Pulmonary Drug Delivery via Reverse Perfluorocarbon Emulsions: A Novel Method for Bacterial Respiratory Infections and Acute Respiratory Failure

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ABSTRACT

Pulmonary Drug Delivery via Reverse Perfluorocarbon Emulsions:
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by

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Inhaled drug delivery is currently the gold standard for treating many respiratory diseases. However, improved treatments are needed for lung diseases like Cystic Fibrosis (CF) and Acute Respiratory Distress Syndrome (ARDS), where mucus or fluid build-up in the lung limits ventilation and, thus, delivery of inhaled drugs. Delivery is most needed in the diseased or damaged regions of the lung, but if an area is not ventilated, inhaled drug will simply not reach it. To overcome this, this research proposes delivering drugs to the lungs within a perfluorocarbon (PFC) liquid. The lungs will be filled with a reverse emulsion containing a disperse phase of aqueous drugs within the bulk PFC and then ventilated. The PFC functions as both a respiratory medium, providing gas exchange, and as a delivery vehicle, providing a more uniform deposition of drugs. After treatment, the highly volatile PFCs are exhaled, returning the patient to normal respiration.

This technique improves upon current therapies as follows. First, drugs are delivered directly to where they are needed, yielding higher concentrations in the lung and lower systemic concentrations. Second, PFCs are ideal for washing out lung exudate and mucus. The low surface tension and high density of PFC allows it to easily penetrate plugged or collapsed alveoli, detach infected mucus from the airway walls, and force

these fluids to the top of the lungs where they can then be removed via suction. Mucus and exudate removal should allow drugs to penetrate previously plugged airways during emulsion delivery and subsequent treatment with inhaled therapies. Thus, drug delivery via emulsion would be used as a pre-treatment to enhance inhaled or systemic drug therapy. Third, PFC's anti-inflammatory properties help return to normal lung function.

This research examines two applications of this technology: delivery of antibiotics to combat respiratory infections (antibacterial perfluorocarbon ventilation, APV) or delivery of growth factors to enhance alveolar repair (perfluorocarbon emulsions for alveolar repair, PEAR). This work represents an in-depth analysis of the emulsions used during APV and PEAR. Initial efforts evaluated emulsion efficacy under *in vitro* setting that better simulated lung *in vivo* antibiotic delivery. The subsequent studies utilized an *in vivo* rat model of bacterial respiratory infection to validate the effects of emulsion on pharmacokinetics and to assess APVs potential treatment benefits. Lastly, *in vitro* methods of cellular response assessed the utility of delivering growth factors in PEAR.

Significant advancements were made in optimizing the emulsion as a viable means of pulmonary drug delivery. Final efforts resulted in a promising emulsion formulation that overcame the quick transport of tobramycin away from the lung and successfully reduced pulmonary bacterial load *in vivo*. *In vitro* applications of PEAR showed the emulsions posed a significant barrier to the availability and, thus, the biological effect of lysophosphatidic acid growth factors. Further *in vivo* work is required to improve APV's efficacy over conventional treatments and to determine PEAR's feasibility and efficacy in promoting lung repair.

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Introduction

1.1 Motivation and Objectives

Improved treatments are needed for lung diseases like Cystic Fibrosis (CF) and Acute Respiratory Distress Syndrome (ARDS), where mucus or fluid build-up in the lung limits ventilation and gas exchange. In CF, a defective gene causes the secretion of a thickened, more viscous mucus that impairs mucociliary clearance and leads to recurrent or chronic bacterial infections, a prolonged inflammatory state, airway plugging, and an accelerated decline in respiratory function.[1], [2] These chronic or repeated infections and the resulting lung inflammation are what causes the patients' decline in respiratory function.[3] In ARDS, a lung injury, typically an infection, initiates a severe inflammatory response that damages the lung epithelium and microvasculature, resulting in edema filling the alveoli and impeding normal gas exchange.[4], [5] This edema disrupts surfactant effects, causing changes in lung compliance and local atelectasis.[6]

The current standard of treatment for these respiratory diseases and infections is systemic and/or inhaled drug delivery. However, systemic delivery of antibiotics requires higher blood concentrations to achieve therapeutic levels in the lung, increasing the risk of systemic toxicity.[7] Inhaled treatment of both antibiotics and growth factors achieves direct delivery to the lung, producing greater pulmonary drug concentrations with lower systemic concentrations.[8], [9] However, inhaled delivery is several limited by the narrow range of particle sizes required to effectively reach the source of disease.[10] As a result,

the majority of inhaled drugs are deposited in the mouth and throat.[11]–[14] Furthermore, diseased areas of the lung have a greater degree of airway obstruction, like edema or mucus, and thus receive a lesser degree of ventilation and inhaled drugs.[11], [12] Inhaled drug delivery thus lies in a frustrating paradox: drug necessary to recover normal respiratory function cannot be delivered without elimination of edema / mucus, but these pathologies cannot be eliminated without recovery.

The goal of this research was to develop an improved method of drug delivery that overcomes the disadvantages of current treatment options. We developed a means to maintain direct delivery to the lungs but remove the dependence on airflow by filling the lung with a perfluorocarbon liquid (PFC). Liquid PFCs are an excellent respiratory medium, uniformly filling injured lungs and maintaining gas exchange. This treatment, termed antibacterial perfluorocarbon ventilation (APV) for lung infections and perfluorocarbon emulsions for alveolar repair (PEAR) for lung injuries, involves filling and subsequent ventilation of the lungs with aqueous drugs emulsified within PFC. Such an application represents a relatively unexplored means of treatment and a new use of the specific emulsifiers used in this work. Therefore, the goal of this work was to characterize the effects of various emulsion formulations on the utility of APV/PEAR treatment. Evaluations included the emulsion's physical properties, bactericidal and reparative abilities, and pharmacokinetics and efficiency as a means of drug delivery.

1.2 Bacterial Respiratory Infections in Cystic Fibrosis

Acute lower respiratory infections cause more disease and death than any other infection in the United States.[15] Systemic or inhaled antibiotics can effectively treat many of these

cases, however, improved treatment is still needed in the case of infection during lung disease, such as Cystic Fibrosis. Cystic Fibrosis (CF) is characterized by a thickening of the respiratory mucus and a proinflammatory airway environment that make these patients susceptible to bacterial lung infections and limit the effectiveness of current antibiotic therapy.

Under normal physiological conditions, the airway surface is lined with a liquid consisting of a biphasic film: an aqueous sol (or periciliary) layer immediately atop of the airway epithelial cells followed by a more gel-like mucus layer. The sol layer, a purely viscous layer (5-10 μm), surrounds the cilia of the epithelial cells, acting as a lubricant to allow their high-frequency beating.[3], [16] The mucus layer, a viscoelastic layer (0.5-10 μm), is composed of water, ions, lipids, proteins, and high molecular weight glycoproteins called mucins.[3], [17] Mucins have a rod-like structure (500-900 nm long, 10 nm wide) and are cross-linked into an entangled three-dimensional network by disulphide bonds.[18] It is this network that gives mucus its non-Newtonian, viscoelastic behavior, exhibiting characteristics of both an elastic solid (resists flow) at low shear stress and a viscous fluid (flows) at higher stresses. The mucus layer along with the movement of the underlying cilia are critical to the innate immune system in the airways. When inhaled particulate or pathogens deposit on the surface of the airways, they become entrapped in the adhesive mucus layer. The beating cilia lining the epithelium then propel the overlying mucus toward the oropharynx where it can be expectorated or swallowed.

The mucus environment is significantly altered during lung disease. In CF, abnormally viscous mucus rheology impairs mucociliary clearance. This retention of mucoid secretions leads to a prolonged inflammatory state, airway plugging, recurrent or

chronic bacterial infections, and an accelerated decline in respiratory function.[1] CF is a multi-system disorder, affecting secretory epithelia in the lungs, salivary glands, pancreas, liver, kidneys, sweat ducts and reproductive tract, however the vast majority of morbidity and mortality is associated with lung disease.[19]

1.2.1 Clinical Definition and Diagnosis of Cystic Fibrosis

Most CF patients are diagnosed at birth as part of the national mandatory screening of newborns, with two-thirds of patients diagnosed by 1 year of age.[20] Screening algorithms for CF rely on testing for high levels of a pancreatic protein, immunoreactive trypsinogen (IRT).[21] The ultimate diagnosis of CF include either genetic testing or positive sweat chloride tests (CF salt levels in sweat: >60 mEq/L; healthy salt level in sweat: < 30 mEq/L) and one of the following: typical chronic obstructive pulmonary disease, documented exocrine pancreatic insufficiency, or positive family history. A number of other tests are used to either confirm or refute a diagnosis of CF, including chest and abdominal radiography, nasal electrolyte transport tests, pulmonary function tests, inflammatory markers in bronchoalveolar lavage fluid, and sputum microbiology.

Clinical Presentation

Given the median age at diagnosis is 6-8 months, the clinical presentation, severity of symptoms, and rate of disease progression in the organs involved varies widely.[20] Typically, individuals with CF have normal lung function in utero, at birth, and after birth, before the onset of infection and inflammation. Shortly after birth, many infants acquire a lung infection, which incites an inflammatory response. Airway inflammation is the

hallmark of lung disease in patients with CF, with some studies suggesting inflammation presents even in the absence of infection.[1], [22]–[24] GI tract presentation in fetuses and neonates may be in the form of bowel obstructions, steatorrhea, and/or failure to thrive. Patients younger than 1 year may present with wheezing, coughing, and/or recurring respiratory infections and pneumonia. Patients diagnosed later in childhood or in adulthood are more likely to have sufficient secretion of pancreatic enzymes and often present with chronic cough and sputum production. Approximately 10% of patients with CF remain pancreatic sufficient; these patients tend to have a milder course.[25]

1.2.2 Pathophysiology of Cystic Fibrosis

The genetic basis of CF is well established to be mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene that codes for an apical membrane chloride channel principally expressed by epithelial cells. The CFTR protein actively transports chloride and bicarbonate toward the airway surface, secondarily bringing water with these ions. Mutations in this gene cause airway acidification and impaired ion movement across the epithelium, resulting in the hyperabsorption of water at the airway surface. Mucus dehydration during CF causes an increase in the relative fraction of glycoproteins, resulting in a significant increase in mucus viscosity and adhesivity.[26], [27] Airway acidification also increases surface-liquid viscosity.[28] It is believed that elevated levels of DNA in the mucus due to inflammatory cell necrosis also induce changes in rheology during such diseases.[3], [29] These rheological abnormalities result in significantly impaired mucociliary transport and inefficient cough clearance.[3] Additionally, an inflammation-induced conversion of water-secreting sol cells to mucus-

secreting cells causes a hypersecretion of mucus, resulting in a mucus thickness much larger than that of healthy individuals.[30] This also causes a depletion of the sol layer, resulting in mucus encroaching upon the cilia of the epithelial cells, further impairing clearance of the abnormally thick mucus already present in excessive amounts.[30], [31] The inability to properly clear mucus represents a critical breakdown in the innate immunity of the lung and, leaving these patients vulnerable to respiratory infection.

Bacterial infections are common in CF patients, with infections typically beginning during childhood.[2] Common and early acquisition of infection for CF patients is thought to be due to impaired mucociliary clearance, increased binding ability of bacteria to CF respiratory epithelial cells, and impaired ingestion of bacteria by CF host immune cells.[2] Bacterial respiratory infections during CF can be due to a variety of organisms, with some of the most common being *Pseudomonas aeruginosa (PA)*, *Staphylococcus aureus*, *Haemophilus influenza*, and *Stenotrophomonas maltophilia*.[32] *PA* is widely considered the most common and damaging bacterial pathogen during CF. Over 70% of US CF patients are chronically infected with *PA* by early adulthood and it is nearly impossible to eradicate.[9], [33], [34] *PA* is a Gram-negative bacterium found in moist settings throughout the environment. *PA* is thought to enter the lower airways through the mouth or nasal passage and then bind to components of the mucus layer residing on top of the cilia.[34] *In vitro* studies have shown that *PA* has the unique ability (not observed in other pathogens evaluated) to adhere to mucins as well as exhibit a chemotactic response to CF mucin.[35] After the initial infection, *PA* typically undergoes a phenotypic adaption in which the organism converts to a mucoid variant characterized by the copious formation of exopolysaccharide alginate-coated microcolonies, commonly referred to as the biofilm

mode of growth.[11], [33], [34] This form of *PA* is nearly uniformly present during chronic lung infection in CF patients.[34] Growth within the thick biofilms has been shown to grant a significant degree of resistance against antibiotics to the *PA* cell, with organisms within biofilm withstanding 20 to 1,000 times greater concentrations of antibiotic than that required to kill the nonmucoid form.[36], [37] Additionally, the alginate matrix provides a degree of protection against the native immune response as well.[38]

Although the inflammatory response is an integral part of innate immunity, it can often play a detrimental role during lung disease or injury. A change in mucus rheology and production impairs host respiratory defense mechanisms, thereby presenting an opportunity for bacterial pathogens to colonize within the airways. These chronic or recurrent bacterial infections result in periods of prolonged inflammation with significantly increased neutrophil presence in the airways and pulmonary circulation.[39] During binding and phagocytosis of a pathogen, neutrophils release a number of enzymes and reactive oxygen species to aid in the degradation of the foreign matter. The release of these molecules can be harmful to host bodily tissues. Consequently, much of the epithelial damage present during CF can be attributed to neutrophil-derived proteases and oxidants.[40], [41] This damage to the respiratory mucosa, along with increased secretions, further hinders mucociliary clearance, and thus perpetuates infection.[42] Such a cycle is described by the “vicious circle” hypothesis (Figure 1.1).

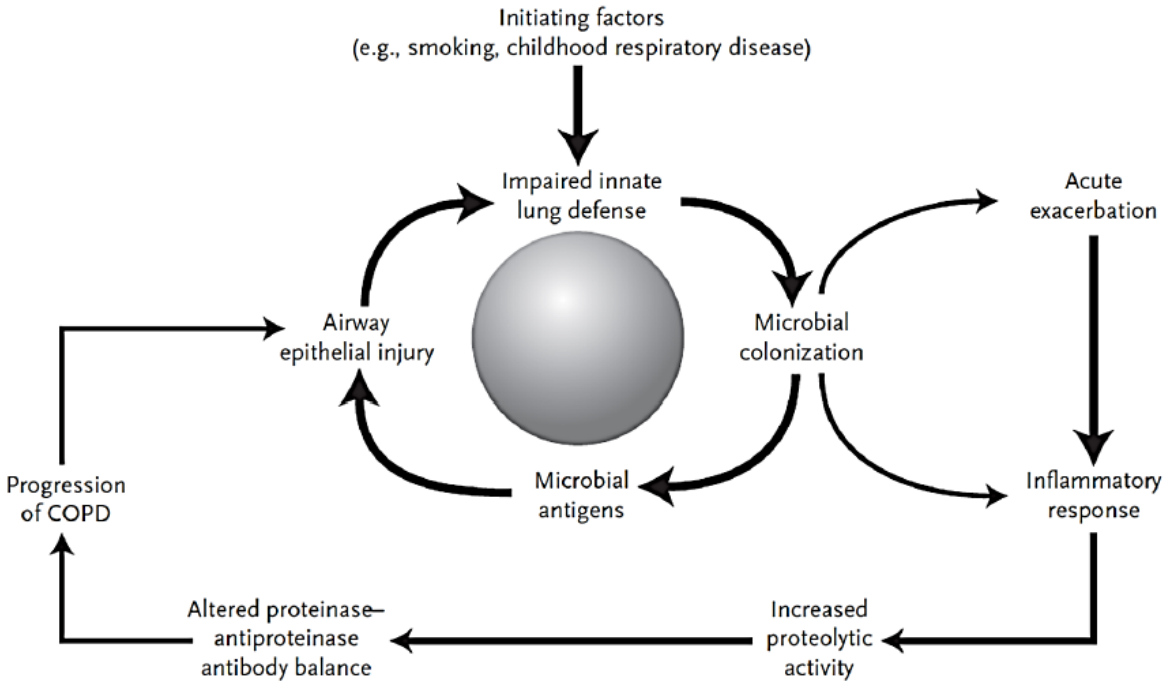

Figure 1.1 Schematic of the "vicious circle" hypothesis.[42]

1.2.3 Epidemiology

Incidence and Mortality

CF is the most common lethal hereditary (autosomal-recessive) disease in Whites. Its estimated heterozygote frequency in Whites is up to 1 in 36; offspring of heterozygote parents have a 25% chance of developing CF.[43] In the United States, Whites have the highest prevalence (1 per 3500) followed by Hispanics (1 per 9500), African Americans (1 per 17000), and then Asian Americans (1/31000). There are currently about 30,000 people living with CF in the United States and approximately 1,000 new cases are diagnosed each year.[44]

The life expectancy for patients with CF has greatly increased in the past years.[45] Thirty years ago, CF patients were not expected to reach adulthood, however, progress in medical and surgical treatment options have improved life expectancy to 80%

of patients reaching adulthood. About half of the patients are older than 18, and the median age of survival for a person with CF is currently 33.4 years, with patients living into their fifties or sixties.[32], [46] *PA* infection is one of the primary contributors to the short life expectancy of CF patients, with one study showing more than five times lower mortality rates for uninfected patients as compared to infected patients.[47] Most deaths result from progressive and end-stage lung disease.

Living with CF

While the diagnosis of CF is often confirmed while the patient is still a child, the onset of the symptoms becomes progressively more apparent over time. Children with CF tend to remain healthy when medicated until adulthood; as lung function declines, the patients often become disabled. Studies have shown adolescents suffer an increased likelihood of psychiatric problems, such as depression, and of scoring poorly on physical functioning measures of quality of life.[48] The increase in these symptoms is usually accompanied by the transition toward taking care of their own treatments. As mentioned previously, respiratory diseases are the most common sequela and involve inflamed and damaged airways and chronic infections, which ultimately end in cor pulmonale and end-stage lung disease.[49] In addition to a steady decline in function, these infections lead to exacerbations of the disease state. A pulmonary exacerbation of CF is usually identified by an increase in cough and sputum and a decrease in pulmonary function. Each year, 27% and 44% of patients under and over 18 years of age, respectively, experience an exacerbation.[32] The average hospital stay per exacerbation is 8 - 10 days.[50] Adults tend to experience additional health challenges including CF-related diabetes and other

complications of the digestive system, osteoporosis, renal disease, cancers, drug allergies and toxic effects, and complications associated with lung transplantation. In addition, over 95% of men with CF are sterile.[49]

Economic Impact of CF

Long-term estimates of CF care costs reported an average annual total cost of $24,768. The majority of the cost are due to outpatient drug costs (53%) and hospitalizations (32%).[51] Treatment costs range from the cost-saving home antibacterial therapy to the cost-intensive recombinant human DNase treatment.[52] Healthcare costs are dependent upon age, with cost being twice as high for adults than for children, the degree of disease severity, and other factors. Lifetime direct costs of CF are estimated at $200,000 - $300,000. The total annual medical cost of treating these patients is $630 million.[53]

1.3 Current Treatment for Cystic Fibrosis

A new era of precision medicine in CF therapeutics has begun with the prescription of small molecules that successfully target the underlying defects and improve CFTR function in a genotype-specific manner. Despite these advances, respiratory infection remains one of the primary contributors to the short life expectancy of CF patients.[47] As previously discussed, the series of persistent infection and inflammation lead to eventual respiratory failure. Thus, the primary goal of treatment is maintaining lung function as near to normal as possible by clearing airways of mucus and controlling respiratory infection.

1.3.1 Airway Clearance Techniques

The rationale for why airway clearance techniques (ACTs) are used in CF is well documented.[54], [55] The underlying theory is that facilitating secretion clearance will reduce bacterial load, decrease infection and inflammation of the lungs and thus reduce airway damage, ultimately delaying the disease process.[56] A number of new airway clearance devices have been developed with the aim of improving efficiency and optimizing outcomes in CF, including chest physical therapy by hand; forced expiratory technique; positive expiratory pressure masks; high-frequency oscillation using a Flutter device; and high-frequency chest compression using a thAIRapy Vest. Despite several studies, there is still no evidence to suggest that one airway clearance technique has superiority over any other.[54] The only clear conclusion is that there may be some short-term benefit to airway clearance compared to no airway clearance.[55]

1.3.2 Antibiotics

Chronic *PA* lung infection is caused by biofilm-growing mucoid strains. Biofilms can be prevented by early, aggressive antibiotic prophylaxis or therapy, and they can be treated by chronic suppressive therapy. Both the bacterial strains present and the intended delivery method determine the type of antibiotic to be used during respiratory infection treatment. Tobramycin, azithromycin, and aztreonam lysine are the most commonly used antibiotics for treatment of infections during CF.[32] tobramycin and azithromycin have similar bactericidal mechanisms which involves binding to bacterial ribosomes and thereby inhibiting the translation of mRNA in to essential proteins. Alternatively, aztreonam lysine inhibits synthesis of vital polymers in the bacterial cell wall. In order for

these, or any, antimicrobial agent to be effective, the agent must reach the site of infection and remain there for a sufficient length of time.[9] This task is an especially challenging one in the case of respiratory infections, particularly for infections overlaid with preexisting lung disease. Systemic (intravenous, IV) administration and inhaled delivery are currently the primary means of antibiotic delivery during bacterial respiratory infection.

Systemic Delivery

Systemic delivery grants the largest degree of freedom with regards to drug selection, as nearly all antibiotics can be delivered in this manner. However, systemic delivery has several disadvantages with regards to effective delivery to the site of infection.[9] When administered IV, antibiotics must first travel throughout the bloodstream before arriving at the pulmonary circulation. Thus, delivery may be limited in any disease or injury in which pulmonary blood flow is compromised. Antibiotics are believed to move from the blood into the bronchial space via diffusion along a concentration gradient.[57] Once in the lung, the antibiotics must then penetrate respiratory mucus to reach the infection. Due to the effect of sputum binding on antibiotic availability to the bacteria, endobronchial antibiotic concentrations of 10 to 25 times the minimum inhibitory concentration are required in order to be effective.[58], [59] In addition, many of the antibiotics used (including aminoglycosides such as tobramycin) have exhibited poor diffusion across lipid membranes and penetration of the respiratory sputum.[34], [58], [60] For these reasons, large IV doses must be given in order to be effective at the site of infection, resulting in high serum concentrations that can increase the potential for nephrotoxicity and ototoxicity.3

Inhaled Delivery

Inhaled aerosolized delivery of antibiotics offers an attractive alternative to systemic administration in that delivery is specifically targeted at the lung. A suspension of drug-containing solid particles or liquid droplets in air (or other gases) is inhaled by the patient. A variety of devices have been created to achieve inhaled delivery.

Metered dose inhalers refer to devices that deliver a specific mass of drug for each actuation of the device. Such delivery is achieved with liquids via a pressurized metered dose inhaler (pMDI) which uses liquefied gas propellants (typically hydrofluoroalkanes) to emit droplets of liquid drug that can be inhaled by the patient. Metered doses of dry drug formulations are achieved with a dry powder inhaler (DPI). These devices rely on the patient's own inspiratory effort to pull drug from the dosing chamber and as such require fairly high inspiratory flows to be used effectively. Dry powder drug formulations used with DPIs require shorter treatment times (one to two breaths) and much less cleaning and thus represent an attractive alternative to nebulized delivery. Dry powder antibiotic formulations of both tobramycin and colistimethate sodium (colistin) have recently been developed and are currently approved for use in CF patients.

Nebulizers, on the contrary, refer to continuous delivery devices that deliver aerosolized drug at a constant flow while the patient continuously breathes over periods of up to 30 minutes. Aerosolization of the liquid drug in this setting is typically achieved through the use of an air jet, ultrasonic sound waves, or a vibrating mesh. Nebulizers tend to offer a wider variety of deliverable drugs than metered dose inhalers but also tend to be larger and less portable. Nebulizers also tend to be less efficient than metered dose inhalers due to drug emitted during exhalation that is not inhaled, although some newer

nebulizers have incorporated features to pause delivery during exhalation. The first inhaled antibiotics were delivered via a nebulizer, with aerosolized tobramycin being the first approved by the Food and Drug Administration (FDA) in 1997. Currently approved nebulized antibiotics include aztreonam lysine and colistin in addition to tobramycin. Parenteral formulations of gentamicin, ceftazidime, and amikacin are also commonly used “off-label” via nebulized delivery.[63] As previously mentioned, nebulizers tend to require long delivery times (up to 30 minutes) and thus are often considered a significant inconvenience to patients. Additionally, nebulizers require frequent and thorough cleaning in order to maintain proper functionality and avoid microbial growth. Issues such as these are thought to contribute to poor patient adherence and thus affect the practical effectiveness of nebulized treatment.

As one may expect with a more targeted delivery approach, inhaled antibiotics have been shown to produce higher intrapulmonary concentrations while limiting absorption into the blood relative to systemic administration. One study showed that inhaled nebulized antibiotics produced as much as 14 times greater antibiotic concentrations in the sputum with seven times lower systemic levels relative to IV delivery.[9] A study comparing nebulized tobramycin with a dry power formulation (via a DPI) showed larger lung depositions, similar central to peripheral distributions, and similar serum profiles for the powder formulation relative to nebulized tobramycin.[64]

Although inhaled antibiotics have shown benefits over systemic treatment and have been proven effective against many cases of infection, significant shortcomings still exist, especially in the setting of CF. The use of inhaled delivery in these patients is inherently flawed due to its innate dependence on airflow. Poor ventilatory capacity due

to both irregular physiology and mucus production significantly hinders effective delivery to the most diseased regions of the lung. In the case of CF, mucus plugs are primarily composed of negatively charged glycoproteins and DNA, causing positively charged aminoglycosides (such as tobramycin) to potentially bind to these compounds and be rendered unavailable.[65], [66] During chronic infection with *PA*, bacteria is thought to reside in airway generations all the way down to the bronchioles.[11] Thus, the high sputum production and mucus plugging typically present within the bronchi of the infected host often prevents distal drug deposition in the regions of highest infection.[11], [12] By relying on the convection of air to distribute the delivered drug throughout the lung, inhaled delivery preferentially treats the well ventilated, typically less burdened, regions of the lung. This is supported by research showing that a lower forced expiratory volume in CF patients correlated with a smaller proportion of delivered drug reaching the periphery.[12]

The narrow range of aerosolized particle sizes required to effectively penetrate the lower airways presents further challenges.[10], [11] Aerosolized particles should be in the size range of 1-5 μm in order to deposit in the lower airways.[67] Particles with a mass median aerodynamic diameter (MMAD) of greater than 5 μm tend to precipitate within the conducting airways.[10] Particles with a MMAD of less than 1 μm have a greater chance of being exhaled.[67] Even if the average particle diameter is 2-4 μm, a larger range of particle sizes is always present, resulting in less than ideal deposition. Studies quantifying aerosol drug deposition in the lung are quite variable but have shown that 25-95% of emitted drug never reaches the lung, with the majority deposited in the oropharynx and delivery device.[11]–[13], [68] Deposition tends to be even worse in very young children with reported values as low as 2% lung deposition.[69] Patients on a mechanical ventilator

also present additional challenges in achieving effective inhaled delivery. Lung deposition of nebulized drug during mechanical ventilation is approximately 20% of the delivered dose, with the majority of drug deposited in the delivery device and ventilator tubing.[70], [71] Of the drug that does reach the lung during inhaled delivery, the majority is confined to the central lung (defined as the central 2/3 of the total lung), with one study showing only 16% of pulmonary tobramycin being deposited peripherally in CF patients.[12] The nonuniform intrapulmonary distribution of delivered antibiotics resulting from these challenges can lead to the inability to clear the infection as well as promote the development of antibiotic resistance.[34], [72] Given the seriousness of these infections, improved methods are needed for antibiotic delivery.

Chronic inflammation, impaired mucociliary transport, and poor ventilation and drug delivery make it difficult to eliminate lower respiratory infections. It would thus be desirable to improve techniques to i) apply antibiotics more uniformly within the lung, including distal and plugged airways where infected mucus can pool, ii) break up and remove thick, infected mucus layers that leads to decreased gas exchange, and iii) reduce inflammation that further weakens mucociliary transport. One possible means accomplishing this is through the use of PFC as an antibiotic delivery vehicle.

1.4 Acute Respiratory Distress Syndrome

Acute Respiratory Distress Syndrome (ARDS) is characterized by the sudden failure of the respiratory system after an Acute Lung Injury (ALI) most commonly due to direct injury—pneumonia, aspiration of gastric contents—or indirect lung injury—shock, sepsis, major trauma, or high-risk surgery [4], [73]. The pathology of ARDS involves severe

inflammatory damage to the lung epithelium and microvasculature, resulting in proteinacious edema filling the alveoli and impeding normal gas exchange.

1.4.1 Clinical Definition and Diagnosis of Acute Respiratory Distress Syndrome

The nonspecific features of ARDS, as well as its vague clinical definition, make defining, diagnosing, and thus treating ARDS a challenge. ARDS was initially defined in 1994 by the American-European Consensus Conference as "an acute condition characterized by bilateral pulmonary infiltrates and severe hypoxemia in the absence of evidence for cardiogenic pulmonary edema."[74] Pulmonary infiltrates are substances denser than air (i.e., pus, blood, protein) that linger within the parenchyma (functional tissue) of the lungs. Usually, these infiltrates are pulmonary edema which collect in the distal air spaces and decrease gas exchange, causing hypoxemia.[6] Hypoxemia is determined by the ratio of the partial pressure of oxygen in the patient's arterial blood (PaO_2) to the fraction of oxygen in the inspired air (FiO_2). According to the 1994 definition, a PaO_2/FiO_2 ratio less than 200 is characteristic of ARDS, and a PaO_2/FiO_2 ratio less than 300 is characteristic of ALI. For reference, the normal PaO_2/FiO_2 = 100 mmHg/0.21 ≈ 500. The Berlin definition of ARDS was drafted in 2012 to provide a more specific classification of ARDS (see .1).[75] Major changes included categorizing severity into three groups based on PaO_2/FiO_2, redefining "acute" to less than "7 days from the predisposing clinical insult," and eliminating the use of unreliable estimates of left atrial pressure to differentiate between ARDS and cardiogenic pulmonary edema.[76]–[78]

Characteristic	The AECC definition 1994	The Berlin definition 2012
Onset	Acute	≤7 days from the predisposing clinical insult
Radiographic abnormality	Bilateral infiltrate on frontal chest radiograph	Bilateral opacities on radiograph or computed tomography scan not fully explained by effusion, atelectasis, or nodules
Noncardiogenic source of pulmonary edema	No clinical evidence of elevated left atrial pressure, or, a pulmonary capillary wedge pressure < 18 mmHg	Respiratory failure not fully explained by cardiogenic pulmonary edema or volume overload
Oxygenation	PaO_2/FiO_2 ratio Acute lung injury: ≤300 Acute respiratory distress syndrome: ≤200	PaO_2/FiO_2 ratio with ≥5 cm H_2O positive end-expiratory pressure (PEEP) Mild ARDS: 201–300 Moderate ARDS: 101–200 Severe ARDS: <100
Predisposing condition	Not specified	If none identified, then need to rule out cardiogenic edema with additional data (eg, echocardiography)

Table 1.1 American-European Consensus Conference (AECC) definition of acute lung injury and the Berlin definition of acute respiratory distress syndrome.[76]

Since ARDS is a clinical diagnosis, there are no specific laboratory abnormalities beyond the expected disturbances in gas exchange and radiographic findings. Bilateral pulmonary infiltrates can be noted on chest radiographs almost immediately after the onset of impeded gas exchange.[4] Physical findings often are nonspecific and include tachypnea, tachycardia, and the need for a high FiO_2 to maintain oxygen saturation. ARDS mimics cardiogenic pulmonary edema, acute eosinophilic pneumonia, acute interstitial pneumonitis, cryptogenic organizing pneumonia, and diffuse alveolar hemorrhage.[76] Various diagnostic modalities are used, such as chest-imaging studies, echocardiography, catheterization, and bronchoscopy to differentiate these conditions from ARDS. Primary pneumonia (bacterial, fungal, or viral) is the most common cause of ALI/ARDS; severe sepsis from pneumonia or a non-pulmonary infectious source is the second most common.[73], [79] The physical examination must identify causes of sepsis, as septic patients lack an obvious source of lung injury, and ARDS will not resolve without resolution of the sepsis first.[4] Other important major causes of ALI/ARDS include aspiration of gastric contents and hemorrhage and shock following major trauma.

