Poly(propylene oxide)-poly(ethylene oxide) block copolymer mediated rescue of diseased blood-brain barrier

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Dedication

To my family.
Abstract

Brain microvascular endothelial cells (BMECs) comprise the blood-brain barrier (BBB), which serves as the vital interface between the vasculature and the central nervous system and controls transport into and out of the brain. During many pathological conditions, BBB dysfunction causes or exacerbates neurological injury. Amphiphilic block copolymers of poly(propylene oxide) (PPO) and poly(ethylene oxide) (PEO) have been extensively employed in biomedical applications. One specific type of PPO-PEO block copolymers called poloxamer 188 (P188) has demonstrated wide utility as a cell membrane stabilizer in various disease settings, including in animal models of neurological injuries where P188 treatment improved BBB function. While animal models have substantiated the use of P188 for protecting the BBB and biophysical studies have enlightened our understanding of how P188 interacts with lipid bilayers, how P188 specifically affects the function and phenotype of the BMECs has not be thoroughly investigated. Furthermore, researchers have mainly focused on P188, but exploration of other hydrophilic dominant PPO-PEO block copolymers is warranted.

In this work, we utilized an in vitro model of the BBB to specifically interrogate the impact of P188 and related PPO-PEO block copolymers to damaged BBB. Human induced pluripotent stem cell-derived BMECs were used as an in vitro model of the BBB to study the state of the BBB in childhood cerebral adrenoleukodystrophy and ischemic stroke. We established that P188 and PPO-PEO diblock copolymers can protect or rescue the BBB during certain disease conditions, leading to mitigation of disease phenotype and improved barrier function. To investigate the localization of a PPO-PEO diblock copolymer in cells, we employed confocal fluorescence microscopy and established
groundwork for fluorophore-free, live-cell imaging via confocal Raman microscopy. Elucidation of the cellular responses to PPO-PEO block copolymer treatment of damaged BMECs in vitro could bridge the gap between studies using non-living biophysical systems and in vivo studies to allow for translational research that may enable the development of therapeutic solutions for neurological diseases.
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Chapter 1. Introduction

1.1. Blood-Brain Barrier

The central nervous system (CNS) consumes approximately 15-20% of the body’s oxygen and glucose intake, while concurrently requiring strict preservation of homeostasis.\textsuperscript{1–3} The delivery of nutrients and oxygen to the CNS is accomplished by an extensive vascular network, and the blood-brain barrier (BBB), composed of brain microvascular endothelial cells (BMECs), forms the interface between the microvasculature and the CNS for regulation of transport into and out of the brain.\textsuperscript{3} The study of the BBB has a long history starting in 1885 when Ehrlich described that water-soluble dyes injected into the bloodstream did not stain the brain, which was attributed to a lower affinity for the dyes in the brain.\textsuperscript{4} The presence of a barrier was hypothesized by Bield and Kraus in 1898\textsuperscript{5} and Lewandowsky in 1900\textsuperscript{6} after observations that injections of cholic acids or sodium ferrocyanide had no pharmacological effect when administered intravenously (i.e., to the bloodstream) but did have an effect when administered intraventricularly (i.e., to the cerebrospinal fluid in the brain), leading Lewandowsky to introduce the term “blood-brain barrier”.\textsuperscript{4} In 1913, Goldmann reported that trypan blue, a water-soluble dye, could stain the brain if injected directly into the cerebrospinal fluid but did not stain the brain if injected intravenously,\textsuperscript{7} supporting that limited permeation from the circulatory system to the brain, not dye binding affinity, is responsible for these observations. It was not until 1967 that evidence of the endothelium constituting a barrier was published, when seminal work by Reese and Karnovsky using electron microscopy revealed the presence of continuous tight junction proteins in between brain endothelial
cells of mice that could block intercellular passage of horseradish peroxidase. Since then, technological advances in conjunction with an increased stake in treating neurological diseases have fueled our understanding of the BBB in health and in disease.

Figure 1.1. (A) Schematic of neurovascular unit. Cross-sectional view of brain microvessel shows BMECs that form the lining of the vessel, with neighboring pericytes and astrocytes. (B) Lateral view of BMECs. The presence of junction proteins in the intracellular space and efflux transporters restrict transport through the BBB.

The BBB is part of the neurovascular unit (NVU), which requires its parts to work cohesively for proper maintenance and function of the BBB. Pericytes present at the capillary level of the vasculature and astrocytes are vital for maintenance of BBB integrity (Figure 1.1.). The BBB is a highly polarized structure with distinct distribution of lipids, glycoproteins, receptors, and transporters between the apical (blood facing) and basolateral (brain facing) membranes of the BMECs. This aids in the function of the BBB as a vital physical, transport, and metabolic barrier between the vasculature and the CNS. The physical barrier is constructed by a network of intercellular junctional proteins, including adherens, gap, and tight junctions. Tight junction proteins, including occludin and claudins, are connected to the cytoskeletal network via zonula occludens
scaffolding proteins.\textsuperscript{18,19} The transport barrier is established by a system of transporter proteins including efflux transporters and solute carriers to selectively allow certain molecules to pass through the BBB into or out of the brain.\textsuperscript{20,21} The metabolic barrier is formed by intracellular enzymes that can metabolize specific molecules in passage.\textsuperscript{22,23} Overall, these attributes mean that paracellular and transcellular permeability through the BBB is highly regulated, with approximately 98\% of small molecule drugs unable to pass the BBB.\textsuperscript{24}

Disruption to the BBB is implicated in many neurological diseases and injuries.\textsuperscript{25,26} BBB dysfunction has been reported in neurological diseases due to mutations in genes such as \textit{SLC2A1}, \textit{MFSD2A}, and \textit{SLC16A2}, which are genes that encode different transporter proteins.\textsuperscript{27–29} In these monogenic neurological diseases, clinical symptoms include development delay and/or intellectual disability. Neurodegenerative diseases that are not clearly associated with a genetic mutation including Parkinson’s Disease, amyotrophic lateral sclerosis, multiple sclerosis, Alzheimer’s disease, and Huntington’s disease are also associated with BBB breakdown.\textsuperscript{30–34} Disruption of the BBB occurs during acute brain injuries such as stroke, traumatic brain injury, and epilepsy.\textsuperscript{35–38} In these cases, opening of the BBB can lead to an inflammatory response that worsens brain injury.\textsuperscript{39,40} Even though BBB permeability can increase during diseased conditions, penetration of drugs through the BBB can still be limited, which presents a major challenge to drug delivery to the CNS for treatment of neurological diseases, and researchers are actively developing strategies for enhanced drug transport including controlled opening of the BBB or fabrication of drug delivery vehicles that can efficiently transport drugs to the brain.\textsuperscript{41} Other tactics for treatment of neurological disorders seek to repair damaged BBB,\textsuperscript{26,41} with increasing
interest in development of neurotherapies that target the BBB as consensus grows that time and extent of BBB damage are important variables for clinical outcome.\textsuperscript{42-44}

A strategy to protect or rescue BBB function during diseased conditions utilizing a synthetic polymer is explored in this thesis. In the last decade, several reports have demonstrated that treatment with poloxamer 188, an amphiphilic triblock copolymer of poly(ethylene oxide) and poly(propylene oxide), in animal models of intracerebral hemorrhage, traumatic brain injury, and ischemic stroke could mitigate effects of BBB damage.\textsuperscript{45-47} To establish a solid context behind poloxamer 188, background on poloxamers will first be discussed before delving into the history of poloxamer 188 applications and our current understanding of its mechanism of action as a therapeutic agent.

1.2. Overview of Poloxamers

Poloxamers are amphiphilic nonionic surfactants (surface active agents) and are triblock copolymers with a poly(propylene oxide) (PPO) core block flanked by symmetric poly(ethylene oxide) chains (PEO) (Figure 1.2).\textsuperscript{48} Block copolymers are composed of two or more covalently connected polymer chains (or blocks). As the architecture of the connectivity can vary, as well as other polymeric parameters such as chemical composition and degree of polymerization of each block, there are a plethora of parameters that can be accessed for the desired properties.\textsuperscript{49} Also called by trade names, Pluronic ® (BASF), Lutrol ® (BASF), and Synperonic (Croda), poloxamers were first patented in the early 1950s by Wyandotte Chemicals Corporation\textsuperscript{50,51} and were the first block copolymers to be commercially produced.\textsuperscript{52}
Block copolymers in solvent can self-assemble into various structures, with self-assembly behavior depending on the solubility differences between blocks, polymer concentration, and temperature. The system of interest in this thesis is biological, so the relevant parameters include water as the solvent and a temperature of 37°C, and further discussion will focus on these parameters. Above the critical micelle concentration (CMC) at a constant temperature, poloxamers will self-assemble in aqueous solutions such that the relatively hydrophilic PEO block will form the micelle corona while the relatively hydrophobic PPO block forms the micelle core to minimize interactions with water. The critical micelle temperature (CMT) is the temperature at which molecules will self-assemble into micelles at a certain concentration. The CMC of poloxamers decreases with an increase in temperature as PEO and PPO exhibit lower critical solution temperature behavior in water, meaning that a critical point on the phase boundary exists at which two phases exist above this point (i.e., higher temperature) and one phase exists below this point (i.e., lower temperature). As temperature increases, breaking of hydrogen bonds between water and the ether oxygens of the polymer results in a decrease in PPO and PEO solubility in water.

Self-assembly behavior of poloxamers has been widely studied and key trends in how composition impacts CMC have been identified. CMC values of poloxamers decrease
with an increase in length of the PPO block,\textsuperscript{60,61} and this dependence is stronger than the change in CMC with varying PEO length, wherein the CMC increases with an increase in the PEO block length.\textsuperscript{62} The CMC of poloxamers of similar PPO/PEO composition is inversely proportion to the overall molecular weight.\textsuperscript{60,62} Importantly, reported CMC values of poloxamers can vary over orders of magnitude.\textsuperscript{63} This discrepancy can be due to differences in experimental techniques used to measure the CMC or lot-to-lot variations of commercially produced poloxamers. Further, the CMC is not a sharp transition point as unimeric polymer chains can coexist with micelles over a relatively wide range of temperature.\textsuperscript{64} The same argument applies for the CMT.

More than 30 poloxamers are commercially available with varying molar masses and degrees of amphiphilicity, a parameter that can be quantified by the hydrophilic-lipophilic balance (HLB) and defined by equation 1.1, where $M_{\text{hydrophilic}}$ and $M_{\text{hydrophobic}}$ refer to the molecular weight of the hydrophilic and hydrophobic segments of the molecule, respectively.\textsuperscript{65} Some manufacturers use equation 1.2 to calculate the HLB values of poloxamers, where $N_{\text{PO}}$ and $N_{\text{EO}}$ refer to the number of repeat units in the PO and EO blocks, respectively.\textsuperscript{66} Table 1.1 outlines properties of select poloxamers. Poloxamers are named with two or three digits following the word “poloxamer,” wherein the digits allude to the composition of the molecule: the first two digits multiplied by a factor of 100 indicate the molecular weight of the PPO core and the last digit multiplied by a factor of 10 specifies the weight percent of PEO. For example, P188 has a PPO core with a molecular weight of 1,800 Da and 80 wt% PEO. When naming poloxamers with the trade names, Pluronic, Lutrol, or Synperonic, a prefix letter of “L”, “P”, and “F” before the numbers signifies the physical state of the pure copolymer as liquid, paste, or flakes, respectively. The first one
or two digits multiplied by a factor of 300 specify the approximate molecular weight of the PPO core, and the last digit multiplied by a factor of 10 denotes the weight percent of PEO:

\[
HLB = 20 \times \frac{M_{\text{hydrophilic}}}{M_{\text{hydrophilic}} + M_{\text{hydrophobic}}} \quad 1.1
\]

\[
HLB = -36.0 \times \frac{N_{PO}}{N_{PO} + N_{EO}} + 33.2 \quad 1.2
\]

**Table 1.1.** Properties of select poloxamers

<table>
<thead>
<tr>
<th>Poloxamer</th>
<th>Pluronic</th>
<th>Molecular weight(a) (kDa)</th>
<th>(N_{PO}^a)</th>
<th>(N_{EO}^{a,b})</th>
<th>HLB(^a)</th>
<th>CMC at 37(^{\circ})C (^c) (mM)</th>
<th>CMC at 40(^{\circ})C (^d) (mM)</th>
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\(^{a}\)Specified by BASF\(^{62,68}\)

\(^{b}\)Number of repeat units in one block

\(^{c}\)Reported by Batrakova et al.\(^{62}\)

\(^{d}\)Reported by Alexandridis et al.\(^{60}\)

The wide-ranging utility of poloxamers in industries extending from personal care to medicine can be largely attributed to their self-assembly behavior and tunability via modulation of polymeric parameters. In the biomedical field, certain applications of poloxamers including their use as thermosensitive hydrogels\(^{69}\) and drug delivery carriers\(^{70}\)
take advantage of the poloxamers’ micellization properties. On the other hand, depending on the PPO/PEO ratio and overall molecular weight, poloxamers in their unimeric state have been employed as chemosensitization agents for cancer therapy\textsuperscript{71–73} and cell membrane stabilizers.\textsuperscript{74–76}

1.3. Applications of Poloxamer 188

Of particular interest to this thesis is poloxamer 188 (P188) that has 80 wt\% PEO and an overall molar mass around 8 kDa. Early reports of P188 applications include its use as an ingredient in experimental intravenous fat emulsions\textsuperscript{77} and as a surfactant in blood oxygenators.\textsuperscript{78} In the 1970s, P188 was approved by the Food and Drug Administration (FDA) for use as a skin wound cleanser as P188 was found to be nontoxic to white and red blood cells and did not affect white blood cells’ function while helping to prevent wound infection.\textsuperscript{79} P188 was also previously approved by the FDA to be used as a surfactant in artificial blood called Flusol-DA.\textsuperscript{80} P188 has been used as a viscosity modifier in real blood\textsuperscript{81,82} and for treating blood circulation through cardiopulmonary bypass.\textsuperscript{83} In clinical settings, P188 has been examined for use as a hemorheological and antithrombotic agent. Additionally, P188 has been utilized as a cell culture additive to act as a shear protectant or cyropreservative, and P188 has been demonstrated to stabilize damaged cell membranes. In subsequent sections, we highlight the clinical trials that are useful in informing our understanding of the safety of P188 injections in humans and biomedical applications that are enlightening for our understanding of P188 interactions with cells.
1.3.1. Clinical applications of P188

P188 was investigated as a potential therapy for sickle cell disease patients, who experience painful vaso-occlusive crisis due to abnormal red blood cells that adhere to vascular endothelium and cause impeded blood flow. P188 was thought to reduce adherence of sickled red blood cells to vascular walls for improved hemorheology. Clinical trials to test the efficacy and safety of treatment of sickle cell patients with P188 (branded as “RheothRx”) for reduction of pain and length of vaso-occlusive crisis were conducted. Patients received a dosage of 300 mg/kg P188 for 1 h followed by a dosage of 30 mg/kg/h for 47 h, which was well tolerated and led to a reduction in duration of painful episodes, duration of hospital stay, requirement of analgesic, and average reported pain intensity.

Moreover, due to evidence that P188 could influence blood rheology as discussed above and could accelerate thrombolysis, P188 was evaluated in clinical trials as an adjunctive therapy in patients with myocardial infarction receiving thrombolytic therapy. More detailed discussion on the use of P188 for treatment of myocardial infarction can be found in a recently published review. Safety and efficacy studies for P188 treatment in patients with acute myocardial infarction were conducted, with study groups between 100 and 150 participants and dosages ranging from 150-300 mg/kg/h for the first hour of treatment and 15-30 mg/kg/h for the subsequent 47 hours. A study with 114 patients reported that P188 treatment resulted in significantly smaller infarct sizes, greater myocardial salvage, improved median ejection fraction, and reduced incidence of reinfarction. However, in a subsequent study with approximately twice the number of patients, P188 treatment did not significantly decrease infarct size or mortality rate and
unfortunately resulted in the occurrence of renal dysfunction in older patients with pre-existing renal conditions.\textsuperscript{93}

Low molecular weight impurities in P188 were later found to be the cause of the effects on renal function, leading to the development of purified P188 (alias vepoloxamer or MST-188), which demonstrated improved renal safety and tolerability.\textsuperscript{95} Biodistribution studies for purified P188 found that the polymer was distributed throughout the bodies in rats injected with \textsuperscript{14}C-labeled P188 for 48 h at a dose of 14,400 mg/kg/day.\textsuperscript{96} Continuous intravenous infusion of purified P188 given over 48 hours to healthy persons was well tolerated, and the studies reported that P188 was mainly cleared renally within 1 week after the termination of P188 infusion.\textsuperscript{96,97} Treatment studies of sickle cell disease patients with purified P188 were conducted but found to be only moderately efficacious in alleviating painful episodes.\textsuperscript{95,98,99} Ultimately, phase III clinical trials failed to pass efficacy endpoints\textsuperscript{100} but helped to demonstrate the safety of the purified P188 in humans.\textsuperscript{101}

1.3.2. Biomedical applications of P188

P188 has perhaps seen the greatest success as an additive for biomanufacturing processes, which require large-scale bioreactors that subject cells to fluid-mechanical stresses.\textsuperscript{102} Since the 1950s, scientists have looked to chemical additives as a method to protect cells against damage from agitation and aeration in bioreactors,\textsuperscript{103} with the first report of utilizing P188 as such protectant in 1960.\textsuperscript{104} While other additives have been explored for this purpose, the surfactant of choice is P188 as a chemically defined medium is preferred.\textsuperscript{105} However, batch-to-batch variability of P188 has led to inconsistent results in terms of shear protection,\textsuperscript{106,107} and as with most biological systems, there have been
reports of cell type dependence on the efficacy of P188 as a protective additive in bioreactors.103,108,109 Nevertheless, P188 has been largely beneficial with the cell types most frequently used in biomanufacturing, such as Chinese Hamster Ovary (CHO) cells.110,111

Researchers have mainly relied on empirical knowledge to guide design decisions regarding surfactant additives as the mechanism of action is not completely understood. Overall, protection by P188 is thought to be conferred in two ways: (1) physical protection due to lower liquid surface tension and thus decreased adsorption of cells to bubbles;112–115 (2) biological protection via P188 interaction with the cell plasma membrane.116–118 Plasma membrane fluidity is an important parameter in the P188 interaction as in vitro studies have reported that P188 can induce a decrease in the cell plasma membrane fluidity as a protection mechanism against shear stress.118 A screening of various poloxamers and inverted poloxamers (PPO-PEO-PPO) added to a sparged bioreactor with insect cells reported that efficacy in protecting cells correlated with the HLB of the polymers.119,120 Interestingly, PEO homopolymer of 8 kDa molar mass was able to enhance cell growth only in a certain type of bioreactor.119 Murhammer and Goochee posited that polymer mediated protection against agitation and sparging in bioreactors is in part due to the PPO block of poloxamers and reverse poloxamers “anchoring” into the cell membrane, allowing the PEO block to form hydrogen bonds with water outside of the cell membrane.119

Using confocal fluorescence microscopy, Gigout et al. investigated the localization of fluorescently tagged P188 in chondrocytes and CHO cells, which showed differential responses.121 Images taken after 1.5 h of incubation with the fluorescently tagged P188 showed the molecules near the plasma membrane in chondrocytes. After the same incubation duration, the fluorescent P188 molecules were co-localized with endosomes and
lysosomes in CHO cells. After 18 of incubation, fluorescently tagged P188 molecules were in endosomal or lysosomal compartments in both cell types. The authors hypothesized that shear protection mediated by P188 may be in part due to P188 in intracellular vesicles that could affect the cell’s mechanical properties.\textsuperscript{121}

Moreover, P188 has been used in cryopreservation medium in pursuit of a chemically defined solution without serum.\textsuperscript{122-124} When mammalian cells were frozen in a solution containing P188 and then subsequently thawed, cell viability was improved compared to cells frozen in a solution containing serum instead of P188.\textsuperscript{122} The mechanism of action for the increased viability upon thawing was attributed to cell membrane stabilization by P188.\textsuperscript{122} P188-containing freezing medium was once commercially available as FILOCETH\textsuperscript{TM}-media. Further, Kerleta et al. have demonstrated that the use of medium containing P188 increased the proliferation rate of Caco-2 cells upon cryopreservation/thawing and subcultivation.\textsuperscript{123} The authors also measured via micropipette aspiration that the membrane tension of the Caco-2 cells increased when incubated with P188 for 60 min for enhanced attachment.\textsuperscript{123} P188 has also been found to inhibit ice formation during freezing of a chemically defined, dimethyl sulfoxide-free cryopreservation medium for human induced pluripotent stem cells.\textsuperscript{124}

1.3.3. P188 as a membrane sealing agent

There have been numerous animal studies investigating the therapeutic benefit of P188 in various disease scenarios thought to stem from the stabilizing interactions of P188 with damaged cell plasma membranes. Membrane resealing via P188 in cells has been postulated to be mediated by a decrease in membrane tension as P188 treatment enabled
membrane resealing in cells treated with tetanus toxin, which prevents exocytosis at sites of membrane disruption and blocks membrane resealing. P188 has been widely studied as a potential therapy for Duchenne muscular dystrophy (DMD) in which absence of the cytoskeletal protein dystrophin leads to progressive skeletal muscle weakness and cardiomyopathy. In dystrophic muscles, membrane sealing by P188 is thought to prevent the entry of extracellular calcium ions into cells. A working model postulates that P188 interaction with damaged cell membranes can help restore membrane barrier function and prevent intracellular calcium overload to facilitate the cell’s endogenous membrane repair response, after which the polymer may extricate away from the membrane. In mice models of DMD, P188 treatment improved cardiac function, respiratory function, cardiac ischemia-reperfusion injury, mechanical stress, and reduced muscular atrophy. In a canine model of DMD, P188 reduced myocardial fibrosis and prevented left-ventricular remodeling. Based on these preclinical efficacy results, P188 is currently being tested in phase II clinical trials for treatment of DMD patients as a supplemental treatment to the current standard of care therapies for improved skeletal and cardiac muscle function. Application of poloxamer based muscle membrane stabilizers for DMD was recently reviewed.

Moreover, therapeutic efficacy of P188 has also been explored for acute injuries. In a porcine model of myocardial infarction, intracoronary infusion of P188 immediately upon reperfusion improved recovery. Additionally, the study suggested that altered blood viscosity leading to improved tissue perfusion does not explain the reduction in cellular and mitochondrial injury as PEO homopolymer altered blood viscosity similarly to P188 but PEO treatment did not improve recovery in the animals with myocardial
infarction. In a model of chronic heart failure in rats, P188 treatment improved left ventricular function but did not alter blood pressure or heart rates, suggesting that the improvement in clinical outcome was not due to alterations in hemodynamics. These studies support that the hemorheological effects of P188, which initially prompted the clinical trials for treatment of patients with myocardial infarction, do not explain all therapeutic outcomes from P188 treatment of myocardial infarction and that cell membrane stabilization plays a role.

Many more reports of P188 mediated improvement of cellular health have been documented. P188 treatment of electropermeabilized muscle improved membrane integrity. In rat models, P188 enhanced survival and diminished tissue damage upon hypotensive resuscitation; reduced alveolar cell death in lung injury caused by ventilation; and alleviated wound-deepening after second-degree burns. P188 administration upon mechanical trauma preserved glycosaminoglycan content in rabbits; enhanced chondrocyte viability in rabbits and bovine explants; and protected spinal cord integrity in guinea pigs. In short, P188 has exhibited protective efficacy in a wide variety of pathophysiological environments.

1.4. Mechanism of Interaction

1.4.1. Investigations of poloxamer interaction with model membranes

In an effort to understand the interaction between poloxamers and cell membranes, researchers have employed biophysical models of phospholipid monolayers and bilayers. In the context of membrane resealing, cell membrane tension, which has shown to be an
important factor for P188 mediated cell membrane repair,\textsuperscript{125,126} is typically not recapitulated in lipid membrane models since living cells have a cytoskeleton network that provide mechanical support and results in a baseline surface tension.\textsuperscript{150} Nevertheless, biophysical studies allow the use of analytical methods that provide valuable quantitative information that are often impractical or entirely impossible with biological samples.

Studies with lipid monolayers in Langmuir isotherms coupled with fluorescence microscopy demonstrated that P188 inserted into the monolayer, changing the phase behavior and morphology, at surface pressures less than or equal to 22 mN/m at 30°C and was expelled out after insertion when monolayer surface pressure was increased.\textsuperscript{151} Similar trends of insertion and elimination were observed for poloxamers 108, 238, and 338, which have the same PEO weight percent as P188 but different overall molecular weights, but poloxamers with larger PPO blocks required lower surface pressure for insertion.\textsuperscript{152} The authors hypothesized that because P188 does not insert into the monolayer above 22 mN/m, the polymer may only interact with damaged portions of the membrane and is removed after healing. Moreover, Monte Carlo simulations found that as the ratio of the PPO block length to PEO block length increased, the pressure required for the poloxamer to be squeezed out of the lipid monolayer increased.\textsuperscript{153} X-ray reflectivity and diffraction studies of lipid monolayers at 30°C showed that P188 molecules phase separated from ordered lipids, forcing lipids to pack tightly, and were exuded out from the monolayer upon further compression.\textsuperscript{154} Atomic force microscopy of lipid monolayers upon P188 insertion supported that morphology of the monolayer changed only when the lipid packing density was below the insertion threshold.\textsuperscript{155}
As lipid monolayers only model the outer leaflet of the plasma membrane, lipid bilayers, either as supported lipid bilayers (SLB) or vesicles, have also been used for studying membrane-polymer interactions. Findings from molecular dynamics simulations found that P188 could seal pores on lipid bilayers but had little interaction with lipid bilayers that had no defects.\textsuperscript{156,157} Degree of hydrophobicity has a significant influence on the interaction of poloxamers with intact lipid membranes, as studies utilizing vesicular lipid membranes established that lipid membrane interactions with more hydrophobic poloxamers led to an increase in the rate of transmembrane lipid flip-flop,\textsuperscript{158,159} membrane permeability,\textsuperscript{160,161} and membrane rigidity.\textsuperscript{162–164} These trends have been supported by computational studies.\textsuperscript{165,166} Microcantilever and fluorescence recovery after photobleaching experiments with SLBs found strong temperature sensitivity and higher solubility of poloxamers with longer PPO blocks in the SLB, leading to stronger association with the SLB and inhibition of lipid diffusion.\textsuperscript{167} Zhang \textit{et al.} employed pulse field gradient nuclear magnetic resonance (NMR) spectroscopy to quantify the binding of poloxamers to liposomes and observed an increase in binding with increasing molecular weight and hydrophobicity.\textsuperscript{168}

There have been reports of membrane stabilization by PEO homopolymers, but at higher concentrations or molecular weights than would be needed for PPO-PEO block copolymers.\textsuperscript{169,170} Cellular studies have noted a dependence on polymer-mediated cellular protection depending on weight percentage of PEO.\textsuperscript{74} This necessitates that the relative hydrophilic-hydrophobic balance of the polymer along with the hydrophobic block length govern the polymer-membrane interaction.\textsuperscript{164,171,172} Utilization of dynamic light scattering and isothermal titration calorimetry to study poloxamer protection of liposomes revealed
that adsorption of hydrophilic poloxamers on the membrane provided protection against induced lipid peroxidation.\textsuperscript{173} Studies using \textsuperscript{1}H Overhauser dynamic nuclear polarization NMR spectroscopy to analyze local hydration dynamics revealed that P188 adsorption onto the lipid bilayer hindered surface hydration dynamics and intrabilayer water diffusivity, whereas poloxamer 181 (P181) augmented intrabilayer water diffusivity.\textsuperscript{174} However, time of incubation is an important factor as a study with giant unilamellar vesicles loaded with fluorescent dyes and subjected to hypo-osmotic stress exhibited that P188 incubation times on the order of hours moderately impeded the release of the fluorescent dye, which the authors attribute to the adsorption of the polymer chains on the vesicles.\textsuperscript{175} However, with prolonged incubations on the order of days, P188 acted as a permeabilizing molecule and disrupted lipid packing.

Lee and co-workers proposed a mechanism of initial adsorption of the polymer onto the membrane followed by insertion for poloxamer interaction with lipid bilayers.\textsuperscript{173–175} The model postulates that poloxamers, irrespective of their hydrophobicity, adsorb onto the lipid membrane, but the kinetics of insertion depend on hydrophilic-hydrophobic balance of the poloxamer. A relatively hydrophobic poloxamer like P181 with 10 wt% PEO will rapidly embed below the lipid head groups due to preferred interaction of the PPO blocks with the lipid tails and short PEO blocks that cannot maintain the entire molecule on the membrane surface. Within 7 h, the entire polymer would be inside the bilayer. A relatively hydrophilic poloxamer like P188 will maintain on the membrane surface in the same time frame and would require much longer incubation times on the order of days for full insertion of P188 into the bilayer.\textsuperscript{175} This is supported by investigations of the adsorption kinetics of P188 and PEO homopolymer of similar
molecular weight as P188 onto a phospholipid SLB at 37°C via surface plasmon resonance that reported similar slow kinetics and adsorption onto the SLB by PEO and P188.\textsuperscript{176}

Kim \textit{et al.} employed neutron reflectively and atomic force microscopy to probe the spatial distribution of P188 and PEO homopolymer of similar molecular weight as P188 in SLBs. The 4.5 mM polymer solutions were flowed over the SLBs at 37°C, and both polymers were observed to fully insert into bilayer with uniformity in the lateral plane.\textsuperscript{177} In contrast, Firestone \textit{et al.} utilized X-ray scattering to probe insertion depth of P188 and observed P188 to weakly insert into the headgroup of lipids in multilamellar lipid vesicles.\textsuperscript{163,178} This study found that when the length of the PPO block approaches the dimensions of the acyl chains of the lipid bilayer, the poloxamer will span the entire lipid bilayer; otherwise, poloxamers with shorter PPO blocks (including P188) will yield partial insertion of the PPO block into the lipid bilayer. The conflict in the reports may stem from differences in sample preparation as the polymer was pre-mixed with the lipid molecules before formation of the bilayer in the latter study. Regardless, the insertion profiles of PEO homopolymers and P188 remain unresolved.

