer cell apoptosis following the delivery of Dox to brain metastases of breast cancer, while sparing normal brain cells from harm.

7. Developed a DTX-loaded lipid-PMAA-PS 80-g-St nanoparticle system which exhibited high loading efficiency of DTX and the ability to deliver DTX across the BBB to brain metastases of triple-negative breast cancer. DTX-NP increased the blood circulation time by 5.5-fold and the AUC$_{0-24h}$ in tumor-bearing brain by 5-fold compared to the clinically used DTX formulation Taxotere®. Treatment with the DTX-NP formulation significantly delayed tumor growth by 11-fold and prolonged median survival of tumor-bearing mice by 94% compared to an equivalent dose of Taxotere®, without inducing histological changes in the major organs.

7.2 Limitations of the Work and Future Directions

Although substantial contributions to the field of nanotechnology-based drug delivery have been made in this thesis, there are some limitations in the studies which require further investigations.
7.2.1 Extension of Implantable Closed-Loop Insulin Delivery Device Efficacy

While the work in this thesis significantly extended the working lifetime of the chemically driven closed-loop insulin delivery device, further improvements would be desired to see future translation of this technology. Device efficacy is currently limited by insulin depletion and degradation of the glucose-responsive membrane. Therefore methods to refill or replace reservoir insulin can be pursued to prevent insulin depletion. Alternatively a larger insulin reservoir or a more concentrated ‘replenishing’ insulin supply using crystalline insulin can be considered.

To combat degradation of the glucose-responsive membrane, improvements to the structure and material of the present membrane can be pursued. Materials like albumin, form which the current membrane is formed, is easily degraded in the body. Modifying the membrane system with inorganic or hydrophobic additives may be pursued to reduce susceptibility to enzymatic degradation. Various non-degradable materials such as PMMA or mesoporous silica have been used in controlled insulin delivery, however these materials would have to be redesigned with a glucose-responsive focus and compatibility with insulin.

7.2.2 Dual-Hormone Insulin/Glucagon Therapy

An ideal glucose-responsive system for regulating blood glucose levels would consist of both insulin regulation and glucagon counter-regulation. Simultaneous use of both hormones would provide a means to self-correct glycemic levels using the opposing hormone when the glucose target is not met. Benefits of such a system have been realized using electromechanical closed-loop insulin delivery systems and have demonstrated advantages in the clinic. A two-device system can be envisioned in which one device provides glucose-responsive insulin delivery while a second device provides the opposite glucose-dependent glucagon delivery. Challenges to this system, different from current dual-hormone systems, include development of a stable glucagon formulation suitable for use with implants.

7.2.3 Controlled Drug Delivery for Type 2 Diabetes

The present system has demonstrated efficacy for the treatment of T1D. However this system may be redesigned and optimized for use in T2D, which represents a much larger patient
population. Design requirements such as insulin dosage and glucose-response would have to be optimized for patients with T2D.

7.2.4 *In Vivo* Assessment of Efficacy in Other Metastatic Breast Cancer and Brain Tumor Models

This work developed two BBB-penetrating nanoparticle systems capable of delivery both hydrophilic and hydrophobic drugs and imaging agents to brain metastases of triple-negative breast cancer. However much of this work has relied on the use of the MDA-MB-231 triple negative cell line. To assess the efficacy of the currently nanosystems in the treatment of brain metastases of breast cancer, additional cell lines should be studied. Additional triple negative breast cancer cell lines including MDA-MB-436 and MDA-MB-468, as well as HER2+ breast cancer cell lines such as BT-474 may be suitable models. This technology may additionally be useful for the treatment of primary brain tumors such as glioblastomas.
Chapter 8
Appendices