Clinical Presentation

Within 12 - 48 hours after the initial lung injury (during the exudative phase), patients experience dyspnea, or difficulty in breathing, during activities, which quickly progresses to dyspnea while inactive. [4], [5] This insufficient gas exchange, often combined with severe sepsis, leads to multisystem organ failure, which almost doubles mortality rates.[80] Since ARDS is not a predictive disease, patients enter the hospital in a critical state and physicians focus on stabilizing the patient first before attempting to diagnose ARDS, determine if injury is direct or indirect, and administer the appropriate treatment for the underlying condition, like sepsis or trauma.

1.4.2 Pathophysiology of Acute Respiratory Distress Syndrome

ARDS progresses over three overlapping phases: acute/exudative, proliferative, and fibrotic. The exudative phase (Figure 1.2) persists for the first 1 to 6 days post injury and is characterized by prominent interstitial and alveolar edema due to increased alveolocapillary (capillary endothelial cells and alveolar type I and II epithelial cells or pneumocytes) permeability.[6], [81] Injury to the endothelium is the most important initial cause of ALI/ARDS. Resident macrophages and damaged tissue cells release inflammatory signals that trigger diffuse and rapid inflammation of the pulmonary vasculature.[82]–[84] The activation and degranulation of neutrophils accumulated in the vasculature leads to release of toxic mediators like reactive oxygen species, and proinflammatory cytokines that increase vascular permeability. Resident pulmonary mast cells and basophils release histamine in response to antigen mediated cross-linking of IgE receptors. Histamine modulates a variety of immune responses, including the

production of inflammatory cytokines and histamine scavenging has been shown to reduced endotoxin-induced acute lung inflammation.[85] Platelets may also play a role in endothelial injury, however the mechanism between platelet- and neutrophil-related injury is poorly understood.[84], [86]–[88] Nevertheless, endothelial damage alone is not enough to cause ARDS; some degree of epithelial damage must be present.[89]

Neutrophils and leukocytes are recruited to the lung tissue, accompanied with erythrocytes and proteinacous edema from the vasculature. This rapid influx of neutrophils is characteristic of the acute phase and is thought to be a major contributor to epithelial damage. [89]–[91] However, neutrophil influx may not directly cause epithelial damage and bulk fluid transport. Furthermore, ARDS has been shown to occur in neutropenic patients.[92] Rather, it is likely the toxic mediators released as a result of neutrophil degranulation and apoptosis that are harmful in ARDS.[81], [93], [94] In particular, neutrophil enzymes elastase and collagenase degrade extracellular matrices, leading to further alveolocapillary damage and permeability.[95] This damage causes type I and II pneumocytes to slough off the alveolar wall and necrose. This not only creates paracellular gaps, but epithelial apoptosis may be an important factor in lung injury.[96]–[99]

With damage to both the capillary endothelium and alveolar epithelium, accumulation of edema in the air space ensues and gas exchange is disrupted. In a healthy lung, type I and II pneumocytes would create a miniosmotic gradient to reabsorb edema from the air space into the interstitium where net clearance can occur through lung lymphatics, pulmonary microcirculation, and bulk flow into the pleural space.[98], [100], [101] However, the epithelium is damaged in ARDS and edema resolution is slowed. This

edema disrupts surfactant effects, causing changes in lung compliance and local atelectasis. In a healthy lung, type II pneumocytes lining the alveoli secrete a phospholipid surfactant that considerably lowers this surface tension, thereby decreasing the work of expanding the lung during inspiration. However, edema significantly reduces the presence and function of these surfactants, thus increasing the work and shear needed to recruit and expand alveoli. Fibrin and proteins from the edema form hyaline membranes throughout the lung and hypoxia-induced intrapulmonary shunting raises pulmonary vascular resistance.[81]

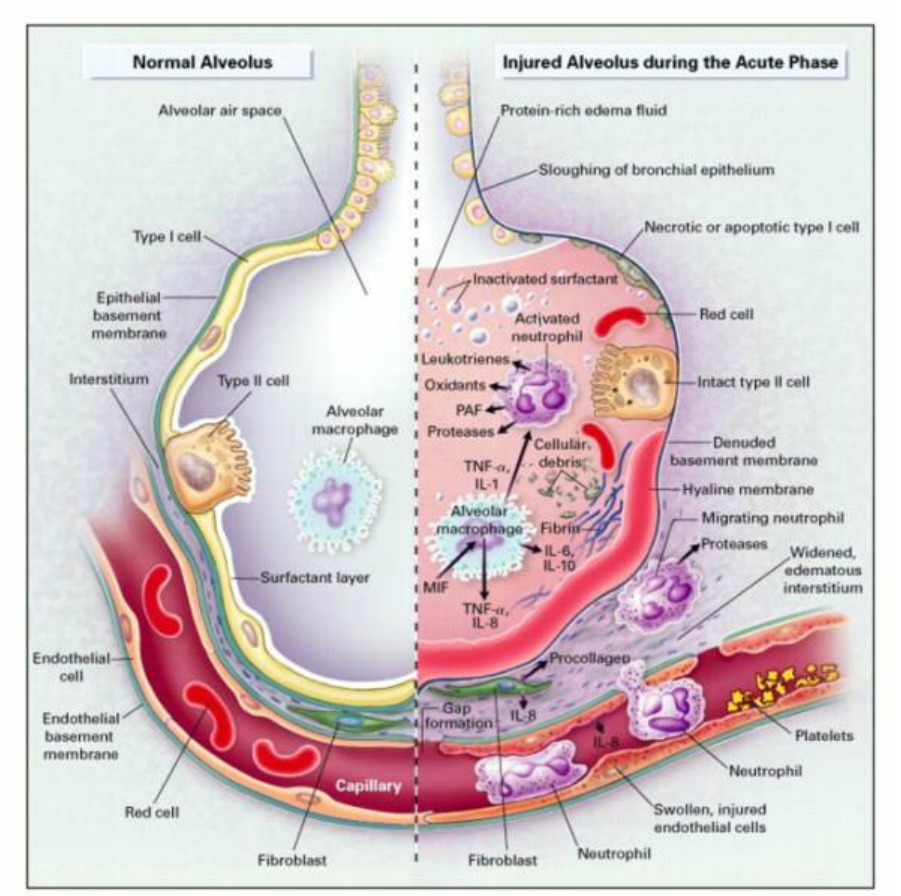

Figure 1.2 Multiple cellular responses and mediators contribute to alveolar–capillary membrane injury (right-hand side) and the transition from normal alveolar structure and function (left-hand side) in the acute phase of ALI/ARDS.[6]

During the proliferative phase (the next 7-14 days), both resident and recruited macrophages internalize neutrophil proteases and phagocytose entire neutrophils to prevent apoptosis.[82], [83], [102] Their phagocytic activity also helps remove debris in the airway and degrade hyaline membranes. In response to the epithelial damage, initial repair events include the proliferation and migration of type II pneumocytes and fibroblasts over the exposed basement membrane.[81] Fibroblasts secrete a collagen-rich extracellular matrix material into the alveolar edema. Type II pneumocytes migrate and proliferate over the organizing matrix, sealing in small areas of edema and converting it into a newly developed interstitial layer. Although the entire alveolus is not lost, the more permanent collagen layers greatly alters the elastic properties of the lung and is the main contributor to fibrotic pathological changes. The critical division between functional and pathological repair may be determined by the extent of basement membrane damage.[103], [104] Thus, the timing and balance between type II pneumocyte and fibroblast migration is crucial in directing healing toward the resolution of edema or the development of fibrosis.

The third, irreversible fibrotic phase (after 14 days) is characterized by scar tissue and cyst formations that drastically decrease lung compliance and gas exchange.[83], [98] The acute neutrophilic infiltrate usually resolves, leaving more mononuclear cells and alveolar macrophages in the alveoli.[83], [102] The degree of epithelial injury, proliferation of type II pneumocytes, and removal of pulmonary edema are all critical in the determining the severity of ARDS.

1.4.3 Epidemiology

Incidence and Mortality

In 2014, patients with ARDS represented approximately 23% of hospitalized, mechanically ventilated patients, a significant increase from the 5% noted in 2004. [76], [105], [106] The 2014 study showed the prevalence of mild ARDS was 30.0%; of moderate ARDS, 46.6%; and of severe ARDS, 23.4%.[106] However, one-third of patients presenting initially with mild ARDS will likely progress to moderate or severe ARDS.[75], [107] In 2000, the age-adjusted incidence of ALI was 86.2 per 100,000 person-years.[107] Increasing with age, ARDS incidence reached 306 per 100,000 person-years for people in aged 75-84 years. An estimated 190,000 Americans still suffer from ARDS annually.[108]These numbers are likely a gross underestimate given the difficulty of diagnosing ARDS because of the nonspecific features of this condition. Ferguson, et al. identified that only 48% of patients with autopsy-proven ARDS had a diagnosis of ARDS noted in their charts.[109] With the number of ARDS cases projected to double by year 2020, there is a strong push to develop a clinically beneficial therapy that not only decreases mortality, but also the length of ventilator days, and time spent in the hospital.[110] Although worsening oxygenation is a risk factor for ARDS mortality, patients generally die from multisystem organ failure or progressive underlying illness; only a minority of patients (13-19%) die from refractory respiratory failure.[111], [112]

The mortality rate of ARDS was once 64-68% in 1978. [111], [113], [114] However, that rate decreased to 32% in 1990's and to as low as 26% in 2005 due to the incorporation of low tidal volume (protective) ventilation.[115] However, among patients who receive protective ventilation, mortality rates plateau at 30-40%.[116], [117]

Living with ARDS

Unfortunately, of those who survive, ARDS exacts significant long-term damage on the body; ARDS is one of the most common reasons for admission to a long-term ventilator rehabilitation unit.[118] Patients under prolonged ventilation typically suffer from muscle atrophy and blood clots, in addition to, malnutrition, dehydration, intestinal bleeding and infection.[4] Even after the exudative phase of ARDS resolves, most patients require a lengthy period to be weaned from mechanical ventilation and to regain diaphragmatic muscle strength.[119] Prolonged mechanical ventilation via tracheostomy and invasive hemodynamic monitoring increase the risk for nosocomial infections, including in-line sepsis and ventilator-associated pneumonia (VAP).[4] The incidence of VAP in ARDS patients may be as high as 55% and appears to be higher than that in other populations requiring mechanical ventilation. Many patients develop long-term neuromuscular, cognitive, and psychological symptoms, often suffering from post-traumatic stress disorder.[120], [121] Their health-related quality of life is significantly below normal.[122], [123] One study showed survivors retained diminished respiratory function, had abnormal 6-minute walking distances, and only 49% had returned to work after one year.[124] This same group of patients five years after recovery from ARDS continued to demonstrate exercise impairment and decreased quality of life.[120]

Economic Impact of ARDS

ARDS patients are hospitalized on average 30.6 days.[125], [126] Thus, adult patients on prolonged (> 2 weeks) acute mechanical ventilation comprise one-third of all adult mechanically ventilated patients, consume two-thirds of hospital resources allocated to

mechanically ventilated population, and are nearly twice as likely to require a discharge to a skilled nursing facility or be re-hospitalized. [110], [127], [128] The expected national bill in 2020 is $60 billion for costs associated with mechanical ventilation, given the expected doubling of ARDS cases.[128] ARDS also has a substantial financial strain on the patient and their families. Most patients require a tracheostomy for prolonged mechanical ventilation, increasing the effective cost of intensive care upwards of $24,000 per patient, not including wages lost during the month long hospital stay.[125], [129] In addition, nearly half of survivors are jobless one year after hospital discharge, and are estimated to have lost an average of $27,000 in earnings.[124], [130]

1.5 Current Treatment for Acute Respiratory Distress Syndrome

ARDS cannot be predicted by physicians and has a rapid onset, the timeline for intervention begins once the patient is in a critical state. Restoring sufficient gas exchange is a priority to avoid multiple organ failure. Stabilizing the patient with fluid administration is next, followed by identifying sites of sepsis or injury that are the underlying causes. Because infection is often the underlying cause of ARDS, early administration of broad spectrum antibiotics is essential, along with careful assessment of the patient to determine potential infection sources. Sepsis-associated ARDS does not resolve without such management.[4] Once the described treatment steps are completed, nothing else is done to help resolve ARDS faster. This hard truth motivates many researchers to develop pharmacological treatments aimed at promoting the resolution of ARDS.

1.5.1 Ventilation Strategies

Non-invasive Ventilation

Noninvasive positive-pressure ventilation (NIPPV) or high-flow nasal cannula is the first-line approach to stop the progression of multiple organ failure, as intubation and mechanical ventilation lead to more complications.[131]–[133] High-flow nasal cannula utilizes heated humidification and large-bore nasal prongs for oxygen delivery at flows up to 50 L/min. NIPPV is usually given by full facemask. High-flow nasal cannula has been shown to improve 90-day mortality compared to standard oxygen and NIPPV, even though all three modes had the same incidence of need for intubation/mechanical ventilation.[134] According to Thille, intubation rates are below 35% in mild ARDS patients.[133] However, 84% of patients with severe ARDS required intubation, when non-invasive methods proved ineffective at improving oxygenation.

Mechanical Ventilation

Mechanical ventilation strives to maintain oxygenation while avoiding oxygen toxicity. High oxygen concentrations lead cells to increase production of harmful hydrogen peroxides and free radicals.[135] The physiologic manifestations of oxygen toxicity include decreases in gas exchange and lung compliance and capacity. Therefore ventilation treatments aim to maintain oxygen saturation between 85-90% (PaO_2 of 55-80 mmHg) and reduce FiO_2 to less than 65% within the first 48 hours.[4] Several complications are associated with mechanical ventilation. ARDS does not affect the lung homogenously, therefore, the healthier, more compliant regions receive a disproportional amount of tidal volume during ventilation. This causes overdistension injury (volutrauma)

to previously functional lung and can cause cyclic lung recruitment and collapse, which increases shear-stress forces in the alveoli.[136] For these reasons, mechanical ventilation is associated with ventilator-associated lung injury (VALI).[4] Studies in the 1970s and 1980s had suggested the previously accepted levels of tidal volumes (12 - 15 mL/kg) and airway pressures (> 30 - 40 cmH_2O) compounded ALI/ARDS injuries, yet not until studies in 1998 and 2000 were protective ventilator strategies proven to drastically decrease mortality and severity of lung injury.[137]–[141] Its protective effect reduces alveolocapillary injury, lung inflammation, and accelerates resolution of alveolar edema.[142], [143] Therefore, current recommendations are for a low tidal-volume of 6 mL/kg, with adjustment to as low as 4 mL/kg if needed to limit the inspiratory plateau pressure to < 30cm H_2O.[4] Additionally, moderate-to-high levels of positive end-expiratory pressure (PEEP) are utilized to combat cyclic recruitment by reducing lung collapse at end expiration and improving oxygenation.[136]

Several other protective ventilatory measures have been introduced to improve oxygenation and alveolar recruitment while preventing VALI, including continuous positive airway pressures, pressure- versus volume-controlled ventilation, high frequency oscillatory ventilation, inverse ratio ventilation, extracorporeal membrane oxygenation, and permitted hypercapnia with acidosis management.[4] However, outside of low tidal-volume ventilation, the actual effects of these protective measures are negligible on ARDS mortality rates for the majority of patient populations.[144], [145] Both respiratory support and alveolar repair are needed to improve patient outcomes.[146]

1.5.2 Pharmacological Treatments

Recent ARDSnet clinical trials have proven that ARDS survival requires supporting the respiratory function of the patient while limiting further lung damage and thus evoking long-term improvements in alveolar epithelial repair.[146] Growing understanding of the pathogenesis of ARDS has led to improved therapeutic options to speed up its resolution, including anti-inflammatories, anticoagulants, surfactant therapy, and growth factors that decrease inflammatory damage, promote healing of the epithelium and microvasculature, or resolve the edema in the alveoli. Despite the many pharmacological therapies, none have been effective at reducing the mortality of ARDS using current delivery methods.

Therapies for Hypoxemia

During the exudative phase, the primary goal for treatment is to improve V/Q matching. These include vasodilators to increase blood flow, selective vasoconstrictors to potentiate hypoxic vasoconstriction, exogenous surfactants to reduce alveolar surface tension, anti-coagulants to antagonize thrombus formation, and β-2 agonists to reduce edema.

Vasodilators are used to increase blood flow to ventilated alveoli in combination with selective vasoconstrictors to further constrict under-ventilated regions and redirect blood flow. Nitric oxide and prostacyclin have potent vasodilatory effects and are used in some centers to reverse hypoxic pulmonary vasoconstriction, prevent leukocyte adhesion, and improve V/Q matching.[147] However, they have failed to show a significant survival benefit in several multicenter studies.[148], [149] Angiotensin receptor blockers reduce alveolar vasoconstriction, vascular permeability, and fibrosis and, thus, decrease ventilator-induced lung injury in animal models.[150] Furthermore,

administration of angiotensin receptor blockers on discharge were associated with reduced mortality in acute respiratory failure patients.[151] These data have encouraged future clinical trials. The cytokine interferon-β has been shown to increase capillary endothelial barrier function *in vivo*.[152] Clinical trials are underway.

Exogenous surfactant has been administered to replace the native, dysfunctional surfactant and reduce alveolar surface tension and inhibit the onset of intense pulmonary inflammation. However, the several clinical trials that have been conducted to test the efficacy of exogenous surfactant have shown that surfactant has no effect on survival rates nor does it provide any significant clinical benefit.[153]

Anticoagulants are used to decrease thrombus formation and fibrin deposition and increase pulmonary blood flow by reducing intravascular coagulation and neutralizing other soluble compounds like platelet-activating factor and complement factors using anti-coagulants and inhibitors of platelet aggregation. Inhaled heparin can reduce fibrin deposition, and one study suggested heparin may increase the number of ventilator-free days.[154] The pre-hospital administration of the potent anti-platelet aspirin has been investigated and associated with a reduction in subsequent ARDS incidence.[155]–[159]

β-2 agonists, like salbutamol, have the potential to improve alveolar fluid clearance in ARDS by upregulating sodium transport mechanisms in alveolar epithelial cells. The transport of sodium from the alveolus into the basal laminae creates a mini osmotic gradient that promoted the resorption of water into the interstitium. Fluid clearance from the alveolar space is crucial for the successful resolution of ARDS. However, large clinical trials showed increased mortality, hypothesizing that β-agonists may have a harmful cardiac effect resulting in a poorer outcome.[160]–[163]

Therapies for Inflammation or Oxidant Injury

The inflammatory response is key to the resolution of ARDS. However, a sustained inflammatory response leads to the destruction of the endothelial and epithelial barrier and thus, the accumulation of edema. This is caused by the deleterious pro-inflammatory cytokines, chemokines, growth factors, and reactive oxygen/nitrogen species released by activated leukocytes and resident lung cells. Accordingly, several therapies with anti-inflammatory agents or antioxidants have been developed to complement interventions targeting V/Q abnormalities. Investigations are underway for agents targeting specific inflammatory mediators or pathways including antibodies or soluble receptors for tumor necrosis factor, IL-8, and CD40 ligand; receptor antagonists for IL-1 and targeting agents like N-acetylcysteine and recombinant superoxide dismutase for oxidant injury.[81], [164], [165]

Anti-inflammatories such as cytokine and elastase inhibitors aim to decrease the recruitment and activation of additional inflammatory cells. A recent analysis has shown silvelestat, a neutrophil elastase inhibitor, to have no effect on short-term mortality and a worse outcome for 180-day mortality.[166], [167]

Antioxidants were administered to patients with ALI in order to convert harmful reactive oxygen species from phagocytic activity into inert molecules. There were no differences in mortality, but the treated groups showed a reduction in duration of ALI and less extrapulmonary organ failure.[168]

Statins**,** HMG CoA-reductase inhibitors, have a range of physiological effects beyond cholesterol reduction, including anti-inflammatory actions and endothelial function modulation.[169] A small ARDS trial, suggested a potential role for statin treatment

showing benefit in pulmonary and non-pulmonary organ dysfunction with no excess of adverse events in the intervention group.[170] Larger trials are currently ongoing.[171]

Immuno-nutrition supplements like vitamin D, E, and C have been shown to have immunomodulatory effects. Models of ALI demonstrate that intra-tracheal administration of vitamin D can reduce neutrophil recruitment to the lung.[172], [173] The anti-oxidants Vitamins E and C have been shown to reduce the days of mechanical ventilation, intensive care requirements, and the incidence of extra-pulmonary organ failure in patients with ARDS.[174]

Corticosteroids, which have broad anti-inflammatory activity, have been found to be ineffective and even potentially harmful in early exudative ALI/ARDS.[175] However, corticosteroids are discussed later for potential use in the fibroproliferative phase of lung injury.

Therapies for Fibroproliferation

Interventions in the fibroproliferative phase of ALI/ARDS must primarily address pathophysiological elements of remodeling, repair and fibrosis as opposed to acute issues of V/Q matching. Current understanding of fibroproliferative lung injury suggests that therapeutic agents need to enhance repair (e.g., angiogenesis and alveolar secondary crest formation) while inhibiting fibroblast proliferation, differentiation, and interstitial matrix deposition. Fibroblast activation, migration, proliferation and collagen production in lung injury are augmented by cytokines, growth factors, and enzymes including transforming growth factor beta, platelet derived growth factor, tumor necrosis

factor, matrix metalloproteinases, and their inhibitors. These therapies are still in the developmental stage and have not been tested clinically in ALI/ARDS.[81]

Corticosteroid therapy is aimed at decreasing inflammation by blocking inflammatory cytokines and promoting the antifibrotic effect of cortisol. Therapeutically, both high-dose and moderate-dose steroids have failed to demonstrate efficacy in ARDS. An ARDSnet trial showed no effect of prolonged treatment: patients were liberated from mechanical ventilation earlier, but were more likely to resume assisted ventilation.[176] In addition, initiation of treatment after 14 days of ARDS was associated with a harmful effect. However, the role of low-dose corticosteroids in established ARDS remains uncertain, with one study demonstrating prolonged low-dose therapy reduces severity of lung injury.[177] Despite a systematic review and meta-analysis, the role of steroids in ARDS remains unclear.[160], [178], [179]

Growth Factors

Several groups have begun to examine the use of various growth factors to improve alveolar repair.

Hepatocyte growth factor (HGF) is secreted by mesenchymal cells and acts as a multi-functional cytokine mainly on epithelial cells.[165] Intratracheal HGF after bleomycin-induced injury reduced fibrosis and increased bronchial epithelial and alveolar epithelial proliferation in animal trials.[180] To date, no clinical trials have studied the effects from the administration of exogenous HGF as a treatment for any lung disease.

Epidermal growth factor (EGF) receptor activation is crucial for the repair of the lung epithelium. Therefore, EGF, may expedite the reparative process in the lungs.[165] Thus far, no clinical studies have studied the effects of EGF on patients with ARDS.

Keratinocyte growth factor (KGF) is secreted by mesenchymal cells and fibroblasts, but the KGF receptor is only expressed on epithelial cells. KGF has been shown to increase alveolar type II epithelial cell proliferation, spreading and motility, fluid transport, and resistance to mechanical and oxidant-induced injury.[181], [182] A phase II trial has commenced investigating the efficacy and safety of intravenous KGF (palifermin) in ARDS.[183]

Granulocyte macrophage colony stimulating factor (GM-CSF) is a novel agent that has been shown to play an important role in the development and homeostasis of alveolar macrophages. A small randomized phase-II study of GM-CSF in patients with severe sepsis and ALI/ARDS showed an improvement in oxygenation.[184]

Stem cells exhibit anti-inflammatory, immunomodulatory and reparative effects, largely mediated through secreted growth factors, although cell to cell contact between stem cells and alveoli also mediates important effects.[185], [186] Animal models of ARDS have shown survival to increase after direct treatment.[187]

Lysophosphatidic acid (LPA) is a serum-derived, phospholipid growth factor involved in proliferation, migration, and cytokine secretion. LPA treatment enhances pulmonary epithelial cell barrier function and induces alveolar cell migration and expression of epidermal growth factor receptors.[Zhao and Natarajan 2013] Exogenous LPA has also been shown to mediate regulation of Th2 cytokine decoy receptors giving

it an anti-inflammatory role in lung inflammatory diseases.[Zhao and Natarajan 2013] There are currently no clinical trials for LPA as a treatment for ARDS.

Even though most of these drugs have some benefit in increasing oxygenation, decreasing ventilator days, or decreasing neutrophil accumulation, the fact that they show no significant difference in mortality leads to discontinuation of the trials. Currently implemented pharmacological interventions have acted to mediate the symptoms associated with ARDS but have little to no effect on the pathophysiology of the disease and its progression.

As discussed, in ARDS, inhaled drug delivery to injured alveoli is far more difficult. Delivery is most needed within the deeper regions of the lung in damaged alveoli, but edema in these regions limits ventilation and thus inhaled drug delivery as well as severely dilutes any drug delivered out of the blood into the alveolar space. However, survival requires supporting the respiratory function and limiting further lung damage. It would thus be desirable to improve techniques to i) uniformly apply growth factors and anti-inflammatories in the damaged alveoli where inflammatory secretions can pool, ii) remove inflammatory exudate that leads to decreased gas exchange, and iii) reduce inflammation that further weakens epithelial barrier function.

1.6 Liquid Ventilation with Perfluorocarbons

Many of the complications associated with respiratory distress can be mitigated in proportion to the reduction of interfacial tension and ventilatory requirements.[188] Therefore, the concept of maximally reducing surface tension by filling the lungs with a liquid has been explored through liquid ventilation. Liquid ventilation (LV) is a respiratory

support method utilizing liquid perfluorocarbons (PFCs) as a means to enhance gas exchange while reducing lung damage. PFC liquids are clear, odorless fluorinated hydrocarbons in which the hydrogen atoms have been replaced by fluorine atoms; for Perflubron (perfluorooctyl bromide) a bromine atom is added as well (see Figure 1.3). The majority of clinical LV trials have used Perflubron, although a number of PFC liquids are suitable for LV.[189]–[194] Much of the animal research in LV utilized FC-770 (previously FC-77), a mixture of perfluorocycloether and perfluorooctane.[195]–[198] PFCs are highly stable due to their strong covalent carbon-fluorine bonds. Thus, they are chemically and biologically inert, undergoing no enzymatic changes nor metabolism in the kidneys or liver.[199] Due to their nonpolarity, PFCs have low intermolecular forces which are responsible for many of the properties of PFC that are critical to LV. PFCs tend to have vapor pressures higher than water, evaporating at or below body temperature; low surface tension (12 - 18 dyne/cm), effectively penetrating small airways and alveoli and working like surfactant to improve lung compliance; and high gas solubility, dissolving gases via insertion into an intermolecular site within the PFC.[199]–[201] The carrying capacity of PFC can be more than twice that of blood for oxygen (35-70 ml O_2/dL blood at 25°C) and for carbon dioxide (122-255 mL CO_2 per dl of PFC).[202] Other vital characteristics of PFCs for use in LV include a greater density than body fluids (nearly twice as dense as water) enabling PFC to descend to the dependent regions of the lungs and re-open areas of atelectasis and that elimination of intact PFC molecules occurs by evaporation during exhalation or transpiration through the skin.[188] Most PFCs have a similar kinematic viscosity to water and are insoluble with water and lipids.[200] As such, PFCs are immiscible with virtually all physiological substances other than gases.

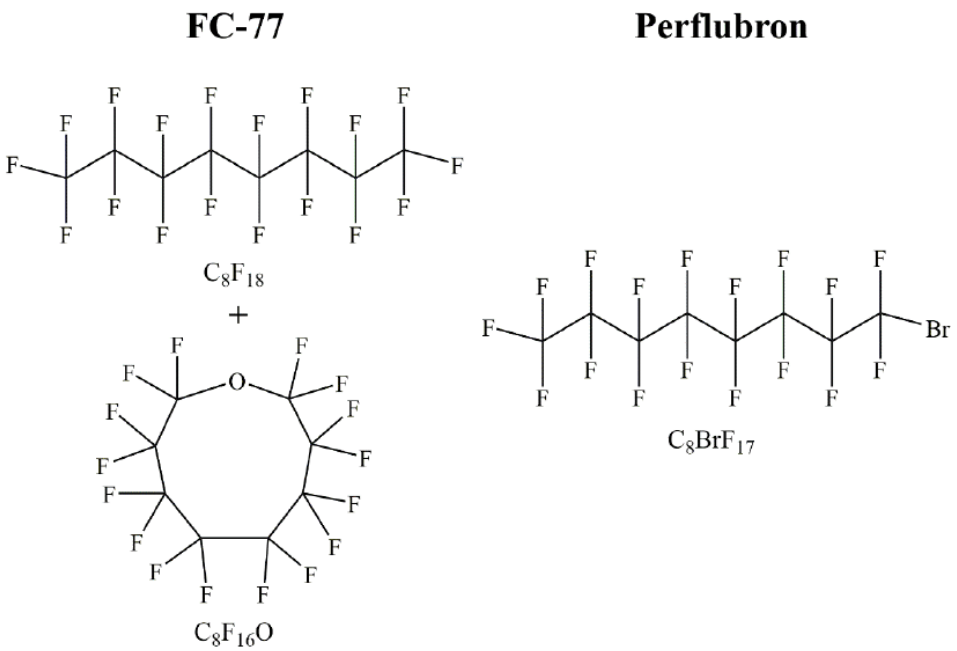

Figure 1.3 Molecular structures of FC-77 and Perflubron.

The concept of using liquid PFC to support respiration was first demonstrated by total immersion of mice in PFCs nearly 50 years ago.[203] Early experiences with LV also included gravity-assisted ventilation to an intubated animal.[200] In general, because PFC liquids are more dense and viscous than gas, with slower spreading and higher diffusion coefficients, LV techniques had to be refined.[204] In its more recent use, two modes of LV were developed—partial liquid ventilation (PLV) and total liquid ventilation (TLV)—which mechanically assist the movement of tidal volumes to and from the lungs to support pulmonary gas exchange (see Figure 1.4). During PLV, the lungs are partially

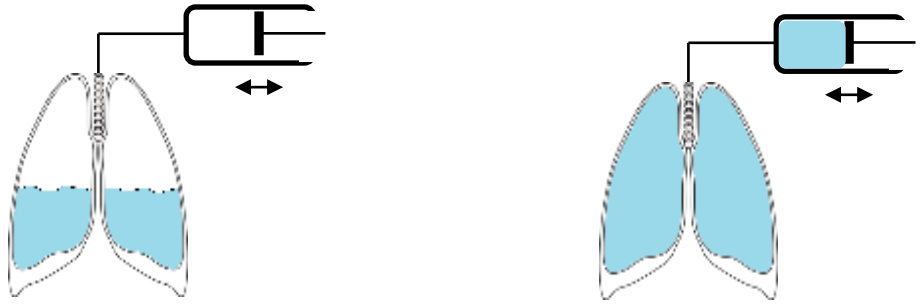

Figure 1.4 Representation of partial and total liquid ventilation.

filled with liquid PFC (typically to end-expiratory volume) and gas ventilated with a conventional ventilator. In this setting, oxygen-rich air is mixed with PFC during each breath, preserving gas exchange (oxygenation and CO2 removal). During TLV, the lungs are completely filled with liquid PFC and tidal volumes of PFC are moved into and out of the lungs typically using a piston pump. In this setting, a parallel, extracorporeal circuit continually achieves gas exchange within the PFC, utilizing a pump to regulate flow, an oxygenator, a heater, and a condensing system to recapture and filter PFC. A schematic of such a setup is shown in Figure 1.5. Due to the need for specialized equipment during TLV, PLV is considered much easier to implement in to clinical practice.