Most studies summarized in this section utilized simplistic model membranes that do not fully capture the complex mix of different lipids and lipoproteins that constitute real cell membranes. To address this issue, using pulse field gradient NMR spectroscopy, Zhang \textit{et al.} probed how poloxamer binding to liposomes changes when liposomes have different cholesterol content, curvature, and lipid composition.\textsuperscript{179,180} These studies determined that polymer binding to liposomes decreased with a decrease in curvature, and polymer binding decreased with an increase in cholesterol content, with cholesterol having a more significant impact.\textsuperscript{179} Further, lipid headgroups and degree of unsaturation of lipid
alkyl chains were discovered to influence polymer binding.\textsuperscript{180}

1.4.2. Exploration of diblock architecture

Diblock copolymers of PPO and PEO have been found to have membrane stabilizing effects in biophysical, cellular, and animal models.\textsuperscript{63,74,168,181} The diblock architecture offers an opportunity to modulate the end group on the PPO block, which has been demonstrated to play a significant role. PPO-PEO diblock copolymers were able to protect liposomes against lipid peroxidation, and PPO-PEO diblock copolymers of similar block lengths but different end groups on the PPO block performed differently, with the tert-butyl end group being more effective than the methyl end group.\textsuperscript{63,182} In a DMD mouse model, PPO-PEO diblock copolymer treatment provided protection against contraction-induced force loss if the end group on the PPO was hydrophobic (tert-butyl) but not if the end group was less hydrophobic (methoxy or hydroxyl).\textsuperscript{181} Molecular dynamics simulations indicated that partitioning of PPO-PEO diblock copolymers in the lipid bilayer depended on the end-group of the PPO block, as the PPO block encroached towards the center of the bilayer if the PPO end group was a hydrophobic tert-butyl moiety but stayed near the polar interface of the lipid bilayer if the PPO end group was a hydroxyl group.\textsuperscript{181} Small angle X-ray scattering experiments comparing the interaction of P188 with PPO-PEO diblock copolymers revealed that diblock copolymers with sufficient PPO chain length interacted with the lipid bilayer to produce a well-ordered lamellar phase, suggesting that the diblock polymer architecture allowed for enhanced interfacing (or anchoring) of the PPO block with the acyl chain region of the lipid bilayer.\textsuperscript{183}

Based on these findings, the “anchor and chain” model was proposed: a
hydrophobic end group on the PPO block increases the overall hydrophobicity of the polymer without significantly affecting the overall molar mass and thus acts as an anchor in the lipid bilayer without disrupting the lipid bilayer.\textsuperscript{63,130,181} Sufficient length of the PEO block is required to sustain the block copolymer at the membrane surface. A summary of how molecular parameters dictate polymer-membrane interactions is illustrated in Figure 1.3.

\textbf{Figure 1.3.} Schematic illustrating the interaction of PPO-PEO block copolymers with lipid bilayer as a function of molecular parameters. Figure from Houang \textit{et al.}\textsuperscript{130} (© The Authors 2018), which is licensed under the Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/).

1.5. P188 as a Therapeutic in Neurological Diseases

Of particular interest to this thesis are the animal studies related to neurological disease models. In a model of traumatic brain injury (TBI), adult mice injured with controlled cortical impact were given intravenously injections of P188 65-70 min post injury and were observed to have reduced cellular permeability in the cortex and
hippocampus.\textsuperscript{184} However, increase in BBB permeability after injury was not mitigated by P188 treatment.\textsuperscript{184} Another study reported that P188 treatment could reduce BBB permeability, neuronal death, and motor and cognitive defects in mice pretreated with P188 via intravenous tail injection 30 min before TBI induction.\textsuperscript{45} A study investigating secondary brain injury after TBI found that rats treated with P188 10 min and 4 hours post-injury had less tissue loss and macrophage infiltration.\textsuperscript{185} Another study of TBI treated adult rats with P188 intravenously 2 h after controlled cortical impact, and treatment resulted in decreased tissue loss, improved function, and less inflammation compared to injured, but untreated, rats.\textsuperscript{186}

Mice with intracerebral hemorrhage had reduced injury volume, improved BBB function, and alleviated neurological symptoms when given an intravenous tail injection of P188 1 h after surgery.\textsuperscript{47} Wang \textit{et al.} reported that P188 treatment of hemorrhaged mice led to suppressed levels of inflammatory matrix metalloproteinases (MMP) and hypothesized that P188 protection is associated with prevention of MMP-mediated tight junction protein degradation, though the mechanism of prevention remains unclear.\textsuperscript{47} Intracisternal injection of P188 in a rat model of intracranial hemorrhage led to smaller lesions but the protective effect was lost when the brain analyzed 7 days post injury.\textsuperscript{187} P188 treatment before induction of cortical spreading depression in mice brain protected neuronal membranes.\textsuperscript{188}

In a model of ischemic stroke, mice impacted with a middle cerebral artery occlusion (MCAO) were treated with P188 via tail vein injection 10 minutes after MCAO and found to have reduced infarct volume and improved motor behavioral defects.\textsuperscript{189} Similarly, another study found that P188 treatment of male mice induced with MCAO was
able to reduce BBB permeability, infarct volume, brain edema, and motor deficits when injected in the tail vein 5 min before reperfusion. When the injured mice were given daily intraperitoneal injections of P188 for 3 weeks, brain atrophy, neurological functions, and survival rates were improved. Intravenous administration of P188 30 min after MCAO in rabbits resulted in increased blood flow in areas of severe or moderate ischemia but little effect in other areas; however, blood viscosity and hemodilution were unaffected, leading to the hypothesis that P188 improves blood flow by diminishing adhesive interactions between proteins and cells. In summary, P188 treatment provided rescue or protection of neurological function and health in various disease scenarios, and several studies have demonstrated that intravenously administered P188 can help rescue the BBB.

1.5.1. *In vitro* investigations

Researchers have employed *in vitro* studies to gain mechanistic understanding of how P188 interacts with cells under neurological injury-mimicking conditions. P188 improved viability of neurons subjected to oxygen-glucose deprivation (OGD) as a model for ischemic stroke and decreased apoptosis and prevented necrosis in PC2 cells under mechanical stress. Hippocampus neurons under excitotoxicity or oxidative stress were kept viable by P188 treatment, even if the treatment came 8 h after the induction of stress. P188 interaction with the neurons led to an increase in cell surface area, suggesting P188 insertion into plasma membrane. Further, P188 was able to block lipid peroxidation and was suggested to be a scavenger of reactive oxygen species. Interestingly, P188 had a reduced effect on improving cell viability of neurons injured by staurosporine compared to other toxins. The authors attributed this to the fact that staurosporine does not affect
cellular membrane integrity and thus supports that P188-mediated neuronal protection is via blocking and repairing damage to the cell membrane.\textsuperscript{192}

Several studies signify the involvement of the mitochondria in P188-mediated cellular protection. Mechanically injured neurons had improved mitochondrial and lysosomal membrane integrity with P188 treatment.\textsuperscript{193} P188 treatment of murine primary cortical neurons during OGD led to decreased death, disruption of mitochondrial membrane potential, mitochondrial release of cytochrome C, activation of caspase-3.\textsuperscript{189} The same effects were observed in a different study with rat hippocampal neurons, though in this study by Shelat \textit{et al}., P188 treatment was given after OGD and could inhibit apoptosis even when the cells were treated 12 h after OGD.\textsuperscript{194} Additionally, Shelat \textit{et al}.
reported that P188 interferes with BAX translocation to the mitochondria, an early step for mitochondria outer membrane permeabilization (MOMP), which leads to apoptosis.\textsuperscript{195} Via super resolution microscopy, direct interaction of fluorescently labeled P188 with mitochondria of rat hippocampal neurons after 45 min of OGD and 2 h incubation with the fluorescently tagged P188 was observed; few P188 molecules were present in the control condition.\textsuperscript{196} Wang \textit{et al}.
also determined that P188 can interact with isolated mitochondrial to prevent MOMP.\textsuperscript{196}

Finally, mouse brain endothelial cells under mechanical trauma in an \textit{in vitro} model of TBI and treated with P188 had lower levels of MMP-2 and MMP-9 activity and gene expression, which are markers for inflammation.\textsuperscript{197} P188 tagged with fluorescent TAMRA dye molecules incubated with mouse brain endothelial cells also showed internalization of the molecules (time of incubation was not reported).\textsuperscript{197} Interestingly, in cells injured by microcavitation, fluorescently tagged P188 molecules were present throughout the
cytoplasm and also co-localized with the nuclei. It should be noted that a control condition with the endothelial cells incubated with the TAMRA dye showed internalization of the dye throughout the cytoplasm. The authors postulate that P188 interactions inside the cells and nuclei as well as in between the cells may mediate the observed protective effects of P188.

1.6. Objectives of this Work

Though the use of P188 in clinical and biomedical settings has a long history, the mechanism of action for different applications, including for cell membrane stabilization, is incompletely understood. In the context of cell membrane stabilization, this may be partly attributed to the diversity of cells/tissues and diseased states to which P188 treatment has been applied. Further, the non-specific and inherently weak interactions between the polymers and cells make investigations challenging. For neuroprotection facilitated by P188, cellular responses induced by PPO-PEO block copolymer interaction with the BBB have not been thoroughly investigated. Most in vitro studies investigating how P188 mitigates neurological dysfunction have focused on neurons. Given the importance of the BBB and that there is in vivo evidence that P188 treatment can lead to improved BBB function in neurological injuries, there is a need for direct interrogation of P188-mediated rescue of the BBB.

While insights gained from biophysical studies provide valuable knowledge into the interaction between lipid bilayers and poloxamers, they fail to capture the dynamics and heterogeneity of living cells and tissues. Additionally, studies utilizing model membranes have been limited to damages such as lipid peroxidation and hypo-osmotic
stress, but in living systems, diseased states are far more diverse both in their induction and consequences. Interaction behavior of poloxamers with lipid bilayers does not always correlate with that of poloxamers with living cells. For instance, Grozdova and co-workers showed that poloxamer binding efficiency with cells increased with increasing PEO block length, but partitioning into artificial lipid bilayers decreased with increasing PEO block length.\textsuperscript{198} It is also important to recognize that \textit{in vitro} models cannot recapitulate all events of a whole animal. \textit{In vivo}, P188 has been reported to have antithrombotic,\textsuperscript{88,89} anti-inflammatory,\textsuperscript{185,199,200} hemorheological,\textsuperscript{87,201} and membrane stabilizing\textsuperscript{130,141} effects. Thus, the overall therapeutic efficacy of P188 may only be explained by the synergy of all observed events,\textsuperscript{190,202} which cannot be fully simulated \textit{in vitro}.

Nevertheless, \textit{in vitro} systems capture the dynamics of a living system at a cellular level and provide a powerful tool to probe biological responses as a result of specific perturbations. The use of \textit{in vitro} models may be especially important for studying poloxamer-BBB interactions as BMECs are highly specialized cells with an added level of complexity due to intercellular connections that form the barrier which are hard to mimic with biophysical models. \textit{In vitro} models also provide an easier platform in which to screen various polymers to search for the “best-in-class” molecule. \textit{In vivo} evidence suggests that there are better candidates than P188 for BBB protection. For example, in a mouse model of TBI, an alternate membrane sealing polymer, Kollidon VA64, was found to be more efficacious in resealing cell membranes at a fifth of the concentration required for P188. Kollidon VA64 treatment also led to reduced BBB permeability, whereas P188 treatment was unable to rescue BBB function.\textsuperscript{184} Therefore, using an \textit{in vitro} model of the BBB, we sought to study the BBB under diseased conditions and investigate how P188 and related
block copolymers affect diseased BBB.

1.6.1. Methods to study the blood-brain barrier

As was the case for the initial studies that led to the discovery of the BBB, researchers have historically relied on animal models to study the BBB. However, in addition to issues with scalability, phenotypic differences, especially regarding transporter expression and function,\textsuperscript{203,204} in the BBB of different species make human based models more appropriate when investigating the BBB with the ultimate goal of translating findings to human therapies. Thus, researchers have developed various \textit{in vitro} models of the BBB by culturing BMECs in a platform such as a cell culture insert (e.g., Transwells) in which BMECs can be cultured atop a permeable membrane (Figure 1.4). Due to the polarization of the BMECs, culture on a Transwell creates apical and basolateral compartments, mimicking how one side of the cells faces the blood while the other side faces the brain \textit{in vivo}.

\textbf{Figure 1.4.} \textit{In vitro} model of BBB. Cell culture inserts (e.g., Transwells) are used for \textit{in vitro} models of the BBB wherein BMECs are cultured on a permeable membrane such that the apical and basolateral compartments are mimetic of the blood and brain side of the barrier, respectively.
Primary BMECs of bovine, rat, mouse, or porcine origin have been used for *in vitro* models, but the challenge of species differences as mentioned previously remains. Human primary BMECs have also been used for *in vitro* BBB models, but sources are limited and once they are taken *ex situ*, they lose their *in vivo* functionalities and characteristics. Immortalized human BMEC lines are available but do not have effective barrier function. The transendothelial electrical resistance (TEER), a measure of barrier tightness, of the aforementioned *in vitro* models are an order of magnitude lower than estimates of TEER across the BBB *in vivo*. Overall, these models do not possess key *in vivo* BBB characteristics, which include high TEER and proper expression of junctional and transporter proteins.

1.6.2. Human induced pluripotent stem cell-derived brain microvascular endothelial cells

The introduction of human induced pluripotent stem cell (hiPSC) technology has sparked great promise for therapeutic medicine as well as personalized *in vitro* modeling. Pluripotent stem cells have the ability to develop into any cell type in the body under the appropriate conditions. Human donor cells, such as fibroblasts, can be induced to be pluripotent by reprogramming certain transcription factors to produce hiPSCs. The first report of such transcription factors that were shown to induce pluripotency were Oct3/4, Sox2, Klf4 and c-Myc, which are now termed the “Yamanaka factors”. Subsequently, other combinations of factors have been demonstrated to induce pluripotency. Since hiPSCs are generated by reprogramming donor cells, they can be derived from any individual, opening the door for genetic disease modeling, personalized medicine, and tissue engineering applications.
In 2012, a method to differentiate human pluripotent stem cells into BMECs was introduced. In this method, endothelial cells with brain-specific phenotypes are differentiated from hiPSCs by co-differentiation with neural cells. The mixed population is then purified by seeding onto a matrix of collagen IV and fibronectin, which will only allow the endothelial cells to adhere. Subsequent modifications to the protocol have been introduced, including the addition of retinoic acid, which has been shown to enhance endothelial and BBB properties, a directed differentiation approach using chemically defined medium, an accelerated differentiation protocol, and alteration of extracellular matrix coating. With high TEER and low solute permeability, hiPSC-derived BMECs possess key markers of the BBB including proper expression and function of crucial efflux transporter proteins. Since their inception, hiPSC-derived BMECs have been utilized for various studies of the BBB in health and in disease. Recent analyses of these model cells via RNA sequencing indicated that they contain endothelial and epithelial cell transcripts. Nevertheless, cell structure and function of these cells closely mimic that of the BBB in vivo. Consideration of these factors has led to the observation that the cells would be more appropriately named “hiPSC-derived BMEC-like” cells. For simplicity, hiPSC-derived BMEC-like cells will be referred to as “iBMECs” throughout this thesis.

1.6.3. Outline of thesis

In this work, we utilized an in vitro system to study the BBB under specific diseased conditions and evaluate biological responses to treatment with P188 and related block copolymers. Application of iBMECs facilitated two routes of disease modeling: (1) study
of a monogenic disease utilizing patient-derived hiPSCs to differentiate into iBMECs and
(2) study of acute injuries via exposure of iBMECs to a disease mimicking microenvironment. The former method is discussed in Chapter 3 where examination of the BBB in childhood cerebral adrenoleukodystrophy (ccALD) is described. To model the BBB function of ccALD patients, we differentiated patient-derived hiPSCs into iBMECs and compared their function, phenotype, and genotype with iBMECs differentiated from hiPSCs from healthy individuals. Barrier function quantification, immunocytochemistry, electron microscopy, and RNA-sequencing analyses provided support for the hypothesis that the BBB function in ccALD is inherently compromised. Importantly, this investigation was one of the first reports utilizing patient-derived hiPSCs to study the impacts of a neurological disease on the BBB. Further, we found that treatment of the ccALD-iBMECs with a PPO-PEO diblock copolymer during development led to an improvement in barrier function.

In Chapter 4, we address another method of disease modeling to explore ischemic stroke. We investigated the effects of oxidative stress, a major source of damage during ischemic stroke, on the BBB, utilizing hydrogen peroxide (H$_2$O$_2$) as the insult. Preliminary work to screen poloxamers and PPO-PEO diblock copolymers was done. Impaired barrier function of H$_2$O$_2$-challenged iBMECs could be mitigated by treatment with a PPO-PEO diblock copolymer, which also prevented actin stress fiber formation and an increase in intracellular calcium concentration. This platform could be used to strengthen our understanding of how PPO-PEO block copolymer treatment affects BMECs under oxidative stress.

Application of confocal fluorescence and Raman microscopy for the purpose of
visualization of PPO-PEO block copolymers in iBMECs is outlined in Chapter 5. A diblock copolymer of PPO and PEO was modified to conjugate the end of the PEO block with Alexa Fluor 647. Confocal fluorescence microscopy experiments showed that the polymers were internalized in iBMECs within 2 h of incubation. To circumvent the issue of possible non-native interactions of the polymer with the cell due to the presence of the fluorophore, an alternative live-cell imaging method was explored. Preliminary work shows promise that confocal Raman microscopy could be a fluorophore-free and more quantitative method to probe the interaction between PPO-PEO block copolymers and live cells.

In Chapter 6, we describe additional experimental methods to induce iBMEC damage that mimic events of an ischemic stroke, including neurotransmitter toxicity, hypoxia/reoxygenation, and oxygen-glucose deprivation (OGD). Preliminary experiments were conducted with a co-culture of iBMECs and human primary astrocytes under OGD with P188 treatment, which suggested that synergistic interactions of P188 with the NVU need to be considered.
Chapter 2. Materials and Methods

Materials and experimental methods utilized throughout this thesis are discussed here. Cell culture methods are first described, followed by immunocytochemistry and fluorescence imaging methods. Then, anionic polymerization of block copolymers and polymer characterization methods and results are outlined. Experimental techniques specific to work in individual chapters are discussed in the respective chapters.

2.1. Differentiation of Human Induced Pluripotent Stem Cells

2.1.1. Culture of iBMECs on collagen IV and fibronectin

Human induced pluripotent stem cells (hiPSCs) were differentiated into brain microvascular endothelial cells (iBMECs) using a previously established method. For completeness and clarity, the differentiation method and timeline are summarized here. hiPSCs were detached with Accutase (STEMCELL Technologies) and seeded on a 6-well plate coated with Matrigel at a density between 45,000 – 60,000 cells/well in TeSR-E8 with 10 µM Y-27632 (STEMCELL Technologies). The medium was changed to fresh TeSR-E8 daily until the culture reached 20 – 30% confluency (approximately 30,000 cells/cm²). The differentiation was started by changing the medium to unconditioned medium (UM). The day that the medium is switched from TeSR-E8 to UM is Day 0. On Days 1 – 5, the medium was changed daily with fresh UM. On Day 6, the medium was changed to endothelial cell (EC) medium with 20 ng/mL basic fibroblast growth factor (bFGF; PeproTech) and 10 µM all-trans retinoic acid (RA; Millipore Sigma). EC medium consisted of human serum free endothelial cell medium (heSFM; Thermo Fisher Scientific)
with 1% platelet poor plasma derived serum (Biomedical Technologies). The same medium formulation was utilized through Day 8. On Day 8, cells were subcultured onto collagen IV and fibronectin coated well plates, Transwells (Corning), or µ-slides (ibidi) depending on the assay. For all experiments in this thesis, Transwells with a polyethylene terephthalate membrane and 0.4 μm pore size were utilized. On Day 9, the medium was changed to EC medium without bFGF nor RA. Preparation of UM and collagen IV and fibronectin coating concentration and methods are detailed in Stebbins et al.225

2.1.2. Culture of iBMECs on laminin 511-E8

Later experiments were conducted with iBMECs cultured on laminin 511-E8 following a modified differentiation protocol.218 The same procedure as outline in the previous section was followed until Day 6. On Day 6, the medium was changed to EC medium with 20 ng/mL bFGF and 10 μM RA where the EC medium consisted of heSFM with 1% fetal bovine serum (Thermo Fisher Scientific). The same medium formulation was utilized through Day 8. On Day 8, cells were subcultured onto collagen IV and fibronectin coated 6-well plates to purify the cell population. Two hours later, the cells were detached with Accuaste and subcultured again onto well plates, Transwells, µ-slides, or glass-bottomed chambers (Nunc) coated with laminin 511-E8 (EMD Millipore). Cultureware were coated with 1 μg/cm² laminin 511-E8 and incubated at 37°C for 1 h, at room temperature for 3 h, or at 4°C overnight before use. On Day 9, the cell culture medium was changed to EC medium without bFGF nor RA.
2.2. Immunocytochemistry and Fluorescence Microscopy

Unless otherwise noted, cells were fixed by adding ice-cold methanol to the cells, and the cells were then incubated for 15 min at room temperature and subsequently washed 3 times with DPBS (without calcium nor magnesium). Fixed cells were blocked for 1 h at room temperature with 10% normal goat serum in DPBS. Primary antibody was diluted in 10% normal goat serum in DPBS and incubation was done either at room temperature for 1 h or overnight at 4°C on a rocker. The cells were washed with DPBS 3 times (5 min per wash) and then incubated with secondary antibody diluted in 10% normal goat serum in DPBS for 1 h at room temperature in the dark on a rocker. Cells were washed with DPBS 3 times (5 min per wash) and stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 5 min to label the nuclei. Cells were washed once more with DPBS for 5 min before imaging. Antibody information is outlined in Table 2.1 and Table 2.2. All reagents mentioned in this section were from Thermo Fisher Scientific. Imaging was done with the EVOS FL Auto Imaging System with a 10X objective that has a numerical aperture of 0.25 or a coverslip-corrected 20X objective that has a numerical aperture of 0.75. The excitation and emission wavelengths of the light cubes on the microscope are summarized in Table 2.3.

<table>
<thead>
<tr>
<th>Target antigen</th>
<th>Antibody species</th>
<th>Clone or product no.</th>
<th>Dilution</th>
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<tbody>
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<td>Claudin-5</td>
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<tr>
<td>GLUT-1</td>
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<td>Occludin</td>
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<td>331500; clone OC-3F10</td>
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<td>Rabbit</td>
<td>RB10333P</td>
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<td>Rabbit</td>
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Table 2.2. Secondary antibodies used for immunocytochemistry. All antibodies are from Thermo Fisher Scientific.

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<td>Alexa Fluor 594 (A-11005)</td>
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</tr>
<tr>
<td>Rabbit</td>
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Table 2.3. Excitation and emission wavelengths of microscope light cubes

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<tr>
<td>Invitrogen Texas Red</td>
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2.3. Polymer Synthesis

Diblock copolymers of poly(propylene oxide) (PPO) and poly(ethylene oxide) (PEO) were synthesized by sequential ring-opening anionic polymerization following established techniques necessary for air and water free environments. Detailed procedures regarding reaction setup and monomer purification are described elsewhere.63,226,227 The reaction scheme is outlined in Figure 2.1. Alumina-dried tetrahydrofuran (THF) was used as the solvent for all steps of the polymerization. PPO was synthesized first with potassium tert-butoxide as the initiator, and the PO monomer was purified using n-butyl magnesium. The polymerization was carried out for 48 h, and the reaction was terminated with excess acidic methanol. The polymerization was conducted in the presence of 18-crown-6 ether (2:1 ratio of crown ether to initiator), which can complex with the potassium cations to favor the presence of the propagating free ion and reduce side reactions.228–230 Upon termination of the reaction, the polymer was filtered and water washed with the polymer dissolved in hexane to remove potassium salts and crown ether complexes. The polymer was recovered by freeze drying in benzene.

In preparation for PEO chain extension, potassium naphthalenide was prepared by
first adding potassium to a dried flask under dynamic argon flow. THF was added to the
flask; a representative preparation consisted of 250 mL THF for 1 g potassium. Under
argon flow, naphthalene was added to the flask at a 10% molar excess relative to potassium.
The flask was pressurized to 5 psi argon, and the flask was sealed and stirred at room
temperature for at least 12 h before use, resulting in a dark green solution. THF was added
to a dry reactor containing PPO polymer, and the reactor was placed in a 40°C water bath
(the temperature was maintained during the reaction until termination). To re-initiate the
PPO chains, potassium naphthalenide was added dropwise to the reactor via needle and
syringe until the polymer solution maintained a light green color for at least 30 min. Then,
EO monomer, purified by distillation from a solution containing with n-butyl magnesium,
was added to the reactor. After 24 h, the reaction was terminated with excess acidic
methanol and the diblock copolymer was purified by filtration. The final product was
recovered by freeze drying in benzene. Diblock copolymers are named as \( t\)-P\(_x\)E\(_y\), where the
“t” stands for the tert-butyl end group on the PPO block and \( x \) and \( y \) refers to the number
average degrees of polymerization of the PPO and PEO blocks, respectively.

\[
\text{(A) } O^- K^+ + n \left( \begin{array}{c} \Delta \end{array} \right) \xrightarrow{H^+} O\{\begin{array}{c} \Delta \end{array}\}_n H
\]

\[
\text{(B) } O\{\begin{array}{c} \Delta \end{array}\}_n H \xrightarrow{1. m} O\{\begin{array}{c} \Delta \end{array}\}_m H
\]

**Figure 2.1.** Anionic polymerization reaction scheme. (A) Initiation, propagation, and
termination of PPO block. (B) Reinitiation of PPO, propagation, and termination of PEO
block.
2.4. Polymer Characterization

Polymer solutions in deuterated chloroform (CDCl$_3$; Cambridge Isotope Laboratories, Inc) at a concentration of 20 mg/mL were prepared for $^1$H nuclear magnetic resonance ($^1$H-NMR). NMR spectroscopy was performed on a Bruker AX-400 (400 MHz). Representative NMR spectra are shown in Figure 2.2. For dispersity characterizations, size exclusion chromatograph (SEC; Agilent; calibrated with polystyrene standards) was utilized with polymer solutions of 2 mg/mL in THF. Polymers were characterized by matrix-assisted laser desorption/ionization mass spectroscopy (MALDI; AB SCIEX TOF/TOF 5800) to establish the number average ($M_n$) and weight average ($M_w$) molecular weights and dispersity ($D = M_w/M_n$). MALDI samples were prepared by mixing 0.2 mL of the matrix solution (30 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) in THF), 5 µL of the salt solution (1 mg/mL silver trifluoroacetate in THF), and 0.1 mL of the polymer solution (3 mg/mL in THF). As an example, an SEC trace and MALDI spectrum of P188 and $f\text{-P}_{15}\text{E}_{190}$ are shown in Figure 2.3. For the P188 data (Figure 2.3 A and B), there exists a shoulder on the SEC trace, and the MALDI spectrum shows a bimodal distribution; these features are due to the presence of diblock contaminants in commercially produced poloxamers.\textsuperscript{231,232} Thus, $M_n$ and $D$ values of the commercial triblock copolymers determined from MALDI data only take the larger molecular weight peaks into consideration. A summary of the polymer characterization data is presented in Table 2.4.
Figure 2.2. Representative $^1$H-NMR spectra of (A) poloxamer (P188) and (B) PPO-PEO
diblock copolymer ($t$-P$_{15}$E$_{190}$) with accompanying proton assignments. Residual solvent
(CDC$_3$) peaks are present around $\delta=7.2$ ppm.
Figure 2.3. Representative SEC and MALDI data of commercially available poloxamers and synthesized PPO-PEO diblock copolymers. (A) SEC trace and (B) MALDI spectra of P188. (C) SEC trace and (D) MALDI spectra of ζ-P$_{15}$E$_{190}$. SEC eluent was THF, and CHCA matrix was used for MALDI.
Table 2.4. Polymer characterization

<table>
<thead>
<tr>
<th></th>
<th>$M_{n,1}\text{a}$ (kDa)</th>
<th>$M_{n,2}\text{b}$ (kDa)</th>
<th>$D\text{b}$</th>
<th>$w_{\text{PEO,1}}\text{a}$</th>
<th>$w_{\text{PEO,2}}\text{d}$</th>
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</tbody>
</table>