8.1 Supplementary Material to Chapter 4

8.1.1 Glucose Challenge Test in Diabetic Rats

To examine the *in vivo* response of microporous membrane-protected devices, a short-term glucose challenge test was conducted in both diabetic rats and healthy rats, which served as a control. 60 µm microporous membrane devices were prepared as previously described in Chapter 4.3, however with an altered glucose-responsive plug composition, which incorporated SiO₂ nanoparticles into the albumin membrane matrix (32.8% BSA, 4.35% GOX, 1.66% CAT, 35% NIPAM/MAA NPs, 8.73% MnO₂ NPs, 17.5% SiO₂ NPs). The devices were filled with 50 mg/mL insulin solution and implanted subcutaneously in STZ-induced diabetic rats, or healthy rats. Intraperitoneal administration of a glucose bolus (1g/kg dextrose) in the diabetic rats on day 5 and day 10 after implantation resulted in rapid elevation of blood glucose levels followed by a gradually returned to euglycemia within 60 minutes without inducing hypoglycemia (Figure 52). This response closely mimicked the blood glucose concentrations in healthy rats receiving the same treatment.

![Day 5 Glucose Challenge](image1)

**Figure 52.** Blood glucose concentration in normal and STZ-diabetic rats with implanted glucose-responsive insulin delivery devices (60 µm membrane device filled with 50 mg/mL insulin) after glucose challenge (i.p. administration of 1g/kg dextrose) at t=0. Error bars represent standard deviation (n=3). Measurements were taken 5 and 10 days following device implantation.
8.2 Supplementary Material to Chapter 5

8.2.1 LDL Receptor Mediated Endocytosis of PMAA-PS 80-g-St Nanoparticles in Breast Cancer Cells

To explore the putative mechanism of PMAA-PS 80-g-St NP cell uptake by LDL receptor mediated endocytosis, MDA-MB-231 cells were treated with nanoparticles in the presence and absence of human recombinant receptor associated protein (RAP), which antagonizes the ligand binding of the low-density lipoprotein receptor (LDLR) family. MDA-MB-231-luc cells were seeded at densities of 7,000 cells per well in a 96-well plate and allowed to grow for 24 hours in 200 µL of growth medium at 37° in 5% CO₂ atmosphere. Cell nuclei were then treated 30 µL of Hoechst 33258 (1:100 dilution) for 30 minutes to fluorescently label the cell nucleus. The cells were then washed 3x with PBS and incubated in 200 µL culture medium with 3 µL NP or 3 µL NP with 1 µM human recombinant RAP (Calbiochem), a LDLR family antagonist, for 4 hours. Following a 3x wash with PBS, fluorescent images of the cells were acquired at 40x magnification using a GFP, RFP, and DAPI filterset to visualize the nanoparticles, doxorubicin, and cell nuclei respectively.

Terpolymer nanoparticle uptake was readily observed at 4 hours post incubation with MDA-MB-231-luc cells, a triple negative human breast cancer cell line which exhibits high LDLR cell surface expression (Figure 53. Cellular uptake of fluoresceinamine-labeled ter-polymer NP loaded with dox at 4 h into MDA-MB-231-luc cells. Top row: cells incubated with nanoparticles. Bottom row: cells incubated with nanoparticles and RAP. Scale bar represents 100 µm.). Nanoparticles localize adjacent to the nucleus but are absent from the nucleus itself. Doxorubicin can be seen throughout the cytoplasm and highly concentrated in the nucleus. Co-incubation of the terpolymer nanoparticles and RAP with the MDA-MB-231-luc cells resulted in significantly less nanoparticle uptake, suggesting that LDLR play a role in mediating terpolymer NP uptake in these cells. Nevertheless, some nanoparticles can be observed within the cell indicating that another mechanism may be responsible for the nanoparticle uptake in these cells.
Figure 53. Cellular uptake of fluoresceinamine-labeled ter-polymer NP loaded with dox at 4 h into MDA-MB-231-luc cells. Top row: cells incubated with nanoparticles. Bottom row: cells incubated with nanoparticles and RAP. Scale bar represents 100 µm.
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