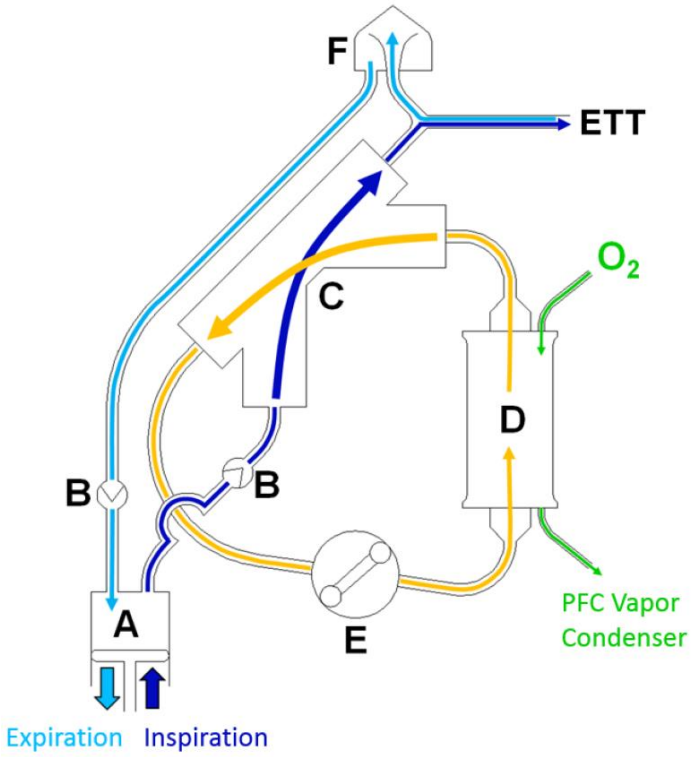

Figure 1.3 Schematic of TLV circuit showing piston (A), one-way valves (B), mixing chamber (C), heater/oxygenator (D), roller pump (E), bubble trap (F), and endotracheal tube (ETT). Continuous flow through the conditioning circuit is shown in yellow while the inspiratory and expiratory flow to and from the patient is shown in dark and light blue, respectively.[195]

Following LV, PFCs are either suctioned from the lung or left to evaporate. The majority of PFC left in the lung evaporates within hours, the remaining trace amounts are cleared within seven days of instillation via evaporation during exhalation.[205] The rate of evaporative PFC clearance from the lung has been shown to be largest immediately following administration and steadily decline thereafter.[206] Additionally, clearance tends to occur in the nondependent regions of the lung first. When the lung is filled completely, animals can breathe spontaneously after a few hours.[191], [205] Small amounts of PFC may diffuse from the lung into the circulation, but due to its immiscibility with water, the majority of PFC in the blood and tissues is dissolved in fatty tissues.[188], [207] The amount of PFC in the blood has been shown to be less than 1% of the administered dose after PLV.[206] Virtually all delivered PFC is believed to ultimately leave the body via evaporation through the lung or transpiration through the skin.[200], [207] Even PFC delivered to the systemic circulation in the form of an emulsion for use as an imaging agent or blood substitute has been shown to be cleared via expired air after phagocytosis by reticuloendothelial macrophages.[208] Although trace amounts of PFC remain for relatively long periods of time, there has been no evidence of any negative consequences.[200], [209] PFC toxicity has been studied in animals and in patients for periods up to 10 years without evidence of significant adverse effects after use in respiratory applications or as blood substitutes.[210]–[214]

The effectiveness of LV has been evaluated for a variety of respiratory conditions. Treatment with PLV in the setting of ALI/ARDS and other respiratory failures has demonstrated improved survival and respiratory function in both animals [215]–[230] and humans.[189]–[194], [231] Treatment with TLV has also demonstrated improved

outcomes in similar lung injury or disease settings in animals.[195], [232]–[235] These observed improvements following treatment with LV are a result of a variety of mechanisms, the most common being a marked improvement in gas exchange.[189], [190], [194], [195], [216]–[222], [224], [225], [231], [233] The degree of pulmonary shunting in an injured lung has been found to be significantly decreased following treatment with LV.[191], [216], [218], [221], [231], [233] This effect is likely due to both the recruitment of atelectatic regions as well as the displacement of aqueous exudate (e.g. fluid, mucus, meconium) in the alveoli impeding gas exchange. The low surface tension of PFCs allow them to easily penetrate and fill the lower airways, recruiting previously collapsed or deflated alveoli.[191], [193], [232] In addition, PFCs displace the aqueous exudate in the peripheral airways and alveoli via buoyant forces (higher density than and immiscibility with water). The aqueous fluid is typically redistributed throughout the surface area of the lung or transported to the central airways where it can be removed via suctioning.[191], [228] Both of these actions result in increased alveolar surface area available for gas exchange, thereby decreasing shunting. Furthermore, LV may redistribute pulmonary blood flow from the dependent regions, typically experiencing the greatest degree of consolidation and atelectasis, to the nondependent regions of the lung, thereby improving V/Q relationships.[228] All of these factors likely contribute to the significantly improved gas exchange and lower the risk of oxygen toxicity: better oxygenation decreases need for higher inspired O_2 concentrations.

Treatment with LV may also reduce the risk of VALI by maintaining the alveoli in an expanded state, and thus, reducing shear forces needed for alveolar recruitment. This is manifested as a significant improvement in lung compliance.[190], [191], [193], [194],

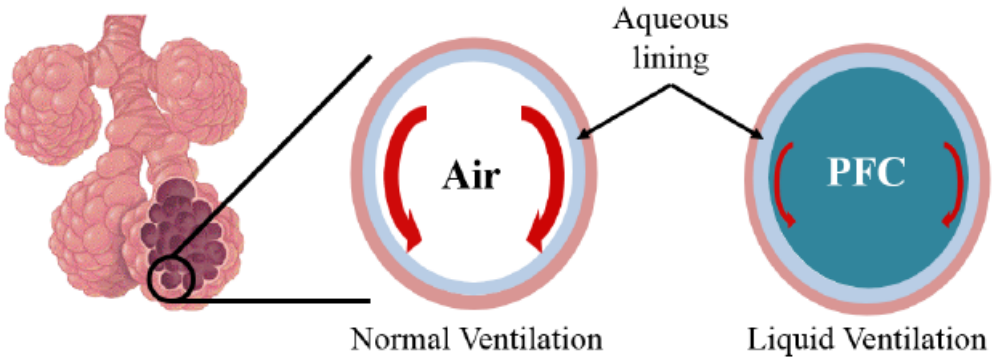

Figure 1.4 Schematic showing reduced resistive effects of interfacial tension on alveolar expansion during liquid ventilation. Red arrows denote forces due to interfacial tension.

[216]–[220], [231]–[233] The interface between air and the liquid film lining the alveoli results in a surface tension that works to minimize this interfacial area, thereby resisting alveolar expansion (Figure 1.6). In a healthy lung, surfactants considerably lower this surface tension, thereby decreasing the work of expanding the lung during inspiration. However, significantly reduced presence and function of endogenous pulmonary surfactants has been shown during lung diseases such as CF and ARDS.[6], [236] During LV, the lungs are filled with PFC and the air-liquid interface is either partially or completely eliminated, being replaced with an aqueous-PFC interface of a much lower interfacial tension (Figure 1.6). Thus, lung volume recruitment is achieved at much lower pressures during LV and lung compliance is increased. In addition, it has been shown that LV may enhance surfactant synthesis and secretion in the lungs relative to conventional gas ventilation, possibly also contributing to the improved lung compliance observed during LV.[237] The low air-PFC surface tension allows PFC to homogenously fill and expand dependent regions of the lung that are often minimally ventilated during gas ventilation likely also contributing to the enhanced gas exchange associated with LV.[216], [221]

Although LV requires lower ventilatory settings due to the increased gas exchange and lung compliance, high PEEP must still be applied to minimize pneumothorax, a

reported complication of LV.[238]–[240]. High PEEP increases oxygenation and may avoid shear stress in nondependent, non-PFC-filled alveoli.[240], [241] Other reported adverse effects include reduced venous return during TLV and both respiratory and metabolic acidosis due to ineffective CO_2 removal. The high viscosity of PFC compared to gas and the small CO_2 diffusion coefficient in PFC make CO_2 removal challenging. This can be reduced by the use of custom ventilators or lung maneuvers, like the breath-hold.[213], [241]–[243]

Despite the large number of positive data surrounding LV, the use of PFCs during LV remains unapproved by the FDA. The first application of LV in humans was performed as TLV in infants in 1989.[244] In the mid to late 1990s, a number of clinical trials were performed by Alliance Pharmaceutical in an effort to receive FDA approval for the use of Perflubron during PLV in adult ARDS patients.[244] These efforts culminated in a Phase III, multi-center clinical study that evaluated PLV with Perflubron relative to conventional mechanical ventilation in 311 adult ARDS patients.[238] The results of this study showed no improvement in 28-day mortality or number of ventilator-free days for PLV relative to conventional mechanical ventilation. It should be noted that although PLV showed no benefit over the control group, both groups exhibited better survival than was widely accepted at the time of the trial.[244] Additionally, safety data from the trial showed that Perflubron was well tolerated by patients. Even so, due to the lack of treatment benefit relative to existing methods shown in this study, the FDA ultimately failed to grant approval for Perflubron and Alliance Pharmaceutical subsequently withdrew all effort and funding in this endeavor. Considering the abundance of positive data surrounding LV in animal models and small-scale clinical trials, the negative outcome of Alliance's Phase III

trial is somewhat puzzling. The discrepancy between this trial and most other work with LV has been attributed to factors ranging from poor and inconsistent clinical implementation of PLV during the Phase III study to a general lack of congruency between animal disease models and real-world patients.

1.7 Effects of Perfluorocarbon on the Native Immune System

Growing evidence suggests PFC exposure can significantly reduce pulmonary inflammation and injury.[197], [245], [246] *In vivo* exposure to PLV during lung injury demonstrated a pulmonary decrease in proinflammatory IL-1 and IL-6 (possibly removing a stimulus for IL-10), lipid mediators such as thromboxane A2, mRNA expression of pulmonary adhesion molecules (P-selectin and ICAM-1) in lung tissue, neutrophil recruitment (independent of IL-8), white blood cell count, capillary leak, as well as a decrease in serum TNF-α, thus reducing systemic sequelae of acute lung injury and inflammation. [192], [196], [198], [247], [248] Furthermore, modes of ventilation that lower evaporative losses of PFC, such as low-bias flow oscillation, were associated with improved lung injury score.[249] This finding is critically important for applications in which patients with respiratory infections or sepsis will undergo LV. A temporary and local reduction of the inflammatory response in the airways of these patients may be conducive to a return to normal mucociliary clearance in CF, normal alveolocapillary barrier function in ARDS, and normal respiratory function overall. However, sustained impairment of the innate immune system could also hinder the patient's ability to control or ultimately clear the infection after treatment. In order to account for these anti-inflammatory effects, an in-depth understanding of PFC's effects on the innate immune system is vital.

The means by which PFC exposure decreases the innate immune response is still not completely understood. It is sometimes difficult to discern a clear cause for the observed anti-inflammatory effects of PFC *in vivo.* The suppressed inflammatory response could be due to a direct effect of PFC or due to an improved disease state or increased pulmonary function.[248] For this reason, much of the work investigating the mechanism by which PFC exposure dampens the immune response has been done *in vitro*. Multiple studies have shown that *in vitro* exposure to PFCs for a matter of hours can cause human neutrophils to have a decreased chemotaxis response.[250], [251] Studies have also shown decreased levels of activation for stimulated (1h with lipopolysaccharide) human macrophages (measured by levels of pro-inflammatory cytokines such as IL-1, IL-6, and TNF) after exposure to PFC.[252], [253] Additionally, a two-hour exposure to PFC has been shown to decrease the production of reactive oxygen species by alveolar macrophages.[254] A reduction in levels of pro-inflammatory cytokines secreted by stimulated macrophages combined with a decrease in the ability of neutrophils to respond to chemokines likely results in significantly reduced neutrophil recruitment.

However, the underlying mechanism by which PFC exposure causes these effects is still not completely understood. Almost all studies with neutrophil and macrophage exposure to PFC have shown little to no difference in cell viability between PFC-exposed cells and controls. This observation implies that the inhibition of a basic metabolic process causing accelerated cell death and, therefore, decreased levels of activation is an unlikely explanation. One initial speculation proposed that the observed effects could be explained by the presence of a physical coating of PFC surrounding cells, thereby interfering with the interaction between stimulant-containing cells and target cells.[255] However, other

studies have shown that decreased chemotaxis effects are observed even after PFC-exposed neutrophils were washed with fresh buffer before being exposed to a stimulus.[250] This suggests that the anti-inflammatory effects are related to a sustained chemical effect rather than the presence of a physical barrier. Another proposed theory is that low levels of PFC diffusing in to the cellular membrane of human leukocytes exert inhibitory effects on transmembrane signaling.[256] In support of this theory, one study has shown that the in vitro cellular effects of various PFCs increase proportionally with PFC lipid solubility.[256] Additionally, by shearing PFC-exposed cells and separating the membrane and cytoplasmic fractions, it was shown that any PFC content present was associated with the membrane fraction. This data suggests that PFCs may have a nonspecific effect caused by PFC localizing in the lipid bilayer of the cellular membrane, resulting in a generalized protective or dampening effect on a variety of membrane-associated responses to activation. Another study focused on the effects of PFC on the Syk pathway, a signaling system located early in a series of events leading to phagocytosis. Results showed that incubation with PFC reduced tyrosine phosphorylation of Syk, resulting in a corresponding reduction in phagocytosis of opsonized sheep erythrocytes.[250] This phenomenon could be explained by PFC-induced alterations at the cell membrane causing disruptions in the transmembrane signaling process leading to Syk phosphorylation. Although evidence exists for PFC-induced modifications of the cellular membrane being responsible for the immune effects associated with PFC, skepticism still exists. One study examined the stimulant-receptor interaction of concanavalin A (ConA) on the cell surface of lymphocytes and monocytes exposed to PFC.[257] PFC itself did not induce cellular activation but it significantly inhibited

activation during ConA stimulation. Neither the amount of cell surface-bound ConA nor the overall pattern of ConA receptor rearrangement was altered by PFC. Therefore, a PFC-induced alteration of stimulant-receptor interaction on the surface membrane does not seem to be the cause of attenuated cell activation. Furthermore, multiple studies have observed an attenuated response by PFC-exposed neutrophils to stimulation with phorbol myristate acetate.[251], [254], [255] Phorbol myristate acetate evokes a cellular response through an intracellular mechanism, more specifically, activation of the protein kinase C pathway. This observation cannot be fully explained by the suggested modified interaction between a stimulant and cellular membrane receptor, possibly suggesting a combination of both intra- and extracellular effects.[253], [254]

1.8 Perfluorocarbon-based Drug Delivery

PFC-based pulmonary drug delivery has been explored for a variety of drugs[258], including vasoactive drugs[259], plasmids[260], [261], pulmonary surfactant[219], [226], [262] insulin[263], and antibiotics[264]–[269]. PFCs represent an attractive medium for drug delivery not only due to their unique application during LV, but also due to their chemical stability. The inert nature of PFCs limits potential interactions with the drug that could hinder the drug's mechanism of action or efficacy. However, the manner in which the drugs are added to the PFC phase presents a challenge. Due to the hydro-, lipo-phobic nature of PFC, a simple mixture of PFC and most drug solutions will result in the less dense aqueous/oil phase quickly separating and rising to the liquid surface. The earliest efforts at PFC-assisted delivery used simple mixtures of drug solutions and PFC, relying solely on bulk flow turbulent mixing to disperse an aqueous phase throughout the

PFC.[264], [266], [268], [269] Although *in vivo* studies have clearly demonstrated that the PFC-assisted administration of aqueous solutions results in a homogeneous distribution of the drug within the lung, the phase separation of the drug solution and the PFC make this approach less useful, both from a pharmaceutical and a clinical point of view.[259], [266], [268], [269] A method of PFC-based delivery able to achieve sustained dispersion of drug in the PFC phase would likely result in increased therapeutic potential. Accordingly, multiple efforts have been made to utilize a drug-in-PFC suspension.

1.8.1 Soluble Drugs and Prodrugs

The most straightforward approach to use PFCs as vehicles for drug delivery is to increase drug solubility in the PFC and administer this solution directly to the lung. Perfluorooctyl bromide is an atypical PFC and is miscible with some organic compounds.[270]–[272] Additionally, a prodrug approach involves the modification of the parent drug with a promoiety to increase solubility in the PFC. The prodrug is expected to partition into the lung tissue after administration via chemical or biological degradation.[272] One study has synthesized prodrugs of nicotinic acid, a precursor of NAD which has been proven beneficial against acute lung injury.[273], [274] *In vitro* evaluation showed enzymatic hydrolysis of the nicotinic acid prodrugs allowed the parent drug to readily partition into cells with low levels of toxicity.[275]

1.8.2 Solubilizing Agents

Solubilizing agents are another possibility to enhance the solubility of drugs in PFC. Solubilizing agents interact noncovalently with a drug to form a PFC-soluble complex.

However, several limitations make it unlikely that solubilizing agents will be useful for clinical applications.[276] Success with this approach has been limited to drugs with phenolic hydroxyl groups and requires high solubilizing agent to drug ratios for dissolution.

1.8.3 Solid-in-Perfluorocarbon Dispersions

Another delivery scheme utilized solid, crystalline antibiotic microparticles suspended within PFC.[264], [265], [267], [268] These suspensions were typically created by spray-drying a mixture of antibiotic, saline, PFC, surfactants, and bulking agents to create porous microparticles with a fluorophilic shell. These microparticles form a stable suspension within a bulk PFC phase. Studies evaluating the pharmacokinetics of LV with these suspensions have shown the treatment's ability to significantly increase pulmonary antibiotic concentration relative to IV or intramuscular (IM) delivery, while still maintaining non-toxic serum concentrations.[265], [268] Treatment efficacy of these suspensions in the setting of a bacterial respiratory infection has also been evaluated. In one study, various treatments employing different antibiotic delivery mechanisms were initiated one day after rats were intratracheally inoculated with *Streptococcus pneumonia*.[264] Treatment with the microparticle-PFC suspensions showed significantly increased survival over a 10-day period relative to IM delivered antibiotics alone, but no advantage over other forms of antibiotic administration coupled with LV (IM or addition of unemulsified antibiotics to PFC). This suggests that the survival benefit may have been due to the anti-inflammatory properties of PFC or perhaps better oxygenation in consolidated lung regions.

1.8.4 Reverse Water-in-Perfluorocarbon Emulsions

An alternative method of drug addition involves suspending the drug in aqueous form (*i.e.* emulsified aqueous droplets in a continuous PFC phase) rather than as solid particles. The use of aqueous drugs rather than solid particles presents both potential advantages as well as new challenges. In theory, if aqueous drug solutions were able to be added during emulsion preparation immediately prior to delivery, a much broader selection of drugs (or even combination of drugs) could be utilized as compared to dry particle suspensions. Furthermore, these dosage forms would be easier to manufacture and potentially capable of encapsulating close to 100% of drug.[277] As previously mentioned, the preparation process required for dry particle suspensions is labor-intensive and thus a desired drug must undergo this individualized process well in advance and likely in a specialized facility. The reduced preparation associated with a liquid-in-liquid emulsion would likely translate to decreased overall costs as well. Additionally, having the drug phase present within the PFC mixture in an aqueous solution may also allow for more controllable kinetics. In the case of either dry particle suspensions or liquid emulsions, once the drug comes in to contact with an aqueous surface in the lung (*i.e.* bacterial biofilms, respiratory secretions, or the epithelium) it will likely be transported into or along that surface via passive diffusion.[263], [278] By having the drug present in a solution within the PFC mixture, one is able to control the concentration of that solution and, thus, better control drug kinetics within the lung. Ultimately this would theoretically allow for more freedom and customization in the treatment process.

Conversely, by including an aqueous phase in the PFC mixture, new mechanisms of instability are introduced. The primary stability concern within a suspension of

dispersed solids is coagulation, or clumping, of the solid particles. For fluid-fluid dispersions, additional methods of de-mixing may be present. The types of phase separation present can depend on many factors, but the most important are likely the size of the dispersed droplets, the density difference between the two phases, and the solubility of the dispersed phase within the continuous phase.[277] In the case of micron-sized, aqueous droplets within a continuous PFC phase, creaming of the less dense aqueous droplets will result in stratification of the two phases and is likely the most dominant form of phase separation. As creaming stratifies the mixture, bringing the droplets in closer vicinity to each other, the average thickness of PFC film separating each droplet decreases until it reaches a critically thin thickness and droplet coalescence occurs. Ostwald ripening, another de-mixing phenomenon in which smaller droplets dissolve in the continuous phase and redeposit on to larger droplets, is also present. This molecular diffusion of water is facilitated by the low cohesive forces in liquid PFCs.[279]

Few studies were found to have previously attempted the use of an emulsion of aqueous drugs in PFC rather than a suspension of solid particles.[263], [280] One study assessed the effect of intranasal instillations (1 mL/kg) of insulin-loaded emulsion on lung tissue integrity and blood glucose levels in mice. Although emulsions did not alter lung tissue integrity, mice exhibited decreased body weight within the 3–4 days that followed the first instillation. This decrease was, however, reversible within a few days. Additionally, insulin-loaded emulsions decreased blood glucose levels. These emulsions were stable over a two-week period. This study demonstrated the potential of the reverse PFC emulsion stabilized with dimorpholinophosphates (F8H11DMP) to systemically and safely deliver drugs, including peptides, upon lung administration. Another study

demonstrated the use of antibiotic-loaded emulsions in conjunction with LV.[280] These emulsions were formed using natural bovine surfactant as the emulsifier. PLV (10-20 mL/kg) with antibiotic emulsions in healthy rabbits resulted in significantly higher intrapulmonary antibiotic concentrations and lower peak serum antibiotic concentrations relative to IV administration. However, the bactericidal capacity and stability of such emulsions were not evaluated.

1.9 Perfluorocarbon Emulsion Ventilation

Lung diseases like CF and ARDS would significantly benefit from improved techniques that i) apply drugs more uniformly within the lung, including distal and plugged airways where secretions can pool, ii) reduce inflammation that further weakens lung function, and iii) remove mucus or edema that leads to decreased gas exchange. However, this goal is nearly impossible due to a paradox, that the drug necessary to recover normal respiratory function cannot be delivered without resolution of edema or mucus, but these cannot be resolved without that same recovery. In order to break through the paradox, we propose a treatment with perfluorocarbon emulsion ventilation (PEV).

During PEV, the lungs are filled with emulsions featuring disperse (< 2.5 vol%) micro-droplets of aqueous antibiotics and/or pharmacotherapies within PFC liquids. Antibacterial perfluorocarbon ventilation (APV) is the specific application of PEV using antibiotic-loaded emulsions for treatment of respiratory infections, as seen in CF. APV supports the respiratory function of the patients while treating the overlaying infections with therapeutic doses of antibiotics, thus reducing the development of drug resistant strains. Perfluorocarbon emulsions for alveolar repair (PEAR) is the specific application

of PEV using emulsions loaded with antibiotics and reparative pharmacotherapies for the treatment of acute respiratory failure, as seen in ARDS. PEAR not only supports the respiratory function of the patients, but also treats the underlying infection and/or injury, and limits further lung damage by promoting alveolar repair. PEV incorporates the necessary improvements to current delivery techniques as mentioned above. First, drug delivery is targeted to the lungs, yielding higher concentrations in the lung and lower systemic concentrations and risk of toxicity.

Although studies have shown inhaled antibiotic therapy to generate higher antibiotic levels in the sputum with lower systemic antibiotic absorption, its delivery is dependent on airflow. [8], [9], Therefore, inhaled drugs are unable to achieve therapeutic doses in the damaged, occluded, poorly ventilated regions of the lung where it is needed most. In contrast, PFCs have the unique ability to homogenously fill the injured lung, allowing PEV to achieve a more uniform distribution of drug throughout the lung, including dependent, atelectatic, and edematous regions typically unreachable via inhaled therapy. Second, PFCs anti-inflammatory properties may promote lung healing and reduce risk of chronic inflammation; inflammation during CF further weakens mucocilliary transport and during ARDS further weakens epithelial barrier function. Lastly, the tidal flow of liquid emulsion during PEV should actively remove infected mucus from airway walls in CF and inflammatory exudate from intra-alveolar spaces in ARDS. Considering the significant role that mucus and edema play in the progression of CF and ARDS, respectively, the debridement of abnormally thick mucus, biofilms, necrotic cells, and inflammatory exudate from the airways should be a primary aim of treatment.

Experiments have consistently demonstrated that LV mobilizes and transports thick secretions into the trachea such that they can be suctioned. This includes inhaled materials such as meconium that are even more tenacious than infected mucus. During spontaneous PFC breathing in rats, “thick, tenacious secretions” were removed from the lung.[281] Inflammatory exudate was mobilized and suctioned in patients with ARDS and meconium, which has a greater surface tension than infected mucus (205 vs. 72-81 dyne/cm), were washed from neonatal lungs.[188], [190], [191], [282]

The ability of PFC to mobilize these fluids from the lung can be better understood by examining the fluid mechanics of mucus flow. During any gas or liquid flow, the ability to detach and remove mucus from the airways is dependent on shear stress, surface tension, and gravitational forces on the mucus and the viscosity, elasticity, and mucus layer thickness.[27], [283], [284] Under fluid flow, shear stress causes mucus to bunch toward the airway lumen (Figure 1.7). When sufficient thickness is generated, the mucus detaches and moves into the bulk fluid flow. Mucus detachment increases with shear magnitude, shear duration, and mucus thickness. Mucus detachment is also greater in unhealthy, more elastic mucus than in healthy, less elastic mucus.[284], [285] Thus, thick layers of tenacious mucus will bunch and detach under shear that only causes thin, healthy mucus to flow along the airway. This is why coughing does little to remove normal mucus but is effective against thick mucus in disease states. Lastly, mucus detachment

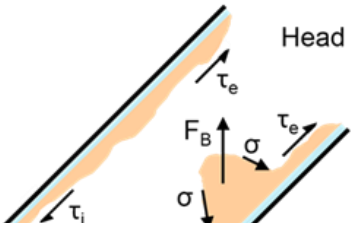

Figure 1.5 Airway depicting forces acting upon mucus. τ_i and τ_e are the shear stresses during inspiration and expiration, σ is the surface tension resisting mucus deformation, and F_B is the buoyant force due to the density difference between PFC and mucus.

is impaired by surface tension at the mucus-air interface, which stabilizes mucus and keeps it from detaching.[27] In addition, a lesser shear stress will be necessary to clear mucus during PEV, as the mucus surface tension is reduced by the presence of emulsion due to the aqueous nature of the mucus. Once mucus or exudate is mobilized, it can be transported out of the lungs. This movement is aided by the buoyant force upon the secretions; the density of PFC is nearly twice that of water and mucus. At the same time, this technique will not remove significant pulmonary surfactant. First, surfactant is not soluble in PFC.[281] Second, it should flow along the airway rather than detach: the surfactant layer is only about 0.1 μm thick, inelastic, and has a far lower viscosity than mucus.[286] Moreover, PFC velocities are very close to zero in the alveoli and thus alveolar surfactant will experience almost no shear. Accordingly, studies clearly demonstrate that pulmonary surfactant is not removed after up to three hours of PFC ventilation, and lung compliance returns to normal after PFC evaporation.[281], [287] Thus, patients have been transitioned to conscious, spontaneous breathing with normal lung function after LV.[189]–[194], [231]

These PEV treatments would be performed similarly to traditional total or partial LV. During total PEV, the patient is still sedated and intubated, but the lungs would be completely filled and ventilated with emulsion (~30 mL/kg). PEV would need only a short duration of treatment (< 2 hours) to ensure appropriate gas exchange while delivering drugs and removing edema or infected mucus. Since the lungs would be completely filled, the lung tissue would be in constant contact with the oxygenated and therapeutic emulsion. During partial PEV, the lung would only be partially filled with emulsion (approximately end-expiratory volume, ~10-20 mL/kg) and then gas ventilated using a

conventional ventilator. Oxygenation of the emulsion, and thus the alveoli, is maintained, but the lung surface would experience a lesser degree of tidal emulsion flow compared to total PEV. The entire lung would be in contact with the emulsion at the end of each expiration, but the larger conducting airways would be gas filled the remainder of the respiration cycle. At the conclusion of either treatment case, some portion of emulsion and any dislodged fluid or mucus would be suctioned out of the lung with the remainder left to evaporate under normal ventilation. This would leave behind a dose of drugs in the dependent and previously occluded airways. Since patients would probably be under sedation and paralysis, PEV is envisioned as a one-time adjunctive treatment to systemic or inhaled delivery. As, inhaled delivery clearly presents a less invasive, more convenient method of drug delivery compared to PEV, it should be attempted prior to PEV in virtually all cases. PEV is a more drastic treatment modality that could greatly improve morbidity and mortality in severe cases in which inhaled delivery is simply insufficient. Consequently, PEV is best suited for patients who are already on a ventilator. Respiratory bacterial infections in CF are common and often exacerbate the disease state to the point that mechanical ventilation is required; ALI often progresses to moderate/severe ARDS during which mechanical ventilation is required. Such patient populations would significantly benefit from PEV with very little added procedures or discomfort.

There have been no previous attempts to deliver growth factors. Insulin-loaded PFC emulsions have been examined, however, not in conjunction with LV. Although PFC has previously been examined as a means of pulmonary antibiotic and insulin delivery, our approach differs in multiple ways. None of the previous approaches examined LV in conjunction with drug delivery in a true emulsion using an appropriate emulsifier. As

discussed in Section 1.8, previous attempts of antibiotic delivery in PFC used a simple mixture of PFC and aqueous antibiotics, a spray-drying process resulting in a suspension of solid microparticles in PFC, or PFC emulsions stabilized with natural bovine surfactant. Delivery of the two phases without a stabilizing process or agent likely fails to sustain uniform therapeutic antibiotic concentrations in a large portion of the lung. The solid microparticle suspensions entail a costly and timely spray-drying process that has failed to show improved treatment efficacy over unstabilized mixtures.[264] The use of a liquid emulsion can likely decrease the cost of such a treatment as well as allow for delivery of a wider array of drugs with more controllable kinetics. Previous attempts to formulate an emulsion of aqueous antibiotics and PFC used natural bovine surfactant (composed of phospholipids) as the emulsifier.[288] Due to the lipophobic nature of PFC, natural pulmonary surfactant is not an ideal emulsifier.

The emulsions utilized in PEV feature disperse (< 2.5 vol%) micro-droplets of aqueous antibiotics and/or pharmacotherapies within PFC liquids. Due to the immiscibility of PFC and aqueous drugs, dispersion of the aqueous droplets is achieved by sonicating a mixture of PFC, aqueous drug, and fluorosurfactant. The hydrophilic and fluorophilc groups on the fluorosurfactant preferentially accumulate at the PFC–aqueous interface, temporarily stabilizing the droplets. These fluorinated surfactants must meet stringent requirements of having a minimal toxic effect, especially when delivered during an active immune response; producing a biologically inert interior surface to reduce biodegradation of any drug; and providing stability to the droplets to prevent separation. Consequently, there are few safe and effective fluorinated surfactants for these applications.[289], [290] The fluorosurfactant in this work utilizes a fluorinated synthetic oil based on

hexfluoropropylene oxide, more specifically a high-molecular weight Krytox 157FS oil (MW = 7250 Da; DuPont, Wilmington, DE). A triblock copolymer was formed utilizing a central polyethylene glycol (PEG, MW = 1000 Da) block with two Krytox 157FS ends (i.e. FSH-PEG-FSH, MW = 15,500 Da).[289], [291] The copolymer was formed by converting Krytox 157FS, a perfluoroether with carboxylic acid functionality, to an acid chloride which is then reacted with polyoxyethylene diamine. The molecular structures of both the unmodified Krytox 157FS and the copolymer (referred to as FSH-PEG) are shown in Figure 1.8. Further analysis and formulation must be developed to investigate cytotoxicity and *in vivo* clearance of our custom made fluorosurfactant. However, similarly structured Krytox-PEG copolymers have exhibited favorable biocompatibility when used to encapsulate mammalian cells or small multicellular organisms within aqueous microcompartments surrounded by PFC.[289], [292] However, further studies are required to determine cytotoxicity *in vivo* after PFC evaporates, leaving behind the fluorosurfactant inside an aqueous or PFC droplet.