$\text{a}$Specified by manufacturer.
$\text{b}$Determined by MALDI mass spectroscopy.
$\text{c}$Determined by SEC.
$\text{d}$Determined from molar ratios by $^1$H-NMR spectroscopy.
$\text{e}$For triblock copolymers, calculated from number-average molecular weight ($M_{n,2}$) and weight fraction of PEO ($w_{\text{PEO,2}}$). $N_{\text{EO}}$ represents the number of repeat units of EO in one PEO block. For diblock copolymers, calculated from end-group analysis of $^1$H-NMR spectra.
*Synthesized by Dr. Karen Haman$^{63}$
†Calculated from $^1$H-NMR

2.5. Dynamic Light Scattering

Dynamic light scattering experiments were done to determine size distributions of the polymers in solution. Polymers were dissolved in HBSS with calcium and magnesium (Thermo Fisher Scientific) and filtered through 0.2 µm filters before measurement. A Brookhaven BI-200SM system with a 637 nm laser at a 90° scattering angle was utilized, and the samples in glass tubes were placed in a decahydranaphthalene (decalin) bath. For all measurements, the temperature of the decalin bath was set to 37°C and the samples were incubated in a 37°C bath for at least 20 min before the measurement. Scattering data were
collected for 5 min. Data were analyzed by performing the regularized positive exponential sum (REPES) inversion on the obtained autocorrelation function, $C(\tau)$, to extract the decay constant ($\Gamma$) distribution. The relationship between $\Gamma$ and the diffusion coefficient ($D$) is given by Equation 2.1,

$$D = \frac{\Gamma}{q^2} \tag{2.1}$$

where $q$ is the scattering vector,

$$q = \frac{4\pi n}{\lambda} \sin \left(\frac{\theta}{2}\right) \tag{2.2}$$

in which $n$ is the solvent index of refraction, $\theta$ is the scattering angle, and $\lambda$ is the wavelength of incident light. Thus, the $\Gamma$ distribution can be converted to a distribution of hydrodynamic radii ($R_h$) by the Stokes-Einstein relation:

$$R_h = \frac{k_B T}{6\pi \eta D} \tag{2.3}$$

where $k_B$ is the Boltzmann’s constant, $T$ is the temperature, and $\eta$ is the viscosity of the solvent. As an example, the autocorrelation function of a 1 mM $t$-P$_{15}$E$_{190}$ solution and the corresponding fit by the REPES algorithm are displayed in Figure 2.4 along with the $R_h$ distribution profile. The $R_h$ distribution curves of other samples are compiled in Figure 2.5.
Figure 2.4. Dynamic light scattering data of PPO-PEO diblock copolymer. (A) Autocorrelation function with REPES fit and (B) $R_h$ distributions of 1 mM $f_{-}P_{15}E_{190}$ in HBSS
Figure 2.5. $R_h$ distributions of 1 mM polymer solutions in HBSS.
Chapter 3. Modeling the Blood-Brain Barrier of Childhood Cerebral Adrenoleukodystrophy Patients and Rescue of Function by PPO-PEO Diblock Copolymer Treatment

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3.1. Introduction

The molecular mechanisms responsible for the onset and progression of childhood cerebral adrenoleukodystrophy (ccALD) remain poorly understood. ccALD is one form of X-linked adrenoleukodystrophy (X-ALD), an inherited metabolic storage disorder affecting 1 in 17,000 individuals. X-ALD is caused by mutations in the ABCD1 gene which codes for the ABCD1 protein. ABCD1 is a peroxisomal transporter protein responsible for transporting very long-chain fatty acids (VLCFAs) from the cytosol into the peroxisome for subsequent beta-oxidation. Mutation type and location are not predictive of phenotype, as the same ABCD1 mutation can lead to clinically distinct phenotypes. A more frequent and less severe phenotype, adrenomyeloneuropathy (AMN), presents with demyelination in the long tracts of the spinal cord and progressive axonopathy, usually around the third or fourth decade of life. Heterozygous females will
develop similar symptoms by age 60.\textsuperscript{243–245} ccALD, the most rapidly progressing phenotype, occurs in boys ages 2-12 and is characterized by sudden inflammatory demyelination in the brain and death within a few years.\textsuperscript{246,247} ccALD affects about 40% of males with an \textit{ABCD1} mutation.\textsuperscript{248,249} MRI observation of gadolinium enhancement in the brain remains the only method to detect this progression.\textsuperscript{250–254} Infections or head trauma have been described as initiators of the conversion from AMN to ccALD, but typically no extrinsic factor can be identified.\textsuperscript{255–257} Current treatment for ccALD includes hematopoietic cell transplant (HCT), but this must be performed at the earliest stages of the disease.\textsuperscript{245,247,258,259}

Much attention has focused on VLCFAs in the search for alternative treatments. While the accumulation of VLCFAs appears to directly contribute to symptoms of AMN, how VLCFAs contribute to the onset or progression of ccALD is unclear.\textsuperscript{260,261} VLCFAs accumulate in many tissue types in X-ALD patients, but this accumulation is not predictive of clinical phenotype.\textsuperscript{262,263} Furthermore, dietary regimens or treatments aimed at reducing the accumulation of VLCFAs (e.g., “Lorenzo’s oil”) cannot prevent ccALD onset,\textsuperscript{264–266} just as immunosuppression cannot prevent the cerebral inflammation seen during ccALD progression.\textsuperscript{267,268} Other biomarkers have been investigated for their potential correlation with ccALD conversion including mitochondrial defects, AMP-activated protein kinases, reactive oxygen species (ROS), and oxidative stress.\textsuperscript{248,269–273} Antioxidant activity levels of superoxide dismutase in blood plasma have been found to decrease prior to and during cerebral diagnosis.\textsuperscript{274} Treatment with the antioxidant \textit{N}-acetyl-L-cysteine improves survival of patients with advanced ccALD undergoing HCT,\textsuperscript{275} and oxidative stress levels decrease in patients after HCT.\textsuperscript{276} A phase II clinical trial testing a cocktail of antioxidants
on patients with AMN was recently completed.\textsuperscript{277} The treatment was deemed to be safe and well-tolerated by AMN patients, and some biomarkers of oxidative damage and inflammation measured in the patients’ plasma samples were reduced to normal levels with treatment, prompting the investigators to call for phase III trials.\textsuperscript{278} Identification at the molecular level of defects underlying the rapid blood-brain barrier (BBB) breakdown seen in ccALD would enable the development of strategies aimed at preventing the onset and progression of ccALD.

The initial BBB breakdown is thought to be mediated by immune cells (specifically T-cells and to some extent B-cells) translocating from the blood into the brain.\textsuperscript{279,280} Until recently, however, little attention has been paid to the brain endothelium constituting the BBB.\textsuperscript{281} X-ALD lacks a suitable mouse model to study the BBB, as mice lacking ABCD1 only develop symptoms of AMN.\textsuperscript{282} A human model of the BBB is difficult to obtain, as primary cells isolated from human brain biopsies are not readily available and tend to de-differentiate upon removal from the \textit{in vivo} microenvironment.\textsuperscript{205} To address these challenges, we utilized patient-derived human induced pluripotent stem cells (hiPSCs) to differentiate into induced brain microvascular endothelial cells (iBMECs) as an \textit{in vitro} model of the BBB.\textsuperscript{214,215,225,283} This system has been used to model the BBB of other neurological diseases such as Huntington’s,\textsuperscript{284} Alzheimer’s,\textsuperscript{285} and Allan-Herndon-Dudley\textsuperscript{286} and to model bacterial interaction with the BBB.\textsuperscript{287} Use of this system provides a unique opportunity to study the BBB of ccALD patients and to ask whether there are differences in barrier function compared to WT controls.

Additionally, this same system can be used to investigate potential therapeutic interventions to improve defects in barrier function. We hypothesized that treatment of
diseased iBMECs with block copolymers of poly(propylene oxide) (PPO) and poly(ethylene oxide) (PEO) may improve barrier function as poloxamer 188 (P188) has been demonstrated to provide cell membrane stabilization for a panoply of cell and tissue types under various stresses. A systematic *in vitro* screening of PPO-PEO diblock copolymers identified the polymer \( \ell\text{-P}_{16}\text{E}_{182} \) to be the most efficacious in stabilizing myoblasts under hypo-osmotic stress and isotonic recovery.\(^{74}\) Thus, in addition to the commonly used P188, we hypothesized that \( \ell\text{-P}_{16}\text{E}_{182} \) may also improve iBMEC function.

In this study, we used a previously established directed differentiation protocol to derive iBMECs from WT- and ccALD-iPSCs. This enabled us to model the BBB of ccALD patients and to examine potential differences in barrier function specific to ccALD. P188 and a PPO-PEO diblock copolymer, \( \ell\text{-P}_{16}\text{E}_{182} \), were investigated for their potential to improve BMEC integrity. Testing of these two block copolymers with this BBB model is a new avenue of investigation for X-ALD. Improvements in barrier function produced by amphiphilic block copolymers have implications for translation into a treatment for preventing the onset of ccALD by improving the BBB integrity of X-ALD patients. Translating the results from this study has the potential to reduce the number of individuals with X-ALD who develop deadly and rapidly progressive ccALD.

3.2. Experimental Methods

3.2.1. Derivation and culture of iPSCs

Normal and ccALD iPSC lines (Table 3.1) were used.\(^{288,289}\) Cell lines were reprogrammed using retroviral gene delivery using the reprogramming factors OCT4,
SOX2, KLF4, and c-MYC (Addgene) (WT1, WT2, ccALD1, ccALD2, ccALD3) or obtained from American Type Culture Collection (ATCC) (WT3 = ACS-1024). Cells were derived from somatic cells on irradiated MEF cultures and transferred to Matrigel (Corning) and E8 Medium (Thermo Fisher Scientific) or TeSR-E8 (STEMCELL Technologies) for additional feeder-free expansion and maintenance. All cell lines tested negative for Mycoplasma contamination via a MycoAlert™ Mycoplasma Detection Kit (Lonza). With the exception of the cell line obtained from ATCC, all cell lines were authenticated using genetic fingerprinting and were also found to be karyotypically normal. Differentiation of WT- and ccALD-iPSCs to iBMECs was done following the protocol outlined in §2.1.1.

<table>
<thead>
<tr>
<th>iPSC line</th>
<th>Sex</th>
<th>Derived cell type</th>
<th>Delivery method</th>
<th>Reprogramming factors</th>
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<tr>
<td>ccALD1</td>
<td>Male</td>
<td>Fibroblasts</td>
<td>Retrovirus</td>
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</tr>
<tr>
<td>ccALD2</td>
<td>Male</td>
<td>Fibroblasts</td>
<td>Retrovirus</td>
<td>OCT4, SOX2, KLF4, c-MYC</td>
</tr>
<tr>
<td>ccALD3</td>
<td>Male</td>
<td>Keratinocytes</td>
<td>Retrovirus</td>
<td>OCT4, SOX2, KLF4, c-MYC</td>
</tr>
<tr>
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<td>Keratinocytes</td>
<td>Retrovirus</td>
<td>OCT4, SOX2, KLF4, c-MYC</td>
</tr>
<tr>
<td>WT2</td>
<td>Male</td>
<td>Urine cells</td>
<td>Retrovirus</td>
<td>OCT4, SOX2, KLF4, c-MYC</td>
</tr>
<tr>
<td>WT3</td>
<td>Male</td>
<td>CD34+ bone marrow cells</td>
<td>Sendai virus</td>
<td>OCT4, SOX2, KLF4, c-MYC</td>
</tr>
</tbody>
</table>

3.2.2, Immunocytochemistry

iBMECs were subcultured onto µ-slides (ibidi) and fixed 2 days later. Immunocytochemistry methods are outlined in §2.2.
3.2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Two days after subculture onto 6-well plates, iBMECs were detached with 0.05% trypsin-EDTA and formed into cell pellets by centrifugation. Total RNA was extracted using an RNeasy Mini Kit (Qiagen) following the manufacturer’s protocol and quantified using a NanoDrop® ND-1000. cDNA was generated from 1 µg of RNA using Omniscript reverse-transcriptase (Qiagen) and oligo-dT primers (Thermo Fisher Scientific). RT-PCR was performed using the GoTaq Green Master Mix (Promega) and PrimePCR primer sets (Bio-Rad) (Table 3.2). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. Gel electrophoresis of RT-PCR products with a 2% agarose gel was used to analyze transcript amplification and imaged with the BioRad ChemiDoc Imager.

Table 3.2. Primers used for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Vendor</th>
<th>ID Number</th>
</tr>
</thead>
<tbody>
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<td>GAPDH</td>
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</tr>
<tr>
<td>CDH5</td>
<td>BioRad</td>
<td>qHsaCID0016288</td>
</tr>
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<td>SLC2A1</td>
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<td>qHsaCID0022232</td>
</tr>
<tr>
<td>ABCB1</td>
<td>BioRad</td>
<td>qHsaCID0020960</td>
</tr>
</tbody>
</table>

3.2.4. Trans-endothelial electrical resistance

iBMECs were seeded onto Transwell filters. TEER was measured daily starting 24 h after subculture utilizing the EVOM2 voltohmmeter with STX3 chopstick electrodes (World Precision Instruments). TEER was measured on an empty Transwell filter coated with collagen IV and fibronectin, and this value was subtracted from the TEER of the cell.
monolayer each time. TEER values were normalized by the surface area of the Transwell filter.

3.2.5. Sodium fluorescein permeability

iBMECs were seeded onto Transwell filters. An empty Transwell filter coated with collagen and fibronectin was utilized to measure the permeability of the membrane only. After a complete medium change, the cells were incubated at 37°C, 5% CO₂ for 1.5 h. TEER was measured before and after the medium change to confirm monolayer equilibration. Medium from the apical chamber was aspirated and replaced with cell culture medium containing 10 µM sodium fluorescein (Millipore Sigma). Every 30 min for 2 h, 150 µL aliquots were extracted from the basolateral chamber and replaced with 150 µL of fresh medium. At 2 h, a 150 µL sample was extracted from the apical chamber and then fluorescence intensity was measured on a BioTek Synergy H1 multi-mode microplate reader at excitation of 485 nm and emission of 530 nm. The permeability values were calculated using the slope of clearance volume as a function of time. The permeability values of the iBMECs only are reported and were calculated from the total permeability and permeability of the blank Transwell. Details of the calculations can be found in Stebbins et al. and Perrière et al.²²⁵,²⁹⁰

3.2.6. Rhodamine 123 accumulation

Accumulation of rhodamine 123, a P-glycoprotein (P-gp) substrate, was measured in the absence and presence of a P-gp inhibitor cyclosporin A to quantify P-gp efflux potential. iBMECs were seeded onto 24-well plates. Cells were pre-incubated with or
without 10 µM cyclosporin A (Millipore Sigma) in HBSS (with calcium and magnesium; Thermo Fisher Scientific) for 1 h at 37°C, 5% CO₂. Next, all cells were incubated with 10 µM rhodamine 123 (Millipore Sigma) in HBSS for 2 h at 37°C, 5% CO₂. Following the incubation steps, cells were lysed using RIPA buffer (Millipore Sigma) and fluorescence intensity was measured on a BioTek Synergy H1 multi-mode microplate reader at excitation of 485 nm and emission of 530 nm. Unlysed cells from a parallel setup were dissociated with Accutase (Thermo Fisher Scientific) and counted using the Countess II FL Automated Cell Counter to normalize the fluorescence on a per cell basis.

3.2.7. Analysis of tight junction continuity

For quantitative analysis of iBMEC integrity, the percentage of cells expressing frayed tight junctions was counted using iBMECs immunolabeled for occludin. Cells were defined as having frayed tight junctions if any cell-cell contact point appeared discontinuous. A blinded analysis in which three different people each counted fifteen separate frames and 12,530 total junctions was used to obtain a percentage of frayed tight junctions for both the ccALD- and WT-iBMECs.

3.2.8. Electron microscopy

iBMECs were seeded onto 6-well plates. Two days after subculture, the cells were fixed with 1 mL of 2.5% glutaraldehyde (Electron Microscopy Sciences) in 0.1 M sodium cacodylate for 1 h at room temperature. Using cell lifters to detach the cells while preserving cell-cell junctions, cells were collected in microcentrifuge tubes and stored in fresh fixative solution for pelleting via centrifugation. Following 3 washes with 0.1 M
sodium cacodylate buffer, cells were post-fixed with 1% osmium tetroxide (Electron Microscopy Sciences). Cells were dehydrated in acetone and subsequently embedded with Embed 812 resin (Electron Microscopy Sciences). A Leica UC6 Ultramicrotome (Leica Microsystems) was used to section the embedded samples. A JEM 1400 Plus transmission electron microscope (JEOL LTD) and AMT Capture Engine Version 7.00 (Advanced Microscopy Techniques Corp.) were used to analyze and image the samples.

3.2.9. RNA sequencing

Two days after iBMECs were seeded onto 6-well plates, cells were detached with 0.05% trypsin-EDTA. Total RNA was isolated from WT- and ccALD-iBMECs using an RNeasy Mini Kit (Qiagen) following the manufacturer’s protocol. RNA with an RNA Integrity Number (RIN) score $> 8$ was used for library generation with the TruSeq Stranded mRNA Sample Preparation kit (Illumina). Paired-end 150 bp length reads were generated using an Illumina MiniSeq. Low quality bases were trimmed using Trimmomatic (enabled with the optional "--qualitycontrol" option and a 3bp sliding-window trimming from the 3' end requiring minimum Q16). The remaining reads were mapped to hg19 using Tophat2. The featureCounts program in the R SubRead package was used to generate a transcript abundance file for input into the R package edgeR to identify differentially expressed genes. Ingenuity Pathway Analysis\textsuperscript{291} was used for network analysis and Gene Ontology\textsuperscript{292,293} for pathway analysis.
3.2.10. Oil-Red-O staining and image analysis

iBMECs were seeded onto 12-well plates. Two days later, cells were fixed with 10% formalin for 20 minutes, subsequently dehydrated with 60% isopropanol, and incubated with Oil-Red-O (Millipore Sigma) for 10 minutes before being washed 4 times with deionized water. Images were captured using a Nikon Eclipse TS100 inverted light microscope connected to a Unitron Microscopes Lumenera® Cameras AU-310-CMOS Infinity 1 camera. To measure the abundance of lipid droplets from the Oil-Red-O stained images, a custom MATLAB script was used to quantify the number and intensity of red pixels. Red pixels were defined on the HSV (hue, saturation, value) scale as having hue between 0.833 and 0.073, saturation between 0.300 to 1, and value between 0 and 1. The MATLAB Color Thresholder tool was used to mask as black any pixels not defined as red. Average intensity was measured by summing the value in the red channel of the RGB scale for each pixel, while the average number of pixels was calculated as the total number of red pixels.

3.2.11. Polymer treatment

Working solutions of polymers were prepared by dissolving P188 and t-P16E182 in 1X DPBS without magnesium or calcium (Thermo Fisher Scientific) to a concentration of 12 mM and sterilized via filtration. Chapter 2 contains information on polymer synthesis and characterization. For polymer treatment during development, the polymers were added to the culture on Day 3 of the differentiation protocol. The polymer working solutions were diluted to a concentration of 0.5 mM or 1 mM in the culture medium, and the resulting solution was added to the culture during standard medium change. The control received
medium with DPBS such that all conditions had the same volume of DPBS in the medium. After 24 h, the medium was changed in accordance with the differentiation protocol, effectively removing any excess polymers. On Day 8, the iBMECs were subcultured onto Transwells for TEER measurements or 12-well plates for Oil-Red-O staining. For polymer treatment post-differentiation, iBMECs were subcultured onto Transwells on Day 8; on Day 9, a small aliquot of the P188 or t-P16E182 working solutions was added to the apical chamber such that the final concentration of polymers in the apical chamber was 1 mM. A corresponding volume of DPBS was added to the control cells.

3.2.12. Statistical analysis

Data are presented as mean ± standard error (SEM) with n defined in figure legends. P-values were determined using an unpaired Student’s t-test. Statistical analysis was performed using GraphPad Prism.

3.3. Results

3.3.1. Directed differentiation of WT- and ccALD-iPSCs into iBMECs

A previously published protocol\textsuperscript{225} was used to direct the differentiation of iPSCs into iBMECs from three clinically confirmed cases of ccALD and three WT controls. Immunofluorescence and RT-PCR demonstrated that both patient and control iBMECs expressed the requisite endothelial markers PECAM-1 and VE-cadherin (CDH5), the tight junction markers claudin-5 and occludin, and the BBB markers P-glycoprotein and GLUT-1 (SLC2A1) (Figure 3.1). Expression of \textit{ABCB1}, which encodes the efflux transporter P-
gp, was decreased for one of the ccALD-iBMEC lines (Figure 3.2a). Thus, we employed a rhodamine 123 accumulation assay to check the P-gp efflux potential of the ccALD-iBMECs (Figure 3.2b). Normalized accumulation after inhibiting P-gp with cyclosporin A (CsA) was lower for the same iBMEC line in which we noticed the decreased \textit{ABCB1} expression (102.7 ± 2.3) compared to the other ccALD-iBMEC lines (152.7 ± 23.4 and 167.1 ± 19.0) as well as the WT-iBMECs lines (258.2 ± 16.2, 220.8 ± 26.4, and 204.7 ± 20.2).

\textbf{Figure 3.1.} iBMECs express the requisite endothelial, tight junction, and BBB markers. \textit{(a)} Representative immunocytochemistry (WT1 and ccALD2). iBMECs from ccALD patients and WT controls express PECAM1, GLUT1, Claudin-5, and occludin. No qualitative difference was observed between the WT and ccALD-iBMECs. \textit{(b)} RT-PCR (all WT and ccALD lines). iBMECs from ccALD patients and WT controls express \textit{CDH5} (VE-cadherin) and \textit{SLC2A1} (GLUT1).
Figure 3.2. P-glycoprotein (P-gp) expression and function. (a) All WT- and ccALD-iBMECs express ABCB1, which codes for efflux transporter P-gp. (b) P-gp function was assessed with Rhodamine 123 accumulation assay, which showed functional P-gp for all iBMECs except ccALD2-iBMECs. Differences in normalized accumulation between no inhibitor and CsA inhibited samples are statistically significant (p < 0.05) for all cell lines with the exception of ccALD2-iBMECs. Fluorescence intensity is normalized by cell density, and accumulation is independently normalized to the corresponding control (no inhibitor). Four biological replicates used (n = 4).

3.3.2. ccALD-iBMECs have impaired barrier properties

To investigate functional differences between the ccALD- and WT-iBMECs, we used trans-endothelial electrical resistance (TEER) to measure the barrier integrity of the iBMECs on Days 1-4 following subculture onto Transwell filters. At all days measured, we found a statistically significant difference (p < 0.0001) in TEER between the ccALD- and WT-iBMECs (peak TEER on Day 2 of measurement: 2592 ± 110 Ω•cm² compared to 5001 ± 172 Ω•cm² for the ccALD-iBMECs and WT-iBMECs, respectively) (Figure 3.3a). Additionally, permeability of sodium fluorescein was measured to be 1.85 ± 0.19 x 10⁻⁵ cm/min for the ccALD-iBMECs and 1.50 ± 0.31 x 10⁻⁵ cm/min for the WT-iBMECs (Figure 3.3b). The difference in permeability is not statistically significant despite the substantial difference (~2400 Ω•cm²) in TEER between the WT- and ccALD-iBMECs;
however, our results are consistent with previous studies that report sodium fluorescein permeability values on the order of $10^{-5}$ cm/min for BMECs with TEER greater than 2000 $\Omega \cdot \text{cm}^2$ and with reports that demonstrate that small molecule passive permeability does not correlate strongly with TEER above certain TEER thresholds.

Figure 3.3. ccALD-iBMECs are functionally distinct from WT-iBMECs. (a) Trans-endothelial electrical resistance (TEER) is significantly decreased in the ccALD-iBMECs compared to the WT-iBMECs at all experimental time points. Data compiled from three independent experiments with nine biological replicates each (all iBMEC lines used) (n = 27). *p < 0.0001. (b) Passive transport as measured by sodium fluorescein permeability is slightly increased in the ccALD-iBMECs compared to WT-iBMECs. All iBMEC lines tested with three biological replicates each (n = 9). (c) Examples of frayed junctions indicated by white arrows on occludin immunolabeled images of WT1- and ccALD3-iBMECs. (d) Quantification of percent frayed junctions in WT1- and ccALD3-iBMECs indicates that WT-iBMECs have fewer frayed junctions than ccALD-iBMECs. Results of nine biological replicates with five technical replicates each shown (n = 45).
To examine potential differences in tight junction organization, we employed a
frayed junction analysis. The ccALD-iBMECs had more frayed junctions (p < 0.01)
compared to the WT-iBMECs (37 ± 3% versus 25 ± 3%) (Figure 3.3c, d). Overall, iBMECs
from ccALD patients appear to form a less intact cellular barrier that permits increased
passive transport of ions as well as small molecules. This defect in barrier integrity may
result from mislocalization of tight junction proteins between cells.

3.3.3. Lipid droplets accumulate in ccALD-iBMECs

To assess any structural differences between the ccALD-iBMECs and WT controls,
we performed transmission electron microscopy (TEM) of ccALD- and WT-iBMECs
using cross-sections of fixed and pelleted cells. Numerous and large lipid droplets were
present in the ccALD-iBMECs, with fewer and smaller lipid droplets in the WT-iBMECs
(Figure 3.4a, Figure 3.5). To quantify the abundance of lipid droplets in the iBMECs, Oil-
Red-O staining was used. This histological stain is specific to neutral lipids and does not
stain the polarized phospholipids of the cell membrane. Lipid droplets are stained bright
red, and image analysis can be used to quantify either the amount (total number of red
pixels) or intensity (redness of the red pixels) of red in micrographs. Quantification of the
lipid deposition in ccALD- and WT-iBMECs revealed a significant increase (p < 0.005) in
lipid abundance in the ccALD-iBMECs compared to the WT-iBMECs (Figure 3.4b, c).
The average intensity of red pixels in images of Oil-Red-O stained WT-iBMECs was
calculated to be 1.8 ± 0.5 \times 10^6 compared to 5.4 ± 1.0 \times 10^6 for the ccALD-iBMECs. The
average number of red pixels was calculated to be 1.1 ± 0.2 \times 10^4 for the WT-iBMECs and
2.8 ± 0.4 \times 10^4 for the ccALD-iBMECs (Figure 3.4c). Very few lipid droplets were seen in
the iPSCs and no statistical difference was observed between the ccALD-iPSCs and WT-iPSCs in the number of red pixels (22 ± 8 and 35 ± 11, respectively) or the intensity of red pixels (4.2 ± 2 x 10³ and 6.4 ± 2 x 10³, respectively) (Figure 3.6). VLCFA accumulation was not observed in the ccALD-iBMECs via TEM. The presence of an increased amount of lipid droplets in the ccALD-iBMECs compared to the WT-iBMECs that arises upon differentiation (i.e., is not present during the iPSC stage) is a difference that potentially contributes to the decreased barrier integrity of the ccALD-iBMECs.

**Figure 3.4.** ccALD-iBMECs accumulate more lipid droplets than WT-iBMECs. (a) Comparison of transmission electron micrographs of WT1- and ccALD3-iBMECs show increased lipid droplet accumulation in ccALD-iBMECs. Some lipid droplets are outlined in red. (b) Representative images of Oil-Red-O stained WT3 and ccALD1-iBMECs. Raw images on left and masked images on right. (c) Quantification of intensity and number of red pixels in images of Oil-Red-O stained iBMECs indicate increased lipid droplet accumulation in ccALD-iBMECs compared to WT-iBMECs. Oil-Red-O staining images of all iBMEC lines were used for quantification using three biological replicates for each cell line (n = 9).
**Figure 3.5.** Additional TEM of WT1-iBMECs (above) and ccALD3-iBMECs (below) at varying magnifications showing increased lipid droplet accumulation in ccALD-iBMECs.
Figure 3.6. Oil-Red-O staining and quantification of WT and ccALD-iPSCs. (a) Oil-Red-O staining of WT and ccALD-iPSCs show little to no lipid droplet accumulation. (b) Quantification of intensity and number of red pixels in images of Oil-Red-O stained iPSCs show no difference in lipid droplet accumulation in ccALD-iPSCs compared to WT-iPSCs. All cell lines were used for quantification with two biological replicates each (n=6).