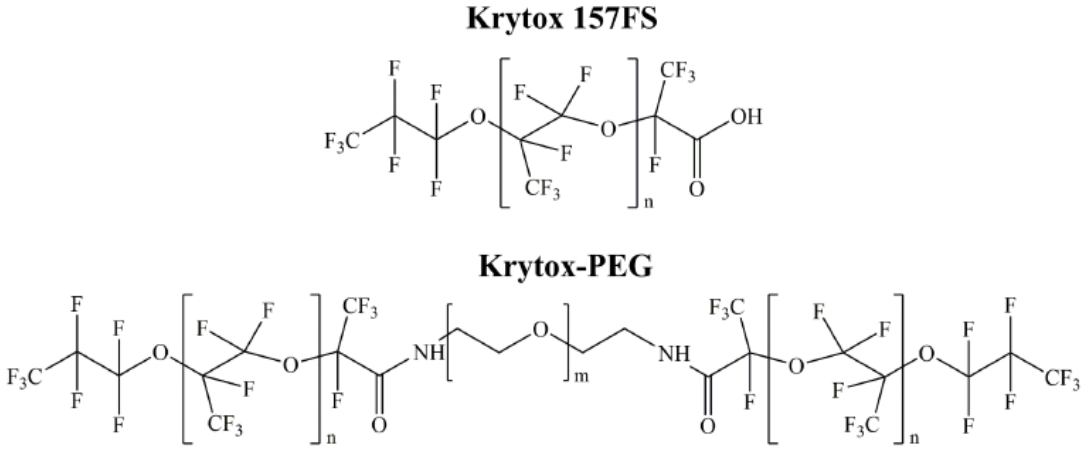

Figure 1.6 Molecular structures of the fluorosurfactant used as an emulsifier in the water-in-PFC emulsions (n = 41, m = 22).

In addition to creating a true emulsion, PEV focuses on removing lung mucus and exudates to maximize treatment. LV mobilizes lung exudates for removal, either by suction (PLV) or within ventilator mucus traps (TLV).[190], [191], [195], [281] However, this aspect of LV has not been evaluated in the treatment of respiratory infections and inflammatory injuries. For these reasons, we believe our approach differs from previous attempts and presents the potential for significantly improved treatment.

Previous *in vitro* work determined that the accumulation of fluorosurfactant at the aqueous surface of intended delivery impairs droplet deposition and, thus, delivery.[293] This partly explains why prior studies in rats using almost fifty times more fluorosurfactant resulted in significant tobramycin remaining in the lungs (up to 22 times that of aerosolized delivery) when the majority of emulsion was expected to have evaporated.[294], [295] Furthermore, these previous *in vivo* studies showed lower V_{aq} (lower total fluorosurfactant delivered) resulted in quicker and larger drug absorption into the systemic circulation, thereby increasing drug delivery. Since then, our lab has determined an optimal fluorosurfactant concentration that sufficiently emulsifies droplets without severely limiting drug availability in an *in vitro* setting. However, the physical mechanics by which drug is delivered from aqueous droplets within the emulsion to the aqueous surfaces of the lung (i.e., epithelial lining, biofilm, or mucus) is still not well understood. This is seen in the fact that despite the use of high fluorosurfactant concentrations, previous *in vitro* studies still showed emulsions were bactericidal, which was not reflected in later *in vivo* attempts where the drug is trapped in the emulsion.[293]

1.10 Summary of Study

This research set out to determine (1) if tobramycin-loaded, water-in-PFC emulsions could be formulated such that they were an effective means of antibiotic delivery and therapy during APV treatment of bacterial respiratory infections *in vivo* and (2) if lysophosphatidic acid-loaded, water-in-PFC emulsions could be used as a means of growth factor delivery and therapy of injured alveolar cells *in vitro*. This work represents an in-depth analysis of the emulsions used during *in vitro* and *in vivo* evaluations of APV and PEAR. Initial efforts characterized the fluid properties of the emulsions; maintaining the physical properties of liquid PFC is critical to the efficacy of APV/PEAR. Thus, evaluating the effects of the emulsification process on these properties is a necessary first step in determining their utility. The next phase involved evaluating the bactericidal capabilities of the APV emulsions using an *in vitro* setting that better simulated lung *in vivo* antibiotic delivery, including convective capillary transport. *In vivo* methods were then used to validate the pharmacokinetics and efficacy of antibiotic delivery via APV with one emulsion formulation. Lastly, our APV findings were translated to PEAR emulsions as a proof-of-concept to deliver growth factors *in vitro* and evoke cellular repair.

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CHAPTER 2

The Role of Convective Tobramycin Transport on Biofilm Bacterial Killing by Aqueous Tobramycin in Reverse Perfluorocarbon Emulsions

2.1 Introduction

Antibacterial Perfluorocarbon Ventilation (APV) is proposed as a short-term treatment that addresses the limitations of inhaled antibiotic delivery. Improved delivery methods are perhaps needed most during acute exacerbations of diseases such as cystic fibrosis and chronic obstructive pulmonary disease. During APV, the lungs would be filled with an emulsion containing aqueous antibiotics dispersed within perfluorocarbon (PFC) liquids (i.e. water-in-PFC) and ventilated for a short period of time (< 2 hours).[1]–[3] Such a treatment would improve antibiotic distribution and penetration in plugged and poorly ventilated regions of the lung as well as mobilize infected mucus for removal from the airways.[4]–[7] Since water and water-soluble drugs are immiscible in PFC, the FSH-PEG fluorosurfactant (chemical structures shown in Figure 1.8) is added to stabilize the droplets and maintain a uniform spatial distribution. Emulsion formulations are, therefore, characterized by their aqueous volume percent (V_{aq}, mL H_2O/mL emulsion), aqueous drug concentration (C_{aq}, mg/mL H_2O), total drug concentration (C_t, mg/mL emulsion) as defined by C_{aq} and V_{aq}, and fluorosurfactant concentration (C_{fs}, mg/mL H_2O). A change in any of these variables can affect the bactericidal capability of the emulsion to varying degrees.

Previous *in vitro* studies using *P. aeruginosa* biofilms have determined that maximizing C_{aq} optimally increases bacterial killing to a far greater extent than increasing V_{aq} and that optimizing C_{fs} increases antibiotic delivery relative to previously used formulations.[5], [6] However, these studies are physically unlike the lung, including the complex diffusional and convective transport processes that define antibiotic concentration in the biofilm. The optimization of APV for an *in vivo* setting, thus, requires an understanding of the time varying behavior during not only delivery to and but also transport away from the lung epithelium.

Therefore, the purpose of this study was to determine the effect of (1) emulsion formulation and (2) “blood” tobramycin levels on bacterial biofilm killing using an *in vitro* setting with a more accurate transport of the aqueous emulsion payload to and from the biofilm. To accomplish this, a two-chamber bioreactor was built that mimics antibiotic transport across the biofilm/lung and into the bloodstream where it is carried away. A biofilm was grown upon a permeable membrane within the bioreactor and exposed to emulsions of varying formulations on the “lung airway” side and to circulating tobramycin concentrations on the pulmonary capillary “blood” side to mimic *in vivo* pharmacokinetics.

2.2 Methods

2.2.1 Emulsion Preparation

The FSH-PEG fluorosurfactant was synthesized as previously described elsewhere. [6], [8] Tobramycin–loaded, water-in-PFC emulsions were prepared in 10 mL batches. First, FSH-PEG was dissolved in 350-580 µL of liquid PFC at an aqueous fluorosurfactant concentration (C_{fs}) of 2 mg/mL H_2O. The PFC used throughout these studies was

perfluorocycloether/ perfluorooctane (FC-770; 3M Inc., St. Paul, MN, USA). FC-770 has been used in previously published studies on partial liquid ventilation (PLV). [9]–[12] For eventual clinical translation, a PFC and fluorosurfactant with a higher level of purity and larger amount of documented *in vivo* safety data would be used. Next, tobramycin (X-Gen Pharmaceuticals Inc., Horseheads, NY, USA) was dissolved in 20 - 250 µL of sterile water to aqueous drug concentrations (C_{aq}) of 0, 40, or 500 mg/mL and added to the PFC solution to a final volume of 600 µL. The mixture was then sonicated (Model S-450D, 20 kHz, 3.2 mm diameter microtip; Branson Ultrasonics, Danbury, CT, USA) at 200 W/cm^2 for 20 seconds (pulse on: 5 seconds, pulse off: 2 seconds). The mixture was then added to 9.4 mL of PFC for a final volume of 10 mL and re-sonicated at 200 W/cm^2 for 60 seconds continuously. The resulting emulsions (pre-treatment) had aqueous volume percents (V_{aq}) of 0.2, 0.6, or 2.5% and total drug concentrations (C_t) of 0, 1, or 3 mg/mL emulsion (Table 2.1). The Blank Emulsion (BE) has no antibiotic and was used as a fluorosurfactant control. Emulsions were consistently prepared in 10-mL batches in an ice bath to minimize PFC evaporation during sonication and batch to batch variability.

Aqueous volume percent, V_{aq} [%]	Aqueous drug concentration, C_{aq} [mg/mL H_2O]	Total drug concentration, C_t [mg/mL emulsion]
2.5%	40	1
0.2%	500	1
0.6%	500	3
Blank Emulsion (BE), 0.6%	0	0

Table 2.1 Emulsion formulations (C_{fs} = 2 mg/mL H_2O).

2.2.2 Bioreactor Design and Construction

The bioreactor was machined from clear polycarbonate. Each bioreactor was comprised of a biofilm/“lung” side and a pulmonary capillary “blood” side (Figure 2.1A). The biofilm

chamber had dimensions (length × width × height) of 40 × 4.4 × 5.6 mm. The “blood” chamber had dimensions of 40 × 4.4 × 8.4 mm. The increase in height is due to the groove for gasketing. A female luer connector (Part #:96442, Qosina, Ronkonkoma, NY, USA) was inserted into each side of the bioreactor, sealed with silicone glue, and allowed to dry overnight. A T-connector with swivel male luer and two female luer locks (Part #: 80061, Qosina, Ronkonkoma, NY, USA) was connected to the luer at the inlet of the biofilm side and the outlet of the “blood” side. The bioreactor with attached connectors was gas sterilized using ethylene oxide along with non-vented male luer caps, 1/16” tygon tubing attached to a male luer lock to Barb Connector (Part #: 11533, Qosina, Ronkonkoma, NY, USA), and the cellulose membrane, which served as a biofilm substratum (47 × 17 × 0.105 mm; 0.025 µm pore size, Millipore Sigma, Billerica, MA, USA). The bioreactor was then assembled (Figure 2.1B) aseptically with the porous cellulose membrane placed between the two chambers. The membrane represents the air-blood barrier. The inlet and outlet tubing for the biofilm/“lung” side was attached and placed in growth media (1 part Tryptic Soy Broth with 1% glucose and 5 parts water (1:5 TSB)) and a waste bottle, respectively. The “blood” side was capped off.

2.2.3 *In Vitro* Assessment of Anti-Biofilm Activity

A cystic fibrosis–derived mucoid strain of *Pseudomonas aeruginosa* (*PA;* ATCC 27853) was selected for its biofilm-forming capacity. A *PA* biofilm was grown on the cellulose membrane within the bioreactor as follows. The bioreactor circuit (tubing and both

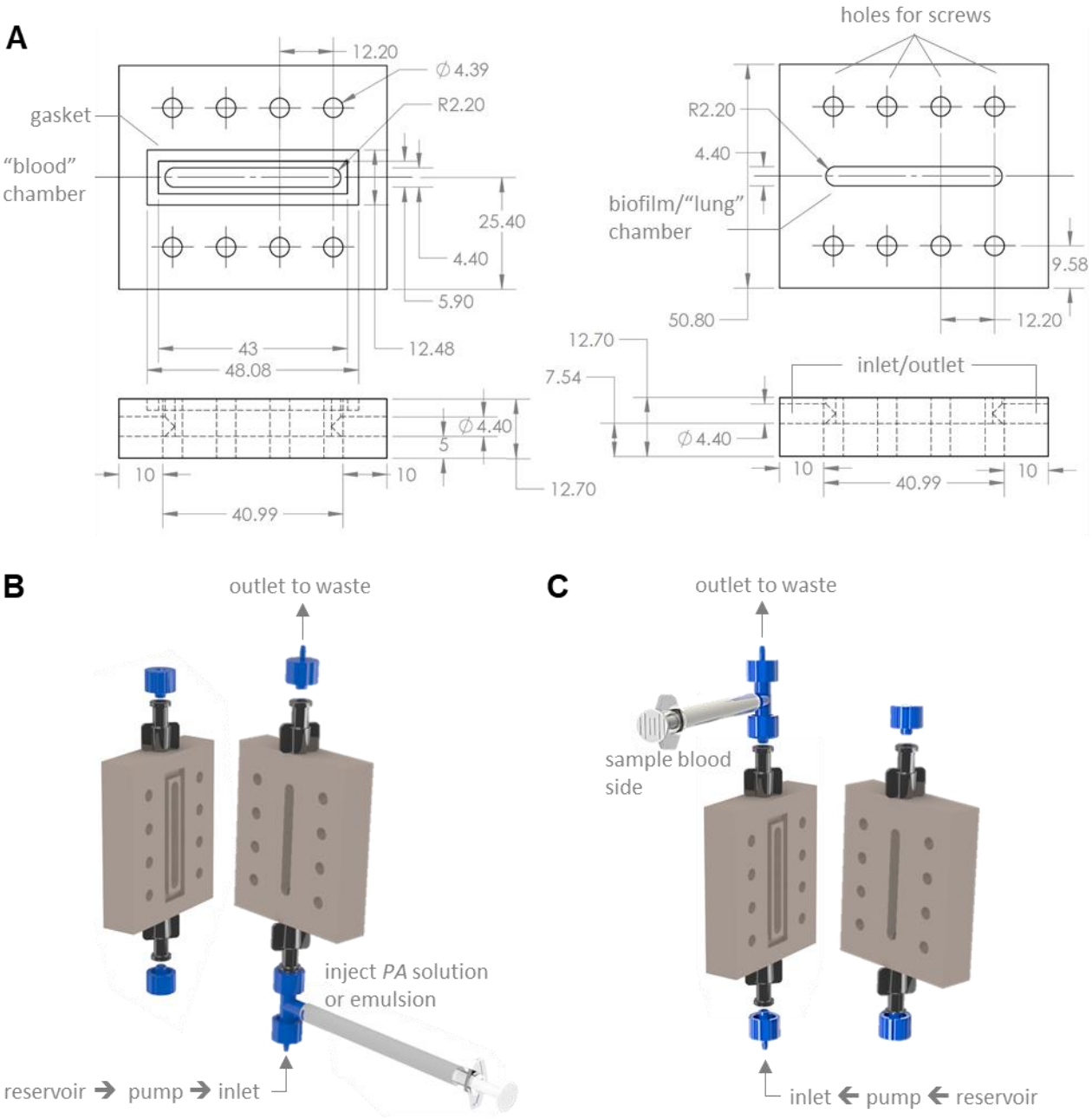

Figure 2.1 A. Bioreactor schematics in mm. **B.** Bioreactor setup for PA overnight growth under flow on the biofilm/"lung" side and stagnant "blood" side. **C.** Bioreactor setup for stagnant treatment on the biofilm/"lung" side and flow on the "blood" side.

chambers) was primed with 1:5 TSB. After priming, the "blood" side was left stagnant and closed to the atmosphere to limit evaporation (Figure 2.1B). A 3-mL aliquot of *PA* at an optical density (OD) of 0.1 at 600 nm was injected into the biofilm side of the bioreactor and left to attach for 1 hour without flow inside a 37°C incubator. The bioreactor remained

in the incubator throughout the duration of the experiment. Thereafter, the 1:5 TSB was flowed through the biofilm side in a single-pass for 21 hours at 0.2 mm/s using a peristaltic pump (Watson Marlow 205S; Watson-Marlow Inc., Wilmington, MA, USA). The pump was placed at the inlet to push the media through the chamber and reduce bubble formation as opposed to being placed at the outlet and pulling the media through the chamber.[13] Diluted growth medium was used to stress the bacteria and promote the biofilm-forming phenotype.[14] The porosity of the membrane was chosen to limit bacterial migration through the membrane.[15]

After biofilm growth, both chambers of the bioreactor were treated simultaneously for 2 hours: the biofilm side was treated with 1.8 mL of either 1:5 TSB, pure PFC, or emulsion (Table 1) and the "blood" side was exposed to various velocities (v_b) of "blood" tobramycin concentrations of either zero or a time varying concentration mimicking *in vivo* pharmacokinetics (C_b) (Table 2.2). First, to assess the role of blood flow (v_b) on tobramycin convection, we measured bacterial killing by a given emulsion under stagnant and physiological blood velocities (v_b). The blood velocity through systemic capillaries and the pulmonary capillary bed is v_b = 0.03 cm/s and v_b = 0.2 cm/s, respectively.[16], [17] The lower velocity was used simply for comparison purposes. The 2.5% V_{aq} (C_{aq} = 40 mg/mL, C_t = 1 mg/mL) was selected for comparison to its performance in our previous studies.[4]–[6] The 0.2% V_{aq} (C_{aq} = 500 mg/mL, C_t = 1 mg/mL) was selected because it is better suited for *in vivo* use. A lower V_{aq} (<1 vol%) and higher C_{aq} is more physiological because it leads to faster uptake in the serum and a quicker drop in concentrations, which has been shown to protect kidney function.[5], [18]–[22] In addition, it decreases the total fluorosurfactant delivered since high concentrations are cytotoxic.[6] Furthermore, these

two emulsions allowed us to determine the effect of decreasing V_{aq} (increasing C_{aq}) on bacterial killing and tobramycin transport when C_t is held constant.

	Treatment Groups						
"Blood" Velocity, v_b [cm/s]	1:5 TSB	PFC	2.5%	0.2%	0.6%	BE+C_b	0.6%+C_b
0 (stagnant)	X	X	X	X			
0.03 (systemic capillaries)			X	X			
0.2 (pulm. capillary bed)	X	X	X	X	X	X	X

Table 2.2 Bioreactor conditions to determine the effect of transport of tobramycin on bacterial killing using various emulsions and flow rates.

Second, to assess the effects of emulsion formulation under the higher, more challenging, simulated pulmonary blood flow, we measured bacterial killing of formulations with higher C_t at v_b = 0.2 cm/s. If the goal for short-term treatment of CF exacerbations is to achieve serum concentrations of approximately 30 µg/mL and our previous *in vivo* emulsions only peaked at 10 µg/mL with a dose of 10 mg/kg, then tripling the antibiotic dose to 30mg/kg should attain the desired peak because aminoglycosides have a linear relationship between the dose and the serum peak concentration. [4], [5], [18]–[22] Thus, the new physiological formulation will have C_t = 3 mg/mL emulsion and V_{aq} <1 vol% (V_{aq} = 0.6%, C_{aq} = 500mg/mL, C_t = 3mg/mL).

Lastly, to assess the effect of therapeutic blood tobramycin concentrations (C_b) at high flows, we measured bacterial killing after exposure to both therapeutic concentrations in the "lung" (0.6% V_{aq}) and in the "blood" (C_b = 30 µg/mL for the first 30 minutes of treatment and then 12 µg/mL for the last 90 minutes) at v_b = 0.2 cm/s. *In vivo*,

a nontrivial concentration of tobramycin collects in the serum during emulsion delivery to the lungs.[4], [5] Thus, biofilms are exposed to emulsified antibiotic in the lungs as well as antibiotic that has filtered through the alveolar wall and is recirculated in the blood. This known blood concentration profile of tobramycin (C_b) is based on recent studies on effective tobramycin doses (peak of 30 µg/mL within 30 minutes and trough of 1 µg/mL after 18 hours).[20]–[22] Since we aim to determine if the blood tobramycin concentration (C_b) plays a significant role in bacterial killing versus just "lung" side biofilm exposure, we also analyzed the effect of C_b alone. A blank emulsion (BE) having the same formulation but no antibiotic (V_{aq} = 0.6%, C_{aq} = C_t = 0 mg/mL) was on the biofilm side to allow for some aqueous surface to form on the biofilm (BE+C_b).

Immediately after preparation, the emulsion was filled into a syringe, and connected to the inlet side of the bioreactor. The pump was stopped, and the inlet tubing was clamped off. The test medium (emulsion, 1:5 TSB, or PFC) was injected at 0.2 mm/s so as not to shear off the biofilm. Once filled, both the inlet and outlet tubing on the biofilm side were disconnected from the circuit and capped to limit evaporation. In addition, the bioreactor sat vertically inside the incubator in order to eliminate any effect of emulsion creaming on antibiotic delivery (Figure 2.1C). On the "blood" side, if stagnant, the chamber was filled with saline and recapped. If under flow, the inlet tubing was connected via a luer and placed in a reservoir of saline with varying tobramycin concentration (0, 12, or 30 µg/mL) and the outlet tubing delivered to a waste bottle (see Table 2.2). The tobramycin solutions flowed through the "blood" side in a single pass, and, every 15 minutes, the outlet of the bioreactor was sampled (200 µL) and assayed for the tobramycin concentration. After a two-hour treatment, the membrane was aseptically

removed, and gently rinsed in 6.5 mL of Hank's Buffered Salt Solution (GibcoHBSS with Ca^{+2} and Mg^{+2}, Thermo Fisher Scientific, Waltham, MA, USA) to remove any planktonic bacteria. The remaining, post-treatment emulsion and, in stagnant cases, "blood" side saline solution was collected to assess tobramycin levels in addition to the "blood" samples collected during flowing conditions.

2.2.4 Image Analysis of Biofilm Viability

The rinsed membrane was cut in half lengthwise and imaged with confocal laser scanning microscopy. Briefly, after rinsing with HBSS, biofilms were stained with the Live/Dead Kit (Molecular Probe, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's directions, rinsed, immersed in HBSS and immediately examined with a Leica DM RXE microscope attached to a TCS SP2 AOBS confocal system (Leica Microsystems, Exton, PA) using a 63X water immersion lens. Images were analyzed using the Leica LCS software. Live bacteria are bright green specs; dead bacteria are bright red specs; and dying bacteria are bright yellow specs (colocalization of green and red). The porous membrane sequestered some of the stain and produced a background fluorescence. A filter was used to remove this background, but a hazy background of red (see Figure 2.3D) or green (see Figure 2.5D) can still be seen in some images. The other half of the membrane was assayed for bacterial viability as confirmation of treatment. To account for the inhomogeneous biofilm growth and the possible shearing of biofilm during halving, experiments used for imaging were excluded from the quantitative data set.

2.2.5 Quantitative Analysis of Bacterial Viability

After a rinse in HBSS, whole membranes were placed in fresh TSB (10 mL) and vortexed three times for 1 minute with a 30 second rest in between cycles to remove the adherent biofilm.[23] The viability of recovered biofilms was quantified as the change in optical density (OD) over 6 hours using previous methods.[4] In addition, the recovered biofilm was serially diluted onto Luria-Bertani (LB) agar plates to count colony forming units per mL broth (CFUs).

2.2.6 Emulsion Delivery

To characterize the amount of tobramycin delivered from the emulsion, the concentration of tobramycin was measured in the pre-treatment emulsion, the post-treatment emulsion after re-sonication, the "blood" (both stagnant and flowing), and the HBSS after the rinse. Re-sonication settings were 200 W/cm^2 for 20 seconds (5 seconds pulse on, 2 seconds pulse off). To extract the tobramycin from the emulsion, an inverted emulsion (continuous aqueous phase with dispersed PFC phase) was prepared as described elsewhere.[6] Briefly, immediately after emulsion sonication, a 240 µL sample of emulsion was diluted with 8 mL of sterile water and re-sonicated at 200 W/cm^2 for 1 minute, resulting in an inverted emulsion, which was then centrifuged at 3000 x g for 20 minutes in order to separate the PFC and aqueous phase. The resulting aqueous phase should have a theoretical tobramycin concentration of 30 µg/mL (C_t = 1mg/mL emulsion) or 90 µg/mL (C_t = 3mg/mL emulsion) if 100% is retrieved. Prior to testing, the 90 µg/mL aqueous phase was further diluted (1:2) to yield a theoretical maximum concentration of 30 µg/mL.

The presence of active aqueous tobramycin in the inverted emulsion, the “blood”, and the HBSS rinses was quantified using the agar well diffusion method as described elsewhere by many researchers.[6], [24]–[26] Briefly, molten LB agar was dispensed (30 mL/dish) into square, polystyrene petri dishes with a grid (Model FB0875711A; Thermo Fisher Scientific, Pittsburgh, PA, USA) on a flat surface. Note that accurately dispensed volumes are important because the resulting agar depth affects assay consistency. The dishes were allowed to cool at room temperature and stored at 4°C if not used immediately after preparation. Next, 500 µL of *PA* (OD_{600} = 0.1) in TSB was used to inoculate the surface of LB agar plates. After allowing the plates to dry at room temperature, wells with a radius of approximately 3.4 mm were made within the agar for each plate. Aqueous samples were loaded into wells (75 µL/well) within the inoculated agar in addition to standard solutions of known tobramycin concentration. A set of standards was included on each individual plate along with the experimental sample to be measured in order to minimize measurement variability due to differences in agar thickness between plates. The plates were then incubated for 24 hours at 37°C before circular inhibition zones were imaged and measured with ImageJ (US National Institutes of Health, Bethesda, MD, USA). Examples of inhibition zones from such a plate are shown in Figure 2.2A. By interpolation from the inhibition zones of the standard solutions, the concentration of the unknown samples was determined. Similar to previously described methods, linear regression analysis of the standards was obtained by plotting the log transformation of the known tobramycin concentrations as a function of inhibition zone radii (Figure 2.2B).[24] Standard curves typically had an $R^2 \geq 0.96$. The lower limit of detection using these methods was 5 µg/mL. Each sample was measured in duplicate

and averaged to produce a single concentration. Five separate trials with independently prepared emulsions were performed for each formulation evaluated. The theoretical maximums of tobramycin delivered in the pre-treatment emulsion on the biofilm side (1.8 mL) are 1800 µg (0.2% and 2.5% V_{aq}) and 5400 µg (0.6% V_{aq}).

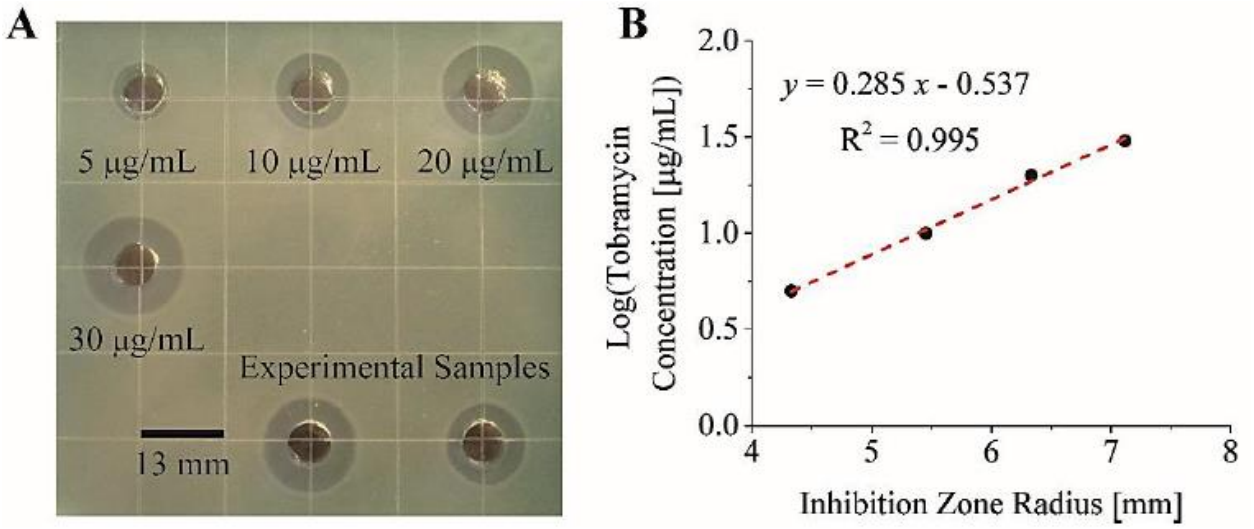

Figure 2.2 A. Example of inhibition zones resulting from the agar well diffusion method. **B.** Example of standard curve used for interpolation during agar well diffusion method.

2.2.7 Statistical Analysis

Statistical differences ($p < 0.05$) in the efficacy of emulsion formulations were evaluated using the post-treatment bacterial OD and the $\log_{10}$(CFU) [referred to as CFUs hereafter]. These differences were determined in SPSS (IBM Corporation, Armonk, NY). A two-way ANOVA and *post hoc* t-tests were used to determine significance between varying flow vs. emulsion formulations. A one-way ANOVA with multiple comparisons applying the Sidak correction was used to determine significance between emulsion formulations at a constant flow. There is an n of 5 or 6 per group. All values are mean +/- standard error.

2.3 Results

2.3.1 Bacterial Growth: A Robust *PA* Biofilm

Typical biofilms recovered from the bioreactor circuit are shown in Figure 2.3. Although images of *PA* biofilms varied in bacterial density (e.g. Fig. 2.3 A and B), they produced similar bacterial counts (data not shown). Biofilms were sufficiently removed from the membrane after vortexing (Fig. 3F). No difference was measured in biofilm formation, OD, nor CFUs between no flow (v_b = 0 cm/s) and flow (v_b = 0.2 cm/s) conditions under 1:5TSB treatment (Fig. 3A, B and C) nor under pure PFC treatment (Fig. 3D and E) treatment (data not shown). Furthermore, treatment with pure PFC showed no effect on biofilm formation compared to the 1:5 TSB control. This was confirmed by OD (1:5 TSB: 1.08 +/- 0.11; PFC: 1.54 +/- 0.24; p = 0.11) and CFUs (1:5 TSB: 5.6 +/- 0.18; PFC: 5.8 +/- 0.09; p > 0.39) via student's t-test.

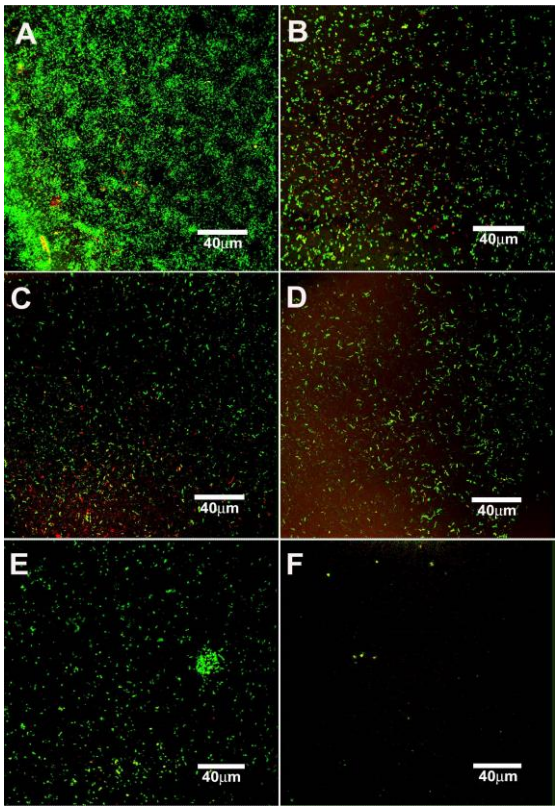

Figure 2.3 Confocal imaging of a typical *PA* biofilm after 21-hour growth followed by a 2-hour treatment on the biofilm side and saline (v_b = 0 or 0.2 cm/s) on the "blood" side. Images **A-E** are pre-vortex, while image **F** is post-vortex. **A.** and **B.** 1:5 TSB control treatment, v_b = 0 cm/s **C.** 1:5 TSB control treatment, v_b = 0.2 cm/s **D.** PFC, v_b = 0 cm/s (red haze is background) **E.** PFC, v_b = 0.2 cm/s **F.** 1:5 TSB control treatment, v_b = 0.2 cm/s, post-vortex, showing most bacteria are removed.