3.3.4. Transcriptome analysis indicates differences in Type I interferon activation and lipid metabolism pathways

To further characterize differences between ccALD- and WT-iBMECs and to elucidate potential mechanisms for the decreased barrier integrity seen in the ccALD-iBMECs, we performed RNA-sequencing of three replicate differentiations for each of our ccALD- and WT-iBMEC lines. Principal component analysis (PCA) separated the WT-iBMECs from the ccALD-iBMECs along the first principal component (Figure 3.7a). Hierarchical clustering of differentially expressed genes (DEGs) (2X fold change, false-discovery rate (FDR) < 0.05) and samples revealed a cluster of genes that were decreased in the ccALD-iBMECs involving the attachment of cells to each other including intracellular attachment between membrane regions (gene ontology (GO): 0022610), while
Type I interferon-activated signaling (GO: 0060337) and insulin-like growth factor receptor signaling (GO: 0043568) pathways were increased in the ccALD-iBMECs (Figure 3.7b). We used Ingenuity Pathway Analysis (IPA) to query upstream regulators of our DEGs. IPA builds a graph-based network from gene expression data and uses this information to predict upstream regulators. The z-score (calculated as the number of standard deviations from the mean of a normal distribution of activity edges using this graph-based network) represents the magnitude of bias in gene regulation that predicts the activity of specific upstream regulators. This analysis revealed upstream regulators involving TGFβ1 signaling, Type I interferon response, and other immune signaling signatures highly activated in the ccALD-iBMECs (IFNG, LPS, TNF, and TGFβ1 z-scores of 5.5, 7.6, 5.6, and 4.6 respectively) (Figure 3.7c). GO analysis was performed on genes differentially expressed between the ccALD- and WT-iBMECs. This analysis calculates a p-value based on enrichment of genes in a particular GO annotation (−log_{10}(p-value) reported as enrichment score for upregulated genes, log_{10}(p-value) reported as enrichment score for downregulated genes). GO analysis on genes upregulated (increased activity) in ccALD-iBMECs indicated an increase in Type I interferon signaling (enrichment score 8.45) and response to lipid pathways (enrichment score 4.34). GO analysis on genes downregulated (decreased activity) in ccALD-iBMECs indicated a decrease in transmembrane and ion transport (enrichment scores -1.58 and -3.5, respectively) (Figure 3.7d). The lipid pathway upregulation is consistent with both the primary ccALD phenotype and our TEM results, while Type I interferon signaling and other inflammatory pathways are secondary and could have many sources.
Figure 3.7. Transcriptome analysis indicates differences in Type I interferon activation and lipid metabolism pathways. (a) PCA mapping of log₂ normalized read counts on global gene expression. The first three dimensions account for 38.3% of the total variance with grouping of individual WT- and ccALD-iBMEC replicates and separation of the experimental and control samples along PC1. (b) Heat map of DEG (n = 1381) on log₂ normalized read counts. Cluster annotations are from gene ontology analysis. (c) IPA upstream regulator analysis of transcriptional regulators predicted by activation z-scores. p-values calculated by Fisher’s exact test using expected and observed genes overlapping with the WT versus ccALD DEGs and all genes regulated by each transcriptional regulator. (d) GO terms of pathways upregulated in ccALD in red with downregulated pathways in green. Data analyzed from three independent experiments with three biological replicates each (n = 9).

3.3.5. Block copolymers reverse impaired barrier integrity and mitigate lipid accumulation

We next investigated whether polymer treatment could rescue the impaired barrier integrity of the ccALD-iBMECs. ccALD-iBMECs were treated with 1 mM of P188 or €P₁₆E₁₈₂ at the end of the differentiation protocol (Day 9) or during development (Day 3). Day 3 was chosen because the cells begin to express endothelial cell markers at this time.
Polymer treatment with either P188 or $t\cdot P_{16}E_{182}$ at the end of the differentiation protocol showed minimal effect on ccALD-iBMEC TEER (Figure 3.8a). However, we saw a significant effect ($p < 0.05$) when the ccALD-iBMECs were treated with the diblock copolymer ($t\cdot P_{16}E_{182}$) during development. The maximum TEER of the ccALD-iBMECs treated with $t\cdot P_{16}E_{182}$ was $3316 \pm 246 \ \Omega \cdot \text{cm}^2$. This was higher than both the untreated and P188 treated ccALD-iBMECs ($2409 \pm 254 \ \Omega \cdot \text{cm}^2$ and $2162 \pm 260 \ \Omega \cdot \text{cm}^2$, respectively; Figure 3.9). The effect of dosage was investigated by treating the ccALD-iBMECs with 0.5 mM or 1 mM $t\cdot P_{16}E_{182}$ on Day 3 of the differentiation protocol, and we observed a larger increase in TEER compared to the control when treated with 1 mM $t\cdot P_{16}E_{182}$ than with 0.5 mM $t\cdot P_{16}E_{182}$ (Figure 3.8b). Notably, the barrier function of the WT-iBMECs was unaffected by polymer treatment as the TEER of the untreated WT-iBMECs ($3074 \pm 127 \ \Omega \cdot \text{cm}^2$) was not significantly different than that of WT-iBMECs treated with P188 ($2998 \pm 50 \ \Omega \cdot \text{cm}^2$) or $t\cdot P_{16}E_{182}$ ($3165 \pm 95 \ \Omega \cdot \text{cm}^2$).

![Figure 3.8](image_url)

**Figure 3.8.** Timing and dosage effect of block copolymer treatment. (a) Addition of 1 mM P188 or $t\cdot P_{16}E_{182}$ on Day 9 of the differentiation protocol had a non-significant effect on TEER of the ccALD3-iBMECs. However, a slight increase in TEER upon treatment with 1 mM $t\cdot P_{16}E_{182}$ was observed. Data compiled from three independent experiments with three biological replicates each ($n = 9$). (b) Maximum TEER of ccALD3-iBMECs treated 1 mM $t\cdot P_{16}E_{182}$ on Day 3 of the differentiation protocol is higher than ccALD3-iBMECs treated with 0.5 mM $t\cdot P_{16}E_{182}$ on Day 3, signifying that treatment efficacy is concentration dependent. Data from three biological replicates ($n = 3$). * $p < 0.005$, ** $p < 0.0005$ with respect to control. # $p < 0.001$ with respect to 0.5 mM $t\cdot P_{16}E_{182}$ condition.
**Figure 3.9.** Diblock copolymer treatment of ccALD-iBMECs during development rescues defective barrier function. Maximum TEER of WT1- and ccALD3-iBMECs treated with 1 mM of P188 or $\het$-P16E$_{182}$ on Day 3 of the differentiation protocol. Treatment with 1 mM $\het$-P16E$_{182}$ resulted in improved ccALD-iBMEC barrier function. Data shown from four independent experiments with three biological replicates each (n = 12).

Additionally, lipid droplet accumulation was decreased in ccALD-iBMECs treated with 1 mM $\het$-P16E$_{182}$ during development (Figure 3.10). Quantification of Oil-Red-O staining images indicated a statistically significant (p < 0.05) decrease in ccALD-iBMECs treated with 1 mM $\het$-P16E$_{182}$ when compared to untreated ccALD-iBMECs. The average intensity of red pixels in images of Oil-Red-O stained ccALD-iBMECs treated with 1 mM $\het$-P16E$_{182}$ was 10.9 ± 1 x 10$^6$ compared to 14.8 ± 1 x 10$^6$ for the control. The average number of red pixels was calculated to be 4.9 ± 0.5 x 10$^4$ for ccALD-iBMECs treated with 1 mM $\het$-P16E$_{182}$ and 6.7 ± 0.5 x 10$^4$ for the control.
Figure 3.10. Diblock copolymer treatment decreases lipid droplet accumulation in ccALD-iBMECs. (a) Representative Oil-Red-O staining of untreated control ccALD3-iBMECs and 1 mM \( \text{t-P}_{16}\text{E}_{182} \) treated ccALD3-iBMECs during development. Raw images shown on left and masked images on right. (b) Quantification of intensity and number of red pixels in Oil-Red-O stained images indicates decreased lipid droplet accumulation in ccALD-iBMECs treated with 1 mM \( \text{t-P}_{16}\text{E}_{182} \) during development. Six biological replicates used for quantification (n = 6).

3.4. Discussion

Our model of the BBB demonstrating that the barrier function is defective and lipid droplets accumulate in iBMECs from patients with ccALD opens the door to new therapeutic avenues aimed at maintaining the integrity of the BBB and preventing the onset of ccALD. In the present work, our findings indicate a significant improvement in barrier function and a decrease in lipid droplet accumulation when ccALD-iBMECs are treated during differentiation with a diblock copolymer with a hydrophobic tert-butoxy end group (\( \text{t-P}_{16}\text{E}_{182} \)). No effect was seen when the ccALD-iBMECs were treated at the same time with P188 or when the ccALD-iBMECs were treated post differentiation with either polymer. Treatment of WT-iBMECs with either polymer during development did not improve barrier function. Response of the ccALD-iBMECs but not the WT-iBMECs to
polymer treatment further highlights that there are fundamental differences between the ccALD- and WT-iBMECs.

Overall, our study demonstrates that one of the intrinsic defects in ccALD is with the integrity of the BMECs that constitute the BBB. These findings are in line with the study by Musolino et al. (2015) in which they knocked down ABCD1 in BMECs and saw mislocalization of the tight junction protein claudin-5. The presence of frayed or discontinuous junctions and its relation to barrier function has been noted in other systems as well. In an in vitro epithelium model, an induced opening of the barrier for drug delivery purposes was marked by both morphological changes in the connectivity of zonula occluden tight junction proteins and a decrease in TEER. In our study of the brain endothelium, we went beyond qualitative observations of tight junction proteins and quantified the integrity of the barrier formed by the WT- and ccALD-iBMECs using TEER. By this metric, we found that the barrier integrity of the ccALD-iBMECs was decreased compared to WT-iBMECs. Musolino et al. also observed an increase in TGFβ1 expression connected to the mislocalization of claudin-5. Interestingly, our transcriptome analysis also indicated increased TGFβ1 activity in the ccALD-iBMECs that could be contributing to the decreased barrier function. With ccALD, in contrast to other demyelinating disorders such as multiple sclerosis, demyelination is thought to precede BBB breakdown. Thus, it is possible that an initial subtle loss in the ability of the BBB to restrict passive transport, as seen with the ccALD-iBMECs in our study, could cause immune cell infiltration and leakage that accelerates demyelination in a feedback loop that eventually results in complete BBB breakdown. In this context, increased matrix metalloproteinases in the cerebral spinal fluid of ccALD patients could also be contributing to further breakdown of
Inherent decreased BBB integrity could also begin to explain why head trauma can initiate the onset of ccALD. The lack of genotype-phenotype correlation is not explained by our model; however, our finding of an inherent decrease in BMEC integrity in ccALD individuals could direct the search for additional environmental or genetic factors specific to the BBB that begin to explain why only a subset of individuals with an \textit{ABCD1} mutation progress to ccALD.

While we did not observe by TEM the classic crystalline aggregates first observed in the adrenal cortex, testis, and white matter of ccALD patients, our finding of increased accumulation of non-pathological lipid droplets in ccALD-iBMECs is novel and warrants further investigation.\textsuperscript{300–302} A study by Schluter \textit{et al.} (2012) showed that VLCFAs can trigger insulin desensitization characterized by oxidative stress and alteration of adipocytokine signaling pathways and chronic inflammation, culminating in changes similar to metabolic syndrome.\textsuperscript{303} Increased insulin-like growth factor receptor signaling in the ccALD-iBMECs indicated by our transcriptome analysis further hints at metabolic dysfunction as a factor contributing to the ccALD phenotype. Another study by van de Beek \textit{et al.} (2017) showed that exposure of X-ALD fibroblasts to VLCFAs resulted in endoplasmic reticulum stress correlated with an increase in lipid droplet deposition.\textsuperscript{304} Both studies suggest that VLCFA accumulation would precede lipid droplet accumulation. A key question that then arises is whether lipid droplet accumulation contributes to the decreased BBB integrity in the ccALD-iBMECs and whether targeting non-VLCFA lipids would have therapeutic relevance in that it could potentially rescue the decrease in BBB integrity of ccALD patients.
Using this system to model the BBB, we achieved physiological levels of TEER. One limitation of our study, however, is that we only investigated one cell type, BMECs. Adding other cell types involved in the neurovascular unit such as pericytes, astrocytes, and neurons to our model could further inform ccALD-specific defects of the BBB. Recently, Neal et al. differentiated hiPSCs with known disease mutations using an updated, chemically defined iBMEC differentiation method and found that the iBMECs possessed enhanced barrier function compared to iBMECs differentiated using a non-chemically defined differentiation protocol. Thus, future studies of ccALD-iBMECs may need to employ updated differentiation methods especially if the methods yield cells with more physiologically relevant characteristics. Nevertheless, the differences we found modeling the BBB using iBMECs were significant and should not be discounted. These differences included a decrease in barrier integrity as well as an increase in lipid accumulation. Both of these findings represent potential biomarkers for brain endothelium health of X-ALD patients and provide a new direction in the search for molecular markers that indicate ccALD onset. Combining the findings from the results of this study with antioxidant therapy currently in clinical trials could provide a much-needed alternative treatment for patients with AMN at risk of converting to ccALD.

With our BBB model, we investigated whether amphiphilic block copolymers can improve defects in barrier function as such polymers have been reported to be able to improve function of many cell and tissue types under various injuries. The application of the diblock copolymer in addition to the widely used P188 was inspired by recent work within our group, which revealed \( \xi \text{P}_{16}\text{E}_{182} \) to be the most efficacious in stabilizing damaged myoblasts \textit{in vitro}. It is interesting that observed enhanced efficacy of \( \xi \text{P}_{16}\text{E}_{182} \)
compared to P188 is consistent with the results of Kim et al. although the cell types and form of damage are vastly different.\textsuperscript{74} In this work, block copolymer concentrations of 0.5 or 1 mM were utilized, which is higher than in the aforementioned work by Kim et al. Though 1 mM polymer concentration was found to be more effective than 0.5 mM in improving barrier function of the ccALD-iBMECs, a more thorough concentration titration experiment should be done to assess whether high concentration of polymer is necessary to engender a therapeutic response. Furthermore, the results of our work showing the decrease in lipid accumulation in ccALD-iBMECs upon treatment with $\text{t-P}_{16}\text{E}_{182}$ suggest a more complex cellular response beyond plasma membrane stabilization that has yet to be fully explored. As the breadth of applications continues to expand, there is a pressing need to elucidate the amphiphilic block copolymer-cell interaction mechanism in order to translate this action into a therapeutic solution. To this end, the \textit{in vitro} disease model presented in this work could provide a platform for studying the mechanism of PPO-PEO block copolymer mediated recovery of cellular function. Since ROS and mitochondrial defects have been implicated in conversion to ccALD,\textsuperscript{272} and P188 has been found to protect mitochondrial function in neurons,\textsuperscript{196} a future route of investigation could involve examining how ROS production and mitochondrial defects affect BBB dysfunction in ccALD and whether PPO-PEO block copolymers act on the mitochondria to improve BBB function. Furthermore, while most researchers have focused solely on P188, there is potential for the design of the polymer to further improve efficacy in restoring function to damaged cells as demonstrated in this work. Elucidating the mechanism of BMEC interaction with the PPO-PEO diblock copolymer will not only engender insight as to how the polymer restores function of ccALD-iBMECs but may also provide a deeper
knowledge as to how the BBBs of ccALD patients are damaged compared to healthy individuals.

At present, there is no suitable *in vivo* model for X-ALD. Nevertheless, as functional *in vivo* models are developed, the work presented here has the potential to be translated to *in vivo* studies. Treatment of ccALD-iBMECs with either P188 or \( \mathbf{t}\cdot\mathbf{P}_{16}\mathbf{E}_{182} \) at the end of the differentiation protocol yielded a slight but non-significant increase in TEER. However, efficacy of polymer treatment at a later stage might be improved upon optimization of pharmacodynamics and pharmacokinetic variables. Furthermore, the superior efficacy of treatment with \( \mathbf{t}\cdot\mathbf{P}_{16}\mathbf{E}_{182} \) when added earlier in the iBMEC differentiation process compared to at the end of the differentiation process suggests that the treatment could be applied at an early stage of BBB development to inhibit the onset and progression of ccALD.

Thus, clinical application might mean using the polymer as a preventative therapy, which requires pre-symptomatic diagnosis of X-ALD. Fortunately, high throughput screening of X-ALD is feasible and reliably identifies affected males. Furthermore, in 2016 the US Department of Health and Human Services recommended that X-ALD be added to the recommended uniform screening panel for state newborn screening programs. Testing of the 4 million infants born each year in the US is predicted to identify around 143 newborns with an *ABCD1* mutation. Early detection will lead to more timely intervention in the form of hematopoietic cell transplant (HCT), which is only advantageous in the early stages of the disease because cerebral inflammation can progress up to 18 months after transplant. Pioneering clinical trials involving the use of Lenti-D for autologous HCT are taking place at several centers around the US and promise to
further reduce severe outcomes associated with allogeneic transplants (such as graft-versus-host disease) and to circumvent issues with finding HLA matched donors (currently, cord blood grafts are used when a suitable donor cannot be found). For those displaying neurological symptoms or MRI abnormalities indicating ccALD onset, the current standard of care for HCT involves fully myoablative chemotherapy, a highly toxic procedure. If a treatment that prevents the onset of ccALD were available, a newborn identified as having an ABCD1 mutation given this treatment may never show symptoms of ccALD conversion and would not need to undergo HCT. In this study, we have shown that amphiphilic block copolymers are one such treatment with the potential to prevent the onset of ccALD and reduce the number of patients needing to undergo HCT.

3.5. Conclusion

Modeling the BBB of ccALD patients using iPSC-derived BMECs indicates that ccALD patients form a less intact BBB. These results open the door for the discovery of brain endothelium-specific molecular markers indicative of the onset of ccALD and for the development of treatment strategies targeted at the brain endothelium that could reduce the number of X-ALD patients who progress to ccALD. One such treatment strategy that we have shown can rescue defective ccALD-iBMECs barrier integrity is PPO-PEO block copolymers. These results have therapeutic implications for preventing the onset of ccALD.
Chapter 4. Modeling Ischemic Stroke Related Damage to BBB and Evaluation of PPO-PEO Block Copolymer Treatment Efficacy

4.1. Introduction

Every year in the United States, approximately 800,000 people experience a new or recurrent stroke, a disease with high mortality and a leading cause of long term disability. More than 85% of stroke cases are ischemic stroke episodes wherein occlusion of a blood vessel interrupts blood flow, reducing the delivery of oxygen and nutrients to a local brain region. Ischemia (occlusion of blood flow) and reperfusion (reestablishment of blood flow) trigger a complex cascade of events including neuronal death, remodeling of the neurovascular unit (NVU), damage to the blood-brain barrier (BBB), and inflammatory response. Prompt reestablishment of blood flow is necessary to minimize cerebral damage, but treatment options for ischemic stroke are limited with options including endovascular procedures to recanalize large occluded vessels or intravenous administration of recombinant tissue plasminogen activator (tPA). tPA is the only FDA approved drug for acute ischemic stroke and is used to accelerate the recanalization of an occluded vessel. However, because of the risks associated with tPA treatment, including increased risk for hemorrhagic transformation (HT), the drug must be administered within 3 hours from the onset of stroke and up to 4.5 hours for eligible patients. The risk for HT upon tPA treatment increases in part because it is associated with damage to the BBB. Given the time restrictions and risks involved, fewer than 5% of stroke patients receive tPA treatment. While clinical outcomes of stroke patients have seen improvement over recent years due to a more standardized system of care,
short- and long-term treatment options are lacking despite widespread efforts to develop neuroprotective therapeutics.

There has been increased interest in development of therapies for stroke that target the BBB as consensus grows that time and extent of BBB damage are important variables for clinical outcome.\textsuperscript{42-44,319} BBB breakdown during ischemia/reperfusion (I/R) is mediated by activated matrix metalloproteinases (MMPs),\textsuperscript{323,324} degradation or alteration of the basement membrane,\textsuperscript{325} increased rate of transcytosis,\textsuperscript{326} disrupted tight junction proteins,\textsuperscript{327,328} and immune cell infiltration.\textsuperscript{329} An important contributor to BBB damage during I/R is oxidative stress, which refers to a cellular state of imbalance between pro-oxidants and antioxidants wherein the level of pro-oxidants exceeds the amount required under physiological conditions.\textsuperscript{330,331} Under physiological conditions, reactive oxygen species (ROS) are important cell response molecules involved in cell growth, cell signaling, and immune responses and are endogenously present in cells as products of metabolic processes.\textsuperscript{332} Cellular ROS include superoxide (O$_2^-$), hydroxyl radical ('OH), peroxynitrite (ONOO$^-$), and hydrogen peroxide (H$_2$O$_2$).\textsuperscript{332} In the endothelium, sources of ROS molecules include the mitochondria, NADPH oxidases, endothelial nitric oxide synthase NOS, and xanthine oxidases (XO).\textsuperscript{333} Concentrations of ROS are normally carefully controlled by endogenous defense mechanisms, but when cells are under oxidative stress, there is significant damage to proteins, lipids, and DNA.\textsuperscript{334-336}

ROS concentration in the brain increases during both ischemia and reperfusion stages of stroke.\textsuperscript{337-341} ROS overproduction is driven by several mechanisms including mitochondrial disruption and increased activity of NADPH oxidases and XO,\textsuperscript{342,343} especially after the onset of reperfusion when oxygen is reintroduced to the affected brain
The BBB is particularly susceptible to oxidative stress due to endothelial cells being enriched with NADPH oxidases, specifically Nox4, and brain endothelial cells having an abundance of mitochondria compared to endothelial cells of other tissues. Immune cells that infiltrate the BBB during I/R can also release ROS, aggravating damage to the BBB. Though the NVU is equipped with antioxidants including glutathione, glutathione peroxidase, glutathione reductase, superoxide dismutase, and catalase, these antioxidants are depleted during I/R without sufficient replacement throughout injury progression. The combination of increasing ROS levels and decreasing ability of endogenous antioxidants to provide defense during I/R contributes to breakdown of the BBB during ischemic stroke. Oxidative stress is also thought to be implicated in neurodegenerative diseases such as Alzheimer’s Disease, and damage to the BBB from oxidative stress may be a factor in exacerbation of neurological diseases. Thus, investigating and developing therapies that target the BBB under oxidative stress may have larger implications besides therapy for acute injuries, especially as stroke patients are at a higher risk for neurodegeneration.

While there have been many attempts to develop antioxidant based stroke therapies, such treatments have not seen much success in clinical trials. As described in Chapter 1, studies have demonstrated that poloxamer 188 (P188) treatment led to improved BBB function during acute cerebral injuries including ischemic stroke, hemorrhagic stroke, and traumatic brain injury, but mechanistic understanding is incomplete. It is unclear whether recovery of BBB function in the aforementioned studies was a primary or secondary result of P188 treatment as it is difficult to uncouple effects of specific interactions in a complex animal model. Additionally, exploration of other block
copolymers of poly(propylene oxide) (PPO) and poly(ethylene oxide) (PEO) has been limited with animal studies since they are low throughput experiments. Thus, we sought to investigate the direct impact of PPO-PEO block copolymers on the BBB during ischemic stroke related damage conditions and to enable screening studies by utilizing human induced pluripotent stem cell (hiPSC)-derived brain microvascular endothelial cells (iBMECs) as an in vitro model of the BBB. An in vitro model allows for specific perturbations to the BMECs such that we can study cellular responses to that perturbation without influence from other systems (e.g., other cells of the NVU).

We hypothesized that treatment with PPO-PEO block copolymers can mitigate effects of oxidative stress, an important instigator of damage during ischemic stroke, on the BBB and utilized H$_2$O$_2$ to induce oxidative stress on iBMECs. Cellular excess of H$_2$O$_2$ has been detected in the affected brain region during reperfusion.$^{358}$ Cellular oxidases generate H$_2$O$_2$ which in turn can produce hydroxyl radicals when reacted with iron and superoxide through the Fenton reaction and the Haber-Weiss cycle.$^{359-362}$ Moreover, myeloperoxidase can facilitate the reaction of chlorine ions with H$_2$O$_2$ to produce hypochlorous acid,$^{363}$ which can oxidize thiol groups in proteins.$^{364}$ Exposure of brain endothelial cells to H$_2$O$_2$ has been previously used as a model for oxidative stress and stroke-related injuries,$^{365,366}$ and exposure to H$_2$O$_2$ has been demonstrated to increase endothelial cell permeability.$^{366-368}$

We characterized the function and phenotype of iBMECs exposed to H$_2$O$_2$ to assess whether the impaired function and phenotype can be attenuated by treatment with PPO-PEO block copolymers. Oxidative stress can interfere with barrier integrity due to reorganization or disruption of cytoskeletal and junctional proteins.$^{352,364,369-371}$ Reduction
in the trans-endothelial electrical resistance (TEER) and reorganization of F-actin, ZO-1, and occludin proteins in endothelial cells challenged by H$_2$O$_2$ have been previously reported.$^{366,368}$ In mature, resting endothelial cells, actin filament (F-actin) bundles are present as cortical actin filaments near cell borders.$^{218,372,373}$ However, when subjected to physiological or pathological stress, formation of actin stress fibers increases.$^{374,375}$ ZO-1 proteins are scaffolding proteins that connect claudin and occludin tight junction proteins to the F-actin cytoskeleton network.$^{376-379}$ The tight junction protein occludin is susceptible to ROS due to the redox-sensitive cysteine residues on the protein.$^{355}$ Increase in permeability through the endothelial cells and cytoskeletal and junctional protein reorganization are in part due to an increase in intracellular levels of calcium under inflammatory conditions.$^{380-382}$ Calcium is a common second messenger involved in various processes including cell proliferation and cell death.$^{383,384}$ At rest, the intracellular calcium concentration ([Ca$^{2+}$]$_i$) in endothelial cells are on the order of 10$^1$ to 10$^2$ nM.$^{382}$ Increase in [Ca$^{2+}$]$_i$ can result from entry of extracellular Ca$^{2+}$ into the cells or release from intracellular Ca$^{2+}$ stores such as the endoplasmic reticulum.$^{385-388}$

Accordingly, iBMECs under H$_2$O$_2$ induced oxidative stress with or without polymer treatment were characterized by measuring the TEER to quantify barrier integrity and by examining organization of F-actin, ZO-1, and occludin using fluorescence microscopy. Intracellular levels of calcium and ROS were measured utilizing fluorescent probes. Moreover, certain poloxamers have been demonstrated to inhibit P-glycoprotein (P-gp) function in bovine BMECs by changing the membrane fluidity and causing energy depletion.$^{389}$ P-gp is an efflux transporter that is vital for proper barrier function in the BBB. Thus, we employed a Rhodamine 123 accumulation assay to quantify P-gp function and
confirm that the PPO-PEO block copolymers considered for BBB protection do not interfere with efflux transporter function. Characterization of H$_2$O$_2$-challenged iBMECs with or without PPO-PEO block copolymer treatment allowed us to examine the direct impact of the polymer interaction on this specific mode of damage to the BMECs and to evaluate treatment efficacy of various PPO-PEO block copolymers.

4.2. Methods

4.2.1. Cell culture and differentiation of hiPSCs to iBMECs

IMR-90-4 hiPSCs (WiCell) were cultured on Matrigel (Corning), maintained in TeSR-E8 (STEMCELL Technologies), and subcultured with ReLeSR (STEMCELL Technologies). Cells were tested monthly for Mycoplasma contamination using the MycoAlert™ Mycoplasma Detection Kit (Lonza) and tested negative each time. Cells were also characterized by genetic fingerprinting at the University of Minnesota Genomics Center and found to be karyotypically normal. Differentiation of hiPSCs to iBMECs was done following a previously published report$^{225}$ and cultured on collagen IV and fibronectin coated well plates, Transwells, or µ-slides depending on the assay (see §2.1.1 for details). Experiments were done 2 days after subculture (Day 10) when the cells reached confluence. For experiments with data shown in Figures 4 - 9, differentiation was done following a modified differentiation protocol$^{218}$ and cultured on laminin 511-E8 coated well plates, Transwells, µ-slides, or glass-bottomed chambers (Nunc) as outlined in §2.1.2. All experiments were done 2 days after subculture (Day 10) when the cells reached confluence. TEER was measured using the EVOM2 voltohmometer with STX2 chopstick electrodes (World Precision Instruments).
4.2.2. Hydrogen peroxide exposure to iBMECs

\( \text{H}_2\text{O}_2 \) (30%; Thermo Fisher Scientific) was diluted in cell culture medium to the desired final concentration by two serial dilutions. Medium from the cell culture was aspirated and replaced with the medium with or without \( \text{H}_2\text{O}_2 \) and incubated in a 37°C, 5% \( \text{CO}_2 \) incubator for 2 h. For experiments with data shown in Figures 1 - 3, \( \text{H}_2\text{O}_2 \) was diluted in human serum free endothelial cell medium (heSFM; Thermo Fisher Scientific) with 1% platelet poor plasma-derived serum (PDS; Biomedical Technologies). For experiments with data shown in Figures 4 - 8, \( \text{H}_2\text{O}_2 \) was diluted in heSFM.

4.2.3. Viability

iBMECs seeded on 12-well plates were utilized for viability studies. After 2 h of \( \text{H}_2\text{O}_2 \) exposure, cells were detached by incubation with 0.5 mL Accutase (STEMCELL Technologies) in a 37°C, 5% \( \text{CO}_2 \) incubator. The cell suspensions were diluted with 0.5 mL cell culture medium (heSFM with 1% PDS) and collected in microcentrifuge tubes, and cell density was determined with the Countess II FL Automated Cell Counter.

4.2.4. Live/Dead staining

iBMECs seeded on 12-well plates were utilized for this study with the Live/Dead Viability/Cytotoxicity Kit (Thermo Fisher Scientific). After 2 h of \( \text{H}_2\text{O}_2 \) exposure, cells were incubated with 2 \( \mu \text{M} \) calcein-AM (live stain) and 4 \( \mu \text{M} \) ethidium homodimer-1 (dead stain) for 30 min at room temperature. Imaging was done with the EVOS FL Auto Imaging
System with the 10X objective. More details concerning the microscope can be found in §2.2.

4.2.5, Polymer treatment

Polymer synthesis and characterization are detailed in Chapter 2. Polymers were dissolved in cell culture medium at a concentration of 12 mM. At either $t = 0$ or $t = 1$ h, a bolus injection of the polymer solution was added to the cell culture such that the final concentration of the polymer was 1 mM. For cells cultured on Transwells, the polymer solution was added to the apical side of the chamber only. For experiments with data shown in Figures 1 - 3, polymers were dissolved in heSFM with 1\% PDS. For experiments with data shown in Figures 4 - 8, polymers were dissolved in heSFM.

4.2.6, Immunocytochemistry and F-actin staining

iBMECs were subcultured onto μ-slides (Ibidi) or Nunc™ Lab-Tek™ II chambered coverglass (Thermo Fisher Scientific). At the end of the H$_2$O$_2$ and polymer treatment experiment ($t = 2$ h), the medium was removed and cells were washed once with DPBS without magnesium or calcium (Thermo Fisher Scientific) and fixed. Experimental details for immunostaining and imaging are discussed in §2.2. For F-actin staining, cells were fixed in 4\% paraformaldehyde for 15 min at room temperature. Fixed cells were permeabilized for 5 min by incubation with 0.1\% Triton X-100 in DPBS on a rocker at room temperature. After 2 washes with DPBS, cells were incubated in DPBS with 0.165 μM Alexa Fluor 488-conjugated phalloidin (Thermo Fisher Scientific) and 1\% bovine
serum albumin for 20 min at room temperature. Cells were washed 3 times with DPBS, stained with DAPI for 5 min, and then washed a final time before imaging.

4.2.7, Fluo-4 intracellular calcium measurement

Cells were seeded on black walled, clear bottomed 96-well plates (Greiner Bio-One) to minimize background autofluorescence. Before starting the H$_2$O$_2$ and polymer treatment, cells were incubated with 2 µM Fluo-4 (Thermo Fisher Scientific) in heSFM in a 37°C, 5% CO$_2$ incubator for 45 min. The H$_2$O$_2$ and polymer treatment experiment was carried out, and at the end of the experiment (t = 2 h), the medium was removed and the cells washed once with HBSS (without calcium nor magnesium; Thermo Fisher Scientific) and replaced with fresh HBSS for the fluorescence reading. Fluorescence was measured on a BioTek Synergy H1 multi-mode microplate reader at excitation of 485 nm and emission of 520 nm. Cells in 1 well per condition were detached from the well plate with Accutase and counted using the Countess II FL Automated Cell Counter to normalize the fluorescence on a per cell basis.

4.2.8, DCFDA intracellular ROS measurement

Cells were seeded on black walled, clear bottomed 96-well plates (Greiner Bio-One) to minimize background autofluorescence. Before starting the H$_2$O$_2$ and polymer treatment, cells were incubated with 25 µM 2’,7’- dichlorofluorescein diacetate (DCFDA; Abcam) in heSFM in a 37°C, 5% CO$_2$ incubator for 45 min. The H$_2$O$_2$ and polymer treatment experiment was carried out, and at the end of the experiment (t = 2 h), the medium was removed and the cells washed once with HBSS (with calcium and magnesium; Thermo
Fisher Scientific) and replaced with fresh HBSS for the fluorescence reading. Fluorescence was measured on a BioTek Synergy H1 multi-mode microplate reader at excitation of 485 nm and emission of 535 nm.

4.2.9. Rhodamine 123 accumulation assay

Cells seeded on 24-well plates were used for this experiment. In each independent experiment, 4 wells were used per condition. HBSS with calcium and magnesium was used as the buffer solution throughout this assay. Cells were pre-incubated in buffer only, 10 μM cyclosporin A (Millipore Sigma) in buffer, or polymer in buffer for 1 h. The supernatant was removed and replaced with the same solutions with the addition of 10 μM rhodamine 123 (Millipore Sigma) for 2 h. Both incubation steps were done on an orbital shaker set at 50 RPM in a 37°C, 5% CO₂ incubator. Next, cells in 3 wells per condition were lysed by incubation with RIPA buffer (Millipore Sigma) for 15 min at room temperature on a rocker, and fluorescence was measured on a BioTek Synergy H1 multi-mode microplate reader at excitation of 485 nm and emission of 530 nm. Cells in 1 well/condition were not lysed and instead dissociated with Accutase and counted using the Countess II FL Automated Cell Counter to normalize the fluorescence on a per cell basis.

4.2.10. Statistical analysis

Data are presented as mean ± standard deviation. The numbers of independent experiments (N) and biological replicates (n) for each experiment are listed in the figure captions. Independent experiments are defined as experiments done with a different batch of differentiated iBMECs, and biological replicates are defined as different wells of cells.
within an independent experiment. Statistical significance was determined using unpaired Student’s t-test or one-way analysis of variance with Tukey post hoc test, and analyses were done using GraphPad Prism.

4.3. Results and Discussion

4.3.1. Optimization of hydrogen peroxide concentration to induce damage to iBMECs

We first evaluated the effect of different concentrations of H$_2$O$_2$ on the barrier integrity (quantified by the TEER) and cell viability of iBMECs. Concentrations above 100 µM were tested based on previous in vitro studies of endothelial cells that demonstrated that permeability increased upon exposure to H$_2$O$_2$ concentrations greater than 100 µM.\textsuperscript{366,367} Reported concentrations of H$_2$O$_2$ in the brain after ischemia and reperfusion span several orders of magnitude,\textsuperscript{390–392} but pathophysiologically relevant concentrations of H$_2$O$_2$ are generally considered to be on the order of 10$^2$ µM.\textsuperscript{350} iBMECs seeded on Transwells were exposed to H$_2$O$_2$ for 2 hours, and iBMECs exposed to 375 µM – 1000 µM H$_2$O$_2$ showed at least a 30% decrease in the TEER compared to iBMECs not exposed to H$_2$O$_2$ (Figure 4.1A). Next, we assessed the viability of the iBMECs after H$_2$O$_2$ exposure. As shown in Figure 4.1B, the viability of iBMECs exposed to 500 or 1000 µM H$_2$O$_2$ was approximately 50% than that of the control, signifying that H$_2$O$_2$ is toxic to the cells above 500 µM H$_2$O$_2$. Thus, H$_2$O$_2$ concentration of 375 µM was chosen for subsequent studies, as this concentration of H$_2$O$_2$ induced damage, but not excessive cell death, to the iBMECs. Live/dead staining was done on iBMECs with or without exposure to 375 µM H$_2$O$_2$ for 2 hours, which confirmed the presence of an intact monolayer for both conditions (Figure 4.1C).
Figure 4.1. (A) Transendothelial electrical resistance (TEER) and (B) viability of iBMECs treated with different concentrations of H$_2$O$_2$ for 2 h. Data are normalized to the control, which were cells not exposed to H$_2$O$_2$. H$_2$O$_2$ disrupted the iBMEC barrier at concentrations above 375 µM and was cytotoxic at concentrations above 500 µM. Data are from 1 independent experiment with 3 biological replicates (N = 1, n = 3). (C) Live (green)/dead (red) staining of iBMECs with or without exposure to 375 µM H$_2$O$_2$ for 2 h. iBMEC monolayer was intact after H$_2$O$_2$ exposure. Images are representative images from 3 independent experiments (N = 3).

4.3.2. Efficacy of polymer treatment in restoring barrier integrity to H$_2$O$_2$-challenged iBMECs

We next looked at whether treatment with PPO-PEO block copolymers of varying parameters could rescue the damaged cells. iBMECs on Transwells were first exposed to 375 µM H$_2$O$_2$ for 1 hour before a bolus addition of a polymer solution to the apical (“blood”) side of the Transwell such that the final polymer concentration is 1 mM. This concentration was chosen based on previous work described in Chapter 3 and other work investigating P188 treatment of endothelial cells. The cells were incubated for another hour before the medium was replaced with fresh medium (see Figure 4.2A for schematic of experimental procedure). The TEER was measured an hour later (t = 3 h) and normalized to the initial
value (Figure 4.2B-E). When the cells exposed to H₂O₂ were treated with the PEO homopolymer, there was no improvement in the electrical resistance (Figure 4.2B). When iBMECs stressed with H₂O₂ were treated with poloxamers with 70 wt% PEO, ability to restore barrier integrity decreased with increasing molecular weight (Figure 4.2C). The same trend was observed for poloxamers with 80 wt% PEO (Figure 4.2D). Finally, two diblock copolymers with approximately the same PPO block length were tested and the polymer with a longer PEO block (90 wt% PEO) was more efficacious in rescuing the barrier function of iBMECs under H₂O₂ stress (Figure 4.2E).

To more confidently assess the structure-function relationship of PPO-PEO block copolymers and iBMECs, additional studies must be done with different polymer concentrations. In this screening, the polymer concentration was 1 mM, at which some of the polymer solutions contain micelles (see Chapter 2). An investigation looking at the efficacy of the block copolymers at lower concentrations where micelles are not present is warranted. Regardless, we continued studies with the best performing polymers (P188 and t-P₁₅E₁₉₀) from this initial screening to assess how the treatment impacts other phenotypes of the iBMECs under H₂O₂ induced stress.
4.3.3. F-actin and ZO-1 organization of H$_2$O$_2$-challenged iBMECs treated with block copolymer

To assess the effects of H$_2$O$_2$ stress and polymer treatment on F-actin and ZO-1 organization in the iBMECs, F-actin staining and immunocytochemistry were done (Figure 4.2).
Alexa Fluor 488-conjugated phalloidin was used to stain actin filaments, which revealed significant decomposition of actin filaments in iBMECs exposed to 375 µM H2O2 compared to the control. No differences were observed between the H2O2-challenged iBMECs with or without P188 or t-P15E190 treatment. ZO-1 staining of iBMECs showed internalization of the proteins in H2O2-challenged iBMECs, but differences between the H2O2-challenged iBMECs with or without P188 or t-P15E190 treatment were not observed. However, the F-actin staining of the control iBMECs (i.e., cells not exposed to H2O2) unexpectedly showed an abundance of stress fibers. Around the time of these experiments, our colleagues demonstrated that the fibronectin and collagen IV extracellular matrix coating in the culture of the iBMECs caused the cells to be in an activated state, which occurs during pathological conditions and causes various responses including stress fiber formation. Thus, the culture method was altered to better mimic the BBB in a healthy state before induction of damage.

**Figure 4.3.** F-actin and ZO-1 staining of iBMECs under H2O2 stress treated with 1 mM P188 or t-P15E190. Examples of internalized ZO-1 proteins indicated with white arrows. Scale bars represent 200 µm for F-actin staining images and 100 µm for ZO-1 staining images. Representative images from 1 independent experiment are shown; 2 additional independent experiments were done to confirm trends.
4.3.4. H₂O₂ damage and polymer treatment effect on barrier function of iBMECs cultured on laminin 511-E8

Laminin 511-E8 (LN 511) was chosen as the extracellular matrix substrate for the culture of iBMECs as it was found that LN 511 can promote a resting phenotype and induce more uniform junctional protein expression. Data presented in the rest of this chapter are from studies conducted with iBMECs cultured on LN 511. With iBMECs cultured on LN 511, the polymer treatment efficacy study after H₂O₂ exposure was repeated with P188 and \( t\) \( \bullet \) \( P_{15}E_{190} \). Initial experiments showed no difference in the TEER of iBMECs under H₂O₂ exposure with or without polymer treatment, so the treatment timeline was altered such that the polymer treatment was given at the beginning of the experiment. Treatment of H₂O₂-challenged iBMECs with 1 mM \( t\) \( \bullet \) \( P_{15}E_{190} \) at \( t = 0 \) resulted in a statistically significant (\( p < 0.005 \)) increase in the TEER compared to H₂O₂-challenged iBMECs with no polymer treatment (Figure 4.4A). Treatment with P188 at both \( t = 0 \) and \( t = 1 \) h or \( t\) \( \bullet \) \( P_{15}E_{190} \) at \( t = 1 \) h did not improve the barrier function of iBMECs under H₂O₂-induced stress.
Figure 4.4. \( \text{H}_2\text{O}_2 \) stress and polymer treatment of iBMECs cultured on LN 511. Data plotted are the TEER measured at \( t = 3 \) h and normalized to the initial value. (A) TEER of iBMECs exposed to \( \text{H}_2\text{O}_2 \) and treated with 1 mM P188 or \( \text{f-P15E190} \) at \( t = 0 \) or \( t = 1 \) h. Data are from 3 independent experiments with 2 technical replicates each (\( N = 3, n = 6 \)). (B) TEER of iBMECs exposed to \( \text{H}_2\text{O}_2 \) and treated with 0.5 mM P188 or \( \text{f-P15E190} \) at \( t = 0 \) (\( N = 3, n = 6 \)). (C) Comparison of TEER of iBMECs under \( \text{H}_2\text{O}_2 \) induced stress treated at \( t = 0 \) with 0.5 mM or 1 mM polymer (\( N = 1, n = 2 \)). Of the conditions tested, treatment with 1 mM \( \text{f-P15E190} \) at \( t = 0 \) to \( \text{H}_2\text{O}_2 \)-challenged iBMECs was the most effective in improving barrier integrity.
The effect of concentration was studied by treating iBMECs exposed to 375 µM H₂O₂ with 0.5 mM P188 or t-P₁₅E₁₉₀ at t = 0 (Figure 4.4B). Treatment with 0.5 mM t-P₁₅E₁₉₀ resulted in a statistically significant (p < 0.001) increase in the TEER compared to H₂O₂-challenged iBMECs. However, the fold change between those two conditions (H₂O₂ only, H₂O₂ and 0.5 mM t-P₁₅E₁₉₀) was less than the fold change between the untreated and 1 mM t-P₁₅E₁₉₀ treated conditions (H₂O₂ only, H₂O₂ and 1 mM t-P₁₅E₁₉₀) shown in Figure 4.4A. This trend is made clearer when comparing the TEER of H₂O₂-challenged iBMECs with 0.5 mM or 1 mM polymer treatment within the same independent experiment. We observed that the 1 mM t-P₁₅E₁₉₀ treatment was more efficacious than 0.5 mM t-P₁₅E₁₉₀ in improving the barrier integrity (Figure 4.4C). Interestingly, micelles are present in a solution of 1 mM t-P₁₅E₁₉₀ (§2.5); nevertheless, this treatment was the most effective of the conditions tested in rescuing the barrier function of the iBMECs under H₂O₂ stress. It is important to note that the characterization of polymer solutions via dynamic light scattering was done in a buffer solution, not cell culture medium, which contains a variety of additives including amino acids and vitamins. Moreover, the effective concentration of the polymer in the cell culture medium may decrease with time as the polymers interact with the cells. Studies investigating the use of P188 as a cell culture supplement in bioreactors have suggested the use of higher concentrations of polymer solutions in bioreactors to take into account the decreasing concentration with time.¹²¹,³⁹³ As mentioned previously, further analyses to explore the concentration effect and the effect of micelles on the cell-polymer interaction are required.

Of the polymers tested, t-P₁₅E₁₉₀ was found to be the most effective in restoring barrier integrity of the damaged iBMECs, and akin to the work discussed in Chapter 3, we
have demonstrated here that a PPO-PEO diblock copolymer with 90 wt% PEO and a tert-butoxy end group on the PPO is more efficacious than P188 in treatment of damaged iBMECs. However, in the oxidative stress model discussed here, the TEER of \( t \)-\( P_{15}E_{190} \) treated iBMECs under \( H_2O_2 \) stress did not recover to the level of the unstressed control, suggesting that there is still room for improvement. Optimization of dosage may result in complete recovery of barrier function, but there may also be opportunity to explore other PPO-PEO block copolymers. Based on previous work, alteration of polymeric parameters such as end-group of the PPO block and architecture of the block copolymers should be considered. Screening studies may lead to the best performing polymer (i.e., one that can improve barrier function at the lowest dosage possible). Furthermore, the \textit{in vitro} model used here only considers the BMECs in isolation from the rest of the NVU in order to understand the direct impact of polymer treatment on BMECs. Ability of different PPO-PEO block copolymers to restore barrier integrity of the BBB may be different if other cells of the NVU are incorporated into the \textit{in vitro} model. Future work that could be done to incorporate more aspects of the \textit{in vivo} NVU microenvironment is discussed in more detail in Chapter 7.

4.3.5. \( H_2O_2 \) damage and polymer treatment effect on F-actin, ZO-1, and occludin organization of iBMECs cultured on laminin 511-E8

With the knowledge that iBMECs under \( H_2O_2 \) stress and treated with 1 mM \( t \)-\( P_{15}E_{190} \) at \( t = 0 \) have improved barrier function compared to damaged iBMECs without polymer treatment, actin filament and junctional protein organization were analyzed via F-actin staining and immunocytochemistry. Although P188 treatment at \( t = 0 \) did not result
in improved barrier function of iBMECs under H2O2 stress, this condition was included for subsequent studies to demonstrate the lack of changes due to polymer treatment compared to H2O2-challenged iBMECs with \( \text{t-P15E}_{190} \) treatment. The control sample (i.e., iBMECs with neither H2O2 nor polymer treatment) displayed a network of cortical actin filaments, while H2O2-challenged iBMECs showed abundant stress fibers (Figure 4.5). F-actin staining images of iBMECs exposed to H2O2 and treated with either 1 mM P188 or \( \text{t-P15E}_{190} \) at \( t = 0 \) show different responses. With P188 treatment, stress fibers were still present (though less prevalent than in the H2O2 only sample), but \( \text{t-P15E}_{190} \) treatment resulted in iBMECs with no stress fibers.

Changes in ZO-1 expression between the different experimental conditions were more subtle. Compared to the control, iBMECs exposed to H2O2 showed instances of internalized ZO-1 proteins or discontinuous junctions (Figure 4.6). iBMECs under H2O2 stress treated with 1 mM \( \text{t-P15E}_{190} \) had less cases of internalized or discontinuous ZO-1 proteins; however, it was not consistent throughout the sample. Occludin staining of iBMECs under H2O2 stress exhibited disruptions as evidenced by the presence of frayed and discontinuous junctions and internalized occludin proteins (Figure 4.6). The instances of frayed and discontinuous junctions and internalized occludin protein were fewer in iBMECs under H2O2 stress and treated with 1 mM \( \text{t-P15E}_{190} \). As suppression of actin stress fibers is a marker of BBB protection and junctional protein organization is vital for proper barrier function,\(^{394,395}\) these results support that \( \text{t-P15E}_{190} \) treatment, but not P188, can protect iBMECs under oxidative stress for improved cellular function.
**Figure 4.5.** F-actin staining of iBMECs with or without exposure to H$_2$O$_2$ and 1 mM polymer for 2 h. Actin stress fibers were present in iBMECs under H$_2$O$_2$ induced stress, but not in H$_2$O$_2$ stressed iBMECs treated with $\tau$-P$_{15}$E$_{190}$. Representative images from 1 independent experiment are shown here; trends were confirmed with 2 additional independent experiments.
Figure 4.6. ZO-1 and occludin staining of iBMECs with or without exposure to H$_2$O$_2$ and polymer for 2 h. iBMECs exposed to H$_2$O$_2$ exhibited discontinuous junctions and/or internalized proteins (examples indicated by white arrows). iBMECs treated with t-P$_{15}$E$_{190}$ showed less mislocalized proteins. Representative images from 1 independent experiment are shown here; trends confirmed with 2 additional independent experiments.
4.3.6. Quantification of intracellular calcium and ROS

Next, we compared \([\text{Ca}^{2+}]_i\) of iBMECs challenged with 375 µM H\(_2\)O\(_2\) with or without polymer treatment utilizing a Fluo-4 fluorescence assay (Figure 4.7). iBMECs challenged with 375 µM H\(_2\)O\(_2\) had higher Fluo-4 fluorescence intensity compared to the control, indicating higher \([\text{Ca}^{2+}]_i\). iBMECs stressed with 375 µM H\(_2\)O\(_2\) and treated with 1 mM \(\xi\)-P\(_{15}\)E\(_{190}\), but not P188, exhibited decreased \([\text{Ca}^{2+}]_i\) that is statistically significant (p < 0.05) compared to iBMECs challenged with 375 µM H\(_2\)O\(_2\). Thus, improved barrier function and lack of actin stress fibers in iBMECs exposed to H\(_2\)O\(_2\) and treated with 1 mM \(\xi\)-P\(_{15}\)E\(_{190}\) may be due to inhibition of increase in \([\text{Ca}^{2+}]_i\). PPO-PEO block copolymer mediated suppression of increased \([\text{Ca}^{2+}]_i\) has been observed in other studies including those of cardiomyocytes under oxygen and glucose deprivation and dystrophin-deficient cardiac myocytes under passive stretching.\(^{75,132}\) The decrease in \([\text{Ca}^{2+}]_i\) in H\(_2\)O\(_2\) challenged-iBMECs treated with \(\xi\)-P\(_{15}\)E\(_{190}\) may be in part due to sealing of leaky plasma membrane to prevent entrance of extracellular Ca\(^{2+}\), which has been hypothesized to be the case for muscle tissues under stress.\(^{129,130}\)
Figure 4.7. Measurement of Fluo-4 fluorescence intensity to quantify intracellular calcium concentration. The \([\text{Ca}^{2+}]_i\) of iBMECs exposed to 375 \(\mu\text{M} \text{H}_2\text{O}_2\) was higher than the unstressed control. When \(\text{H}_2\text{O}_2\)-challenged iBMECs were treated with \(\ell\text{-P}_{15}\text{E}_{190}\), \([\text{Ca}^{2+}]_i\) was decreased compared to untreated iBMECs. Plot shows compilation of 5 independent experiments with 2 or 3 technical replicates each (\(N = 5, n = 14\)). \(\text{ns} = \text{no significance.}\)

Since the \([\text{Ca}^{2+}]_i\) of iBMECs stressed with 375 \(\mu\text{M} \text{H}_2\text{O}_2\) and treated with 1 mM \(\ell\text{-P}_{15}\text{E}_{190}\) was lower than that of stressed iBMECs but without polymer treatment, we hypothesized that the polymer-treated iBMECs may have lower intracellular ROS concentration (\([\text{ROS}]_i\)). DCFDA fluorescence marker was utilized to compare the \([\text{ROS}]_i\) of iBMECs (Figure 4.8). As expected, iBMECs challenged with 375 \(\mu\text{M} \text{H}_2\text{O}_2\) exhibited greater DCFDA fluorescence intensity than the undamaged control, indicating higher \([\text{ROS}]_i\). There was no difference in the DCFDA fluorescence intensities of \(\text{H}_2\text{O}_2\) stressed iBMECs with or without 1 mM \(\ell\text{-P}_{15}\text{E}_{190}\) treatment, suggesting that suppression of intracellular ROS is not a mechanism by which polymer treatment rescues function and phenotype of iBMECs under oxidative stress. This finding is in agreement with studies of
cardiomyocytes under oxygen and glucose deprivation that found P188 treatment is able to increase viability but unable to decrease levels of intracellular ROS. However, in an in vitro model of traumatic brain injury, increase in superoxide levels in brain endothelial cells after mechanical trauma was lessened with P188 treatment. Although the preceding cases are different disease models with different cell types, they prompt additional investigations with the system at hand. Proposed experiments include measurement of the intracellular levels of specific ROS molecules and examination of the effect of polymer treatment on the function of ROS generators (e.g., mitochondria).

![Image](image_url)

**Figure 4.8.** Measurement of DCFDA fluorescence intensity to quantify intracellular ROS. The [ROS]$_i$ of iBMECs exposed to 375 µM H$_2$O$_2$ was higher than the unstressed control. Polymer treatment of H$_2$O$_2$-challenged iBMECs did not result in a decrease in [ROS]$_i$. Plot shows compilation of 3 independent experiments with 3 or 4 technical replicates each (N = 3, n = 10).
Barrier integrity as quantified by the TEER of H$_2$O$_2$-challenged iBMECs and treated with 1 mM $\ell$-P$_{15}$E$_{190}$ was not fully recovered to that of the unstressed control. However, the [Ca$^{2+}$]$_i$ of H$_2$O$_2$-challenged iBMECs treated with 1 mM $\ell$-P$_{15}$E$_{190}$ was similar to the control. Due to the large variance of the data shown in Figure 4.7, other methods to measure [Ca$^{2+}$]$_i$ may be needed to ascertain whether there is a difference in the [Ca$^{2+}$]$_i$ of unstressed iBMECs and H$_2$O$_2$-challenged iBMECs treated with 1 mM $\ell$-P$_{15}$E$_{190}$. However, another factor may be that there are other mechanisms that are involved in disruption of iBMEC function that the polymer treatment does not absolve.

Other possible mechanisms of damage that warrant investigation include lipid peroxidation and MMP activation. P188 treatment to cardiomyocytes after oxygen and glucose deprivation was found to be unable to protect the cells against lipid peroxidation. In rat hippocampal neurons, however, ROS induced lipid peroxidation was inhibited by P188, leading the authors to suggest that P188 may act as ROS scavengers. Yet, Wang et al. argued that poloxamers are not ROS scavengers because if that were the case, their investigation of poloxamer mediated protection of liposomes damaged via lipid peroxidation would have yielded similar results for all poloxamers given that they share the same chemical details, which was not observed. The reported differences in P188-mediated protection against lipid peroxidation necessitate further examinations with the platform discussed in this work. The amount of lipid peroxidation in iBMECs exposed to H$_2$O$_2$ could be measured to assess whether lipid peroxidation contributes to decreased barrier function of the iBMECs and whether polymer treatment mitigates the level of lipid peroxidation. Several methods to quantify lipid peroxidation are readily available including fluorescence measurements of molecules (e.g., C11-BODIPY) that exhibit altered
fluorescence behavior upon oxidation\textsuperscript{396} or spectroscopic detection of lipid peroxidation byproducts (e.g., isoprostanes or malondialdehyde).\textsuperscript{397} Additionally, lipid peroxidation can provoke a reduction in plasma membrane fluidity,\textsuperscript{398} which can be quantified by fluorescence anisotropy and pyrene excimer fluorescence to measure rotational and lateral plasma membrane fluidity, respectively.\textsuperscript{399,400} Prevention of membrane fluidity change upon oxidative stress has been found to be a mechanism by which melatonin protects cells against oxidative stress.\textsuperscript{401} Since PPO-PEO block copolymers can stimulate a modulation in membrane fluidity,\textsuperscript{118,389} investigations into how plasma membrane fluidity of iBMECs changes with H\textsubscript{2}O\textsubscript{2} and PPO-PEO block copolymer exposure may provide supplemental insight as to whether the polymers protect iBMECs under oxidative stress by altering the mechanical properties of the cells.

Furthermore, inhibition of MMP activity has been suggested as a mechanism by which P188 can ameliorate BBB damage in acute injuries.\textsuperscript{47,197} Preliminary work was done looking at MMP gene expression, but differences between control and H\textsubscript{2}O\textsubscript{2}-challenged iBMECs were too small to make a conclusion (data not shown). Measurement of MMP activity, rather than expression, may be a better avenue of investigation. Overall, these studies would allow us to gain a more complete understanding of how treatment with $\mathbf{P}$-P\textsubscript{15}E\textsubscript{190} of H\textsubscript{2}O\textsubscript{2}-challenged iBMECs enables improved barrier function.

4.3.7. P-glycoprotein activity of iBMECs treated with PPO-PEO block copolymers

Finally, a rhodamine 123 (Rho123) accumulation assay was conducted to ensure that P188 and $\mathbf{P}$-P\textsubscript{15}E\textsubscript{190} do not affect P-gp function. A previously published study demonstrated 0.04 mM poloxamer 235 (P235; reported as 0.01 wt% Pluronic P85) to have
strong P-gp inhibition behavior in bovine BMECs.\textsuperscript{389} Although only relatively hydrophobic poloxamers are thought to be inhibitors of P-gp, we wanted to experimentally confirm that P188 and $t$-P$_{15}$E$_{190}$ do not affect P-gp function especially since the effect of diblock PPO-PEO block copolymers on P-gp function has not been characterized before. Rho123 is a cell permeable molecule that is a substrate for P-gp and will accumulate inside the BMECs when P-gp function is hindered. Cyclosporin A (CsA) and 0.04 mM P235 were used as positive controls. As expected, iBMECs treated with CsA or P235 retained more Rho123, as indicated by the higher fluorescence intensity measurement, due to inhibition of P-gp (Figure 4.9). iBMECs treated with P188 or $t$-P$_{15}$E$_{190}$ retained Rho123 at around the same levels as the control, demonstrating no interference of the polymer interaction on P-gp function. Note that there is a slight decrease in the Rho123 accumulation for iBMECs with 0.5 mM $t$-P$_{15}$E$_{190}$; however, this was not observed in all replicate experiments. This study confirmed that P188 and $t$-P$_{15}$E$_{190}$ do not interfere with P-gp function.