2.3.2 Bacterial Killing: Effect of Tobramycin Transport and Emulsion Formulation

Biofilm bacterial loads after 2-hour treatments are shown in Figure 2.4 as either OD or CFUs. Overall, emulsions can limit bacterial growth. However, bacterial killing was reduced when tobramycin was transported away from the biofilm by flowing saline on the "blood" side. The 2.5% V_{aq} emulsion significantly reduced OD (Figure 2.4A) under all three v_b conditions (0cm/s: 0.03 +/- 0.02; 0.03cm/s: 0.19 +/- 0.1; and 0.2cm/s: 0.15 +/- 0.13; $p < 0.01$ for each) compared to the 1:5 TSB control (1.08 +/- 0.11). The emulsion efficacy was slightly reduced with increasing v_b, but the three flow conditions were not significantly different from one another ($p > 0.99$ for all comparisons). Results for the 0.2% V_{aq} emulsion demonstrated a similar trend as the 2.5% V_{aq} emulsion, but with reduced efficacy. The 0.2% V_{aq} emulsion OD was significantly ($p = 0.001$) lower than the 1:5 TSB control when v_b = 0 cm/s, but not under either flow condition ($p = 0.99$ for 0.03cm/s and p=0.2 for 0.2 cm/s). In addition, the OD with v_b = 0 cm/s was significantly lower than that of v_b = 0.03 cm/s ($p = 0.03$), but not that of v_b = 0.2cm/s ($p = 0.91$). Lastly, there was no difference in OD between the emulsion formulations for the same flow rates ($p > 0.2$ for each).

Results for CFUs established the same trends but with the emulsions demonstrating an overall smaller effect on bacterial growth. Figure 2.4B shows the CFUs of 2.5% V_{aq} at v_b = 0 cm/s was significantly lower (1.85 +/- 0.73; $p < 10^{-9}$) than the 1:5 TSB control (5.6 +/- 0.18). The anti-bacterial effect of the 2.5% V_{aq} emulsion under flow was completely lost ($p > 0.97$ for both) compared to the 1:5 TSB control. This was confirmed by confocal images in Figure 2.5. The stagnant condition (panel B) had substantially less living bacteria than the control (panel A), but the flow conditions (panels

C and D) did not. Although Figure 2.5D had more bright red specs, the bacterial counts (OD and CFUs) were no different than 1:5 TSB controls (data not shown). The CFUs for the 0.2% V_{aq} emulsion demonstrated similar trends with reduced efficacy. The CFUs at v_b = 0 cm/s (3.63 +/- 0.14) was significantly lower than the control and both flow conditions ($p < 0.01$ for all). Similarly, the antibacterial effect was lost under flow compared to the control ($p = 0.99$ for both). Lastly, the only situation demonstrating significantly different efficacy between the emulsion formulations was v_b = 0 cm/s, where the 2.5% V_{aq} emulsion had a greater reduction in CFUs than the 0.2% V_{aq} emulsion ($p < 0.01$).

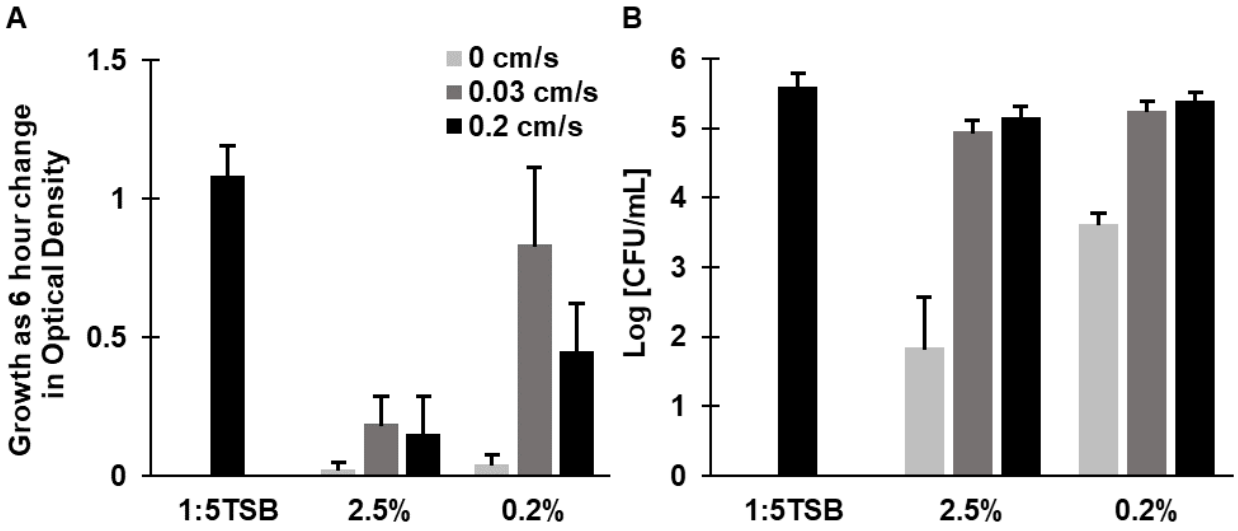

Figure 2.4 Biofilm growth after 2-hour treatment with 2.5 and 0.2% V_{aq} emulsion under stagnant (v_b = 0 cm/s) or flowing conditions (v_b= 0.03 and 0.2 cm/s). **A**. Change in Optical density (600nm) after 6-hour biofilm growth. **B.** Log of CFU/mL after 18-hour incubation.

To determine if this superior efficacy of 2.5% V_{aq} was due to any differences in emulsion delivery, the amount of tobramycin (µg) was measured in the pre- and post-treatment emulsion, the "blood", and the rinse. Both emulsions, on average, delivered similar amounts of tobramycin, retaining 30% (2.5% V_{aq}) and 24% (0.2% V_{aq}) of the total µg of tobramycin after treatment ($p = 0.52$). Also, similar amounts of drug were recovered in the rinses between the two emulsions, 8% (2.5% V_{aq}) and 6% (0.2% V_{aq}) of the total µg

of tobramycin in the pre-treatment emulsion. Under stagnant conditions, the amount of tobramycin that diffused into the “blood” was 2% (2.5% V_{aq}) and 3% (0.2% V_{aq}) of the total µg of tobramycin in the pre-treatment emulsion. Under flow, the concentration of tobramycin that diffused into the “blood” was less than the limit of detection (< 5 µg/mL) for both emulsions at all time points. Therefore, up to 70% of the total tobramycin could have diffused into the “blood”, resulting in a final concentration of less than 2.4 µg/mL, suggesting no difference in total tobramycin delivered between the two emulsions.

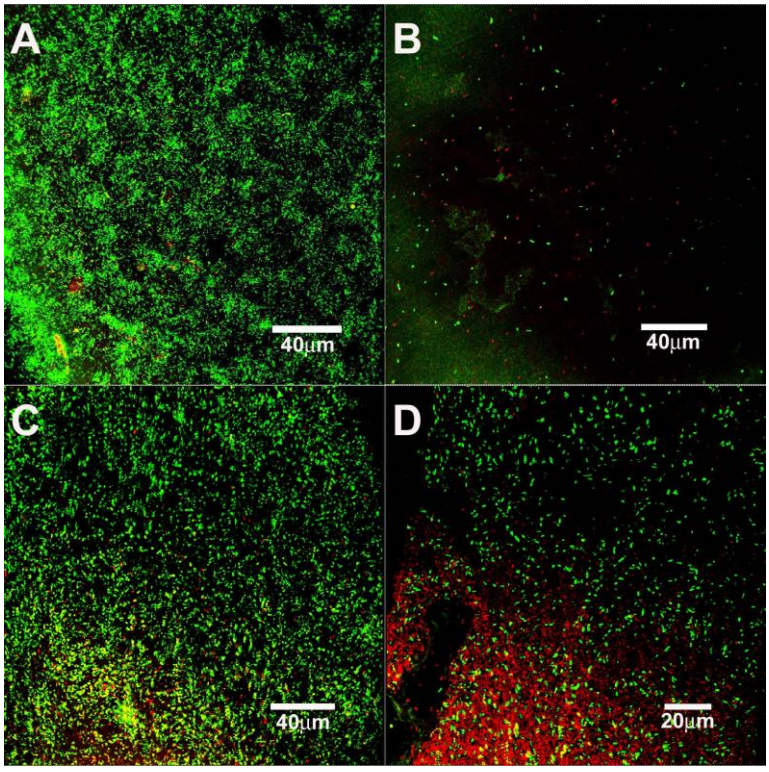

Figure 2.5 Confocal imaging of a typical *PA* biofilm after a stagnant 2-hour treatment on the biofilm side and saline (v_b = 0, 0.03, or 0.2 cm/s) on the “blood” side. **A.** 1:5 TSB control treatment, v_b = 0.2 cm/s **B-D.** 2.5% V_{aq} treatment with v_b = 0 cm/s (**B**), v_b = 0.03 cm/s (**C**), and v_b = 0.2 cm/s (**D**).

2.3.3 Bacterial Killing: Effect of Total Drug Concentration

Figure 2.6 compares how OD (Figure 2.6A) and CFUs (Figure 2.6B) were affected by higher C_t (higher V_{aq} and constant C_{aq}) when treated under physiological conditions (v_s = 0.2 cm/s). After treatment with 0.6% V_{aq}, both OD (-0.006 +/- 0.005, $p < 10^{-4}$) and CFUs (3.62 +/- 0.32; $p < 10^{-4}$) were sigificantly reduced compared to the control. In addition,

0.6% V_{aq} was significantly more bactericidal than the 0.2% V_{aq}, observed under OD ($p = 0.04$) and CFUs ($p < 10^{-3}$).

Since, C_t is three times higher in the 0.6% V_{aq} emulsion, three times the tobramycin was delivered: both emulsions retained a similar percentage of their respective tobramycin, 21% in the 0.2% V_{aq} and 23% in the 0.6% V_{aq}. Almost three times the amount of tobramycin was found in the rinse from 0.6% V_{aq} (12%) compared to the 0.2% V_{aq} (5%, p = 0. 036, student's t-test), but the "blood" side concentrations for both were below the limit of detection at all time points.

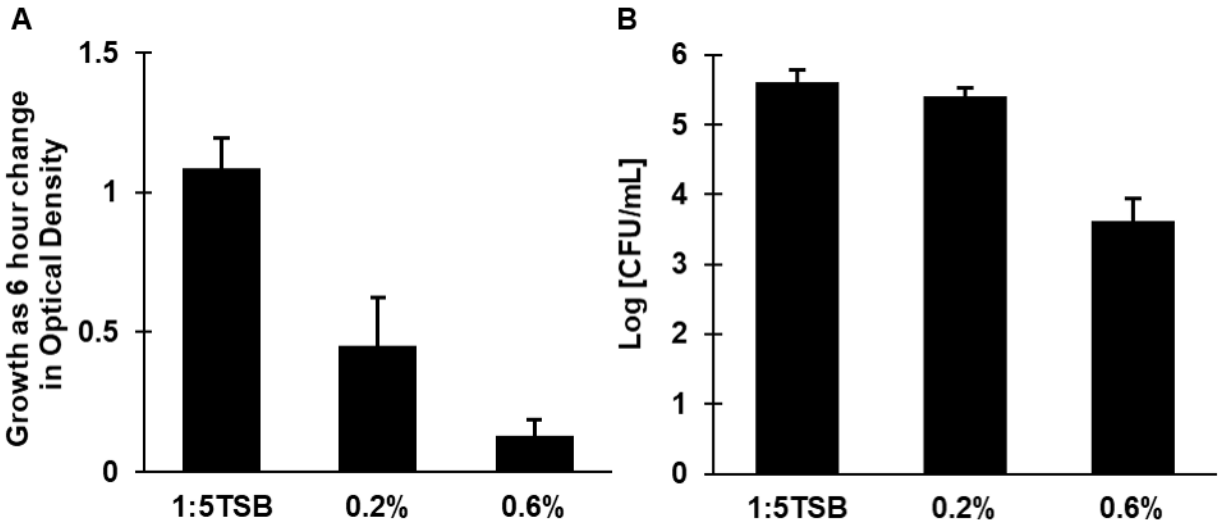

Figure 2.6 Biofilm growth after 2-hour treatment with 0.2% and 0.6% V_{aq} under pulmonary conditions (v_b = 0.2 cm/s). **A**. Change in optical density after 6-hour biofilm growth. **B.** Log of CFU/mL after 18-hour incubation.

2.3.4 Bacterial Killing: Effects of "Blood" Side Tobramycin Concentrations

To further evaluate our emulsion formulation under more realistic *in vivo* conditions, the biofilm was treated with emulsion on the biofilm/"lung airway" side while there is simultaneous, non-zero "blood" side tobramycin concentrations under physiological flows (v_b = 0.2 cm/s). This "blood" side tobramycin profile was C_b = 30 μg/mL for the first 30

minutes of treatment and then 12 µg/mL for the remaining 90 minutes of treatment. Figure 2.7A and 2.7B show treatment with the blank emulsion (BE) on the biofilm/“lung” side and tobramycin in the “blood” side (BE + C_b) significantly reduced the OD (0.03 +/- 0.01; $p < 10^{-10}$) and CFUs (3.62 +/- 0.44; p = 0.02), respectively, compared to the control. In addition, BE+C_b yielded a comparble reduction in OD and CFUs as seen in 0.6% V_{aq}, which only treats the biofilm side (p > 0.99 for both). Accordingly, treatment with tobramycin on both the biofilm and the “blood” side had an additive effect, reducing OD (-0.003 +/- 0.002; $p < 10^{-10}$) and CFUs (1.56 +/- 0.61; $p < 10^{-5}$) compared to the control. All three treatment groups maximized reduction in OD, and thus are not significantly different from one another (p > 0.99). However, the combined treatment of 0.6%+C_b had the greatest reduction in CFUs compared to the other treatement groups (p = 0.01 for both). For comparison, 0.6%+C_b under flow achieved the same reduction in CFUs as seen with the 2.5% V_{aq} (one-third the tobramycin dose and four times the water content) in stagnant conditions.

Although C_b behaved as an infinite bath, no measurable tobramycin accumulated in the blank emulsion (BE) post-treatment. Both the 0.6% V_{aq} and the 0.6%+C_b retained 23% and 26% of the total tobramycin in the post-treatment emulsion, respectively. Additionally, both emulsions had similar amounts of tobramycin washed off in the rinse, 12%came off from 0.6% V_{aq} and 21% from 0.6%+C_b (p = 0.35, student’s t-test). Therefore, the 0.6% V_{aq} delivered the same amount of tobramycin regardless of the presence of tobramycin in the “blood”.

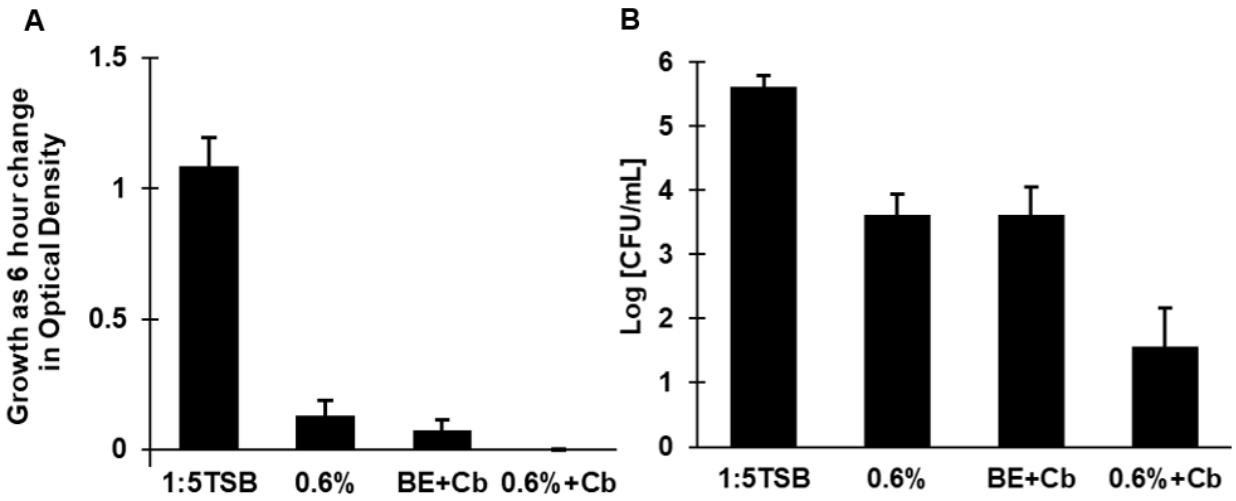

Figure 2.7 Biofilm growth after 2-hour treatment with 0.6% emulsion alone, blank emulsion with C_b alone (BE+C_b), and 0.6% with C_b (0.6%+C_b) under flowing conditions (v_b = 0.2 cm/s). **A**. Optical density after 6-hour biofilm growth. **B.** Log of CFU/mL after 18-hour biofilm growth.

2.4 Discussion

Overall, these results demonstrate that PFC emulsions are capable of significant bacterial killing within the biofilm. However, the results also show that bacterial killing is significantly reduced when tobramycin is convectively transported away from the biofilm chamber, as it would be via the pulmonary capillaries and lymphatic vessels. This diffusion of tobramycin through the biofilm does significantly halt the short-term bacterial growth as is observed in the change in OD after 6 hours, capturing the post-antibiotic effect.[27] However, that exposure to tobramycin does not lead to permanent bacterial killing as is observed in the CFUs after 18 hours, giving the damaged bacteria time to recover. In these studies, the extent to which convective transport of tobramycin away from the biofilm affects bacterial killing depends on the emulsion formation, the tobramycin dose, and the "blood" side tobramycin concentration.

Overall, the higher V_{aq} emulsion caused greater inhibition of bacterial growth at a total tobramycin dose, C_t, of 1 mg/mL. Compared to the 0.2% V_{aq} emulsion, the 2.5% V_{aq}

emulsion retained more of its ability to reduce the OD under flow (Figure 2.4A) and the CFUs in stagnant conditions (Figure 2.4B). These results are similar to previous, static studies when the emulsion was exposed to a highly porous inoculated agar surface.[6] In these settings, higher V_{aq} may allow a larger aqueous surface area and/or thicker aqueous film to spread over the biofilm and, thus, a more prolonged period of aqueous transport from the "airway" side, through the biofilm, and into the "blood". This likely exposes more of the biofilm to therapeutic concentrations, as opposed to smaller, but more concentrated aqueous areas that limits the effectiveness of the tobramycin-loaded droplet to radial diffusion, as would be seen in the 0.2% V_{aq}. Furthermore, the aqueous film from the 2.5% V_{aq} (C_{aq} = 40 mg/mL) may create less of driving force for tobramycin to diffuse down the concentration gradient into the "blood" compared to the 0.2% V_{aq} (C_{aq} = 500 mg/mL). If taken alone, these results suggest that transport away from the lung will eliminate most of the long-term antibacterial effect of the C_t = 1 mg/mL emulsions and make pulmonary delivery of emulsion ineffective. However, it must be remembered that these simulations occur with a constant, "blood" side antibiotic concentration of zero, a far more challenging situation than the normal, *in vivo* setting in which antibiotic from the lungs is transported to the bloodstream, building up the serum tobramycin concentration, and possibly slowing antibiotic transport from the airway lining to the bloodstream.

During *in vivo* delivery of similar emulsions containing C_t = 1 mg/mL, serum tobramycin concentrations have been shown to peak at around 10 µg/mL.[4], [5] The known ideal peak serum tobramycin concentration for intravenous delivery of tobramycin is 30-35 µg/mL.[20]–[22] Thus, higher tobramycin doses should be delivered for optimal effect. Because peak tobramycin concentrations scale linearly with dose, this study

examined if the bacterial killing was regained using emulsions with three times as much tobramycin: 0.6% V_{aq} emulsions with a total tobramycin concentration of C_t = 3 mg/mL.[19] Furthermore, we investigated how much additional bacterial killing would be created by having higher "blood" side tobramycin concentrations to help maintain higher concentrations of tobramycin in the biofilm.

When there is zero "blood" side tobramycin, the 0.6% V_{aq} emulsion demonstrated greater bacterial killing than 0.2% V_{aq}, emulsion, as observed by OD (Figure 2.6A) and CFUs (Figure 2.6B) while under flow. Post-treatment tobramycin concentrations in the emulsion confirmed that the 0.6% V_{aq} emulsion delivered three times as much tobramycin. Furthermore, when there is a more clinically appropriate "blood" side tobramycin concentration (C_b = 30 µg/mL for 30 min, then 12 µg/mL for the remaining 90 minutes) but no tobramycin transport from the emulsion (BE+C_b), there was similar bacterial killing as the 0.6% V_{aq} with flowing saline on the "blood" side (Figure 2.7B). During this case, the biofilm should equilibrate relatively rapidly with the "blood" side tobramycin concentration. The "blood" side acts as a large reservoir of tobramycin in this setting, and we saw little tobramycin delivered through the biofilm to the static emulsion in the "airway."

Combining the advantages of both the higher dose 0.6% V_{aq} emulsion and the expected "blood" side tobramycin concentration during this treatment, the 0.6%+C_b yields an additive increase in bacterial killing (Figure 2.7B). In this case, 0.6%+C_b achieved a 4-log reduction in bacteria compared to the 2-log reduction seen after the 0.6% V_{aq} and BE+C_b, separately. Although there is more "blood" side tobramycin, this does not hinder the emulsion from delivering the same payload as it did with C_b = 0 µg/mL over the 2-hour

treatment (~75% delivered in each case). Although 0.6%+C_b represents an ideal case, this result indicates the significant benefit of dosing the emulsion such that serum tobramycin concentrations reach the clinically ideal peak of 30-35 µg/ml.

Several additional factors not studied here will influence the ultimate clinical efficacy of the emulsion in treating a *Pseudomonas aeruginosa* infection. Clinical bacterial killing is unlikely to achieve such a high level as in this ideal 0.6%+C_b case featuring simultaneous, high concentrations of tobramycin in both the lung and blood compartments. However, *in vivo* treatment will include both multiple rounds of daily treatment and an active immune system. Both should progressively help to clear the damaged biofilm. This immune response might, however, be diminished by PFC's inherent, but poorly understood, anti-inflammatory effect as discussed in Section 1.7. Pure PFC also slightly increases bacterial growth, as seen in our previous studies, likely due to PFCs higher oxygen capacity and *PA* being an aerobic pathogen.[4], [28] This effect, however, is overcome in the presence of antibiotic (see Section 2.3). Lastly, a PFC and fluorosurfactant having a more documented *in vivo* safety record, such as perfluorooctyl bromide (perflubron), would likely be used clinically, and its effect on the immune system and antibiotic delivery may be slightly different. In addition, penetration of tobramycin within the airways, fluorosurfactant concentration, and orientation of lung tissues relative to the airway will change the emulsion's *in vivo* effectiveness. As previously shown, emulsions maintain similar viscosities and interfacial tensions as pure PFC.[4] Thus, emulsions maintain their ability to penetrate plugged airways, mobilize mucus for removal, and delivery drugs within deeper airways.[4], [29], [30]

Previous research has also determined that the optimal drug delivery occurs at an intermediate fluorosurfactant concentration that depends on the fluorosurfactant chemical structure.[6] Below the optimal concentration, the emulsion is too unstable to be prepared and delivered prior to phase separation. Above this, the emulsion droplets are too stable and, thus, do not coalesce with the aqueous surface of the lung. As such, the emulsions in this study were purposely made to be relatively unstable using the optimal fluorosurfactant concentration of 2 mg/ml of water. Despite this, 24-30% of tobramycin is consistently retained in the emulsion in each treatment, regardless of v_b and C_b. This is not surprising as even the optimal fluorosurfactant concentration (2 mg/mL H_2O) was shown to deliver only 57% in an in vitro, agar-well experiment.[6] *In vivo* delivery will be different, however, due to the large surface area of the lung relative to the emulsion volume and the certain delivery of all drug once the PFC evaporates after several hours. *In vivo,* lower V_{aq}*,* would be preferred because it would deliver less total fluorosurfactant to the lung, decreasing the barrier to diffusion that it possesses and enabling higher, faster serum tobramycin peaks.[5]

The emulsions in this study are also not sufficiently stable to measure droplet size and number density, but average droplet diameters for more stable versions of these emulsions are 1.9 +/- 0.2 µm with a droplet number density of 3.5 +/- 1.7 x 10^9 droplets/mL. [4] Aqueous droplets experience creaming due to the greater density of PFC relative to water, and this creaming can affect drug delivery at aqueous surfaces (biofilm, tissue) above or below the emulsion. For this reason, all experiments were done with the bioreactor facing vertically to remove any effect of creaming. *In vivo,* creaming would not create significant regional differences in drug delivery. Droplets in superior regions of the

lung would float into tissues. Droplets in dependent regions could flow into conducting airways but are not likely to travel large distances without contacting lung tissue and coalescing with the aqueous surface.

Ultimately, effective emulsion formulations appear to be possible. A treatment that kills bacteria while enhancing gas exchange could be of significant benefit to any situation in which a lower respiratory bacterial infection is inhibiting normal respiratory function and intubation might be necessary. In these cases, these emulsions could potentially turn mechanical ventilation from a situation in which the bacteria are actively protected by the endotracheal tube to one in which they are actively killed in the process. This work has shown, however, that these emulsions should feature higher emulsions concentrations (3 mg/mL) and achieve significant “blood” side tobramycin concentrations. Therefore, a good starting emulsion formulation for *in vivo* studies should incorporate $C_t \geq 3$ mg/mL to overcome the convective transport of tobramycin away from the lungs; $C_{fs} = 2$mg/mL to maximize drug delivery; and $V_{aq} < 1\%$ to minimize total fluorosurfactant delivered, decreasing toxicity and the barrier to delivery they possess.

2.5 References

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CHAPTER 3

In Vivo Evaluation of Reverse Perfluorocarbon Emulsions for Pulmonary Drug Delivery of Tobramycin in Respiratory Infections

3.1 Introduction

Antibacterial perfluorocarbon ventilation (APV) is proposed as an adjunct treatment that addresses the shortcomings of inhaled antibiotic delivery. In cystic fibrosis (CF), mucus plugging limits ventilation and delivery of inhaled antibiotics near infected regions where antibiotic is most needed.[1], [2] As a result, acute exacerbations of CF are typically treated with intravenous antibiotics, which increase the risk of toxicity.[3] During APV, the lungs are filled with a reverse emulsion containing a dispersed phase of aqueous antibiotics within perfluorocarbon (PFC) liquids (*i.e.* a water-in-PFC emulsion) and ventilated for a short period (< 2 hours). Such treatment would allow for improved penetration and distribution of delivered antibiotic directly to the lung as well as the ability to actively detach and remove infected mucus from the airways.[4]–[7]

To stabilize dispersed water droplets within the immiscible PFC, FSH-PEG (molecular structures shown in Figure 1.8) is used as an emulsifier and added to the mixture prior to mechanical dispersal of the aqueous phase. Previous work utilized a lower molecular weight version of this fluorosurfactant.[8], [9] Furthermore, recommendations from the previous study in Chapter 2 have significantly altered the emulsion formulation from what was previously used toward a lower fluorosurfactant

concentration, lower aqueous volume percent, and higher antibiotic concentration. Thus, the physical properties of these water-in-PFC emulsions are largely unknown. As discussed in Sections 1.6 and 1.9, the ability of PFC to provide adequate respiratory support as well as effectively remove mucus during LV are largely dependent on its physical and rheological properties. Therefore, it is imperative to understand any potential changes that are induced in these properties as a result of the emulsification process.

In addition to providing a means of respiratory support and mucus removal, the emulsion must also be capable of effective antibiotic delivery. As discussed in Section 1.2, bacterial biofilms represent a key pathological feature of chronic airway infections and a significant impediment to effective antibiotic therapy.[1], [10]–[12] Much of the therapeutic potential of APV rests on the ability of the tobramycin-loaded emulsions to achieve effective delivery to the lungs and eradication of bacteria in a biofilm setting. In Chapter 2, we determined that emulsions capable of achieving therapeutic levels in the lung as well as peak serum concentrations of 30-35 μg/mL had a greater impact on bacterial killing.[3], [13]–[16] Thus, an *in* vivo evaluation of pharmacokinetics is necessary to determine if our new emulsion formulation improved tobramycin delivery and/or efficacy compared to previous studies discussed in Section 1.9.

Because this work requires an accurate determination of delivered drug doses, partial APV treatment was used in this study. As previously mentioned, APV can be performed as either partial or total APV. During total APV, emulsion is constantly cycled out of the lung, through a large extracorporeal circuit, and back into the lung. It is, therefore, likely that some degree of drug deposition occurs within the extracorporeal circuit. Additionally, at the end of total APV, some portion of emulsion must be removed

from the lung to transition to partial APV. Procedures such as these make the determination of a total dose of drug delivered to the lung difficult and variable. Partial APV on the other hand involves delivery of a finite volume of emulsion (and thus drug) directly to the lungs. Although during partial APV in a human, some portion of emulsion would be suctioned from the lung, emulsion delivered in this work was not recovered. Suctioning liquid from the airways of a rat has proven to be technically difficult and highly inconsistent. Thus, partial APV (referred to in the remainder of the chapter simply as APV) without recovery of emulsion was used in this work.

Previous studies have shown that these emulsion formulations are effective at killing bacteria in bioreactor settings mimicking the pulmonary airways, including convective capillary transport of tobramycin away from the lung. The purpose of this study was to perform a comprehensive evaluation of a tobramycin-loaded, water-in-PFC emulsion in order to explore the feasibility and efficacy of APV *in vivo*. This work characterized the emulsion's rheology, *in vivo* pharmacokinetics, and bactericidal function during a single APV treatment of a rat *Pseudomonas aeruginosa* pulmonary infection model.

3.2 Methods

3.2.1 Preparation of Bacterial Inoculum

The ideal animal model for this work would mimic the disease characteristics clinically observed in cystic fibrosis (CF) patients during infection. This is primarily the presence of mucoid *PA* growth (*i.e.* biofilms) in the airways, increased production of thick respiratory mucus by the host, as well as a severe airway inflammatory response. To achieve the

characteristics of a chronic infection, the following work utilized a chronic *Pseudomonas aeruginosa* (PA) respiratory infection rat model that was induced using a modified version of a previously established alginate microbead inoculation model.[17], [18] Use of bacteria within alginate provides an additional barrier between the bacteria and the pulmonary environment, thereby typically resulting in a less immediate immune response and more prolonged infection. A cystic fibrosis–derived mucoid strain of PA (ATCC 27853) was selected for its biofilm-forming capacity. Bacteria was grown in tryptic soy broth (TSB) supplemented with 1% glucose at 37°C on a gyratory shaker to mid-log growth. The optical density (OD) of the resulting solution was measured and the number of colony forming units (CFU) per volume of bacterial solution was calculated (assuming 1.78×10^8 CFU/mL at $OD_{600} = 0.1 \pm 0.005$). Ten mL of sterile phosphate-buffered saline (PBS) and approximately 1.1×10^9 CFU (approximately 1-10 mL of bacteria solution depending on its phase of growth) were combined in a 15-mL conical. An example calculation is below:

OD of original bacteria solution is 0.205.

To achieve an OD = 0.1in 1mL, solve for x: (0.205 OD)*(x mL) = (0.1 OD)*(1 mL).

x = 0.488 mL is needed to get 1.78×10^8 CFU from original bacterial solution.

Prepare a sample at this dilution (0.488 mL original bacterial solution plus 0.512 mL sterile TSB) and confirm OD = 0.1 ± 0.005 (if accurate, continue with calculation; if not, remeasure original bacteria solution and repeat calculation and OD confirmation).

The original bacterial solution has (1.78×10^8 CFU) / (0.488 mL) = 3.65×10^8 CFU/mL.

The target bacterial count is 1.1×10^9 CFU to yield 1×10^6 CFU after washing steps.