**Figure 4.9.** Rhodamine 123 accumulation assay to evaluate P-glycoprotein function of iBMECs incubated with select PPO-PEO block copolymers. Representative data from 1 independent experiment with 3 biological replicates are shown; trends were confirmed with 2 additional independent experiments with 3 biological replicates each.
4.4. Conclusion and Future Work

Using hiPSC-derived BMECs, an in vitro platform was developed to study the effect of PPO-PEO block copolymer treatment on the BBB under oxidative stress, an important contributor to BBB damage in ischemic stroke. H$_2$O$_2$ was used to induce oxidative stress in the iBMECs. Barrier function as quantified by the TEER showed that iBMECs exposed to H$_2$O$_2$ and treated with t-P$_{15}$E$_{190}$ have improved barrier function compared to untreated iBMECs. Cytoskeleton and junction protein staining, intracellular calcium concentration measurements, and intracellular ROS concentration measurements were conducted to further characterize the effect of H$_2$O$_2$ stress on the iBMECs with or without polymer treatment. H$_2$O$_2$-challenged iBMECs treated with t-P$_{15}$E$_{190}$ showed little to no actin stress fibers, less discontinuous or internalized junction proteins, and lower intracellular calcium concentration compared to H$_2$O$_2$-challenged iBMECs without polymer treatment. There was no difference in the intracellular ROS levels of H$_2$O$_2$-challenged iBMECs with or without t-P$_{15}$E$_{190}$ treatment. P-gp function of iBMECs was unaffected by neither P188 nor t-P$_{15}$E$_{190}$. Overall, we provide evidence to support that impaired function and phenotype of iBMECs under oxidative stress can be mitigated by treatment with PPO-PEO block copolymers without impacting other vital functions such as efflux transport.

While this work characterized some of the effects of H$_2$O$_2$ stress and polymer treatment on the iBMECs, there is still more to be analyzed in pursuit of a more complete understanding of the mechanism of action. As demonstrated here, one of the benefits of using an in vitro model is that we were able to examine the effect of polymer treatment specifically on the BMECs and examine various aspects of cellular health and function. This platform could be utilized for additional polymer screening studies, for which it may
be sufficient to quantify barrier function via TEER. Importantly, concentration titration studies are needed to elucidate the concentration of PPO-PEO block copolymers required for protection of iBMECs under oxidative stress. Although other in vitro reports investigating the use of P188 as cell membrane stabilizers for diseased endothelial cells also employed near millimolar polymer concentrations, similar in vitro studies with different cell types such as neurons demonstrated therapeutic efficacy of P188 with concentrations less than 100 µM.\textsuperscript{46,189} Elucidation of the concentration effect on the application of PPO-PEO block copolymers on diseased BBB will be important to allow for translation to in vivo studies. Another important aspect to consider for this in vitro platform to inform in vivo studies is the incorporation of physiologically relevant complexities (e.g., dynamic models to account for flow on the luminal side of the BBB; discussed further in Chapter 7) that may impact the response of iBMECs to oxidative stress and the dosage of PPO-PEO block copolymers required for therapeutic efficacy. Overall, enhanced mechanistic understanding of the polymer interaction specifically with the BBB along with screening studies may inform and motivate translational research.
Chapter 5. Confocal Fluorescence and Raman Microscopy to Visualize Localization of PPO-PEO Block Copolymers in iBMECs

5.1. Introduction

As described in previous chapters, the efficacy of block copolymers of poly(propylene oxide) (PPO) and poly(ethylene oxide) (PEO) in improving the integrity of a damaged blood-brain barrier (BBB) has been demonstrated in animal models as well as with cellular models, the latter being the focus of this thesis. An important aspect of a more complete understanding of the mechanism by which PPO-PEO block copolymers restore integrity to the BBB, as well as in other cell types and tissues, is knowledge of the localization of the polymers in cells. This chapter describes work in pursuit of utilizing both confocal fluorescence and Raman microscopy to visualize the localization of PPO-PEO block copolymers with live human induced pluripotent stem cell-derived brain microvascular endothelial cells (iBMECs).

Fluorescence microscopy (FM) has been widely utilized for study of subcellular components as well as detection of exogenous molecules such as polymeric nanoparticles in cells or tissues.\(^{402-404}\) Progress in FM technology including development of a wide array of fluorescent probes, confocal microscopy, and super resolution microscopy have enabled high resolution imaging of biological samples and have made FM a critical tool in the life sciences.\(^{405,406}\) These advancements coupled with widespread commercialization of fluorescence microscope systems have made the technique more accessible to researchers.\(^{407}\) Further, technological developments including chambers for environment control and tools to minimize phototoxicity have made FM live-cell imaging ubiquitous in
biological research.\textsuperscript{408,409} Fluorescence microscopy has previously been utilized to visualize fluorescently-tagged poloxamer 188 (P188) in live cells, which demonstrated intracellular accumulation of the fluorescently-tagged P188 molecules on the time scale of hours.\textsuperscript{121,196,197} However, a major caveat to FM is that fluorescent molecules can affect native cell physiology,\textsuperscript{410,411} and labeling of molecules with a fluorescent dye alters the molecules’ characteristics. PPO-PEO block copolymers pose a unique challenge in that the size of a fluorescent dye can be comparable to that of a polymer chain. The molar mass of fluorescent tags can range from 500 – 1500 Da with commonly used Alexa Fluor\textsuperscript{TM} molecules having a molar mass around 1000 Da.\textsuperscript{412} The addition of the fluorophore can also change the hydrophilic-hydrophobic balance of the polymer, which strongly influences the polymer’s interactions with cells.

An analytical method to circumvent the requirement of a fluorescent tag is Raman spectroscopy, which is a vibrational technique that takes advantage of the unique Raman scattering of chemical bonds to allow for detection of the “chemical fingerprint” of the sample of interest.\textsuperscript{413} Infrared (IR) spectroscopy, a counterpart to Raman spectroscopy, is another analytical tool in which a label is not required. However, a key difference - in the context of live-cell imaging which requires an aqueous medium - between Raman and IR spectroscopy is that water has a weaker signal in Raman than in IR spectroscopy, due to the fact the former technique relies on the polarizability of the molecules whereas IR signal arises from changes in dipole moment.\textsuperscript{413,414} While researchers have utilized Raman spectroscopy to study biological samples since the 1970s,\textsuperscript{415} Raman spectroscopy for live cell imaging applications is a relatively new field and not as widely utilized as FM.\textsuperscript{416} There has been limited work probing endothelial cells with Raman spectroscopy.\textsuperscript{417} Thus, in this
work, we aimed to utilize both fluorescence and Raman microscopy with the goal of visualizing the localization of PPO-PEO block copolymers in iBMECs to gain insight into the polymer-cell interaction. We utilized a confocal system for both fluorescence and Raman microscopy, as in a confocal system, light pathways are focused to eliminate out-of-plane signal, allowing for optical sectioning of the sample. Synthesis of a fluorescently tagged PPO-PEO diblock copolymer ($\ell$-$\text{P}_{15}\text{E}_{190}$, which was utilized in work outlined in Chapter 4) and subsequent confocal fluorescence microscopy live-cell imaging experiments with iBMECs are described. Preliminary experiments with live-cell confocal Raman microscopy are also discussed. Comparison of fluorescence and Raman microscopy analyses may complement each other for more confident investigations into the localization of PPO-PEO block copolymers in cells.

5.2. Materials and Methods

5.2.1. Culture of iBMECs

IMR-90-4 human induced pluripotent stem cells (hiPSCs; WiCell) were utilized. For confocal fluorescence microscopy experiments, hiPSCs were differentiated to iBMECs and cultured on Nunc\textsuperscript{TM} Lab-Tek\textsuperscript{TM} II chambered coverglass (Thermo Fisher Scientific) coated with laminin 511-E8 as described in §2.1.2. Imaging experiments were done 2 days after subculture (Day 10) when the cells reached confluence. Initial confocal Raman microscopy experiments (data presented in Figure 5.7) were conducted with iBMECs cultured on $\mu$-Dishes (ibidi) with a #1.5 glass bottom coated with collagen IV and fibronectin (see §2.1.1 for details). Later experiments (data presented in Figure 5.11) were conducted with iBMECs cultured on $\mu$-Dishes coated with laminin 511-E8 (see §2.1.2).
For all Raman microscopy experiments, culture conditions had to be modified in order to minimize signal from phenol red, a pH indicator additive in cell culture medium. Thus, 3 or 4 days after subculture (Day 11 or 12), the medium was changed to phenol red-free Dulbecco’s Modified Eagle Medium (DMEM; Thermo Fisher Scientific) with 1% fetal bovine serum (FBS; Thermo Fisher Scientific). The imaging experiment was done the following day.

5.2.2. Synthesis of amine-functionalized $\xi$-$P_{15}E_{190}$

Synthesis of $\xi$-$P_{15}E_{190}$ is detailed in §2.3 with characterization information summarized in Table 2.4. The synthesis protocol utilized here was previously optimized by Dr. Karen Haman and Dr. Mihee Kim following work by Mahou et al. The reaction schemes are shown in Figure 5.1. First, the hydroxyl end groups of $\xi$-$P_{15}E_{190}$ were tosylated. Prior to the start of the reaction, the polymer and $p$-toluenesulfonic chloride were freeze dried (separately) in benzene. Starting with an approximate 10 wt% solution of polymer in toluene in the reactor, hygroscopically adsorbed water was removed via azeotropic distillation at 70°C until half of the toluene by volume was removed. The reactor was charged with 5 psi argon before adding $p$-toluenesulfonic chloride and triethylamine (TEA) to the reactor, at a 5X and 10X molar excess relative to the polymer, respectively. The reactor was sealed under 5 psi argon and placed in a 40°C oil bath and stirred for 12 hours. The solvent was removed via rotary evaporation. The polymer was dissolved in dichloromethane (DCM) and water-washed twice, and the product was retrieved by precipitation into cold diethyl ether and freeze dried in benzene.
Next, the tosyl group was substituted for an azide group. Freeze dried sodium azide was added at a 20X molar excess to the reactor already containing dry polymer and the reactor was placed under vacuum. Dimethylformamide (DMF), which was previously dried over 4Å molecular sieves overnight, was added to the reactor via cannula transfer. The reactor was charged with 5 psi argon and placed in a 90°C oil bath; the reaction was continued overnight. The solvent was removed via rotary evaporation, and the polymer was dissolved in DCM and washed in brine twice followed by washing in water twice. The product was then precipitated into cold diethyl ether and freeze dried in benzene. Finally, the azide end groups were reduced to amine groups by Staudinger reaction. Triphenylphosphine at 16X molar excess was added to the reactor containing polymer in tetrahydrofuran (THF). The reaction was done at 55°C under reflux for 3 days. Then, distilled water was added to the reactor and kept under reflux for an additional 3 days. The solvent was removed via rotary evaporation, and the product was purified by precipitation into cold diethyl ether, which was done twice before freeze-drying in benzene.
Figure 5.1. Reaction schemes for fluorophore labelling of PPO-PEO diblock copolymer. (A) Tosylation of $t$-$P_{15}E_{190}$. (B) Azide substitution of $t$-$P_{15}E_{190}$-tosyl. (C) Reduction of azide to amine end groups. (D) Conjugation of $t$-$P_{15}E_{190}$-$NH_2$ with Alexa Fluor 647 succinimidyld ester.
5.2.3. Conjugation of Alexa Fluor 647 to amine-functionalized f-P15E190

The protocol utilized here was previously optimized Dr. Mihee Kim. DMF was dried over 4Å molecular sieves overnight. DMF was added via cannula transfer to the reactor containing dried amine functionalized f-P15E190. The reactor was charged with 5 psi argon and TEA was added via needle and syringe through a septum on the reactor. Alexa Fluor 647 succinimidyl ester (AF647 SE; Thermo Fisher Scientific) was added to the reactor by first dissolving in dry DMF and transferring the solution to the reactor via needle and syringe. For 6 mg of polymer, 3 mL DMF, approximately 500 µL TEA, and 1 mg of AF647 SE were utilized. The reactor was stirred at room temperature in the dark for 5 days to ensure complete reaction. The solvent was removed via vacuum distillation, and 1 mL distilled water was added to the reactor. The reactor was stirred for an additional 5 days at room temperature in the dark. The product was purified by running through a PD-10 desalting column (GE Healthcare Life Sciences) and lyophilized. For fluorescence characterization, AF647 SE and polymer conjugated AF647 were dissolved in HBSS buffer with calcium and magnesium (Thermo Fisher Scientific) at a concentration of 14 µM and added in triplicates to a black walled, black bottomed 96-well plate (Corning). The fluorescence emission spectra, excitation spectra, and maximum intensities were measured on a BioTek Synergy H1 multi-mode microplate reader.

5.2.4. Confocal fluorescence microscopy

iBMECs were labeled with calcein by incubating the cells with 2 µM calcein AM (Thermo Fisher Scientific) for 15 min in a 37°C, 5% CO₂ incubator. All incubation steps and wash steps were done with HBSS with calcium and magnesium. Next, the cells were
incubated with AF647 SE or $\text{t-P}_{15}\text{E}_{190}$ conjugated to Alexa Fluor 647 for 2 h. The cells were washed with HBSS 3 times before adding fresh HBSS to the cells for imaging. Confocal imaging was done with a Nikon TiE stand with an A1Rsi confocal scan head, operated by the NIS-Elements confocal software (Nikon, Japan). A 60X water immersion objective with numerical aperture of 1.2 was used. Excitation for fluorescence images was performed sequentially via 488 and 647 nm lasers. Laser intensity and exposure were kept constant during each imaging session, and FIJI software was used for image processing.

5.2.5. Synthesis of alkyne functionalized P188

P188 was functionalized with alkyne end groups following a report by Hiki and Kataoka describing end-group functionalization of PEO. Potassium naphthalenide was prepared as outlined in §2.3. Dried P188 was added to the reactor, which was then subjected to argon-vacuum cycles to remove air in the reactor. The reactor was pressurized to 5 psi argon after the last cycle. THF dried over an alumina column was added to the reactor to make a 10 wt% solution. Potassium naphthalenide was added dropwise to the reactor via needle and syringe through a septum until the solution retained a light green color for at least 30 min. Next, propargyl bromide (Millipore Sigma) was added to the reactor at a 10X molar excess. The solution was stirred at room temperature for 48 h. The product was purified by precipitation into cold diethyl ether. The polymer was retrieved by filtration and freeze-dried in benzene for the final product.
5.2.6. Synthesis of PPO-PEO with deuterated PEO block

Synthesis was done following the procedure outlined in §2.3 but with deuterated ethylene oxide (dEO; Millipore Sigma). Briefly, tert-butoxy terminated PPO with 15 repeat units of PO (t-P15) was chain extended by re-initiation with potassium naphthalenide. dEO was purified over n-butyl magnesium before adding to the reactor. The polymerization reaction was conducted for 48 h.

5.2.7. Polymer characterization

Polymer characterization methods via $^1$H nuclear magnetic resonance ($^1$H-NMR), $^{13}$C-NMR, size exclusion chromatography (SEC), and matrix-assisted laser desorption/ionization mass spectroscopy (MALDI) are detailed in §0.

5.2.8. Osmotic stress assay

This assay was developed by Kim et al.\textsuperscript{74} based on methods from Martindale et al.\textsuperscript{75} and Houang et al.\textsuperscript{135} C2C12 myoblasts (ATCC) were cultured in DMEM with 20% FBS and 1% Penicillin-streptavidin. Cells were seeded in 96-well plates at a density of 5000 cells per well. Experiments were conducted two days later when the culture was approximately 70-80% confluent. Isotonic buffer (330 mOsm) and hypotonic buffer (74 mOsm) were prepared as described by Kim et al.\textsuperscript{74} and sterilized before use. Medium was removed from the cells and replaced with 330 mOsm isotonic buffer with or without polymer and incubated for 30 min at 37°C, 5% CO$_2$. To induce hypotonic stress, the isotonic buffer was removed and 74 mOsm hypotonic buffer with or without polymer was
added to the cells and incubated for 50 min at 37°C, 5% CO₂. For isotonic recovery, the hypotonic buffer was removed and 330 mOsm isotonic buffer with or without polymer was added to the cells and incubated for 30 min at 37°C, 5% CO₂. Finally, 0.01% Triton X-100 (Millipore Sigma) was added to the cells for lysis. Polymer concentration of 150 µM was utilized for all steps. The cell supernatant solutions removed at each step were collected and stored at 4°C until ready for analysis. The Lactate Dehydrogenase Reagent Set (Pointe Scientific) was used to quantify the level of lactate dehydrogenase (LDH) in the supernatant solutions. The reagents (lactate and nicotinamide adenine dinucleotide) were added to the cell supernatant samples, and a BioTek Synergy H1 multi-mode microplate reader was utilized to measure absorbance at 340 nm over 2 h at 37°C. Data are normalized to the total amount of LDH released to take into account variability in cell density.

5.2.9. Raman spectroscopy

Raman spectroscopy and imaging were done with the WITech Alpha 300R Confocal Raman Microscope with a UHTS300 spectrometer, DV401 CCD detector with 600/mm grating, Nd:YAG laser with 532 nm wavelength, and an 100X objective (numerical aperture of 0.90, Nikon Instruments). Raman spectra of polymer samples were captured by spot spectral measurements. Neat polymer samples or polymer solutions (polymer dissolved in HBSS with calcium and magnesium) were placed on a #1.5 glass coverslip. Data collection was done with the WiTecControl 4.05 software. For each spectrum acquisition, 3 scans were collected for a duration of 20 s each, and data were processed for cosmic ray removal with the WiTec Project 4 software. Spectral data are plotted as intensity normalized to the maximum.
For live-cell Raman imaging, cells were incubated with or without polymer in phenol red-free DMEM. Before imaging, the medium was removed and replaced with HBSS with calcium and magnesium. The dish was filled with enough buffer such that the entire dish could be sealed and turned upside down without any air bubbles present in the dish. This was done so that the microscope objective could be closer to the cells as the microscope objective is in an upright position and has a working distance shorter than the height of the dish. The integration time for each spectral point was 0.5 s for data shown in Figure 5.7 and 0.3 s for data shown in Figure 5.11. Using the WiTec Project 4 software, spectra were processed for cosmic ray removal, and Savitzky-Golay filter (order 7) was applied for smoothing. Chemical maps (i.e., images) were rendered by filtering spectra for wavenumber ranges that correspond to the functional group of interest (Table 5.1).

<table>
<thead>
<tr>
<th>Substance</th>
<th>Wavenumber (cm(^{-1}))</th>
<th>Functional Group/Vibration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass</td>
<td>450 – 550</td>
<td>Si-O-Si bending and stretching(^{421,422})</td>
</tr>
<tr>
<td>Proteins, lipids (cells)</td>
<td>1620 – 1700</td>
<td>Amide I C=O, C=C stretching(^{124,423,424})</td>
</tr>
<tr>
<td>Lipids (cells)</td>
<td>2800 – 3000</td>
<td>CH(_2) stretching(^{423,424})</td>
</tr>
<tr>
<td>P188</td>
<td>780 – 860</td>
<td>C-O-C symmetric and C-C stretching(^{425-427})</td>
</tr>
<tr>
<td>P188-alkyne</td>
<td>2000 – 2200</td>
<td>Alkyne(^{428})</td>
</tr>
<tr>
<td>(t)-P_{15}E_{133}</td>
<td>2050 – 2250</td>
<td>C-D stretching(^{429})</td>
</tr>
</tbody>
</table>

5.3. Results and Discussion

5.3.1. Synthesis and characterization of amine functionalized \(t\)-P_{15}E_{190}

We chose to modify \(t\)-P_{15}E_{190} utilized in the work described in Chapter 4 since this polymer led to improved function and phenotype of iBMECs under oxidative stress. Chemical structures of all polymers described in this chapter are outlined in Table 5.2, and
polymer characterization information is in Table 5.3. Since there are multiple steps to the synthesis of amine functionalized $t$-P$_{15}$E$_{190}$, the polymers were characterized via NMR after each step to confirm successful reaction. The $^1$H-NMR spectra of tosylated $t$-P$_{15}$E$_{190}$ ($t$-P$_{15}$E$_{190}$-tosyl) before and after reaction are presented in Figure 5.2. DMSO-d$_6$ was used as the solvent. The spectrum of the $t$-P$_{15}$E$_{190}$-tosyl displayed a peak at 4.1 ppm, which was not present in the spectrum of $t$-P$_{15}$E$_{190}$. Integration of the peak at 4.1 ppm signified 100% conversion of hydroxyl end groups to tosyl end groups. Next, the tosyl end groups were exchanged to azide groups, and reaction was confirmed with $^1$H-NMR and $^{13}$C-NMR with D$_2$O as the solvent. The $^1$H-NMR spectrum (Figure 5.3B) for $t$-P$_{15}$E$_{190}$-N$_3$ did not contain a peak around 4.1 ppm and the $^{13}$C-NMR spectrum (Figure 5.3C) showed a peak at 50 ppm, signifying absence of the tosyl groups and presence of the azide groups, respectively. Finally, azide end groups were reduced to amine groups. Integration of the peak at 2.9 ppm in the $^1$H-NMR spectrum (Figure 5.4) for $t$-P$_{15}$E$_{190}$-NH$_2$ indicated a 70% conversion to amine end groups.

5.3.2. Synthesis and characterization of Alexa Fluor 647 conjugated $t$-P$_{15}$E$_{190}$

The fluorescence activity of Alexa Fluor 647 conjugated $t$-P$_{15}$E$_{190}$ ($t$-P$_{15}$E$_{190}$-AF647) was compared to AF647 SE, and the excitation and emission spectra are plotted in Figure 5.5A and B, which showed maxima near the wavelengths listed by the manufacturer of AF647 (excitation wavelength of 651 nm and emission wavelength of 672 nm). The maximum fluorescence intensities of AF 647 SE and $t$-P$_{15}$E$_{190}$-AF647 at excitation wavelength of 651 nm and emission wavelength of 672 nm were comparable, which is a sign of a successful conjugation reaction (Figure 5.5C).
**Table 5.2.** Chemical structures of polymers

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t$-P_{15}E_{190}</td>
<td><img src="image1" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>$t$-P_{15}E_{190}-tosyl</td>
<td><img src="image2" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>$t$-P_{15}E_{190}-N_3</td>
<td><img src="image3" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>$t$-P_{15}E_{190}-NH_2</td>
<td><img src="image4" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>P188-alkyne</td>
<td><img src="image5" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>$t$-P_{15}dE_{133}</td>
<td><img src="image6" alt="Chemical Structure" /></td>
</tr>
</tbody>
</table>

**Table 5.3.** Polymer characterization

<table>
<thead>
<tr>
<th>Polymer</th>
<th>$N_{PO}$</th>
<th>$N_{EO}$</th>
<th>$M_n$ (kDa)</th>
<th>$D^\dagger$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t$-P_{15}E_{190}-tosyl</td>
<td>15</td>
<td>190</td>
<td>9.5</td>
<td>1.04</td>
</tr>
<tr>
<td>$t$-P_{15}E_{190}-N_3</td>
<td>15</td>
<td>190</td>
<td>9.4</td>
<td>1.04</td>
</tr>
<tr>
<td>$t$-P_{15}E_{190}-NH_2</td>
<td>15</td>
<td>190</td>
<td>9.4</td>
<td>-</td>
</tr>
<tr>
<td>P188-alkyne</td>
<td>27</td>
<td>150</td>
<td>8.2</td>
<td>1.06</td>
</tr>
<tr>
<td>$t$-P_{15}dE_{133}</td>
<td>15*</td>
<td>133*</td>
<td>7.3*</td>
<td>1.03</td>
</tr>
</tbody>
</table>

$N_{PO}$, $N_{EO}$, and $M_n$ calculated from $^1$H-NMR unless noted otherwise

* Determined via MALDI with CHCA as the matrix

$^\dagger$ Determined via SEC with THF as the solvent
Figure 5.2. $^1$H-NMR spectra of (A) t-$P_{15}E_{190}$ and (B) t-$P_{15}E_{190}$-tosyl in DMSO-d6. (C) Overlay of spectra in the 3 – 4 ppm region to show presence of triplet at 4.1 ppm in the t-$P_{15}E_{190}$-tosyl, indicating successful conversion from hydroxyl to tosyl end groups.
Figure 5.3. $^1$H-NMR spectra of (A) $t$-P$_{15}$E$_{190}$ and (B) $t$-P$_{15}$E$_{190}$-N$_3$ in D$_2$O. Lack of peak near 4.1 ppm in the spectra for $t$-P$_{15}$E$_{190}$-N$_3$ supports absence of tosyl groups. (C) $^{13}$C-NMR spectrum of $t$-P$_{15}$E$_{190}$-N$_3$ in D$_2$O. Peak at 50 ppm indicates presence of azide groups.
Figure 5.4. (A) $^1$H-NMR spectra of $t$-P$_{15}$E$_{190}$-NH$_2$ in D$_2$O. (B) Overlay of spectra in the 1 - 3 ppm region to show presence of triplet peak around 2.9 ppm in the $t$-P$_{15}$E$_{190}$-NH$_2$ spectrum that is not present in the spectrum for $t$-P$_{15}$E$_{190}$-N$_3$ indicating successful conversion from azide to amine end groups.
With the synthesis of \( t\text{-P}_{15}\text{E}_{190}\text{-AF647} \), confocal fluorescence microscopy was utilized to image iBMECs incubated with free AF647 SE dye or with polymer conjugated
to AF647. As shown in Figure 5.6, in iBMECs incubated with either 14 µM or 100 µM \( t \)-P\(_{15}\)E\(_{190}\)-AF647, the fluorescence signals from the AF647 appeared inside the cells. We speculate that \( t \)-P\(_{15}\)E\(_{190}\)-AF647 molecules were present in the cytoplasm as well as in endosomal compartments. The cytoplasmic localization was signified by the yellow signal due to co-localization of the AF647 fluorescence (red) and calcein fluorescence (green).

Calcein AM molecules traverse into the cell and are quickly de-esterified in the cytoplasm to produce calcein molecules, which are fluorescent. Calcein does not penetrate intact membranes\(^{430}\) and thus, does not appear in endosomal compartments (note that if calcein is present in the extracellular space, it can be transported into the cell via endocytosis, in which case calcein would be present in endosomal compartments\(^{431}\)). Thus, the presence of red signal from the AF647 that was not co-localized with the green signal supported the conclusion that some fluorescently tagged polymers were contained in endosomal compartments. Future imaging experiments could be done wherein the endosomal compartments (i.e., endosomes, lysosomes) in the iBMECs are labeled with fluorescent markers to assess whether the \( t \)-P\(_{15}\)E\(_{190}\)-AF647 molecules are inside endosomal compartments. These studies would allow us to assess whether internalization of the PPO-PEO block copolymers is due to permeation of the molecules through the cell membrane or due to interactions with the membrane that are sustained long enough for the polymers to be endocytosed.
Figure 5.6. Orthogonal projection views of confocal fluorescence microscopy images of iBMECs incubated with Alexa Fluor 647 SE or \( t\text{-P}_{15}\text{E}_{190}\text{-AF647} \) (red) at concentrations of (A) 14 µM or (B) 100 µM for 2 h. iBMECs were labeled with calcein (green) prior to incubation with Alexa Fluor 647 SE or \( t\text{-P}_{15}\text{E}_{190}\text{-AF647} \). In all cases, internalization of the fluorescent molecules was observed.

Importantly, the localization behavior of AF647 SE and the polymer conjugated-AF647 were similar at both 14 µM or 100 µM. Hughes et al. have quantified the membrane interaction factor of various fluorescent labels and reported AF647 SE to have a low membrane interaction factor and to have weak interactions with egg phosphatidylcholine vesicles.\textsuperscript{432} AF647 SE molecules were likely to have been endocytosed into the cells since
they were unlikely to have permeated through the membrane given the weak membrane interactions. This was supported by the fact that the signal from the AF647 SE in the above images mostly appear as red, rather than yellow, in the iBMECs incubated with AF647 SE. Thus, it is possible that the AF647 molecule conjugated to the polymer influenced the polymer chains’ localization behavior. Further, the orthogonal projection views in Figure 5.6 showed faint signal on the periphery of the cells; however, it was hard to distinguish from background noise. It is important to note that the localization behavior of both the free AF647 SE and polymer conjugated AF647 may be affected by how the molecules are exposed to the iBMECs. To better mimic in vivo conditions, future work should consider the use of flow chambers or microfluidic devices to flow solutions of the free AF647 SE and polymer conjugated AF647 molecules over the iBMECs.

5.3.4. Confocal Raman imaging of iBMECs with P188

Preliminary studies for Raman imaging of iBMECs were attempted with P188. iBMECs incubated with or without 1 mM P188 for 5 h were used for Raman imaging. Cells were successfully imaged; however, the lack of unique characteristic functional group in P188 (i.e., all functional groups in P188 are also endogenously present in a cell) made it difficult to distinguish signal from P188 from other cellular components. As shown in Figure 5.7, the rendered Raman images of the iBMECs with or without P188 incubation both showed the presence of ether bonds, which were used to identify P188 in the Raman data. Thus, we sought to modify the polymer such that it would give a unique Raman signal in the cellular silent region, which is a region of the Raman spectrum around 1800 – 2800 cm$^{-1}$ that is largely devoid of signal from biological samples.$^{416}$
5.3.5. Raman tagging of PPO-PEO block copolymers

Raman tagging refers to the practice of modifying the molecule of interest to have a chemical moiety with a strong Raman signal. Raman tagging with alkyne functional groups has been successfully employed for molecule visualization in live cells.\textsuperscript{433} Alkyne functional groups have a strong Raman signal around 2100 cm\textsuperscript{-1}, which is near the middle of the cellular silent region. P188 was end-group functionalized with an alkyne group and characterized with $^1$H-NMR and Raman spectroscopy. The $^1$H-NMR spectrum exhibited the presence of the hydrogen(s) on the terminal carbon of the alkyne group and on the carbon adjacent to the oxygen, signifying successful reaction (Figure 5.8A). Integration of the peaks at 2.4 ppm and 4.2 ppm indicated a 70% conversion of hydroxyl to alkyne end groups, which is the maximum conversion reported by Hiki et al.\textsuperscript{420} Raman spectra of the polymers before and after reaction were obtained. The unique alkyne signal was observed around 2100 cm\textsuperscript{-1} in the Raman spectrum of the neat P188-alkyne sample (Figure 5.8B); however, this peak was not present in the aqueous sample (Figure 5.8C). Due to the broadening of the Raman band for the alkyne group in aqueous solutions, detection of the alkyne group peak was thought to be unlikely in cell samples.
Figure 5.7. (A) Schematic of sample set-up. (B) Raman images of iBMECs (control). (C) Raman images of iBMECs incubated with 1 mM P188 for 5 h. Both samples show the presence of ether bonds, which were utilized to identify P188 molecules. Scan area of the samples is a cross-section in the X-Z plane.
Thus, we turned to deuterium labelling, which has also been successfully employed in Raman imaging of live cells, since the characteristic Raman band for carbon-deuterium (C-D) is around 2100 cm$^{-1}$. We turned to a diblock copolymer system for easier synthesis and to parallel the work done with the confocal fluorescence microscopy.