Therefore, (1.1×10^9 CFU) / (3.65×10^8 CFU/mL) = 3.014 mL needed form original bacterial solution to have 1.1×10^9 CFU. This is then added to 6.986 mL of PBS.

The bacteria-PBS mixture was then centrifuged at 3,725 x g at 4°C for 20 minutes. Supernatant was discarded to the taper line of the 15-mL conical (leaving approximately 1.5 mL), and the bacteria were re-suspended in PBS to a final volume of 2.5 mL. The previously described centrifugation and re-suspension step was repeated once more to ensure removal of growth medium from the bacterial suspension. The suspension was serial diluted for quantitative culture on Luria-Bertani (LB) agar plates to ultimately confirm the number of bacteria delivered to each rat. Quantitative cultures were performed on this solution rather than the final alginate solution because they are more accurate and reliable. Two milliliters of bacterial suspension were added to 7.5 mL of previously prepared sodium alginate solution (11 mg/mL; Alginic acid sodium salt, CAS # 9005-38-3; Sigma Aldrich, St. Louis, MO, USA). Droplets of the sodium alginate and bacteria solution were forced through a small-gauge, polyethylene tubing with an inside diameter of 0.58 mm (BD Intramedic Polyethylene Tubing, Model 427411; Becton Dickinson, Franklin Lakes, NJ, USA) while a jet of air was aligned coaxial to the tubing to blow off the droplets. Droplets were blown into a sterile flask containing 40 mL of a cross-linking solution of 0.1 M $CaCl_2$ in TRIS-HCl buffer (0.1 M, pH 7.0) and allowed to cure for 1 hour under continuous stirring at room temperature. The alginate solution was then centrifuged at 3,725 x g at 4°C for 5 minutes. The supernatant was discarded, and the microspheres re-suspended in 25 mL of sterile PBS. Centrifugation was repeated once more to ensure removal of excess $CaCl_2$ solution from the bacterial suspension. The supernatant was discarded, and the microspheres were re-suspended in sterile PBS to a final volume of 8 mL. The final inoculum solution contained 10.31 mg/mL of sodium alginate and an inoculum bacterial concentration of approximately 10^6-10^7 CFU/mL. Equations are below:

Total CFUs/mL = (Averaged CFU count across dilutions) / (0.05 mL plated per dilution)

Total CFUs encapsulated into alginate beads = (Total CFUs/mL)*(2 mL of solution added to alginate solution)

Inoculum bacterial concentration = (Total CFUs encapsulated into alginate beads) / (8 mL final volume of inoculum solution with added PBS)

Inoculum dose of CFUs/lung = (Inoculum bacterial concentration, CFUs/mL)*(0.2 mL delivered to each lung)

The final inoculum was stored at 4°C for at most 2 hours before delivery (0.2 mL) to rats.

3.2.2 Inoculation Procedure

Animal work was approved by the Allegheny Health Network's IACUC. To achieve an infection of 10^6-10^7 CFU/rat lung, specific pathogen-free, male Sprague Dawley rats [n = 42, weight 453 +/- 53 g (mean +/- standard deviation); Taconic, Hudson, NY, USA] were initially anesthetized with a mixture of ketamine hydrochloride [70 mg/kg, intraperitoneal (IP); Hospira Inc., Lake Forest, IL, USA] and xylazine hydrochloride (7.5 mg/kg, IP; Lloyd Laboratories, Shenandoah, IA, USA). Rats were placed in a prone position and orally intubated with a 16-gauge angiocatheter (used as an endotracheal tube). Immediately following intubation, a length of tubing (inner diameter 0.58 mm) was inserted through the angiocatheter such that the tip of the tubing extended approximately 2 mm past the end of the angiocatheter within the trachea. This placed the end of the tubing approximately 2-3 mm proximal to the carina (see Figure 3.1). The appropriate length of tubing to be inserted into the angiocatheter was measured and marked, and then loaded with 200 µL of freshly prepared inoculum using a 1-mL syringe full of air attached to one end of the

tubing via a 24-gauge angiocatheter. Following placement of the tubing in the trachea, the syringe was used to evacuate the contents of the tubing, thereby intratracheally delivering the inoculum. Inoculum doses of 10^6-10^7 CFU/rat were used throughout the trials. Such a dose tends to result in low mortality while also producing a lasting infection. A short period (approximately 5 minutes) of low tidal-volume mechanical ventilation (Model 683; Harvard Apparatus, Holliston, MA, USA; tidal volume (TV) = 2.5 mL/kg bodyweight; respiratory rate (RR) = 30 breaths/minute; positive end expiratory pressure (PEEP) = 3 cmH_2O) followed immediately after inoculum delivery (in the supine position) in order to resuscitate breathing. Following delivery of the inoculum and resumed spontaneous breathing, rats were provided supplemental oxygen until observed to be ambulatory at which point they were returned to housing with free access to food and water. The anti-inflammatory drug carprofen (50mg/mL, subcutaneous (SQ), Pfizer, Groton, Connecticut, USA) was administered subcutaneously (5 mg/kg bodyweight). All rats remained in housing for a period of 24 hours before undergoing treatment. Body weight was measured daily, and animals were observed for signs of pain and distress.

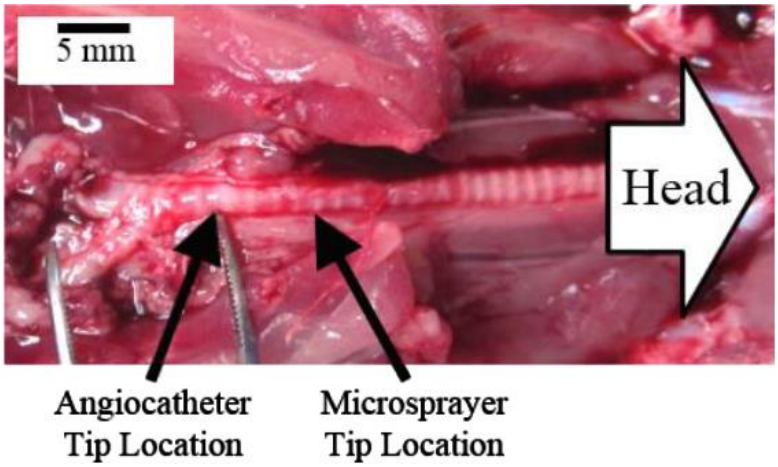

Figure 3.1 Image of intubated rat trachea showing angiocatheter (16-gauge, 1.77” long) and Microspayer (Model IA-1B) tip locations relative to carina during treatment.

3.2.3 Tobramycin–PFC Emulsion Preparation

Tobramycin–PFC emulsions were prepared in 10 mL batches as described previously.[19] Briefly, FSH-PEG at fluorosurfactant concentration (C_{fs}) of 2 mg/mL H_2O (as described in Section 1.9) was dissolved in 9.960 mL of PFC liquid. The PFC used throughout these studies was perfluorocycloether/perfluorooctane (FC-770; 3M Inc., St. Paul, MN, USA). Sterile water with 750 mg/ml of tobramycin (X-Gen Pharmaceuticals Inc., Horseheads, NY, USA) was added to the PFC solution. The two phases were emulsified via sonication (Model S-450D, 3.2 mm diameter microtip; Branson Ultrasonics, Danbury, CT, USA) at 200 W/cm^2 for 60 seconds. The emulsion used for APV in animal studies had a final C_{fs} = 2 mg/mL H_2O (0.08mg FSH-PEG), a final aqueous volume percent (V_{aq}) of 0.4% (40 µL H_2O), and a total drug concentration (C_t) of 3 mg/mL emulsion. The two emulsions used for emulsion characterization also had final C_{fs} = 2 mg/mL H_2O and aqueous drug concentrations (C_{aq}) of 750 mg/mL H_2O but had V_{aq} = 0.2 and 2.5%. Formulations are listed in Table 3.1. All emulsions were then stored at 4°C until either undergoing characterization or being delivered to rats within thirty minutes of preparation.

Aqueous volume percent, V_{aq} [%]	Aqueous drug concentration, C_{aq} [mg/mL H_2O]	Total drug concentration, C_t [mg/mL emulsion]
0.2% (characterization)	750	1.5
0.4% (rat APV)	750	3
2.5% (characterization)	750	18.75

Table 3.1 Emulsion formulations (C_{fs} = 2 mg/mL H_2O).

3.2.4 Emulsion Viscosity

The dynamic viscosities of pure PFC and 0.2% and 2.5% V_{aq} emulsions were measured using a cone and plate rheometer (Brookfield DVII-Pro; Brookfield Engineering

Laboratories, Midleboro, MA) at 37°C and 200 s^{-1} as previously described.[8] Three repeated measurements were taken.

3.2.5 Emulsion Air and Water Interfacial Tension

The surface and interfacial tension of pure PFC and 0.2% and 2.5% emulsions were measured using a DuNouy ring tensiometer with a platinum-iridium ring (Cenco Model 70545; 6 cm circumference; CSC Scientific Company Inc., Fairfax, VA) as previously described.[4] Immediately after emulsion preparation, 10-15 mL of emulsion or pure PFC were placed in a 50-mL beaker and allowed to reach room temperature (22-25° C) before being used for measurements. For water-emulsion or water-PFC interfacial tension measurements, 10 mL of de-ionized, filtered water was introduced on top of the emulsion or pure PFC. Three repeated measurements were taken for each condition.

3.2.6 *In Vivo* Assessment of Emulsion Efficacy

Animal preparation. The most clinically relevant method of evaluation would be to intervene with treatment only after the bacteria has been introduced to the airways and given sufficient time to acclimate as well as evoke a host response (*i.e.* inflammation and mucus production). Considering this, all treatment intervention in this work was initiated no earlier than 24 hours following bacterial delivery. One day after inoculation, all rats were initially anesthetized as described above then continuously infused intravenously via the lateral tail vein with ketamine (1 mg/kg/min) and xylazine (0.03 mg/kg/min) to maintain sedation. All rats were given supplemental 100% oxygen throughout the duration of the experiment. Heart rate and arterial oxygen saturation were monitored via a pulse

oximeter (VetOx Plus 4800; Heska, Loveland, CO, USA). Rat body temperature was monitored and maintained with a homeothermic blanket system (Model 507220F; Harvard Apparatus, Holliston, MA, USA). A throat swab (Peel Pouch Dryswab Fine Tip, Model MW113; Medical Wire and Equipment Co., Wiltshire, England) was performed and cultured to confirm the presence of *PA* in the airways. Following swabbing of the back of the throat, the swab tip was cut off into a microcentrifuge tube containing 1 mL sterile water and stored on ice. The tube was allowed to sit at room temperature for 30 minutes and then vortexed for 30 seconds before a sample (50 µL/sample) was plated on PA-selective agar (Cetrimide Agar Base; Becton Dickinson, Franklin Lakes, NJ, USA) and incubated at 37° C overnight. In the supine position, rats underwent carotid cannulation with a 24-gauge angiocatheter and a tracheostomy with a 16-gauge angiocatheter(used as an endotracheal tube).[20] In an effort to ensure complete delivery of the intended drug dose to the airways, a tracheal tie was implemented in all groups in order to create a seal around the angiocatheter within the airway. This also ensured changes in pulmonary pressures due to the tie were accounted for in all groups. After the tie was tightened around the trachea and the angiocatheter inside of the tracheal lumen, rats were connected to the ventilator in a supine position.

Mechanical ventilation. All rats underwent 6-hour ventilation with tidal volume (TV) = 4-9 mL/kg bodyweight, respiratory rate (RR) = 50-100 breaths/minute, positive-end expiratory pressure (PEEP) = 3 cmH_2O, and 100% oxygen. RR and TV were adjusted to maintain peak inspiratory pressures (PIP) less than 30 cmH_2O. Peak inspiratory pressures were measured continuously by using an airway pressure monitor attached to a side port on the angiocatheter.

Study groups. Rats were assigned to one of four treatment groups: untreated control, aerosolized aqueous tobramycin (positive control, 30 mg/kg), PFC only (no drug, 10 mL/kg), and partial APV (10 mL/kg delivered to a final dose of 30 mg/kg). Untreated control animals were anesthetized, catheterized, and mechanically ventilated, but were given no antibiotic treatment. Aerosolized aqueous tobramycin was administered via a Microsprayer Aerosolizer (Model IA-1B; Penn-Century, Wyndmoor, PA) as previously described.[9] Briefly, prior to tracheostomy, the length of the angiocatheter was trimmed to ensure that the Microsprayer nozzle was sufficiently exposed when fully inserted into the angiocatheter (see Figure 3.1). Following tracheostomy and a brief period of ventilation, the ventilator was disconnected from the trimmed angiocatheter and the Microsprayer was fully inserted into the angiocatheter and tobramycin (30 mg/kg in 250 µL of sterile saline) was delivered intratracheally. Although efforts were made to synchronize aerosol delivery with inspiration, the rats often became temporarily apneic following insertion of Microsprayer and thus the aerosol was sometimes delivered in the absence of inspiratory or expiratory flow. The partial PFC and APV were achieved by instilling preoxygenated PFC (10 mL/kg) and preoxygenated emulsion (10 mL/kg delivered to a final dose of 30 mg/kg), respectively, via a port connected to the angiocather. The PFC or emulsion was instilled during gas ventilation in successive aliquots (2-3 mL/aliquot) with each aliquot instilled over a period of approximately 60 seconds. Gas ventilation was continued for 6 hours in the supine position following treatment; all rats were euthanized via exsanguination of the carotid artery.

Evaluation of physiological status during ventilation. Arterial blood gas was measured from blood samples drawn via the carotid artery to evaluate lung function and

the effectiveness of ventilation and antibiotic therapy and to detect any acid-base imbalance. Measurements were taken at baseline prior to treatment and at 1, 3, and 6 hours post-treatment. Interventions included changes in ventilator settings and administration of sodium bicarbonate (1 - 3 mEq/kg) into the lateral tail vein to mitigate major changes in sO_2 (oxygen saturation), $PaCO_2$, pH, and bicarbonate.

Pharmacokinetic evaluation of serum tobramycin. Blood samples were drawn via the carotid artery at 0, 10, 30, 120, and 360 minutes following delivery of tobramycin. Immunoassay measurements of serum tobramycin concentration were performed by the Laboratory Medicine Department within Allegheny General Hospital (Pittsburgh, PA).

Lung harvest for microbiological assessment. All trials evaluated pulmonary bacterial load at six hours following a single treatment (also initiated at 24 hours post-inoculation). Although the goal of these trials was to evaluate pulmonary bacterial presence immediately post-treatment, necropsy was delayed six hours post-delivery in an attempt to allow adequate time for tobramycin to leave the lungs via systemic absorption. Tobramycin still present in the lung tissue at the time of homogenization may affect the accuracy of subsequent quantitative cultures. Post euthanasia, lungs and trachea were removed intact. The lungs were then thoroughly homogenized (LabGEN 7b Series Portable Homogenizer; Cole Parmer, Vernon Hills, IL, USA) with sterile saline (25 mL/kg bodyweight) for further processing. This resulted in an average volume of 11.3 mL of lung homogenate. This dilution was necessary to ensure that lung tobramycin concentrations were underneath the minimal inhibitory concentration (25 µg/mL, data not shown) following homogenization. This concentration was found by exposing inoculated homogenized lung to various concentrations of tobramycin and counting resulting CFUs.

The homogenizer was immersed in ethanol followed by two separate rinses in sterile water in between samples in order to avoid cross contamination. The second rinse was plated onto PA-selective agar (50 µL/sample) and incubated at 37° C overnight to confirm no cross contamination occurred. A sample (100 µL) of the lung homogenate was then serially diluted and cultured on PA-selective agar (50 µL/sample) and incubated overnight at 37° C in order to quantify the number of viable bacteria present (CFUs/lung). Equations are below:

Total CFUs/mL = (Averaged CFU count across dilutions) / (0.05 mL plated per dilution)

Total CFUs/lung = (Total CFUs/mL)*(25 mL/kg)*(kg bodyweight)

Results are expressed as $\log_{10}$CFU/lung. The presence of tobramycin in the homogenate was measured as previously described.

Alveolar permeability to FITC-dextran. Lung permeability was determined by retention of FITC-dextran in the lung tissue indicating extravasation from the vascular space. One milliliter of FITC-dextran (70kDa) in saline (0.5 mg/mL) was administered directly into the lateral tail vein via the angiocatheter 2 hours prior to euthanasia. During exsanguination from the carotid artery, the heart was exposed, and the superior and inferior vena cava were severed. Ten milliliters of saline were flushed through the right ventricle to clear the lung circulating dextran in the blood. The lungs were removed and homogenized as previously described. The homogenate was centrifuged at 16,161 x g for 30 min. Fluorescence of the supernatant was measured at 485 nm excitation and 525 nm emission in triplicate. A standard curve for fluorescence vs. FITC-dextran concentration was constructed for each experiment. Results are represented as µg dextran per lung.

Cytokines (IL-6 and TNF-α) and chemokine (MIP-2) in lung homogenate. The lungs were removed, homogenized, and centrifuged as previously described. The supernatant was stored at -80°C until measurement. IL-6 (#KRC0061C), TNF-α (#KRC3011C), and MIP-2 (#KRC1022) levels in lung homogenate supernatants were measured according to the manufacturer's directions using commercially available cytokine-specific rat ELISA kits (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Results (µg per lung) are reported as normalized values to a healthy rat.

3.2.7 Statistical Analysis

SPSS (IBM Corporation, Armonk, NY) was used to perform all statistical analysis. A one-way ANOVA and post-hoc analysis were performed with $p < 0.05$ as the level of significance for viscosity, interfacial tension, $\log_{10}$[CFUs/lung] (referred to as CFUs for simplicity), and dextran lung concentrations. A mixed model analysis was performed to examine differences in the serum tobramycin concentrations and ABG measurements with time used as the repeated-measure variable. A student's t-test was performed to examine differences in lung tobramycin content. All reported values are mean +/- standard error.

3.3 RESULTS

3.3.1 Emulsion characterization

To determine if the use of the more stable and less cytotoxic FSH-PEG fluorosurfactant had any effect on emulsion characterization compared to previous work, the viscosity and surface tension were measured.[8], [19] There was no significant difference ($p = 0.99$) in

viscosity between pure PFC (mean +/- standard deviation: 2.30 +/- 0.09 cP), the 0.2% V_{aq} emulsion (2.39 +/- 0.05 cP), and the 2.5% V_{aq} emulsion (2.41 +/- 0.09 cP). Figure 3.2 shows the surface tension significantly increased with increasing water and fluorosurfactant content (mean +/- standard deviation; PFC: 14.1 +/- 0.09 dyn/cm; 0.2% V_{aq}: 14.4 +/- 0.1 dyn/cm; and 2.5% V_{aq}: 15.6 +/- 0.3 dyn/cm; $p < 0.01$ between all groups). In contrast, the water-emulsion interfacial tension decreased with increasing water and fluorosurfactant content. Pure PFC (35.6 +/- 4.4 dyn/cm) and 0.2% V_{aq} (36.4 +/- 3.0 dyn/cm) emulsion were not significantly different from each other ($p = 0.81$), but the 2.5% V_{aq} (16.3 +/- 5.4 dyn/cm) was significantly lower than both ($p < 10^{-3}$).

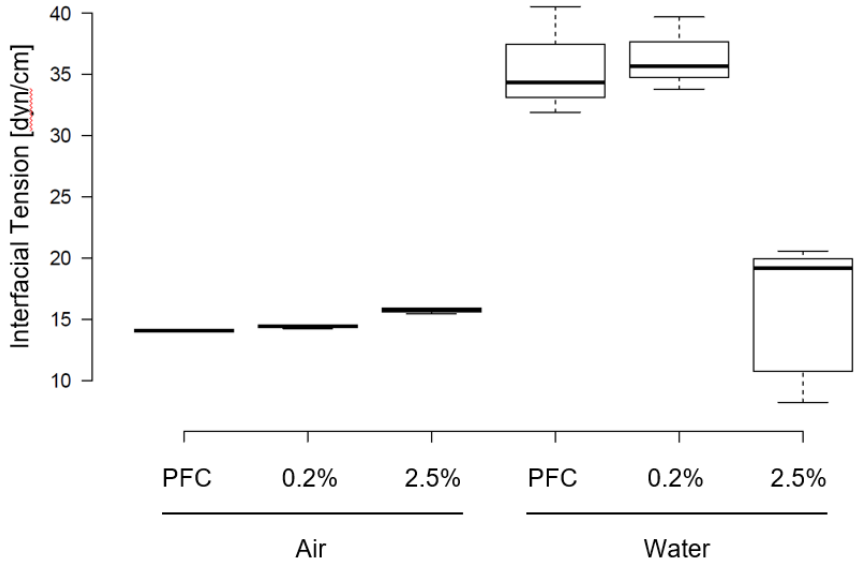

Figure 3.2 Air and water interfacial tensions of PFC and 0.2% and 2.5% V_{aq} emulsions.

3.3.2 Pharmacokinetic Evaluation of Serum Tobramycin

To determine if our new emulsion formulation achieved desired peak serum concentrations between 30-35 µg/mL, blood samples were drawn during the APV and aerosolized treatment groups and are shown in Figure 3.3. Tobramycin delivery via APV produced significantly ($p < 0.04$) lower serum concentrations relative to aerosolized

delivery at all time points except the 6-hour (p = 0.72). Peak concentrations for APV and aerosolized treatments were 18.7 +/- 2.5 and 42.5 +/- 4.5 µg/mL and occurred at 30 and 10 min, respectively. Although APV had lower serum tobramycin concentrations, APV achieved similar tobramycin concentrations in lung homogenate as aqueous aerosolized (p = 0.67). Mean lung homogenate tobramycin levels were 43.1 +/- 4.9 and 47.6 +/- 9.6 µg/lung for APV (n = 10) and aerosolized treatment (n = 7), respectively.

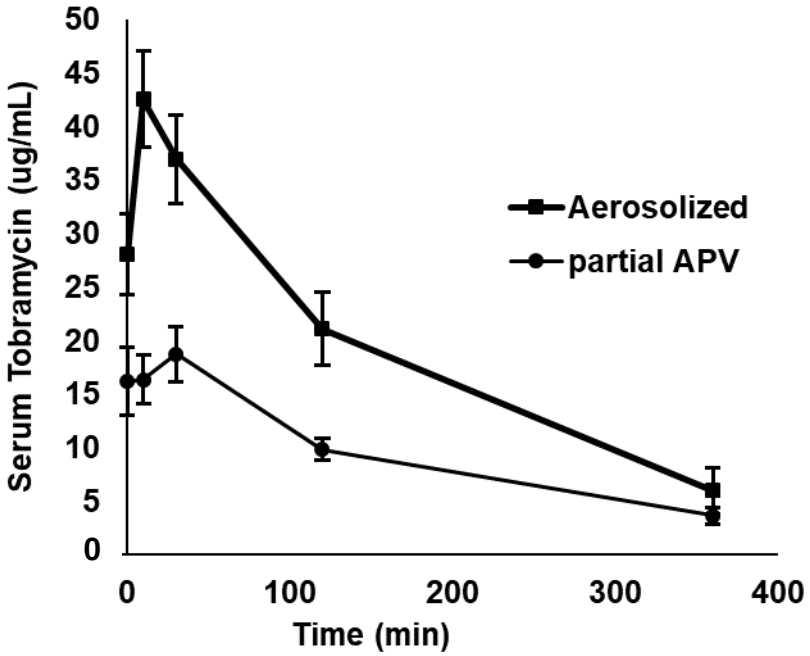

Figure 3.3 Tobramycin concentration of serum at 0, 10, 30, 120, and 360 minutes following tobramycin delivery via aerosolized (■, n = 13) and APV (●, n = 6). Errors bars represent standard error.

3.3.3 Microbiological Assessment of Lung Bacteria

The pulmonary bacterial load delivered to rats (Inoculum) and recovered from rats at 6 hours post-treatment (Post-treatment) is shown in Figure 3.4 as $\log_{10}$(CFUs/lung). The alginate microsphere methods described in this work were shown to produce a consistent bacterial respiratory infection with less than a 3% 24-hour mortality. All groups received a similar dose of inoculum (average 6.6 +/- 0.007; p = 0.99 between groups) and

maintained the bacterial infection at least 24 hours post-delivery, as evidenced by consistently positive throat cultures taken before treatment. In the time following inoculation, rats were typically observed to be lethargic with decreased appetite and moderately labored breathing. However, porphyrin staining around the eyes and nose, a common indicator of stress or sickness in rats, was seldomly observed. At the time of necropsy, viscous, mucus-like exudate was often observed in the angiocatheter (used as endotracheal tube; Figure 3.5A) and airway (Figure 3.5B).

Figure 3.4 shows the pulmonary bacterial load of rats one day post-inoculation and six hours post a single treatment. A one-way ANOVA showed no difference in lung CFUs after treatment with PFC (7.1 +/- 0.09) compared to the untreated control (6.9 +/- 0.1; $p = 0.98$). Both treatments with tobramycin delivered as aerosolized (5.7 +/- 0.16; $p < 10^{-4}$) and APV (6.1 +/- 0.22; $p < 10^{-2}$) significantly reduced lung CFUs compared to the untreated control. APV achieved equivalent bacterial killing to that of conventional aerosolized tobramycin ($p = 0.68$).

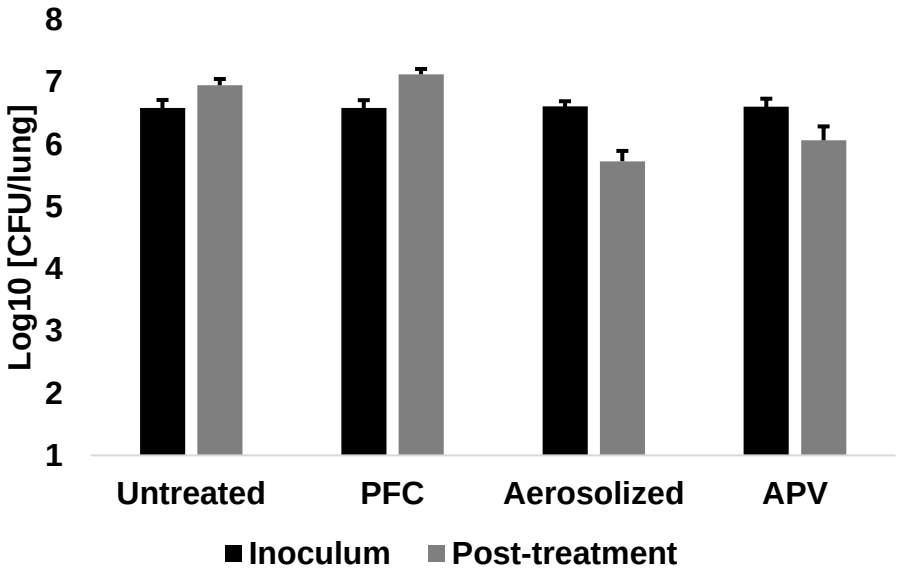

Figure 3.4 Pulmonary bacterial load for groups receiving no treatment (n = 10), PFC (n = 8), aerosolized tobramycin (n = 13), or APV (n = 9). Error bars are standard error.

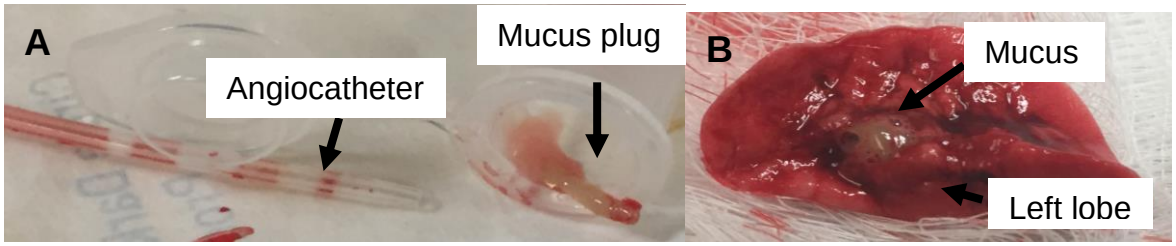

Figure 3.5 Images taken at euthanasia. **A.** Mucus plug removed from angiocatheter (used as endotracheal tube) **B.** Mucus bubbling out of airways.

3.3.4 Physiological Status during Ventilation

Although APV significantly reduced pulmonary bacterial load, a single dose (10 mL/kg) of emulsion left in the lung for 6 hours was not well tolerated. Figure 3.6 depicts the changes in physiological status over time. Figure 3.6A shows PCO_2 values for rats over time; for reference, normal PCO_2 values are (mean ± SE) 47.14 ± 0.89 mmHg in an anesthetized rat.[21] First, we see all groups tended to start off over ventilated with PCO_2 ranging from 31.44 ± 1.17 mmHg in the untreated group to 38.4 ± 3.74 mmHg in the PFC group. PCO_2 levels did not significantly change over the course of treatment in the untreated and aerosolized groups. As for the liquid ventilation groups, PCO_2 levels significantly increased (PFC: 70 ± 11.79; APV: 54.98 ± 8.40; $p < 0.03$) at the 6-hour time point compared to their respective baseline levels.

PCO_2 buildup resulted in a significant decrease in pH at the 6-hour time point (7.15 ± 0.06) for both the PFC ($p = 0.01$) and APV ($p = 10^{-3}$) groups as observed in Figure 3.6B. For reference, normal pH values in anesthetized rats are (mean ± SD) 7.33 ± 0.07.[21] The 6-hour pH in the APV group was significantly lower than the 1-hour ($p < 0.01$) and 3-hour ($p < 0.05$) timepoints.

The high pH in the LV groups was managed by changing ventilator settings (increasing RR and/or decreasing TV, while maintaining PIP < 30 mmHg) and by

administering sodium bicarbonate (1 - 3 mEq/kg). Figure 3.6C shows the administration of bicarbonate (only to animals with high PCO_2 and/or low pH) helped to maintain normal levels of bicarbonate in the PFC and APV groups, whereas the untreated group had a significant decrease in bicarbonate over time ($p < 0.05$); the aerosolized group did not have a significant decrease over time ($p = 0.5$). For reference, bicarbonate levels are 24.81 ± 3.28 mmol/L in anesthetized rats.[21]

Although respiratory acidosis (high PCO_2 and low pH) was prevalent in the LV groups, the major cause of death during APV treatment was decreased oxygenation as seen in Figure 3.6D. Although the PFC group trends toward a decrease in oxygenation, these values were not significantly lower than baseline ($p = 0.29$). Oxygen saturation at the 6-hour timepoint (78.7 ± 8.84) was significantly lower than baseline (97.61 ± 0.45; $p < 0.05$) for the APV group. Mortality rates during treatment in the APV (6 of 18 rats, which all died due to > 0.3 mL of pulmonary edema collected from the lung) and aerosolized (4 of 17 rats, only 1 of which died due to pulmonary edema) groups were higher than mortality in PFC (1 of 9 rats; a separate 2 of the 9 rats had non-fatal pulmonary edema) and untreated (0 of 10 rats, none with pulmonary edema) groups. Figure 3.7 depicts the pulmonary edema removed from the lungs after euthanasia.

3.3.5 Alveolar Permeability

In an attempt to determine how APV increased lung fluid, alveolar permeability was measured. Figure 3.8 shows a trend toward increased accumulation of dextran in the lung space for infected rats compared to healthy rats (15.03 +/- 1.03 µg/lung). Of the

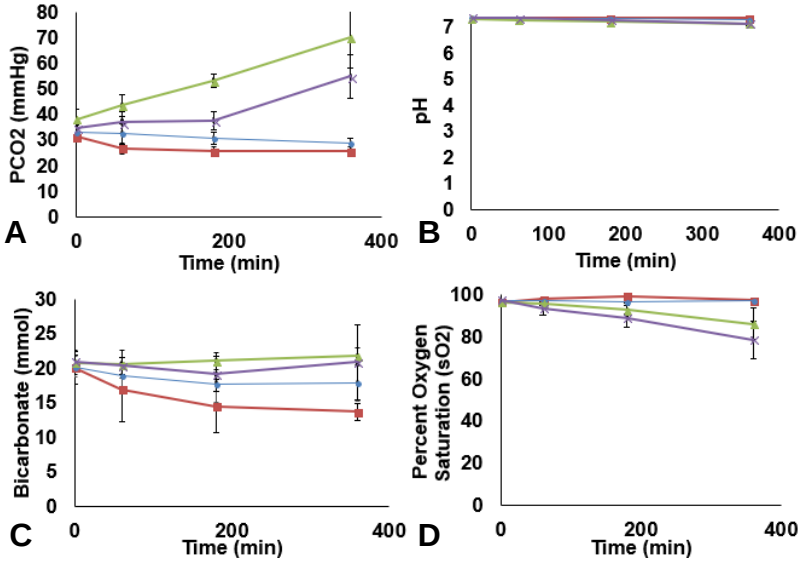

Figure 3.6 Arterial blood gas measurements at 0 minute (baseline before treatment) and 60, 180, and 360 minutes after treatment. **A.** Partial pressure of CO_2 (mmHg). **B.** Blood pH. **C.** Bicarbonate levels. **D.** Oxygen saturation.

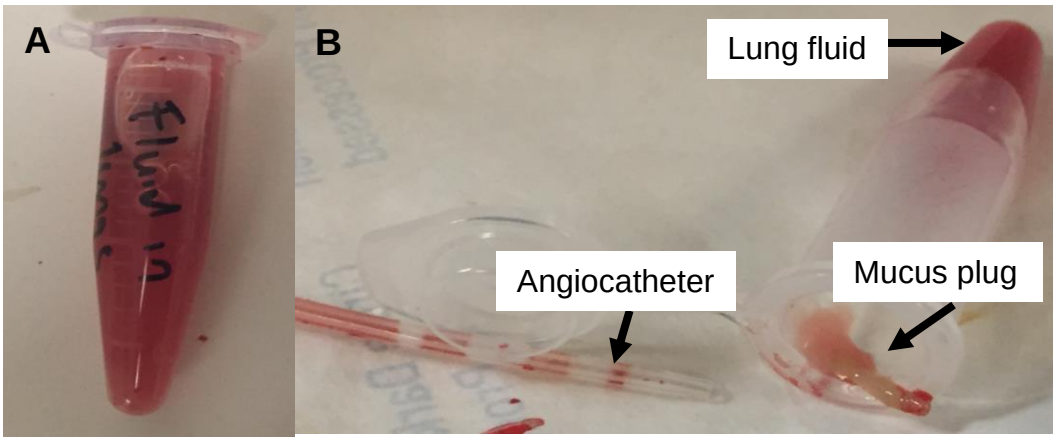

Figure 3.7 A. 1.4 mL of fluid in the lungs. **B.** 0.3 mL of fluid in the lung and a mucus plug removed from the angiocatheter (acting as an endotracheal tube).

rats surviving to 6-hour euthanasia, dextran levels among infected groups—untreated (36.99 +/- 10.9 µg/lung), PFC (42.67 +/- 7.5 µg/lung), aerosolized tobramycin (37.39 +/- 5.59 µg/lung), and APV (34.69 +/- 7.72 µg/lung)—were not statistically different from one another ($p = 0.89$). It should be noted that rats in the APV group with fluid in their lungs did not survive to the 6-hour time point and are not included in the data.

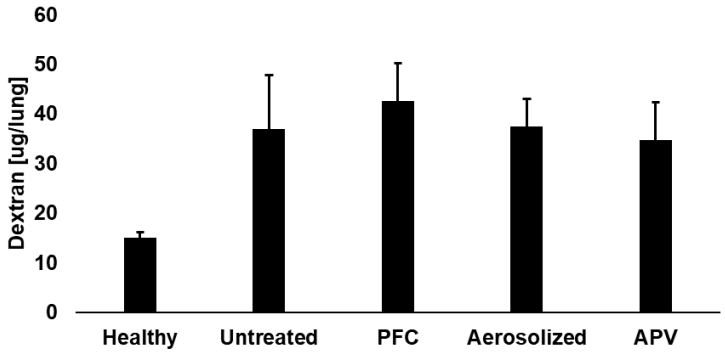

Figure 3.8 Dextran measured in lung space for a healthy group (n = 2) and those groups receiving no treatment (n = 3), PFC (n = 4), aerosolized tobramycin (n = 4), or APV (n = 5). Healthy rats were ventilated for 6 hours and received IV dextran 2 hours before lung harvest. Error bars are standard error.

3.3.6 Inflammatory Markers

To further determine if the excess lung fluid was caused by an increased inflammatory response in the APV groups, cytokines IL-6 and TNF-α and chemokine MIP-2 were measured in lung homogenate. Figure 3.9 shows that all inflammatory markers were increased compared to the healthy lung (fraction > 1); however, there was no significant increase in inflammation across all groups ($p > 0.68$).

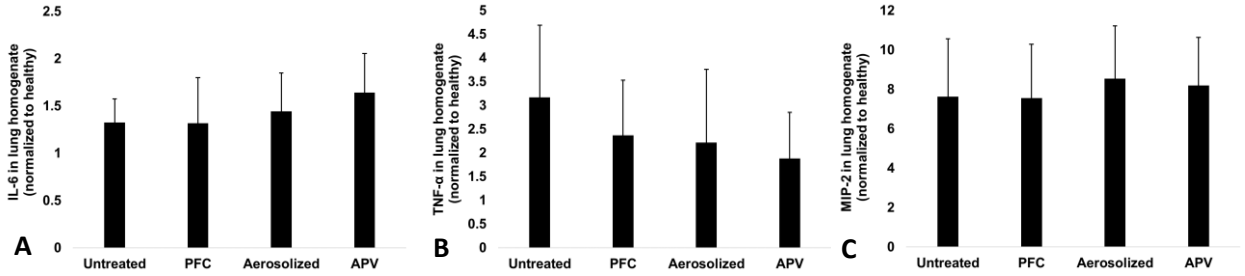

Figure 3.9 Inflammatory markers in lung homogenate. **A.** IL-6 cytokine. **B.** TNF-α cytokine**. C.** MIP-2 chemokine.

3.4 Discussion

The current findings provide evidence of a potential advantage for APV in eradicating a chronic *PA* respiratory infection. Considering the increased understanding of the emulsions that has been gained prior to these trials (see Section 1.9 and Chapter 2), the

observed success is predominantly due to the use of lower fluorosurfactant concentrations and higher total tobramycin concentrations. This enabled a single-dose of tobramycin-loaded PFC emulsion to achieve a similar efficacy in bacterial killing compared to conventional aerosolized tobramycin.

For this emulsion to be effective, it must first preserve PFC's viscosity and low interfacial tensions that allow for uniform filling and effective ventilation.[22] There was no difference in emulsion viscosity compared to that of pure PFC, which is expected given the low aqueous volume percents (V_{aq}). The surface tension increased with increasing water (and fluorosurfactant) content, with pure PFC having the lowest and 2.5% V_{aq} the highest. This is likely due to the increased water and fluorosurfactant creaming to the air-emulsion interface. Neither the water (72 dyn/cm), nor the hydrophilic PEG portion of the fluorosurfactant prefer to interact with non-polar air. However, this increase was not substantial enough to change PFC's flow properties. The water-PFC interfacial tension, in contrast, decreased with increasing water (and fluorosurfactant) content. The water-2.5% V_{aq} emulsion interfacial tension was considerably less than that of the water-PFC. This is likely due to the fluorosurfactant present in the emulsion. Their hydrophilic and fluorophilc groups preferentially accumulate at the water-emulsion interface, causing a decrease in the interfacial tension. Thus, emulsions maintain their ability to penetrate down to the alveolar level. In addition, the lower aqueous interfacial tension (compared to pure PFC) should more effectively penetrate fluid-filled airways and enhance fluid removal.[23], [24]

Second, the emulsion must be able to deliver drug effectively. As discussed in Section 1.9, the emulsions are purposely made to be unstable for effective drug delivery

(optimal fluorosurfactant concentration of 2mg/mL H_2O). Although susceptible to destabilization through coalescence and Ostwald ripening, these emulsions should be stable enough to deliver disperse drug to the lungs immediately after bedside sonication, similar to activating ultrasound contrast agents [e.g., Definity (Lantheus Medical Imaging) or Optison (GE Healthcare)] or preparing emulsions used in chemoembolization.[25]

Third, the emulsion must be bactericidal. Figure 3.4 shows our improved emulsion formulation (V_{aq} = 0.4%, C_{aq} = 750 mg/mL H_2O, C_t = 3 mg/mL emulsion, C_{fs} = 2 mg/mL H_2O) significantly reduced pulmonary bacterial load compared to untreated controls. The successful translation of the positive results observed in the previous study in Chapter 2 to the current *in vivo* treatment scenario can likely be attributed to multiple factors. Previous *in vivo* studies had shown lower V_{aq} (lower total fluorosurfactant delivered) and higher C_{aq} resulted in quicker and larger drug absorption into the systemic circulation, thereby removing it from the site of infection. Our study in Chapter 2 proposed to incorporate higher C_t in order to prolong exposure between the drug and pulmonary bacteria as opposed to increasing C_{fs} in an attempt to slow droplet coalescence and thus delivery. Moreover, previous studies proposed lowering C_{fs} to further increase the availability of emulsified tobramycin, thus significantly lowering pulmonary concentrations and raising serum levels from what was previously observed. With the combination of these changes, APV treatment demonstrated bactericidal functionality and retained similar pulmonary tobramycin concentrations as aerosolized delivery. However, we cannot speak to whether the emulsified tobramycin found in the lung homogenate is as available as the remaining aerosolized tobramycin. The dilution and homogenization method—which releases tissue water—likely broke or inverted the emulsion droplets and

increased the drug's availability during measurement relative to its prior emulsified state. This could have been accomplished in a manner similar to that achieved during the phase inversion and separation process used to quantify tobramycin content discussed in Chapter 2. It should be noted that pulmonary bacterial load was evaluated after a 6-hour ventilation to ensure majority of PFC had evaporated and that sufficient tobramycin was delivered to the tissues and blood to reduce bacterial killing during homogenization. The volume of emulsion remaining in the lungs at the time of euthanasia was not measured in this work. However, a previous study evaluating partial liquid ventilation (PLV) with pure PFC in similarly sized rats noted that the addition of 0.8 mL of PFC every 30 minutes was needed to maintain a constant PFC volume in the lungs.[26] Although the evaporative loss rate for PFC during PLV is known to decrease with time following delivery, clinical studies have shown that the rate remains fairly constant up to eight hours post-delivery.[7] The average amount of emulsion instilled during this study was 4.5 mL. Thus, assuming a near-constant evaporative rate over the duration of the experiment (six hours), one would expect the PFC phase of the emulsion to have largely evaporated at the time of euthanasia and tissue tobramycin measurement. In addition, although efforts were not made to recover or measure any PFC that may have been present in the homogenized tissue, PFC was not typically observed during homogenization or the subsequent analysis. Additionally, although homogenization likely freed additional tobramycin, the dilution process (25 mL/kg) lowered tobramycin concentrations to below minimum inhibitory concentrations (25 µg/mL) for this strain of PA in lung homogenate. Furthermore, homogenate was immediately serially diluted in 10-fold increments to ensure bacterial growth was not affected.

Another goal to get successful killing was to deliver enough drug to achieve therapeutic serum levels of 30 ug/mL. Emulsions did not achieve these concentrations, but still had equivalent bactericidal effects compared to aerosolized delivery. Due to the short resonance time in the lung (as evidenced by the high peak at 10 minutes), it is possible the aerosolized treatment relied more on therapeutic serum levels to decrease bacteria load. It is worth noting that while this peak concentration of tobramycin is quite high, samples were drawn from the carotid artery before becoming diluted in the circulated system, as seen with blood samples drawn more distal to the heart. It is therefore likely, this is an overestimate of serum concentrations. The emulsion treatments had a delayed and lower peak, possibly increasing pulmonary resonance time. As seen in previous studies, emulsions form a barrier to delivery both with slowed diffusion of large aqueous droplets to the airway surface and the fluorosurfactant layer that forms on the aqueous surface.[19], [27] These factors have consistently resulted in our *in vivo* emulsions delivering ~50% less of their payload compared to aerosolized.[19] The ideal case would be to measure the tobramycin concentration at the biofilm/alveolar level. However, best estimates rely upon pulmonary (measured in lung homogenate) and serum concentrations. The drug delivery mechanism of droplet deposit, fluorosurfactant accumulation at the surface, and tobramycin penetration through the lung are not well understood. These mechanisms explain the discrepancy with the aerosolized pharmacokinetics having a greater area under the curve than that of the APV, yet both delivery methods retaining the same amount of drug in the lung.

First, due to lack of solubility of tobramycin in PFC, it is expected that the drug delivery mechanism is dependent upon the diffusion of a droplet near enough to the

aqueous interface to coalesce with it, as the drug is not available to diffuse across PFC alone. It is likely that some portion of droplets undergo this process due to liquid agitation during filling of the lung at the initiation of APV. Following the filling process, droplet motion within the emulsion-filled lung is likely dominated by convective forces induced by the tidal fluid movement of continuous ventilation. During total APV virtually all surfaces within the lung are in constant contact with the emulsion. Conversely, during partial APV the upper airways are gas filled during the majority of the respiratory cycle. As PFC evaporates during treatment, an even greater portion of the lung will lose contact with the emulsion and thus have limited access to the delivered antibiotics. Although this might be addressed by intermittently altering the positioning and inclination of the lungs during ventilation, the experimental setup used in this work did not allow for easy manipulation of the rat's position relative to the ventilator. This limited contact of emulsion with lung tissue is further compounded by the fact that ventilation with PFC has been shown to alter the distribution of pulmonary perfusion to the less dependent regions.[28] Thus, in partial APV, higher blood flow occurs in the upper airways while the antibiotic is in constant contact with the lower airways. However, the similar pulmonary retention between aerosolized and APV delivery would suggest that PFC-induced differences in pulmonary blood flow are likely not the primary cause of the decreased serum uptake in APV compared to aerosolized.

Secondly, both aqueous droplet deposition as well as free fluorosurfactant in the PFC drive the accumulation of fluorosurfactant at the lung surface. As discussed in Section 1.9, the impairment of mass transfer of drug into an aqueous surface is likely due to the fairly quick (effects shown after only 10 minutes of pre-exposure) aggregation of

fluorosurfactant at the aqueous surface.[19] Similar effects were observed *in vivo* during APV pharmacokinetic trials evaluating tobramycin delivery via emulsion utilizing $C_{fs} \geq 30$ mg/mL H_2O (compared to $C_{fs} = 2$ mg/mL in the current study). Results from these studies showed relatively large amounts of drug remaining in the lung, seemingly unavailable for systemic absorption, at a time point at which the PFC phase of the emulsion is expected to have largely evaporated. Although the current study significantly decreased C_{fs}, further work studies need to be performed to ensure optimal C_{fs} for *in vivo* delivery. However, due to the active nature of emulsified tobramycin following recovery via the phase inversion and centrifugation process (described in Chapter 2), the formation of a chemical bond between tobramycin and fluorosurfactant molecules is an unlikely explanation for the observed lack of availability.

Thirdly, the mechanism of tobramycin penetration into lung tissue is not fully understood. Several studies suggest that the positive surface of tobramycin forces it to be actively transported across cells and thus able to saturate cells which leads to a discrepancy between serum and tissue levels.[29], [30] Although higher pulmonary doses of tobramycin are effective, aminoglycosides have been shown to accumulate in the kidneys and ears leading to nephro- and ototoxicity. [31], [32] However, it is this saturation of the kidneys that allows higher, one-time doses of tobramycin to be less toxic than lower, multiple doses. [3], [13], [15] Given the preferred method of a high, single-dose to limit toxicity, we could potentially increase our total delivered dose to ensure we reach therapeutic ranges of 30 µg/mL peak within 30 minutes and a 1 µg/mL trough after 18 hours.[13], [15]

Given that APV demonstrated equivalent bactericidal capabilities to aerosolized tobramycin in spite of having lower serum concentrations, a single APV treatment has the potential to outperform aerosolized delivery for several reasons. First, APV can be delivered at much higher doses (> 30 mg/kg) compared to aerosolized delivery without significant increases in systemic toxicity. Second, as discussed in section 1.9, APV is envisioned as a therapeutic lavage of the lungs. At the conclusion of APV, some portion of the emulsion would be suctioned from the lungs along with a large portion of dislodged mucus or biofilm. This would substantially decrease both the total tobramycin and water delivered to the lung as well as the pulmonary bacteria load. If the C_{aq} (750 mg/mL) and V_{aq} (0.4%) in our study were to be used clinically, a 70-kg patient undergoing PLV with 15 mL/kg of the emulsion (the dose used in previous clinical trials) would receive approximately three grams of tobramycin in 4.2 mL of water instilled into the lung. However, at the completion of therapy, a significant amount of the emulsion would be drained from the lung. Conservatively, estimating that 10 mL/kg is left behind, the delivered dose would be 2 grams in 3 mL of water, only twice as large as the currently recommended daily intravenous dose of 1.05 grams, but with the same level of toxicity.[15] During currently used nebulized tobramycin treatment for cystic fibrosis patients, approximately 10 mL of aqueous antibiotics are delivered to the lung on a daily basis. [Novartis Pharmaceuticals Corporation. TOBI, Tobramycin Inhalation Solution: Prescribing Information, 2014.] Considering that such treatment is generally well tolerated without supplemental oxygen or ventilatory support, the range of V_{aq} values evaluated in this body of work (0.2 – 2.5%) are likely within reason for a patient on mechanical ventilation. Thus, encouraging the use of higher antibiotic concentrations. The expected

benefits of airway clearance during APV were absent in this study as previous experience with rats has shown that suctioning of PFC from the lung is difficult and highly inconsistent. During APV in the current studies, mucus or biofilms were often dislodged (clogged angiocatheters) but were not removed from the airways. Third, the fraction of the administered dose actually delivered to the lungs may be higher during APV than aerosolized delivery due to aerosolized drug loss via exhaled drug as well as deposition in the oropharyngeal region and delivery device.[33] These studies were biased towards higher masses of aerosolized drug reaching the lungs compared to what is typically seen clinically. Aerosolized delivery in this study was performed with the Microsprayer nozzle positioned within the rat trachea. This technique likely results in much greater lung deposition than shown with clinical devices in humans, with little drug deposition in the trachea. Despite better lung deposition, intratracheal placement of the Microsprayer in the rat can sometimes induce apnea, resulting in aerosol delivery in the absence of airflow. The effects of poor ventilation distal to mucus plugging on aerosolized delivery are likely diminished in such a scenario.

Although effective in resolving the bacterial infection, tobramycin treatments were not well tolerated by the inoculated rats. As is typically noted as adverse effects, the LV groups experienced poor CO_2 elimination, mainly due to the high viscosity of PFC compared to gas and a small CO_2 diffusion coefficient in PFC.[34] This led to more pronounced respiratory acidosis compared to the untreated and aerosolized groups. However, it was managed by appropriate setting of the ventilator and sodium bicarbonate administration. The increased mortality during APV was due to pulmonary flooding leading to a decline in oxygenation and eventual death. It is important to note that alveolar

permeability measurements were only conducted on rats that survived to the 6-hour time point. Therefore, permeability data only represents the healthiest of rats and shows no decline in lung permeability compared to untreated controls. However, the high incidence of pulmonary flooding suggests some toxic effect to be present. This toxicity is not due to the process of LV as the PFC group had low mortality (1 of 9), and any exudate in the lung did not lead to early euthanasia. Furthermore, the decrease in saturation in the PFC group could be partially explained by (1) frequent blocking of the endotracheal tube (angiocatheter) due to mucous plugging, impairing gas exchange and requiring frequent clearing or total replacement of the angiocatherter, and (2) the redeposition of washed up mucus into healthy regions as the PFC evaporates. This supports our claim that our disease model was effective at increasing production of thick respiratory mucus and that PFC emulsions can displace mucus plugs. However, our inability to suction this mucus caused decreases in oxygenation. Although APV was affected by similar mucus plugging, the deterioration in lung function has to be attributed something other than LV alone. The toxicity could be a result of fluorosurfactants provoking further inflammatory response in an already injured lung. Previous *in vitro* studies showed current fluorosurfactant concentrations to be nontoxic when applied to alveolar epithelial cells. In addition, other researchers have applied much higher surfactant concentrations to the lung without adverse event.[8], [9], [27] However, the injured lung presents a much more complex environment where primed immune cells in the lung mount an even faster and greater immune response. Although we found no evidence of increased inflammation in the APV group, the long-term biocompatibility of the fluorosurfactants used (or rather any available fluorosurfactants) has not been evaluated. Thus, caution should be used regarding their

use in the lung. Results from this work suggest that our inability to suction inflammatory exudate after a short lung lavage may lead to worsening outcomes when the inflamed lung is exposed to foreign substances.

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CHAPTER 4

In Vitro Evaluation of Lysophosphatidic Acid Delivery via Reverse Perfluorocarbon Emulsions to Enhance Alveolar Epithelial Repair

4.1 Introduction

Acute Respiratory Distress Syndrome (ARDS) is characterized by severe inflammatory damage to the lung alveolocapillary barrier, resulting in the build-up of edema in the alveoli that decreases gas exchange.[1] As discussed in Section 1.5, the ideal treatment for ARDS would both support the respiratory needs of the patient while also enhancing alveolar repair.[2] Liquid Ventilation (LV) with perfluorocarbons (PFCs) was developed as a means to enhance gas exchange while reducing lung damage (see Section 1.6). However, LV could not evoke long-term improvements in alveolar repair. A host of pharmacological treatments (see Section 1.5.2) have been researched to improve alveolar repair, but, to date, none have demonstrated significant reductions in mortality among adults. This is possibly because drugs need to be delivered directly to the injured alveoli where they are needed.[3] Unfortunately, inhaled drug delivery to the alveolar level is difficult since the poor ventilation in the damaged, edematous regions of the lung impedes drug delivery.

To remedy this, we propose Perfluorocarbon Emulsions for Alveolar Repair (PEAR), a drug delivery vehicle that uses reverse water-in-PFC emulsions.[4]–[7] These emulsions contain a dispersed drug-loaded aqueous phase (< 2.5% by volume) that is emulsified within the liquid PFC. The resulting emulsion is delivered to the alveolus by

partially filling the lung with the emulsion and then ventilating with a standard gas ventilator over the emulsion. PEAR is proposed as a superior treatment for ARDS because it supports respiratory function through LV and it can uniformly deliver reparative drugs while removing exudate that impairs gas exchange and dilutes therapeutic concentrations.[8]–[14] In addition, PEAR can reduce inflammation that further weakens epithelial barrier function (see Section 1.7).

The optimal drugs to promote alveolar repair and reduce mortality are not yet known, but could potentially include epithelial and endothelial growth factors, anti-inflammatory drugs, antibiotics, and pulmonary surfactant. The purpose of this paper is to examine this concept through the delivery of one growth factor, lysophosphatidic acid, from PFC emulsion to lung epithelial cells. As mentioned in Section 1.5.2, lysophosphatidic acid (LPA) is a serum-derived, phospholipid growth factor that induces epithelial cell migration and proliferation, and, most importantly, enhances barrier function.[15], [16] After the inflammatory exudate has been washed up and suctioned out following treatment with PFC emulsions, an increase in barrier function would slow the influx of edema and neutrophils, allowing alveolar cells an opportunity to migrate into the wounded area and proliferate to reestablish functional tissue.

Delivery of antibiotics to biofilms via PFC emulsions has been shown to be effective in previous studies, but delivery of a growth factor to affect cellular repair and inflammation has never been attempted using a water-in-PFC single emulsion. In the current study, the effects of delivering LPA to alveolar epithelial cells via water-in-PFC emulsions were compared to that of aqueous LPA at similar concentrations using migration, proliferation, and barrier function assays *in vitro*.

4.2 Methods

4.2.1 Materials

1-oleoyl ($C_{18:1}$) LPA and fluorescein isothiocyanate–dextran (FITC-dextran; 4 kDa) were purchased from Sigma–Aldrich (Milwaukee, WI, USA). The alamar blue cell viability reagent was purchased from Life Technologies (Carlsbad, CA, USA). Bovine serum albumin (BSA) was purchased from New England Biolabs (Ipswich, MA, USA). Crystal violet (0.1% aqueous) was purchased from Ward's Science (Rochester, NY, USA). Luria-Bertani (LB) agar powder was purchased from ThermoFisher Scientific (Waltham, MA, USA). Similar to previous work with water-in-PFC emulsions, the PFC used was perfluorocycloether/perfluorooctane (FC-770) purchased from 3M Inc. (St. Paul, MN, USA) and the FSH-PEG fluorosurfactant was used as described in Section 1.9. Working solutions of fluorosurfactant were prepared with ≤ 5 mg of FSH-PEG in 1 mL PFC.

4.2.2 Cell Culture

The murine lung epithelial cell line MLE-12 cells were purchased from A.T.C.C. (Manassas, VA, USA) and were maintained in HITES medium (Dulbecco's modified Eagle's medium/F-12 medium) complemented with 10% fetal bovine serum in a 37 °C incubator in the presence of 5% CO_2.

4.2.3 Preparation of Aqueous Solutions

Aqueous LPA solutions were prepared fresh in Dulbecco's serum-free medium with 1% BSA immediately before experiments. BSA helps LPA to solubilize in water. Appropriate volumes of 5 mM LPA (in ethanol) were added directly into the serum-free medium + BSA

to prepare 0, 1, 5, 10, 200, and 400 µM aqueous solutions. Uniform dispersion was ensured by vortexing for 2 min. A pH meter was used to ensure aqueous solution would not be harmful to cells. An alternative method is to dry out the ethanol under N_2 then resuspend the remaining LPA in media. (This method would remove the risk of ethanol to the cells, however, it proved unsuccessful for us.) Aqueous crystal violet solutions were either undiluted (i.e. 100%) or diluted to 2.5% v/v in sterile water.

4.2.4 Emulsion Preparation

LPA-loaded, water-in-PFC emulsions were prepared as previously described in 5-mL batches with fixed aqueous volume percent (V_{aq} = 2.5%) and aqueous concentration of fluorosurfactants (C_{fs} = 2mg/mL of water).[5] Briefly, a mixture of PFC (475 µL), aqueous LPA (125 µL), and fluorosurfactant (0.25 mg) was emulsified via sonication (Model VCX 130, 20 kHz, 3.2 mm diameter microtip; Sonics & Materials, INC., Newtown, CT, USA) at 200 W-cm^{-2} for 20 seconds (pulse on: 5 seconds, pulse off: 2 seconds) on ice. This mixture was then added to 4.4 mL of PFC and sonicated at 200 W-cm^{-2} for 60 seconds continuously on ice. Aqueous LPA solutions of 0, 40, 200, and 400 µM were emulsified within the PFC to yield total concentrations of 0, 1, 5, and 10 µM LPA emulsions, respectively. Emulsion formulations are summarized in Table 4.1. The 0 µM emulsion, used to determine the effect of fluorosurfactant on cell behavior, was prepared the same way with a 0 µM LPA aqueous solution (serum-free media + BSA). Crystal violet-PFC emulsions were prepared by emulsifying 250 µL undiluted crystal violet in 9.75 mL of PFC and fluorosurfactant (0.25 mg) to yield a total concentration of 2.5% (v/v) emulsion.

Emulsion Formulations (C_{fs} = 2mg/mL water)	Aqueous volume percent [%]	Aqueous drug concentration [µg/mL water]	Total emulsion drug concentration [µg/mL emulsion]
0 µM	2.5	0	0
1 µM	2.5	40	1
5 µM	2.5	200	5
10 µM	2.5	400	10

Table 4.1 Emulsified LPA formulation

4.2.5 Delivery of Crystal Violet to Aqueous Surface

To determine how emulsion creaming (separation of the less dense aqueous droplets to the top of the emulsion) affected delivery to an aqueous surface (cells, lung epithelium, etc.), molten LB agar (100 µL) was allowed to gel at the bottom of 2-mL microcentrifuge tubes and then exposed to 2 mL of either 2.5% aqueous or 2.5% emulsified crystal violet solutions for 2 hours. The microcentrifuge tubes were oriented such that the gel sat beneath, above, or adjacent to the media (see Figure 4.1). After exposure, the media was removed, and the gel was placed in a clean tube and melted with 1 mL water. The absorbance of 100 µL samples was measured at 590 nm in triplicate.

4.2.6 Scratch Assay for Cell Migration

ARDS is characterized by areas of denuded epithelium. Thus, alveolar repair requires epithelial cells to migrate into these regions. A scratch assay was performed to determine epithelial cell migration. The procedure followed the method of Zhao, et al.[17] Briefly, cell monolayers (1e6 cells) were seeded in 6-well plates, scratched with a 10 µL pipette tip, washed to remove non-adherent cells and cellular debris, and digitally photographed using a phase contrast microscope. Cells were then treated for 2 hours with 1 mL of either i) 0 µM aqueous control, ii) 1 and 5 µM aqueous LPA (positive control), iii) pure PFC (PFC

control), iv) 0 μM emulsion containing no drug (fluorosurfactant control), or v) 5 and 10 μM LPA emulsions. Next, cells were incubated 18 hours in serum-free medium, the media was changed, and the final images were taken. ImageJ was used to quantify the area occupied by cells and thus, the extent of cell migration (see Figure 4.2). The amount of migration is calculated using equation (1)

$$migration = \frac{pre\ migration\ area - post\ migration\ area}{length\ of\ scratch} \quad (1)$$

, where "pre migration area" is the area of the wound before treatment and "post migration area" is the area after treatment and incubation. This data is then presented as the percent increase in migration normalized by cells treated with 0 μM aqueous control, equation (2).

$$\%increase\ in\ migration = \frac{migration_{treatment} - migration_{control}}{migration_{control}}\ x\ 100 \quad (2)$$

4.2.7 Epithelial Permeability Assay with Measuring Dextran Leak

Epithelial barrier disruption leads to the edematous alveoli that is characteristic of ARDS. A rapid increase in barrier function will slow the influx of exudate, enabling epithelial cells to repair. Dextran leak was utilized to determine the effect of aqueous and emulsified LPA (5 and 10 μM) on pulmonary epithelial barrier function. The procedure was followed according to Strengert and Knaus.[18] Briefly, MLE-12 cells were plated at 100% confluence (2.5e5 cells) on permeable inserts containing 0.4 μm pores (24-well plate; Corning, Kennebunk, ME, USA). For 2 hours, the top chamber was treated with 300 μL of either i) 0 μM aqueous control, ii) 5 and 10 μM aqueous LPA, iii) pure PFC, iv) 0 μM emulsion, or v) 5 and 10 μM LPA emulsions. The bottom chamber was filled with 450 μL

serum-free medium. After treatment, the cells were washed with Hank's buffered salt solution (HBSS). Dextran (150 µL) was added to the top chamber at 0.5 mg/mL in HBSS for 1 hour; the bottom chamber was filled with 450 µL HBSS. To further ensure this specific PFC had no lasting effect on barrier function, a separate experiment was conducted. Cells were pre-treated with 200 µL of blank medium or PFC for 2 hours and then exposed to a final mixture of dextran (0.5 mg/mL) and aqueous LPA (5 or 10 µM) in HBSS for 1 hour.

For both experiments, fluorescence levels were measured in the top and bottom (in triplicate) chamber by a fluorescence microplate reader (100 µL) with excitation at 490 nm and emission at 520 nm and were then converted to a dextran concentration (µg/mL) using a standard curve. The concentration of dextran that leaked into the bottom chamber (µg/mL) is indicative of the barrier function, with less dextran leakage implying greater barrier integrity. Dextran leak was normalized by the leak of cells treated with 0 µM aqueous control (equation 3), and the data is depicted as percent increase in barrier function (equation 4).

$$normalized\ dextran\ leak = \frac{dextran\ leak_{treatment}\ (\mu g/mL)}{dextran\ leak_{control}\ (\mu g/mL)} \quad (3)$$

$$\%increase\ in\ barrier\ function = 100 - (normalized\ dextran\ leak * 100) \quad (4)$$

4.2.8 Alamar Blue Assay for Proliferation

Alveolar type II epithelial cells must proliferate in order to repair the denuded alveolar wall and re-establish functional tissue. Proliferation was determined using the alamarBlue assay per manufacturer's instructions. Cells (10,000 cells/well) were seeded in 24-well plates and, after an 8-hour attachment, were starved overnight in serum-free medium.