Figure 5.8. (A) $^1$H-NMR spectrum of P188 before and after alkylation (CDCl$_3$ as solvent) indicates 70% conversion of hydroxyl to alkyne end groups. (B) Raman spectra of neat P188 and P188-alkyne; spectrum of P188-alkyne shows unique alkyne peak near 2100 cm$^{-1}$. (C) Raman spectra of 4.5 mM aqueous solutions of P188 and P188-alkyne. Spectrum of P188-alkyne does not show unique alkyne peak near 2100 cm$^{-1}$.  

Thus, we turned to deuterium labelling, which has also been successfully employed in Raman imaging of live cells, since the characteristic Raman band for carbon-deuterium (C-D) is around 2100 cm$^{-1}$. We turned to a diblock copolymer system for easier synthesis and to parallel the work done with the confocal fluorescence microscopy.
discussed earlier in the chapter and synthesized a diblock copolymer of PPO and deuterated PEO with 90 wt% dPEO (t-P_{15}dE_{133}). The NMR spectrum of the deuterated polymer did not contain a prominent peak at 3.6 ppm, indicative of the absence of PEO based protons (Figure 5.9A). Raman spectroscopy of the deuterated polymer confirmed the presence of deuterium in the molecule with the spectrum showing the characteristic C-D band around 2100 cm\(^{-1}\) (Figure 5.9B). MALDI-TOF was used to determine the overall molecular weight, from which the number of repeat units of the dPEO block was calculated using the known molecular weight of the PPO block before chain extension (Table 5.3). Raman spectra of t-P_{15}dE_{133} at different concentrations were collected (Figure 5.9C). Spectra of polymer solutions at concentrations down to 1 mM clearly showed the presence of the C-D band.

Though the weight percent of dPEO in the synthesized diblock polymer was approximately 90 wt%, the number of dEO repeat units was lower than the number of EO repeat units in the hydrogenated counterpart (133 vs. 190). Thus, we performed a cellular assay to confirm that the cell protection behavior of the deuterated polymer is similar to the hydrogenated counterpart even with fewer repeat units of the EO. Figure 5.10 compares the LDH release from C2C12 myoblasts after hypotonic stress and isotonic recovery with or without polymer treatment. The LDH release of the saline only condition was significantly higher than the LDH release of either the t-P_{15}dE_{133} or t-P_{15}E_{190} treated cells. With confirmation of comparable cell protection efficacy and observation of the unique C-D band in the Raman spectrum in aqueous conditions, we next turned to Raman imaging.
Figure 5.9. (A) $^1$H-NMR of $t$-P$_{15}$E$_{190}$ and $t$-P$_{15}$dE$_{133}$ with CDCl$_3$ as the solvent. (B) Raman spectra of 4.5 mM aqueous solutions of $t$-P$_{15}$E$_{190}$ and $t$-P$_{15}$dE$_{133}$. The spectrum of the deuterated polymer shows the characteristic band for C-D near 2100 cm$^{-1}$. (C) Raman spectra of $t$-P$_{15}$dE$_{133}$ at different concentrations to assess sensitivity of detection of the C-D signal. C-D band is observable down to 1 mM polymer solution.
Figure 5.10. LDH release from myoblasts after hypotonic stress and isotonic recovery with or without polymer treatment. Data are normalized to the total amount of LDH released. LDH release of myoblasts treated with either $t\text{-}P_{15}dE_{133}$ and $t\text{-}P_{15}E_{190}$ are similar, indicating similar protection efficacies of both polymers. Data are presented as mean ± standard deviation and are from 2 independent experiments, with 3 biological replicates each.

Since PPO-PEO block copolymer interactions with damaged cells or lipid membrane models are thought to be more prevalent than interactions with healthy cells or membranes,\textsuperscript{151,196} we tried imaging iBMECs under hydrogen peroxide-induced stress and treated with 1 mM $t\text{-}P_{15}dE_{133}$, following the experimental procedures outlined in Chapter 4. Though the cells could be imaged, the signal from the deuterated polymer was too weak and could not be observed. In order to maximize signal, iBMECs were incubated with a 4.5 mM polymer solution since the concentration of the polymer in or around the cell is likely to be lower than the concentration in the medium. Importantly, there is a mixed population of unimers and micelles above 1 mM $t\text{-}P_{15}E_{190}$ (see §2.5), but because both unimers and micelles of PPO-PEO block copolymers can interact with cells,\textsuperscript{435} using $t\text{-}P_{15}dE_{133}$ concentrations above 1 mM for the purposes of these proof-of-concept studies was thought to be acceptable. iBMECs were incubated with or without 4.5 mM $t\text{-}P_{15}dE_{133}$ for 3 h before imaging. The live cells were successfully imaged as shown in Figure 5.11A. However, the C-D signal could not be observed in either the control (as expected) or the
sample that was incubated with the deuterated polymer. Longer incubation times of up to 24 h with the polymer did not improve signal deconvolution.

Figure 5.11. (A) Rendered Raman images of iBMECs incubated with or without 4.5 mM \( t\)-P\(_{15}\)dE\(_{133}\) for 3 h. Schematic of sample set-up is shown in Figure 5.7A; scan area is in the X-Z plane. (B) Spectrum of iBMEC sample incubated with 4.5 mM \( t\)-P\(_{15}\)dE\(_{133}\) for 3 h in the glass portion of the scan area shows strong band near 500 cm\(^{-1}\) whereas (C) spectrum obtained from the middle of the sample scan area contains characteristic bands for cells and the polymer.
We speculated that one issue may be the cell culture dish, and so we tried a shallower cell culture dish that fit better on the microscope stage. This led to observation of the C-D signal presumably in or around a cell. In iBMECs incubated with 4.5 mM \( t \)-P_15dE_{133} for 3 h, we could see differentiation of signal in the scan area where the glass signal (Si-O-Si) was strong (Figure 5.11B) and where cell and C-D signals were strong (Figure 5.11C). Unfortunately, clear images were not able to be rendered from this data. Although we could not clearly localize the C-D signal in iBMECs, data shown in Figure 5.11B and C lead us to believe that with further optimization of sample preparation or with a different microscope system, confocal Raman microscopy could be utilized as a tool for imaging of PPO-PEO block copolymers in cells.

A limitation of the instrument utilized for Raman imaging in this work is that we were unable to control the sample environment to 37°C and 5% CO_2, which is critical for maintaining cell viability during live-cell imaging. Short spectral integration times had to be used, and although approximately 1 million cells were seeded on the cell culture dish, only 1 or 2 cells could be imaged per sample. Thus, it is likely that we were not obtaining representative data during each imaging experiment. To bypass challenges related to live cell imaging, the cells could be fixed, a method in which cells are chemically preserved most commonly by crosslinking or denaturing of proteins. Cell fixation may lead to changes in protein organization, and there is also the risk that polymer chains loosely bound to the cell membrane may be washed away during the fixation process. Nevertheless, it may allow for enhancement of signal for imaging of the iBMECs with deuterated block copolymers. Moreover, we have initiated a collaboration with a research group that employs a custom-built Raman spectrooscope that could be adapted to make suitable for
live-cell imaging. Preliminary studies have demonstrated higher sensitivity with this Raman instrument as the C-D band could be detected in a 300 µM solution of t-P15dE133. We may also be able to incorporate an environmental control chamber to this instrument, which would accommodate longer scan times of live cell samples without having to fix the cells. With Raman spectral data of enhanced sensitivity, there is great opportunity to explore quantitative analyses to gain deeper insight into polymer-cell interactions without the need for fluorophore labeling.

5.4. Conclusions and Future Work

The work described in this chapter was done with the goal of visualizing the localization of PPO-PEO block copolymers in iBMECs. Synthesis of polymers to be used in confocal fluorescence microscopy and confocal Raman microscopy are described, and results from preliminary imaging experiments are discussed. A diblock copolymer of PPO-PEO with 90 wt% PEO (t-P15E190) was modified to be tagged with Alexa Fluor 647 at the end of the PEO block and subsequently used for confocal fluorescence microscopy experiments. Images demonstrated internalization of both the free AF 647 SE molecules and t-P15E190-AF647 in iBMECs when incubated for 2 h. Future work investigating whether the polymers are present in endosomal compartments should be done to confirm the pathway by which these molecules enter the cells. Furthermore, additional insight could be gained by investigating whether the polymer localization in iBMECs changes when the cells are under stress conditions and under flow conditions. For Raman microscopy experiments, a diblock copolymer of PPO-PEO with 90 wt% deuterated PEO was synthesized. While we were able to image the iBMECs, no clear localization of the
polymers could be determined. Different sample preparation methods including fixation of the cells to allow for longer scan times and use of a microscope system built for cellular imaging may improve the signal-to-noise ratio for clear visualization. In the future, confocal Raman microscopy may provide a technique to image PPO-PEO block copolymers in live cells without the need for a fluorescent label to complement data from confocal fluorescence microscopy experiments.
Chapter 6. Modeling Damage to the Blood-Brain Barrier During Ischemic Stroke Events

6.1. Introduction

Ischemic stroke triggers a complex sequence of events with wide ranging pathological and clinical consequences. In Chapter 4, we considered oxidative stress, a major contributor to blood-brain barrier (BBB) damage during ischemic stroke, and we utilized hydrogen peroxide to induce oxidative stress on human induced pluripotent stem cell (hiPSC)-derived brain microvascular endothelial cells (iBMECs). In the work described here, we aimed to study the impact of additional stresses related to ischemic stroke events on the BBB again using iBMECs. *In vitro* models were utilized throughout this thesis in an effort to bridge the gap between complex *in vivo* models in which biological responses are difficult to deconvolute and biophysical models that often do not encompass the dynamics and heterogeneity of biological systems. Using an *in vitro* model of the BBB allowed us to assess the effects of specific events and whether treatment with poly(propylene oxide) (PPO) – poly(ethylene oxide) (PEO) block copolymers can protect or rescue damaged iBMECs under different modes of damage. The stroke related stresses that are discussed in this chapter are neurotransmitter excitotoxicity, reoxygenation injury, and oxygen-glucose deprivation and reperfusion.

During ischemia/reperfusion (I/R), an increase in intracerebral concentration of certain neurotransmitters instigates opening of the BBB. For example, glutamate concentrations in the brain interstitial space can dramatically increase, with *in vivo* studies using rats reporting as much as a 55 fold increase in concentration. In human patients,
glutamate levels in plasma and cerebrospinal fluid of stroke patients have been measured to be significantly higher than in control subjects.\textsuperscript{440,441} Under physiological conditions, the cerebral concentration of glutamate is regulated by excitatory amino acid transporters (EAATs) to prevent excitotoxicity.\textsuperscript{442} However, under ischemic conditions, dysfunction of EAATs leads to a release of glutamate from neuronal and glial stores.\textsuperscript{443} Studies \textit{in vivo} and \textit{in vitro} have demonstrated that high concentrations of glutamate can disrupt the brain endothelium by activation of \textit{N}-methyl-\textit{D}-aspartate receptors.\textsuperscript{444,445} Another molecule that is released during I/R and affects the BBB is histamine, a vasoactive mediator that can induce an increase in BBB permeability.\textsuperscript{446} Cerebral ischemia prompts mast cells to release histamine from their stores.\textsuperscript{447,448} Increase in BBB permeability caused by a high concentration of glutamate is facilitated by an increase in intracellular calcium concentration.\textsuperscript{444,445} Similarly, vasoactive agents activate pathways that increase the intracellular calcium concentration, interfering with tight junction protein organization.\textsuperscript{449,450} Thus, we examined the effect of glutamate and histamine on the barrier integrity of iBMECs.

Another event during ischemic stroke that disrupts the BBB is reoxygenation injury, which occurs when the ischemic area is reoxygenated after a period of low oxygen (hypoxia) and induces oxidative stress on the BBB \textit{in vivo}.\textsuperscript{451} The effects of oxidative stress on the BBB are discussed more thoroughly in Chapter 4. To model hypoxia and reoxygenation (H/R) on the BBB, a chemical agent to mimic hypoxia and true hypoxia were utilized on the iBMECs and the barrier function of the cells was quantified. Cobalt chloride (CoCl\textsubscript{2}) was utilized as a chemical mimicker of hypoxia. The heterodimer hypoxia-inducible factor 1 (HIF1), consisting of HIF1\textalpha{} and HIF1\textbeta{}, is a regulator of hypoxia signaling, and
transcription of HIF1 leads to the expression of genes related to hypoxia response including those involved in angiogenesis and oxygen transport. While HIF1α is at very low levels during normal oxygen conditions, the subunit accumulates in cells under hypoxia. CoCl2 mimics hypoxic conditions by preventing degradation of HIF1α. To induce true hypoxia in the iBMECs, a hypoxia chamber, wherein the gas composition inside the chamber can be regulated, was utilized.

Furthermore, the major event of an ischemic stroke is the obstruction and subsequent reestablishment of blood flow. This can be modeled in vitro with oxygen-glucose deprivation (OGD) and reperfusion, which is often employed as a method to model a stroke in vitro. The experimental set-up involves the replacement of cell culture medium with medium without glucose and placement of the cell culture in a hypoxia chamber. We also utilized a co-culture of iBMECs and astrocytes for this study as in vitro studies of the effect of OGD on BMECs have demonstrated differential response depending on whether the BMECs are co-cultured with other cells of the neurovascular unit. Astrocytes are vital in the maintenance of the BBB during physiological and pathological conditions. Thus, we wanted to examine whether P188 treatment would have a different response when the iBMECs are co-cultured with astrocytes. Herein, we describe investigations of the effects of stresses to the BBB related to ischemic stroke, including glutamate and histamine excitotoxicity, H/R, and OGD/reperfusion, on barrier function of the iBMECs. For studies with iBMECs under true hypoxia and OGD/reperfusion, we also present preliminary results on PPO-PEO block copolymer treatment of iBMECs with impaired barrier function.
6.2. Experimental Methods

6.2.1. Cell culture and differentiation of hiPSCs to iBMECs

hiPSCs were cultured on Matrigel (Corning), maintained in TeSR-E8 (STEMCELL Technologies), and subcultured with ReLeSR (STEMCELL Technologies). The experiments discussed in §6.3.3 were conducted with iBMECs differentiated from IMR-90-4 hiPSCs (WiCell) and cultured on laminin 511-E8 (§2.1.2). All other experiments were done with iBMECs were differentiated from IMR-90-4 or DF19-9-11T.H hiPSCs (WiCell) and cultured on collagen IV and fibronectin (§2.1.1). Experiments involving glutamate and histamine exposure were done 2 days after subculture with iBMECs seeded on Transwells. Experiments with cobalt chloride were initiated 1 day after subculture and were conducted with iBMECs on Transwells or well plates.

6.2.2. Measurement of barrier integrity

The trans-endothelial electrical resistance (TEER) was measured using the EVOM2 voltohmmeter with STX2 chopstick electrodes (World Precision Instruments). Reported TEER values take into account the surface area of the iBMEC layer and the effects of the medium and membrane on TEER (quantified by TEER measurements of a Transwell coated with the extracellular matrix proteins, but without cells). The sodium fluorescein permeability assay was performed as described by Stebbins et al.225 iBMECs seeded on Transwell filters were utilized to measure the permeability of the iBMEC layer and membrane, and an empty Transwell filter coated with extracellular matrix proteins was utilized to measure the permeability of the membrane only. After a complete medium change, the cells were incubated at 37 °C, 5% CO₂ for 1 h. TEER was measured before
and after the medium change to confirm equilibration of the iBMEC layer. Medium from the apical chamber was aspirated and replaced with fresh medium containing 10 μM sodium fluorescein (Millipore Sigma). Every 15 min for 1 h, 150 μL aliquots were extracted from the basolateral chamber and replaced with 150 μL of fresh medium. At 1 h, a 150 μL sample was extracted from the apical chamber and fluorescence intensities of the aliquots were measured on a BioTek Synergy H1 multi-mode microplate reader at excitation of 485 nm and emission of 530 nm. The permeability values were calculated using the slope of clearance volume as a function of time. The permeability values of the iBMECs only are reported and were calculated from the total permeability and permeability of the blank Transwell. Details of the calculations can be found in Stebbins et al. and Perrière et al.225,290

6.2.3. Measurement of lactate dehydrogenase concentration

Cell supernatant solutions were analyzed with the Lactate Dehydrogenase Reagent Set (Pointe Scientific) to quantify the level of lactate dehydrogenase (LDH). The reagents (lactate and nicotinamide adenine dinucleotide) were added to the cell supernatant samples, and a BioTek Synergy H1 multi-mode microplate reader was utilized to measure absorbance at 340 nm. The concentration of LDH was calculated from the absorbance intensities following the manufacturer’s instructions.

6.2.4. Live/Dead staining

iBMECs seeded on 12-well plates were utilized for this study with the Live/Dead Viability/Cytotoxicity Kit (Thermo Fisher Scientific). Cells were incubated with 2 μM
calcine-AM (live stain) and 4 μM ethidium homodimer-1 (dead stain) for 30 min at room temperature. Imaging was done with the EVOS FL Auto Imaging System with the 10X objective; microscope details can be found in §2.2.

6.2.5. Hypoxia/reoxygenation and polymer treatment

Four or five days after subculture, iBMECs seeded on Transwells were placed in a hypoxia chamber (STEMCELL Technologies) with 1% O₂ and 5% CO₂ and the entire chamber was placed in a 37°C, 5% CO₂ incubator. A petri dish filled with Milli-Q water was placed inside the chamber to keep the chamber humidified. The time point when the cells were placed inside the hypoxia chamber is t = 0. After 18 h inside the hypoxia chamber, the plates were taken out and placed in a 37°C, 5% CO₂ incubator with normal oxygen levels. The control condition was iBMECs in a 37°C, 5% CO₂ incubator with normal oxygen levels throughout the experiment. For polymer treatment, poloxamer 188 (P188) or poly(ethylene oxide) (PEO8K) were dissolved in culture medium at a concentration of 12 mM. Characterization data of the polymers are summarized in §0. After taking the cells out of the hypoxia chamber at t = 18 h, a bolus injection of the polymer solution was added to the cell culture (apical side of the Transwells only) such that the final concentration of the polymer was 1 mM.

6.2.6. Oxygen-glucose deprivation

iBMECs were subcultured onto Transwells and 3 days later, the medium was changed to Dulbecco’s modified essential medium (DMEM) supplemented with 1% fetal bovine serum (FBS). The next day, the medium of the iBMECs was changed to glucose-
free DMEM, and the cells were placed in a humidified hypoxia chamber with 1% $O_2$ and 5% $CO_2$. The time point when the OGD started is $t = 0$. The entire chamber was placed in a 37°C, 5% CO$_2$ incubator for 18 h. Then, the cells were taken out of the chamber and the medium was replaced with DMEM with 1% FBS and placed in a 37°C, 5% CO$_2$ incubator with normal oxygen levels. At either the onset of OGD ($t = 0$) or the onset of reperfusion ($t = 18$ h), the iBMECs were treated with 1 mM P188 in the apical side of the Transwell only. P188 was directly dissolved to a final concentration of 1 mM in either glucose-free DMEM for the $t = 0$ treatment or DMEM with 1% FBS for the $t = 18$ h treatment. The control condition was a culture of iBMECs in a 37°C incubator with normal oxygen levels throughout the experiment; the cells were kept in DMEM with 1% FBS and the medium was changed to fresh DMEM with 1% FBS at the same times as when the medium of the non-control cells was changed (i.e., at $t = 0$ and $t = 18$ h).

Co-culture experiments were done with human primary astrocytes cultured in human astrocyte growth medium (Cell Applications, Inc.) on plates coated with gelatin (Millipore Sigma). Astrocytes were cultured until they started to differentiate, which was evident by the change in cell morphology and usually happened after two passages upon thawing. To prepare for co-culture with iBMECs, the astrocytes were seeded onto 12-well plates at a density of 50,000 cells/well. One day prior to the start of the OGD, the media of the astrocytes and the iBMECs were changed to DMEM with 1% FBS. The next day, Transwells seeded with iBMECs were transferred to the well plates where the astrocytes were cultured. After at least 8 h, the iBMECs and astrocytes were placed under OGD and P188 treatment in the same manner as was done for the iBMECs only described above. The control condition was a co-culture of iBMECs and astrocytes in a 37°C, 5% CO$_2$
incubator with normal oxygen levels throughout the experiment; the cells were kept in DMEM with 1% FBS and the medium was changed to fresh DMEM with 1% FBS at the same times as when the medium of the non-control cells was changed.

6.2.7. Cobalt thiocyanate colorimetry assay

Cobalt thiocyanate was prepared from cobalt nitrate and ammonium thiocyanate (Millipore Sigma) and the assay was performed following a report by Ghebeh et al.\textsuperscript{46}\textsuperscript{1} Briefly, the sample solutions were mixed with cobalt thiocyanate, ethyl acetate, and ethanol in poly(propylene) microcentrifuge tubes. The samples were centrifuged at 11,500\textit{g} for 8 min after which precipitates of P188 complexed with cobalt thiocyanate were formed. The solvents were removed by pipette, taking care to not disturb the precipitates settled on the bottom of the microcentrifuge tubes. The precipitate and tube walls were washed by adding fresh ethyl acetate and mixing the tube before centrifuging at 11,500\textit{g} for 3 min. The solvent was removed, and the process was repeated until the supernatant was colorless; 6-8 wash cycles were needed for this process. The pellet was then dissolved in acetone, and the solution was transferred to a cuvette. Absorbance of the solutions at 624 nm was measured in duplicates with a BioTek Synergy H1 multi-mode microplate reader. A standard curve was generated using polymer solutions of known P188 concentration. The standards were prepared in the same medium (DMEM with 1% FBS) as that of the samples to be analyzed (i.e., samples of unknown P188 concentration). A standard curve was generated for each independent experiment; an example standard curve is shown in Figure 6.1.
6.2.8. Statistical analysis

Data are presented as mean ± standard deviation. The numbers of independent experiments (N) and biological replicates (n) pertaining to each assay are listed in the figure captions with independent experiments being defined as experiments done with a different batch of differentiated iBMECs, and biological replicates being defined as different wells of cells within an independent experiment. Statistical significance was determined using unpaired Student’s t-test or one-way analysis of variance with Tukey post hoc test; analyses were done using GraphPad Prism.

6.3. Results and Discussion

6.3.1. Glutamate and histamine exposure

Barrier disruption upon endothelial cell exposure to glutamate or histamine has been previously reported with immortalized human brain endothelial cells or human vascular endothelial cells co-cultured with rat glioma cells.445,446 To examine the effect of excess glutamate on iBMECs, tightness of the barrier as quantified by TEER was measured.
upon exposure to glutamate. A bolus injection of glutamate (Millipore Sigma) dissolved in DPBS (without calcium and magnesium; Thermo Fisher Scientific) was added to the basolateral chamber of the Transwells (i.e., the brain facing side) seeded with iBMECs to a final concentration of 10 mM. *In vivo* concentrations of glutamate in the brain during ischemia have been reported to be on the order of $10^2$ μM.\textsuperscript{462} However, previous *in vitro* studies examining the effect of glutamate on endothelial cells have required the use of 1 – 4 mM glutamate to observe a decrease in the barrier function,\textsuperscript{295,445} and thus, we tested concentrations above 1 mM and found that glutamate concentration of 10 mM was required to induce damage. As shown in Figure 6.2A, after 1 h of incubation, we observed a reduction in the TEER of iBMECs that had been exposed to glutamate compared to the control, which received the same volume of DPBS in the basolateral chamber as the other condition but without glutamate (i.e., sham control). Cells exposed to glutamate exhibited TEER that is 80% of their initial value compared to 92% for the control. After another 1-2 h incubation, the TEER of iBMECs under glutamate stress recovered to values similar to the control (data not shown). This is likely due to the transport of glutamate from the basolateral side to the apical side of the iBMECs, leading to a decrease in the glutamate concentration in the basolateral chamber. EAATs on the brain facing side of the BMECs allow entrance of glutamate from the brain into the BMECs and facilitative transport expels the glutamate from inside the cells into the plasma.\textsuperscript{463–465}

To sustain the damage to the cells, we added bolus injections of glutamate every hour over the course of 6 h and monitored the TEER. Additionally, we considered the effect of the concentration gradient across the cells by adding glutamate in the apical and/or basolateral chambers of the Transwells seeded with iBMECs. With this experimental set-
up, there was a steady reduction in TEER over the course of 6 h (Figure 6.2B). The slight decrease in TEER in the sham control condition (designated as “0mM/0mM”) was likely due to perturbations to the cells with each hourly bolus addition of DPBS. Importantly, the reduction in TEER was observed only in conditions where the concentration of glutamate in the basolateral chamber was higher than the concentration in the apical chamber. This behavior was expected as the glutamate concentration increase on the brain side of the barrier is the source of injury during I/R. Thus, these results demonstrate the ability of this in vitro model to recapitulate the in vivo response of a damaged BBB.

Furthermore, we studied the effect of histamine on the barrier integrity of iBMECs. A bolus injection of histamine (Millipore Sigma) dissolved in DPBS was added to the basolateral chamber of Transwells seeded with iBMECs. The TEER of the iBMECs after 30 min of incubation with 5 mM histamine was approximately 80% lower than the sham control (Figure 6.3A). The concentration of LDH released from the cells was measured to assess whether histamine stimulates damage to the cell membrane. After 30 min of incubation with histamine, there was no difference in the amount of LDH in the cell culture supernatant of the control and the histamine-challenged iBMECs (Figure 6.3B). This behavior was anticipated as histamine is a vasoactive molecule that provokes paracellular opening of the BBB. Additional assays should be done to confirm that there is no cell membrane damage in the iBMECs upon exposure to histamine.
Like glutamate, the decreased barrier function of the iBMECs exposed to histamine was not sustained for more than 1-2 h. Experimental conditions could be modulated as mentioned previously for more sustained damage to the iBMECs. Moreover, the histamine concentration required to induce damage to the iBMECs was higher than expected from what has been previously reported in the literature. As histamine concentrations in the brain increase, activation of the H2 receptors leads to an opening of the BBB mediated by an

Figure 6.2. (A) iBMECs after 1 h incubation with 10 mM glutamate in the basolateral (“brain”) chamber of Transwells exhibited lower TEER than the sham control. (N = 3, n = 9). (B) Glutamate induced damage to the iBMECs could be sustained if bolus injections of glutamate were added hourly to the culture. The damage occurred only when the glutamate concentration was higher in the basolateral chamber. Concentrations are shown as “Apical concentration/Basolateral concentration” in the legend. (N = 1, n = 3). Trends were confirmed with additional independent experiments.

Like glutamate, the decreased barrier function of the iBMECs exposed to histamine was not sustained for more than 1-2 h. Experimental conditions could be modulated as mentioned previously for more sustained damage to the iBMECs. Moreover, the histamine concentration required to induce damage to the iBMECs was higher than expected from what has been previously reported in the literature. As histamine concentrations in the brain increase, activation of the H2 receptors leads to an opening of the BBB mediated by an
increase in intracellular calcium concentrations; with further increase in histamine concentration, H1 receptors are activated, leading to a decrease in BBB permeability via increase in cyclic AMP.\textsuperscript{449,466} Studies with bovine capillary endothelial cells have reported that 1 mM histamine is a high enough concentration to induce tightening of the barrier.\textsuperscript{296} Thus, we would not have expected for 5 mM histamine exposure to iBMECs to provoke an opening of the barrier. This discrepancy may be due to differences in experimental methods including the fact that the aforementioned report exposed histamine to both sides of the endothelial cell barrier.\textsuperscript{446} To support that iBMECs appropriately recapitulate response to histamine as observed \textit{in vivo}, additional studies with higher concentrations of histamine (\textgreater{} 5 mM) should be done, for which we would expect to observe an increase in TEER.

We attempted to evaluate the effect of PPO-PEO block copolymer treatment on iBMECs damaged via glutamate or histamine exposure. Results were inconsistent, sometimes even within a single independent experiment. We speculate that this may be due to the difference in the time scale of action. Glutamate and histamine act on the iBMECs on the order of minutes, whereas the interaction of the polymers with the iBMECs may not be as fast. Nevertheless, the results presented here demonstrate the ability of this \textit{in vitro} model to recapitulate responses of a damaged BBB observed \textit{in vivo} and examples of how the experimental parameters could be adapted.
Histamine exposure disrupted barrier integrity, but not cell membrane integrity, of the iBMECs. (A) TEER of iBMECs after 30 min of incubation with 5 mM histamine in the basolateral chamber was decreased compared to the control. (N = 3, n = 7). (B) There was no difference in the LDH release from iBMECs after 30 min of incubation with or without histamine. (N = 2, n = 6).