Cells were then treated for 2 hours with 300 µL of either i) 0 µM aqueous control, ii) complete HITES medium with 10% FBS (positive control), iii) 5 and 10 µM aqueous LPA, iv) pure PFC, v) 0 µM emulsion, or vi) 5 and 10 µM LPA-PFC emulsions. Cells treated with emulsions were then quickly washed with PFC to remove any remaining FSH-PEG. All cells were incubated for 72 hours in medium (5% FBS); the positive control cells were incubated in complete medium (10% FBS). alamarBlue in serum-free medium (10% by volume) was added to each well (225 µL) and incubated an additional 4 hours. Reduction of alamarBlue was determined by measuring well absorbance in duplicate at 570 and 600nm (λ). Within an acceptable range of cell density and incubation time, the level of reduced alamarBlue linearly increases with the number of living cells; thus, percent difference in reduction in alamarBlue between treated and control cells will be presented as percent increase in proliferation (equation 5)

$$\%increase\ in\ proliferation = \%difference\ in\ reduction$$

$$= \frac{\varepsilon_{600} * A_{570} - \varepsilon_{570} * A_{600}\ treated}{\varepsilon_{600} * A_{570} - \varepsilon_{570} * A_{600}\ control} * 100 \qquad (5)$$

, with ε_{600}=117,216 and ε_{570}=80,586 as the molar extinction coefficients for Alamar Blue at different wavelengths and A_λ being the absorbance values from each wavelength.

4.2.9 Statistical Analysis

Statistical differences ($p<0.05$) in all variables were determined in SPSS (IBM Corporation, Armonk, NY) using a one-way ANOVA with multiple comparisons applying Tukey's method and the Sidak correction. All values are represented as mean +/- standard error unless stated otherwise.

4.3 Results

4.3.1 Delivery of Crystal Violet was Decreased in Emulsions.

To determine the effect of emulsion creaming on delivery to an aqueous surface, the uptake of aqueous or emulsified crystal violet into an agar gel was measured in three orientations. Figure 4.1 shows the difference in emulsified delivery compared to that of aqueous delivery. The 2.5% aqueous dye solution equilibrated with the gel when it was oriented on the top (mean +/- standard deviation: 2.4% +/- 0.3) and side (2.3% +/- 0.1). When on the bottom, however, the gel only took up 56% of dye compared to the top (1.3% +/- 0.05, $p = 10^{-4}$). The delivery of emulsified dye also dependent on orientation. The dye uptake significantly increased from bottom (0.5% +/- 0.1; $p = 10^{-4}$ compared to both side and top orientation) to side (0.9% +/- 0.2; $p = 10^{-4}$ compared to top orientation) to top (1.9% +/- 0.2) as exposure to the rising aqueous droplets became more favorable. Comparing aqueous to emulsion uptake, emulsions delivered 60% less dye than aqueous solutions in both the bottom and side orientations (60 +/- 8%; p = 0.99). However, the emulsion achieved only a 19% reduction in delivery compared to aqueous exposure to the gel on top (19 +/- 7%; $p = 10^{-4}$ compared to side and bottom orientations).

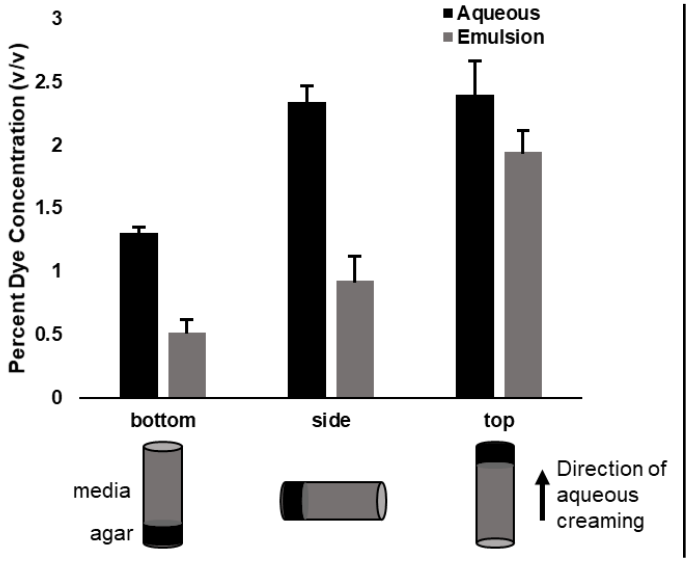

Figure 4.1 Concentration of dye in agar gel after 2-hour exposure to 2.5% aqueous or emulsified crystal violet solution. Error bars are standard deviation (n = 3).

4.3.2 LPA Emulsion Induces Epithelial Cell Migration.

To determine if emulsified LPA retained its effect on epithelial cell migration as seen with aqueous LPA, cells were treated with both aqueous (1 and 5 μM) and emulsified (5 and 10 μM) LPA during a scratch assay. Figure 4.2 depicts typical images of experimental groups, with greater migration into the scratch after 5 μM aqueous treatment.

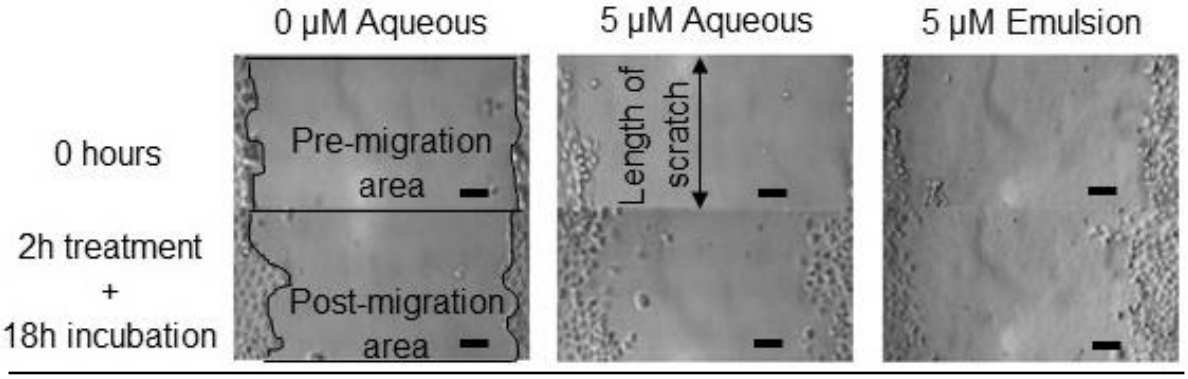

Figure 4.2 Scratch assay of MLE cells. Scale bar equals 15 μm.

Figure 4.3 presents the percentage increase in epithelial cell migration vs. the 0 μM aqueous control. Migration increased with LPA concentration, both in purely aqueous form, as researchers have previously shown, and when delivered via PFC emulsion.[17] Thus, the emulsion was capable of delivering LPA to the cells. For aqueous delivery, percent increase in migration following treatment with 1 μM LPA (12.3 +/- 6.6%) was not significantly greater than that following treatment with 0 μM control (0 +/- 2.9%), but percent increase in migration following treatment with 5 μM LPA (27.6 +/- 8.3%) was significantly greater than that of the 0 μM control ($p < 0.05$). For emulsified delivery, the 10 μM emulsion (26.2 +/- 6.1%) had significantly greater percent increase in migration than the 0 μM aqueous control ($p < 10^{-3}$), but the 5 μM emulsion did not ($p = 0.1$). However, the 10 μM emulsion was not significantly different than the 5 μM emulsion (14

+/- 2.3%; p = 0.5). Treatment with pure PFC alone (-15.8 +/- 2.1%) significantly reduced migration compared to 0 μM aqueous control (0 +/- 2.9%; $p < 0.05$), however, there was no difference between the 0 μM emulsion (-8.1 +/- 4.1%) and 0 μM aqueous control (p = 0.6). Comparing the performance of aqueous to emulsified LPA, there was no difference between the aqueous LPA and emulsified LPA groups. However, approximately twice the concentration of emulsified LPA (10 μM emulsion) was required to achieve the same ~30% increase in migration as demonstrated in 5 μM aqueous.

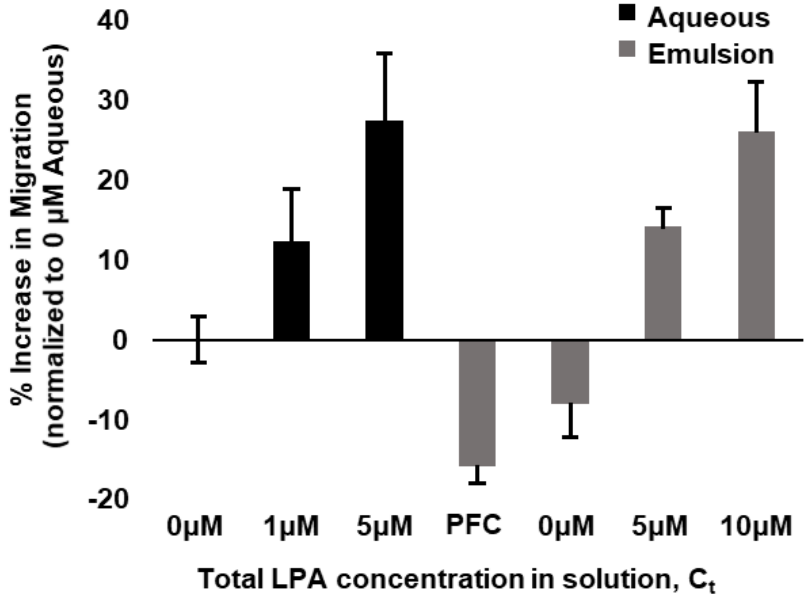

Figure 4.3 Percent increase in proliferation post 2-hour treatment and 72 hour incubation. Error bars are standard error. There was an n = 5 for all groups.

4.3.3 LPA Emulsion does not Enhance Epithelial Barrier Function.

To determine if emulsified LPA retained its effect on epithelial cell barrier function as seen with aqueous LPA, cells were treated with both aqueous (5 and 10 μM) and emulsified (5 and 10 μM) LPA during a dextran permeability assay. Figure 4.4 presents the extent of dextran leak across the epithelium as a percentage of that seen in the 0 μM aqueous control. These results confirm that aqueous LPA increases barrier function.[19] In Figure

4.4A, the 5 and 10 μM aqueous LPA (33 +/- 3% and 42 +/- 4%, respectively) increased barrier function (reduced normalized dextran leak) compared to the 0 μM aqueous control (0 +/- 0.6%; $p < 10^{-5}$ and $p < 10^{-6}$, respectively), but were not significantly different from each other ($p = 0.1$). However, the emulsified LPA had no effect on barrier function compared to the 0 μM aqueous control ($p > 0.95$ for all groups). In addition to Figure 4.4A showing pure PFC alone had no effect on barrier function ($p = 0.99$), Figure 4.4B also shows that pre-exposure to pure PFC did not affect subsequent cellular response to aqueous LPA treatment ($p = 0.99$). There was no difference in barrier function between LPA treated cells with or without pre-exposure to pure PFC ($p > 0.99$).

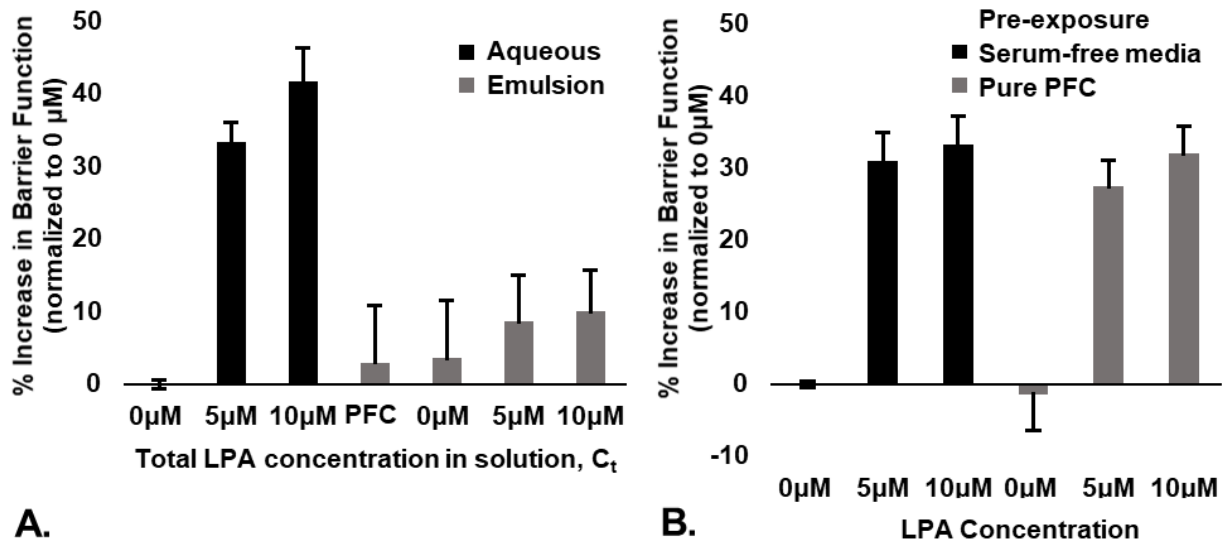

Figure 4.4 Percent increase in barrier function using dextran leak assay. **A**. 2 -hour treatment followed by 1-hour exposure to FITC-dextran (0.5 mg/mL). **B.** 2-hour pre-exposure to serum-free medium or PFC followed by 1-hour simultaneous treatment with aqueous LPA (5 or 10 μM) and FITC-dextran (0.5 mg/mL). Error bars are standard error. There was an n = 6 in panel **A** and n = 4 in panel **B**.

4.3.4 LPA Emulsions do not Induce Cell Proliferation

To determine the effect of LPA on proliferation, alveolar epithelial cells were treated with both aqueous and emulsified LPA. Figure 4.5 shows increasing proliferation with increasing aqueous LPA concentration, but not to the extent of the positive control (HITES medium). The HITES control had significant growth with a mean of 84 +/- 10% ($p < 10^{-6}$), whereas the 5 and 10 μM aqueous LPA had percentages of 16 +/- 5% and 28 +/- 5%, respectively. The 10 μM aqueous significantly increased proliferation compared to the 0 μM aqueous control, but the 5 μM aqueous did not ($p < 0.05$ and $p = 0.3$, respectively). There was no significant difference in proliferation after treatment with PFC or LPA emulsions compared to the 0 μM aqueous control ($p > 0.95$ for all groups).

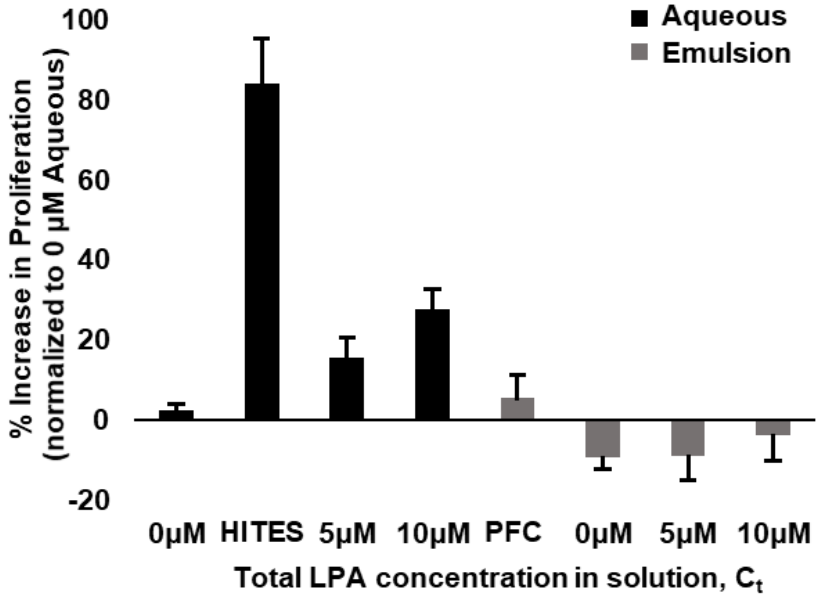

Figure 4.5 Percent increase in proliferation post 2-hour treatment and 72 hour incubation. Error bars are standard error. There was an n = 5 for all groups.

4.4 Discussion

Lowering the mortality of ARDS requires both supporting the respiratory function as well as enhancing alveolar repair. This second requirement is difficult to fulfill as researchers

have yet to discover a drug or combination of drugs that promotes alveolar repair *in vivo.*[20]–[22] In any case, once the optimal drug cocktail is developed, the delivery vehicle must uniformly apply drugs in the diseased regions and either penetrate or remove any edema that has pooled in those regions. This emulsion formulation has already been shown to preserve PFC's viscosity and low interfacial tensions that allow for effective ventilation (see Section 3.3.1), and this formulation already has the optimal fluorosurfactant concentration to deliver drug effectively.[5] Furthermore, our previous research has already shown that antibiotics are delivered to the lungs and significantly reduce bacterial loads after filling with PFC emulsions (see Chapter 3). In addition, others have been able to washout exudate from the lungs of ARDS patients during PFC ventilation.[11] The objective of this paper was then to determine the effectiveness of a PFC emulsion containing one growth factor, LPA, when delivered to epithelial cells.

The effect of emulsified LPA was either diminished or completely hindered in all cases. This was not due to sonication possibly disrupting LPA bioactivity since aqueous LPA sonicated at the same intensity and duration settings as that used to make the LPA emulsion prompted the same cellular response for migration, barrier function, and proliferation as un-sonicated aqueous LPA (data not shown). Thus, the reasons for emulsified LPA's diminished activity are multifactorial, involving both differences in drug delivery and direct effects of emulsion components on the cells.

Previous work has demonstrated 55% drug availability when delivering tobramycin from a similar emulsion to an agar well.[5] Figure 3.2 showed LPA delivery is likely reduced by emulsion creaming in this experimental set-up: the PFC is 1.8 times more dense than water, and thus the aqueous droplets rise up and away from the epithelial

layer at the bottom. This explains why dye uptake increased with orientations that increased exposure to the aqueous droplets in the emulsion. The aqueous condition also showed a decrease in dye uptake when the gel was placed on the bottom. This is possibly because the gel is slightly denser than the water. Gravity pushes the gel toward the bottom, thus increasing surface area of exposure in the top and side orientations. However, the bottom orientation is already at equilibrium and gravity holds the gel flush against the microcentrifuge tube, limiting the exposed surface area. Furthermore, as drug is delivered from the aqueous emulsion droplets to other aqueous media (e.g. agar, cells layers), fluorosurfactant is also delivered to the PFC-aqueous interface. Over time, this stabilizes the interface and hinders further delivery of aqueous droplets.[5] This process likely accelerates with PFC evaporation. Accordingly, the ability of LPA to modify epithelial function is certainly hindered to some degree by drug availability in this experiment. This latter effect would be less of an issue *in vivo.* There, droplet buoyancy is as likely to bring droplets into contact with airway walls as it is to float the droplet out of the alveolus.

Even if LPA is delivered, contact with the other emulsion components could perturb other cellular properties such as receptor binding affinity or subsequent signaling pathways that could limit the effect of LPA. The cellular level effects of PFC and the fluorosurfactant are not well understood, but we have previously shown that there are no toxic effects from this emulsion formulation nor pure PFC on epithelial cells.[5] This is confirmed here in our proliferation and barrier function results. In addition, there was no significant difference between the PFC only group and the 0 µM emulsion group in any of the assays. However, PFC has a direct, negative effect on migration in this *in vitro* setting. Cells migrated less than the control, but there was still positive migration. A PFC with

more documented *in vivo* safety has been developed and would be used for eventual clinical translation (perfluorooctyl bromide, Liquivent, Origen Biomedical, Austin, TX). The fluorosurfactant, however, is custom made and further formulations must be developed to decrease cytotoxicity and increase *in vivo* clearance. LPA, media, fluorosurfactant, and PFC are all being delivered to the cell surface simultaneously via the emulsion; the final effect on the cell is the sum of all these effects.

Despite this, emulsified LPA stimulated epithelial migration. Pure PFC significantly stunted migration; however, the 0 μM emulsion did not. This is likely due to the 0 μM emulsion providing some polar, aqueous environment to the cell surface, such that the cells do not contact the PFC as directly.[23]–[25] Researchers have shown PFC to interfere with the attachment of various cell types to various surfaces, leading to less spreading.[26] Although cells are only exposed to PFC for 2 hours, there may be some residual PFC that disrupts cellular anchorage and migration. However, the emulsified LPA was able to recover and stimulate migration when the LPA concentration was increased by 50%. This was expected taking into account the 60% loss in drug delivery we predict based on the crystal violet experiments.

Unlike with migration, the loss in functionality of emulsified LPA on barrier function cannot be attributed to some hampering effect from PFC or 0 μM emulsion. We have shown that our PFC has no effect on barrier function, as seen elsewhere.[27] In addition, PFC has no after-effect on barrier function, as treatment with aqueous LPA directly following exposure to pure PFC exhibited a similar increase in barrier function as seen when treated with aqueous LPA only. Taking into account the 60% loss in drug delivery we predict based on the crystal violet experiments, the effect of the LPA is likely blunted

by lesser LPA delivery from the emulsion, as is seen with migration. However, the barrier function is not recovered after a 50% increase in concentration. Further experiments are need to determine if PFCs and/or fluorosurfactant interfere with the downstream signaling pathways that are responsible for barrier function.

Lastly, LPA has not previously been shown to increase alveolar epithelial cell proliferation, specifically. However, it has been shown for other cell types including fibroblasts and mesenchymal stem cells.[28]–[30] For alveolar epithelial cells, we see a dose-dependent increase in proliferation with aqueous LPA. The effect is small, however, and not seen at all with the emulsion. As with barrier function, neither PFC nor 0 μM emulsion decreases proliferation and thus reduced drug availability is the predominant issue. Taking into account the 60% loss in drug delivery we predict based on the crystal violet experiments and the small effect LPA has on proliferation, it is possible higher concentrations are needed. This experiment was limited on increasing LPA concentrations because higher LPA concentrations required more ethanol in the aqueous solution. If the ethanol could be successfully blown off with N_2, then higher LPA concentrations could be used without risk to cell viability.

The cellular level effects of drug delivery to the lungs via emulsion are likely to vary based on the drug and the effect it is intended to create. Furthermore, these effects might be different *in vivo* given the greater surface area to volume ratio and the lessened effect of droplet creaming on drug availability. Overall, the benefit of direct delivery to the lungs while ventilating the patient may outweigh the reduced effect or it may be possible to simply increase emulsion concentrations to achieve the desired effect.

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Chapter 5

Conclusions, Limitations, and Future Work

5.1 Conclusions

The presented work explored the use of tobramycin-loaded and lipophosphatidic acid-loaded emulsions during antibacterial perfluorocarbon ventilation (APV) and perfluorocarbon emulsions for alveolar repair (PEAR), respectively. APV treatment improves upon delivery of antibiotics to combat respiratory infections; PEAR treatment improves upon delivery of growth factors to enhance alveolar repair during acute lung injury. This work represents an in-depth analysis of the emulsions used during APV and PEAR. Initial efforts evaluated emulsion efficacy under *in vitro* setting that better simulated lung *in vivo* antibiotic delivery. The subsequent studies utilized an *in vivo* rat model of bacterial respiratory infection to validate the effects of emulsion on pharmacokinetics and to assess APVs potential treatment benefits. Lastly, *in vitro* methods of cellular response assessed the utility of delivering growth factors in PEAR. From these studies, it can be concluded that:

1. The higher molecular weighted fluorosurfactants used in this work (PEGylated perfluoroether copolymers) are capable of sufficiently emulsifying aqueous tobramycin and lipophosphatidic acid within a naturally immiscible perfluorocarbon (PFC) phase. The emulsion preparation process is straightforward and could be performed at the bedside immediately prior to administration.

2. The addition of aqueous drug and fluorosurfactants, along with the remainder of the emulsification process, does not negatively alter the rheological properties of PFC critical to its use as a LV medium. The viscosity and air interfacial tension of the emulsion are not significantly different than that of neat PFC. The aqueous interfacial tension of the emulsion is reduced relative to that of neat PFC, potentially resulting in increased mucus removal capabilities during treatment.
3. Exposure to the tobramycin-loaded emulsions for the intended treatment duration (two hours) results in effective killing of *Pseudomonas aeruginosa* biofilms in a more complex *in vitro* setting. Antibiotic was allowed to diffuse out of the “lung” chamber and into the flowing “blood” chamber, as is observed *in vivo.* Bactericidal effects in this setting increased for emulsion formulations utilizing increased total antibiotic concentrations (C_t), aqueous volume percent (V_{aq}), and aqueous levels of tobramycin on the “blood” side (C_b).
4. Partial APV with emulsions utilizing FSH-PEG at a relatively low C_{fs} (2 mg/mL H_2O) was successful in reducing pulmonary bacterial load in a rat model of chronic lung infection relative to aerosolized tobramycin delivery. Furthermore, APV achieved an equivalent decrease in pulmonary bacterial growth. This result was due to the incorporation of (1) relatively unstable emulsions to maximize drug delivery and (2) the tripling of total antibiotic concentration to overcome the rapid removal in the blood.
5. Exposure to the lipophosphatidic acid-loaded emulsions for the intended treatment duration (two hours) results in variable efficacy on epithelial cells *in vitro.* Migratory effects were present but inhibited, however barrier function and proliferation effects

were completely hindered. In this setting, increased cellular response was observed for emulsion formulations utilizing increased aqueous concentrations (C_{aq}) and/or total concentrations (C_t).

5.2 Limitations and Future Work

The major limitations of this work primarily resulted from the use of a rat model as opposed to a larger animal species. The use of rats severely limited our ability to evaluate the mechanically-induced mucus and biofilm removal effects of APV. The significant role of abnormal mucus and biofilm presence in the progression of bacterial respiratory infection during lung disease is well established. Mucus removal has been previously noted during LV with neat PFC.[1]–[3] Moreover, there is ample theoretical evidence to expect that ventilation with emulsion during APV should result in equivalent or even greater mucus removal effects (discussed in Section 1.9). Mucus and biofilm removal during initial treatment with APV would not only make an immediate positive impact on the disease state, it should significantly increase the effectiveness of subsequent inhaled treatment. Yet all rat treatment trials performed in this work utilized emulsion delivery without subsequent removal, a setting that does not adequately allow for such effects to be manifested. The small airways of rats make suctioning of emulsion, as well as dislodged mucus and biofilm, technically difficult to perform in a consistent and reliable manner. Furthermore, the redistribution of mucus throughout the treatment may have led to the significant decrease in oxygenation as seen in Chapter 3. Future APV work in a larger animal model (likely rabbits, ferrets, pigs, or sheep) would allow for the use of equipment (*e.g.* cuffed endotracheal tubes) and treatment procedures (*e.g.* bronchoscopy) more akin to those used clinically. Additionally, the use of a larger animal

model may afford a more clinically relevant evaluation of a multi-day course of treatment including APV with subsequent aerosolized delivery. Furthermore, larger animals produce a more relevant disease model. Although the rat model discussed in the current work recreates many of the symptoms exhibited during cystic fibrosis (CF) in humans, recent work has made strides in developing genetically-altered CF pig and ferret models that resemble the clinical disease state much more closely.[4], [5] Pigs generated with mutated cystic fibrosis transmembrane conductance regulator (CFTR) genes even demonstrate impaired pulmonary host defenses as indicated by the spontaneous development of mucus accumulation, bacterial infection, and airway inflammation.[6]

Another limitation within these studies was the lack of an ideal experimental testbed to evaluate the reparative capacity of the emulsions used during PEAR. The ability of the emulsions to effectively promote alveolar repair in the setting of lung injury is integral to the treatment efficacy of PEAR. Initial studies evaluating the reparative effects of the emulsions (discussed in Chapter 4) used traditional cellular assays. The primary drawback of these experimental setups is that the cells sit beneath the emulsion, thus allowing creaming of the aqueous drug to severely limit delivery and thus cellular response. A higher cellular surface area relative to emulsion volume may have increased drug delivery. As the PFC evaporated, the height of the emulsion column decreased, bringing the aqueous drug layer closer to the epithelial layer; the shorter the column, the better chance of drug delivery. Based on the experimental setup, the migration assay had a high surface are to volume ratio, while the barrier function and proliferation assays were almost an order of magnitude lower. A more controlled, *in vitro* setting is often ideal during the initial development and optimization phases of a technology such as PEAR. A much

more improved *in vitro* experimental setup would expose a large surface area of cells to emulsion in a manner such that buoyancy effects are neutralized, neither directing drug towards nor away from the cellular surface. Although the physiological scenario would include epithelial surfaces in nearly every orientation relative to gravity, the described setup would represent the most ideal scenario, and thus a useful experimental testbed. The easiest and most relevant method to achieve this may involve emulsion exposure to a portion of excised injured airway or lung.

Although the current work made great strides in optimizing the emulsion formulation, further optimization is likely possible and should be explored. In addition to continuing to optimize the efficacy of APV, further biocompatibility testing should also be pursued in parallel. *In vitro* cytotoxicity results were promising; however, *in vivo* trials showed an increased sensitivity to APV in the injured lung. More in-depth *in vivo* trials should be performed in rats or a larger animal model to assess lung function as well as liver and kidney function at multiple time points following APV treatment. It is worth noting that only a small collection of fluorosurfactants have been evaluated in this application. Although the selection of fluorosurfactants available is somewhat limited, even small changes in the molecular structure and size may have significant effects on treatment potential. Additionally, this work assessed emulsions utilizing only a single type of PFC (FC-770). The current studies represent initial optimization and proof-of-concept work, and thus the use of a single PFC which is readily available and affordable is justified. However, multiple PFCs with varying molecular structures have been used during LV and have even been shown to produce varying cellular responses.[7] The type of PFC used will ultimately affect its interaction with the fluorosurfactants within the emulsion and thus

is a determinant of the resulting emulsion properties. Exploration of the use of other PFC types has the potential to further improve APV treatment and should be pursued.

In a much broader sense, future work should also explore the same basic delivery principles used during APV/PEAR to deliver other therapeutic agents in other disease states. In theory, drug delivery via ventilation with a water-in-PFC emulsion using the methods developed in this work could be used to achieve spatially uniform delivery of any aqueous soluble drug, including stem cells, siRNA, and large proteins. Any disease states in which inflammation is a primary concern would also benefit from the inherent anti-inflammatory effects of PFC. Any treatment in which a primary aim is to lavage the lungs and rid the airways of an aqueous media would benefit from the ability of PFCs to displace aqueous fluid. Commonly performed whole-lung lavages of pulmonary alveolar proteinosis patients in order to rid the lung of lipoproteinaceous material may represent an opportunity for application of this technology. Such a lavage could be performed with emulsion in order to simultaneously deliver therapeutic agents (such as granulocyte macrophage colony-stimulating factor) that are typically given via aerosol following lavage. In addition to treatment of pulmonary diseases, this intrapulmonary method has advantages over systemic delivery for treatment of non-pulmonary diseases. Any disease that would benefit from drugs having a faster systemic uptake and longer half-life could utilize this PFC emulsion delivery mechanism. For example, emulsions have better delivery profiles of large proteins like insulin compared to systemic delivery. The use of LV has also been explored for inducing protective hypothermia after cardiac arrest.[8] This treatment could utilize the drug delivery aspect of PFC emulsions to better treat and/or recover bodily functions during hypothermia. One case showed a patient with

ARDS to undergo hypothermia along with muscle paralysis to reduce total body oxygen consumption.[9] The patient's condition improved under hypothermia, and he was discharged later in a stable condition. In this scenario, PEAR would have a several applications in (1) supporting oxygenation, (2) washing out exudate, (3) calming inflammation, (4) inducing protective hypothermia, and (5) delivering drugs or stem cells to speed lung repair. The general strategy used during APV/PEAR possesses unique treatment capabilities and likely has the potential to improve the efficacy of respiratory and systemic treatments.

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