6.3.2. Utilizing cobalt chloride and true hypoxia to study reoxygenation injury

CoCl₂ and true hypoxia have been demonstrated to disrupt tight junction complexes in iBMECs. To study the effect of reoxygenation injury, we utilized CoCl₂ or true hypoxia and monitored the barrier function of iBMECs after removal of CoCl₂ or reestablishment of normal oxygen conditions, respectively. iBMECs were exposed to CoCl₂ for 5 h before the culture medium was changed to fresh medium, and the TEER was measured for the next two days (Figure 6.4A). The medium on the control sample was changed at the same times as the conditions with CoCl₂. There was approximately a 10% decrease in the TEER 24 h and 48 h after the 5 h treatment of iBMECs with 0.1 mM or 1 mM CoCl₂ compared to the control.

Although the data indicated that CoCl₂ exposure and subsequent removal caused a reduction in the TEER of the iBMECs, another measurement was done to ensure the fidelity of the TEER measurements because the presence of any residual ions from CoCl₂ in the culture medium would significantly affect the TEER measurement. Thus, the
permeability ($P_e$) of sodium fluorescein, a cell-impermeable solute, across the iBMEC monolayer was measured. The permeability coefficient was measured approximately 20 hours after the iBMECs were exposed to CoCl$_2$ for five hours. As expected, the permeability of sodium fluorescein in iBMECs that were previously exposed to CoCl$_2$ was approximately 20-30% higher than that of the control. Further, because CoCl$_2$ is known to cause cell apoptosis, viability was assessed with live/dead staining after 5 h of CoCl$_2$ exposure to establish whether significant cell death was contributing to the observed increase in permeability of the iBMECs. We observed an intact network of iBMECs in all conditions, signifying that the hypoxia mimicking condition did not induce significant cell death. However, there may be more cell death in the period after CoCl$_2$ removal, prompting assessment of viability at later time points in future work. Note that the images of the iBMECs treated with 1 mM CoCl$_2$ appear dimmer than the other conditions; this may be due to quenching of calcein fluorescence by residual cobalt ions.
As a supplementary method to investigate reoxygenation injury on the iBMECs, the cells were placed under true hypoxia. iBMECs were placed under hypoxia for 18 h before reoxygenation. The TEER was tracked and normalized to the value measured before the cells were placed in the chamber (Figure 6.5A). After 1 h of reoxygenation, we observed c. 30% drop in the TEER of the cells that were previously exposed to hypoxia, signifying that the reoxygenation induced damage to the cells. After 24 h of reoxygenation, the TEER of the iBMECs previously exposed to hypoxia recovered to the level of the

**Figure 6.4.** (A) iBMECs were treated with CoCl₂ for 5 h before CoCl₂ was removed from the cell culture. TEER of iBMECs 24 or 48 h after removal of CoCl₂ treatment was decreased compared to the untreated control. Plotted data are from 1 independent experiment with 3 biological replicates (N = 1, n = 3). Trends were confirmed with an additional independent experiment. (B) Sodium fluorescein permeability of iBMECs 24 h after 5 h CoCl₂ treatment was increased compared to the untreated control (N = 1, n = 3). (C) Live (green)/dead (red) staining of iBMECs after 5 h of CoCl₂ treatment. Though a few dead cells were present after CoCl₂ exposure, the cell layer was intact. Representative images from 3 independent experiments are shown (N = 3).
control (Figure 6.5B). We assessed whether polymer treatment could mitigate the decrease in TEER caused by reoxygenation injury. When iBMECs exposed to hypoxia were treated with either 1 mM PEO8K or P188 at the onset of reperfusion (t = 18 h), barrier function was moderately improved compared to cells that had been under hypoxia but were not treated with polymer (Figure 6.5C). The normalized TEER value for the H/R condition was approximately 56% compared to 68% and 69% for the conditions under H/R and treated with P188 and PEO8K, respectively; however, the differences in TEER (H/R vs. H/R P188 and H/R vs. H/R PEO8K) were not statistically significant and additional studies are required.

In this work, we exposed iBMECs to CoCl₂ and analyzed the cells’ functionality and viability after removal of the hypoxia-mimicking agent. Similarly, we studied the functionality of iBMECs after hypoxia and subsequent reoxygenation (H/R). A benefit of utilizing CoCl₂ to mimic hypoxia is that assays can be performed on the cells while the cells are still in a hypoxia-mimicking state, not in the recovery state as was the case for the data shown in this section. Such studies are difficult to implement with cells under true hypoxia as once the cell culture is taken out of the sealed hypoxia chamber, the cells return to normoxic state very quickly due to the short half-life of HIF1α proteins. However, evaluation of cells in the presence of the CoCl₂ may be challenging due to interference of the cobalt ions with the analysis (e.g., quenching of fluorophores). This may be a problem even after removal of CoCl₂ as previous reports have noted that after CoCl₂ removal from an *in vitro* culture, residual CoCl₂ can affect the cells’ response for up to 2 days. Further, it is important to emphasize that CoCl₂ is a hypoxia-*mimicking* agent, and CoCl₂ can more broadly affect transcriptional outputs compared to true hypoxia, and CoCl₂ removal
may not recapitulate the same cellular response as reoxygenation after true hypoxia. Nevertheless, these two experimental methods and conditions that we have demonstrated induces damage to the iBMECs offer potential platforms for testing ability of PPO-PEO block copolymers to protect the iBMECs against reoxygenation injury.

Figure 6.5. (A) Time-course TEER data of iBMECs in normal oxygen conditions (control) or under hypoxia for 18 h (t = 0 - 18h) followed by reoxygenation (t = 18 - 24h). Barrier function of the iBMECs after hypoxia decreased upon onset of reoxygenation. (B) Barrier function of iBMECs exposed to 18 h of hypoxia recovered to that of the control after approximately 24 h of reoxygenation. For the data in (A) and (B), plotted data shows results from 1 independent experiment with 3 biological replicates (N = 1, n = 3). Trends confirmed with additional independent experiments (N > 3). (C) TEER of iBMECs after 18 h hypoxia and 5 h reoxygenation was slightly improved (though not statistically significant) compared to the H/R only condition if treated with 1 mM PEO8K or P188 at the onset of reoxygenation. (N = 2, n = 6).
6.3.3. Oxygen-glucose deprivation and reperfusion

Finally, the impact of OGD and reperfusion on the iBMECs was considered. *In vitro* stroke models with OGD and reperfusion of iBMECs have been reported but using iBMECs cultured on collagen IV and fibronectin,\(^458,473\) which was recently found to cause iBMECs to be in an activated state\(^218\) (further discussed in Chapter 4). The studies presented in this section were conducted with iBMECs cultured on laminin 511-E8. iBMECs were subjected to 18 h OGD before reestablishment of normal oxygen and glucose levels. The TEER of the iBMECs after reperfusion was tracked and normalized to the value measured before the cells were placed in the chamber. As shown in Figure 6.6B, after 18 h of OGD followed by 8 h reperfusion, there was approximately a 50% decrease in the TEER in the cells subjected to OGD and no improvement with P188 treatment. Since other cells of the neurovascular unit are thought to significantly contribute to BBB injury during OGD, we repeated the experiment with iBMECs co-cultured with astrocytes (Figure 6.6A). As expected, we observed a TEER reduction in the cells exposed to 18 h OGD and 8 h reperfusion (Figure 6.6C). Interestingly, there was an improvement in barrier function of the iBMECs when treated with P188 at the onset of OGD (t = 0). Since P188 was administered to the apical side of the Transwells, we hypothesized that P188 may cross the iBMECs to interact with the astrocytes, which may in turn aid the function of iBMECs. Accumulation of \(^{14}\)C-labeled P188 in the brain of healthy rabbits upon peripheral injection has been reported,\(^474\) and augmentation of endothelial permeability due to OGD and reperfusion may allow for P188 molecules to pass through the barrier. If this is the case, we may also see an improvement in the TEER at later times of the iBMECs co-cultured with astrocytes under OGD if treated with P188 at the onset of reperfusion (t = 18 h).
However, this is difficult to confirm with this particular set of experimental conditions due to the recovery of iBMECs under OGD and reperfusion at later time points (data not shown).

![Diagram](A) Schematic illustrating how iBMECs were co-cultured with astrocytes in Transwells and polymer treatment administration to the apical side. (B) TEER of iBMECs after 18 h OGD and 8 h reperfusion was lower than the control and was not mitigated by 1 mM P188 treatment. (N = 4, n = 5). (C) Barrier function of iBMECs co-cultured with astrocytes after 18 h OGD and 8 h reperfusion was decreased compared to the control. When iBMECs were treated with 1 mM P188 at the onset of OGD (t = 0), the barrier function was improved compared to the OGD only condition (N = 4, n = 6).

To assess whether P188 is crossing the iBMECs, the concentration of P188 in the cell culture medium was quantified using a cobalt thiocyanate colorimetry assay developed by Ghebeh et al. Using iBMECs only, the cells were put under OGD for 18 h. P188 was added to the apical side of the Transwells at the onset of reperfusion. The medium was collected at 24, 48, or 72 h of reperfusion, and the concentration of P188 in the medium was measured. The concentration of P188 in the apical chamber decreased as time of
reperfusion increased (Figure 6.7A). In the basolateral chamber, no P188 was detected at 24 h after reperfusion; however, at 48 and 72 h of reperfusion, P188 was detected (Figure 6.7B). If P188 molecules traverse the iBMEC layer to interact with the astrocytes, the data in Figure 6.6C suggest that P188 is in the basolateral chamber within approximately 26 h from the time of P188 addition to the culture. However, no P188 in the basolateral chamber was detected 24 h after addition to the iBMECs in the P188 quantification experiment (Figure 6.7B). This may be due to the latter experiment being done with P188 treatment at the onset of reperfusion instead of at the onset of OGD since the dysfunction in iBMEC barrier function may be different during the OGD phase compared to the reperfusion phase. Another factor may be the sensitivity of the assay and potential adsorption of P188 molecules on the cell culture plasticware. For future investigations, it may be necessary to utilize more sensitive assays than the cobalt thiocyanate colorimetry assay. For enhanced mechanistic insight, future work could examine the rate of P188 transport across the BMECs in a healthy or stressed state and whether P188 transport occurs via transcytosis and/or through the paracellular space.

Because the health of BMECs and astrocytes are closely related, it is difficult to ascertain with the data collected so far whether the observed effect (i.e., improvement in TEER of iBMECs co-cultured with astrocytes under OGD treated with P188 at t = 0) is due to the traversed P188 molecules interacting with the astrocytes or whether there is a synergistic effect of P188 on the iBMECs and the astrocytes. To help answer this question, the viability and phenotype of the astrocytes co-cultured with P188-treated iBMECs under OGD and reperfusion should be characterized. Further, studies with P188 addition directly to the astrocytes in the basolateral chamber could be done. This experiment would also
help to determine whether polymer treatment of the BMECs or the astrocytes is more beneficial for the overall health of the neurovascular unit, which could inform the best method of delivery in animal studies of PPO-PEO block copolymer treatment of acute brain injuries, which has not been systemically studied but shown to influence therapeutic capabilities of P188. For instance, Frim et al. reported that intracisternal delivery of P188 was more effective in protection against glutamate toxicity in rats compared to intravenous delivery; BBB function was not assessed in this report. Interestingly, animal studies of acute neurological injuries that evaluated P188 to have an advantageous effect on BBB function utilized intravenous injections of P188. This may be because P188 interactions with the BBB would occur before interactions with neuronal cells if P188 is injected to the blood vessels. Further elucidation of the transport properties of P188 across the BBB and protective effects of P188 in the vasculature compared to effects in the brain is necessary for the development of P188 as a therapeutic for neurological disorders.

**Figure 6.7.** Concentration of P188 as measured by cobalt thiocyanate colorimetry assay in the apical and basolateral chambers of Transwells seeded with iBMECs as a function of time of reperfusion after 18 h of OGD. (A) P188 concentration in the apical chamber decreased with increase in time of reperfusion. (B) No P188 was detected in the basolateral chamber 24 h reperfusion. P188 was detected in the basolateral chamber after 48 and 72 h of reperfusion. Each time point is from one independent experiment.
6.4. Conclusion and Future Work

In this chapter, we present several experimental platforms that model events during ischemic stroke that damage the BBB, including glutamate and histamine excitotoxicity, H/R, and OGD/reperfusion. We also present preliminary results on the impact of PPO-PEO block copolymer treatment to iBMECs after H/R and OGD/reperfusion. We demonstrated that the barrier function of the iBMECs is disrupted when glutamate or histamine is added to the brain-facing side of the iBMECs. We modeled H/R using CoCl$_2$ and true hypoxia and observed that reoxygenation injury disrupts barrier integrity. Additionally, iBMECs with or without astrocytes were placed under OGD and reperfusion. P188 treatment at the onset of OGD was able to protect against the OGD and reperfusion injury to the iBMECs if the iBMECs were co-cultured with astrocytes. Elucidation of the differences of the cellular responses to PPO-PEO block copolymer treatment depending on whether the iBMECs are in mono-culture or co-culture has the potential to inform \textit{in vivo} studies.

With the exception of the work done with OGD/reperfusion, the iBMECs utilized for the studies were cultured on collagen IV and fibronectin. For future studies, it may be important to modify the culture conditions to mimic a healthy BBB before damage stimulation by conducting experiments with iBMECs cultured on laminin 511-E8. Nevertheless, the platforms outlined in this chapter establish experimental parameters for which damage to the iBMECs occurs as evidenced by a disruption to the barrier function. These damage models could be utilized to further assess the effect of PPO-PEO block copolymer treatment on iBMECs damaged by particular stresses as a potential strategy to better understand what ischemic stroke event(s) is/are targeted by PPO-PEO block copolymer treatment.
Chapter 7. Conclusions and Project Outlooks

7.1. Project Summary

Utilizing an in vitro model of the blood-brain barrier (BBB), we established in this thesis that poloxamer 188 (P188) and poly(propylene oxide) (PPO)-poly(ethylene oxide) (PEO) diblock copolymers can protect or rescue the BBB during certain disease conditions. Application of human induced pluripotent stem cell (hiPSC)-derived brain microvascular endothelial cells (iBMECs) enabled disease modeling with patient-derived hiPSCs as well as modeling of acute injuries to explicitly probe the impact of PPO-PEO block copolymers on damaged iBMECs. Use of an in vitro model also allowed for screening of various PPO-PEO block copolymers for therapeutic efficacy. We utilized confocal fluorescence microscopy to determine the localization of a PPO-PEO diblock copolymer and initiated work for a fluorophore-free, live-cell imaging strategy via confocal Raman microscopy. Insights gained from the work in this thesis may provide essential groundwork that bridges the gap between studies using non-living biophysical systems and in vivo studies to allow for translational research that enables the development of a therapeutic that targets the BBB during neurological injuries.

Chapter 3 described our work in studying iBMECs differentiated from hiPSCs derived from patients with childhood cerebral adrenoleukodystrophy (ccALD) via characterization of phenotype, genotype, and functionality. Compared to wild type (WT)-iBMECs, ccALD-iBMECs had decreased barrier function and increased lipid droplet accumulation, supporting the hypothesis that the BBB function in ccALD is inherently compromised. Treatment of ccALD-iBMECs with a PPO-PEO diblock copolymer, but not
P188, during development reversed these outcomes. This disease model may enable identification of biomarkers indicative of the onset of ccALD and development of BBB-targeting therapies for ccALD.

In Chapter 4, we discussed investigations of the effect of PPO-PEO block copolymer treatment on the BBB under hydrogen peroxide (H₂O₂)-induced oxidative stress. A screening of poloxamers and PPO-PEO diblock copolymers found \( t\)-P₁₅E₁₉₀ to be the most effective in restoring barrier integrity of iBMECs under H₂O₂ stress. When H₂O₂-challenged iBMECs were treated with \( t\)-P₁₅E₁₉₀, the cells had improved barrier function, less actin stress fibers, and lower intracellular calcium concentration. Efflux transporter function of iBMECs was unaffected by neither P188 nor \( t\)-P₁₅E₁₉₀. This platform could be employed for further screening of PPO-PEO block copolymers to parse out effects of various polymeric parameters on efficacy of restoring BMEC integrity upon oxidative stress and to gain a more complete understanding of the mechanism of action of how PPO-PEO block copolymers mediate protection against oxidative stress on the BBB.

Application of confocal fluorescence and Raman microscopy with the goal of visualizing the localization of PPO-PEO block copolymers in iBMECs was detailed in Chapter 5. Alexa Fluor 647 was chemically tagged to the end of the PEO block of \( t\)-P₁₅E₁₉₀ and subsequently used for confocal fluorescence microscopy experiments. Both the free fluorophore and fluorescently tagged \( t\)-P₁₅E₁₉₀ molecules were internalized in iBMECs after a 2 h incubation. For Raman microscopy experiments, a diblock copolymer of PPO-PEO with deuterated PEO was synthesized. While we were able to image the iBMECs via Raman spectroscopy and the carbon-deuterium band indicative of the deuterated block copolymer was present in spectral scans, no clear localization of the polymers could be
determined. This preliminary work shows promise that confocal Raman microscopy could provide a technique to image PPO-PEO block copolymers in live cells without the need for a fluorescent label to complement confocal fluorescence microscopy data.

In Chapter 6, we modeled the BBB during ischemic stroke events that induce BBB dysfunction, including glutamate and histamine excitotoxicity, hypoxia/reoxygenation (H/R), and oxygen-glucose deprivation (OGD)/reperfusion. We also presented preliminary results on the impact of PPO-PEO block copolymer treatment of iBMECs after H/R and OGD/reperfusion. We established experimental parameters for which damage to the iBMECs occurred as evidenced by a disruption to barrier function. Examination of iBMECs with or without astrocytes under OGD/reperfusion revealed differential response to P188 treatment depending on treatment timing and whether the iBMECs were co-cultured with astrocytes. P188 treatment at the onset of OGD protected the iBMECs against the OGD/reperfusion injury to the iBMECs if the iBMECs were co-cultured with astrocytes. The damage models outlined in this work could be utilized to assess the effect of PPO-PEO block copolymer treatment on iBMECs damaged by particular stresses to better understand what ischemic stroke event(s) is/are targeted by PPO-PEO block copolymer treatment.

7.2. Project Outlook

Future investigations specific to work in each chapter are discussed in the respective chapters. A common consideration for the work described in this thesis is the need for polymer concentration titration experiments. To limit parameters, we tested only 0.5 mM or 1 mM polymer solutions for treatment of iBMECs in accordance with other
studies examining endothelial cell treatment with P188. However, order of magnitude lower polymer concentrations have been utilized in other systems, and thus, lower dosages of polymers should be tested with the in vitro systems at hand as lower concentrations may be more physiologically relevant. Additionally, we address in the following subsections more general considerations in pursuit of deeper understanding of PPO-PEO block copolymer mediated rescue of the BBB including enhanced modeling of the neurovascular unit, characterization of transcellular transport, and consideration of the role of endocytosed polymers.

7.2.1. Expanded modeling of neurovascular unit and characterization of BBB function

The work in this thesis utilized a static in vitro model of the BBB, which was adequate for answering questions concerning the direct impact of PPO-PEO block copolymer treatment of BMECs under certain damage conditions. However, as we seek to deepen our understanding and connect our findings to in vivo studies, there is need to enhance our model of the BBB to better mimic relevant in vivo characteristics of the BBB and the surrounding neurovascular unit (NVU). As with any model, what complexities to add will depend on the question at hand. As mentioned in previous chapters, a strategy to more closely mimic the in vivo microenvironment is to utilize co-culture models and/or microfluidic devices. As discussed in Chapter 6, other cells of the NVU can be co-cultured with iBMECs in a static Transwell configuration by culturing pericytes and/or astrocytes in the basolateral chamber. Cells could also be cultured in contact with BMECs by seeding cells on the underside of the membrane support with BMECs on the upper side. Neurons or microglia could be included in the co-culture system as well. Additionally, dynamic
models can be utilized to take flow conditions into account,\textsuperscript{475} since blood flow subjects the BBB to shear stress, which upregulates junctional and drug efflux proteins in endothelial cells.\textsuperscript{476,477} Researchers have employed various microfluidic device designs for “BBB-on-a-chip” applications, with some designs allowing for 3-D culture of iBMECs and/or co-culture.\textsuperscript{219,478–483} With iBMECs in a microfluidic device, PPO-PEO block copolymers could be administered to iBMECs by a flow of polymer solution, which would be more mimetic of how the polymers would come into contact with the BBB \textit{in vivo}.

Additionally, there is need to consider the glycocalyx and transcellular transport in the context of BBB damage and rescue via PPO-PEO block copolymers. The glycocalyx is a negatively charged, thin, and dense layer of carbohydrate-rich molecules on the luminal side of healthy BMECs that contributes to the tightness of the BBB by limiting diffusion of molecules based on their size and electrical charge.\textsuperscript{484–486} There is evidence that the glycocalyx contributes to BBB breakdown,\textsuperscript{484,485} and PPO-PEO block copolymer diffusion through the glycocalyx may influence interactions with the BMECs. However, the glycocalyx of iBMECs has not been characterized, and addition of glycocalyx molecules to iBMECs may be necessary to recapitulate the glycocalyx structure of BMECs \textit{in vivo}.$^{221}$ Future work could include the investigation of the glycocalyx of iBMECs and modification to appropriately mimic \textit{in vivo} conditions.

Finally, most of the functional characterization of the iBMECs in this work has focused on paracellular permeability (e.g., trans-endothelial electrical resistance, cell-impermeable solute permeability, tight junction protein organization). A hallmark characteristic of the BBB is the low rate of pinocytotic activity,\textsuperscript{487,488} and key regulators of BBB function involve suppression of transcytosis.\textsuperscript{489,490} Recent studies have elucidated that
rates of transcellular transport increase during various pathological conditions.\textsuperscript{491,492} Especially relevant to the work in this thesis is that damage to the BBB in mice models of ischemic stroke starts with opening of the transcellular barrier in the early hours of reperfusion followed by opening of the paracellular barrier.\textsuperscript{326,493,494} \textit{In vitro}, oxidative stress on endothelial cells resulted in an increase in transcellular permeability.\textsuperscript{495} Therefore, there is need to study the effect of specific BBB damage conditions on the rate of transcellular transport and to assess whether PPO-PEO block copolymers can inhibit an increase in transcellular permeability.

7.2.2. Probing the significance of intracellular stores of P188

Gigout \textit{et al.} observed that P188 molecules are endocytosed (i.e., consumed into the cell by invagination of the plasma membrane) in Chinese Hamster Ovary cells after 1.5 h of incubation and hypothesized that P188 in intracellular vesicles may contribute to shear protection conferred by alteration of cytoplasmic mechanical properties.\textsuperscript{121} We have also shown evidence that PPO-PEO diblock copolymers may be internalized into vesicles in iBMECs (Chapter 5). The two preceding studies were conducted with cells under no stress, and localization may change depending on the type of stress. For the iBMECs, the localization may also change under flow conditions. These issues prompt additional microscopy studies. There still remains the question of whether internalized PPO-PEO block copolymers play a role in polymer-mediated cell protection.

We propose investigations to assess how protection efficacy of the block copolymers changes depending on the localization of the molecules. Preliminary investigations were done with C2C12 myoblasts, as they are easier to culture than iBMECs,
and our colleagues have previously observed via confocal fluorescence microscopy that fluorescently labeled P188 molecules are internalized after 2 h of incubation in C2C12 myoblasts (data not shown). We utilized endocytosis inhibitors to modulate polymer localization and assessed with a functional assay whether polymer protection efficacy changes with or without endocytosis inhibition. Polymer localization was presumed to be at the cell membrane with endocytosis inhibition and in intracellular compartments without endocytosis inhibition. Endocytosis can be broadly divided into two categories: phagocytosis and pinocytosis. Pinocytosis is further classified into clathrin-dependent and independent pathways, the latter including caveolae-mediated endocytosis. Sahay et al. observed that poloxamer 235 (P235) unimers were internalized via caveolae-mediated endocytosis in Madin-Darby Canine Kidney cells (interestingly, P235 micelles were internalized via clathrin-dependent endocytosis). However, since it is not yet confirmed by which pathway P188 molecules are endocytosed into C2C12 myoblasts, two types of inhibitors were applied. Chlorpromazine and genistein were utilized to inhibit clathrin-dependent and independent endocytosis, respectively, following previous reports employing these chemicals in C2C12 myoblasts. In the presence or absence of the endocytosis inhibitors, the cells were subjected to a modified osmotic stress assay similar to the protocol by Kim et al., utilizing the same buffers as described in Chapter 5, to evaluate the ability of P188 to protect the cells against osmotic stress. The experimental procedure was as follows.

C2C12 myoblasts (ATCC) were seeded at a density of 3000-4000 cells/well in a 96-well plate, and the experiment was conducted two days later. Cells were pre-incubated for 30 min with or without 30 μM chlorpromazine (Millipore Sigma) or 200 μM genistein.
(Millipore Sigma) (the same concentrations of the endocytosis inhibitors were used for the next steps). The cells were then incubated in the isotonic (330 mOsm, pH 7.2) or hypotonic (134 mOsm, pH 7.2) buffer for 2 h with or without 14 μM P188 and with or without the endocytosis inhibitors. Next, the cells were incubated for 1 h in the isotonic or hypotonic buffer with or without the endocytosis inhibitors. Finally, the cells were lysed by Triton X-100. Cells were stored in a 37°C, 5% CO₂ incubator for all incubation steps. The supernatant solutions were extracted at each step, and the level of lactate dehydrogenase (LDH) was determined with the LDH Reagent Set (Pointe Scientific) by measuring absorbance at 340 nm with a BioTek Synergy H1 multi-mode microplate reader. The concentration of LDH was calculated from the absorbance readings following the manufacturer’s instructions.

**Figure 7.1.** (A) LDH release from myoblasts after 2 h in isotonic buffer ± endocytosis inhibitors ± P188 and 1 h in isotonic buffer ± endocytosis inhibitors. Low release levels for all conditions indicates no cytotoxic effect from the endocytosis inhibitors. (B) LDH release from myoblasts after 2 h in hypotonic buffer ± endocytosis inhibitors ± P188 and 1 h in hypotonic buffer ± endocytosis inhibitors. CPZ = chlorpromazine; GN = genistein. Data are plotted as mean ± standard deviation and are from 1 independent experiment with 3 – 4 biological replicates. Trends of data plotted in B were confirmed with an additional independent experiment.
As a control experiment, cells were incubated in an isotonic buffer with or without P188 and then incubated for 1 h in isotonic buffer. A subset of the samples contained chlorpromazine or genistein during both incubation steps. Since the cells were maintained in isotonic buffer throughout the experiment, the amount of LDH released was expected to be low and constant for all conditions if there were no cytotoxic effects. At the end of the 3 h incubation period, cells exposed to chlorpromazine without P188 (labeled “CPZ”) discharged slightly higher levels of LDH compared to cells under other conditions, but all LDH release levels were low, suggesting no significant cytotoxic side effects from the endocytosis inhibitors (Figure 7.1A).

Next, cells were incubated in a hypotonic buffer with or without P188 and then incubated for an additional hour in hypotonic buffer. A subset of the samples contained chlorpromazine or genistein during both incubation steps. As expected, the LDH release from cells that were incubated with P188 was lower than from cells that did not receive P188, signifying that P188 protected the cells from osmotic stress. However, if the cells were incubated with P188 as well as the endocytosis inhibitors, the protective effects disappeared (Figure 7.1B). Since P188 was not present in the second incubation step, intracellular stores of P188 may have provided the observed protection against osmotic stress. In conditions where endocytosis was inhibited, intracellular stores of P188 would not have been generated, leading to no protection against osmotic stress. These results suggest that internalized polymer molecules may play a role in cell protection, though additional replicate experiments must be conducted to confirm these results.

The interpretation of the data above heavily relies on the notion that P188 resides near or on the cell membrane when endocytosis is inhibited and that P188 is present in
intracellular compartments in the absence of endocytosis inhibitors. Thus, follow-up studies for this line of investigation should include confirmation via confocal fluorescence microscopy that PPO-PEO block copolymer internalization occurs via endocytosis (discussed further in Chapter 5) and that P188 localization changes in cells when treated with either chlorpromazine or genistein. This study would also confirm whether P188 endocytosis occurs through a clathrin-dependent or independent pathway. Additional control studies could be conducted with chlorpromazine and genistein, as endocytosis inhibition is known to be highly dependent on cell type and cell lines, and sometimes one agent may inhibit multiple endocytosis pathways.\textsuperscript{499–501} Thus, myoblasts incubated with fluorescently labeled molecules of known endocytic pathways and treated with chlorpromazine or genistein can be analyzed via confocal microscopy or flow cytometry to ascertain that each chemical agent inhibits the respective targeted endocytosis pathway. Fluorescently tagged transferrin and lactosylceramide are often utilized for markers for clathrin-dependent and independent endocytosis, respectively.\textsuperscript{501–503} Depending on the results of these studies (e.g., if chlorpromazine or genistein is found to non-specifically inhibit endocytosis), other endocytosis inhibitors may need to be utilized. Overall, the experiments proposed here may provide important insight into the effect of P188 localization on protection efficacy.
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